Journal of Dairy Research (2016) **83** 317–325. doi:10.1017/S0022029916000455

Separation and quantification of milk casein from different buffalo breeds

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Received 18 December 2015; accepted for publication 20 June 2016

Understanding the milk protein expression profile in different buffalo breeds plays an important role in improving hybrid selection and determining the effects on milk protein synthesis. The aim of this research is to compare the differences in milk protein content, composition and distribution between River buffalo and their crossbreeds for hybrid screening. Four groups of milk samples that included Nili-Ravi (N), Murrah (M), a Nili-Ravi-Murrah crossbreed (M-N), and a crossbreed of river buffalo with local swamp buffalo (C) were collected. The protein composition of the buffalo milk was determined by RP-HPLC. A gel-based proteomic approach consisting of two-dimensional gel electrophoresis coupled with mass spectrometry was utilised for the detailed protein characterisation of milk from different breeds. The results of this analysis showed that the river/swamp buffalo crossbreed (C) displayed the highest content of total protein (4·46%) and κ-casein (11·14%) but the lowest content of α-lactalbumin (6·79%). By selecting 23 different protein spots among the four types of milk that contained the most spots corresponding to κ-casein, β-casein and α_{s1}-casein, correlations between the crossbreeds, protein polymorphism and phosphorylation could be made. The results of this study indicate that crossbreeding a swamp buffalo with a river buffalo has a notable effect on the protein content and composition that may be exploited for producing high-quality raw milk in food technology applications and dairy food production.

Keywords: Swamp buffalo, river buffalo, crossbreed, milk protein, comparative proteomics.

Buffalo milk is the second most commonly produced milk in the world, with approximately 90 billion KG produced each year, accounting for 13% of all milk produced in the world (International Dairy Federation, 2010). It is higher in protein content, fat, lactose, total solids, vitamins and minerals than cow milk. These differences can influence the flavour and taste of buffalo milk, and it also makes buffalo milk a highly suitable ingredient for the manufacture of other milk products such as cheese and yogurt (Menard et al. 2010; Hussain et al. 2011).

China is one of the world's largest producers of buffalo milk, with more than 12 million buffalo raised in China. However, most Chinese buffalo are the native swamp breed with lower milk production than other breeds. Therefore, river buffalo breeds such as Murrah (M) and Nili-Ravi (N) have been introduced from India and Pakistan in an effort to improve milk production through crossbreeding with the native swamp buffalo.

Similar to other mammals, buffalo milk includes caseins and whey protein. The dissimilar distribution of α_{s1}, α_{s2}, β, and κ-casein in buffalo milk proteins compared to Holstein proteins has been reported (Bonfatti et al. 2013). The primary structure of buffalo and bovine α_{s1}-casein is homologous and is made up of 199 amino acids with a theoretical MW of 22·80 kDa (dephosphorylated form). Buffalo β-casein consists of 209 amino acids with a theoretical MW of 24·04 kDa (Ferranti et al. 1998). There are several protein variants found in buffalo milk, such as κ-casein (X1: Ile^{135} and X2: Thr^{135}) and α_{s1}-casein (A: Leu^{178} and B: Ser^{178}), while more than 10 variants were found in cattle protein (Caroli et al. 2009; Bonfatti et al. 2013). The milk protein polymorphism also has some relationship with the breeds, and two β-lactoglobulin variants (A and B) have been detected in Indian and Egyptian buffalo; however, only one variant (B) has been detected in the Mediterranean water buffalo (Patel et al. 2007; Feligini et al. 2009).

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Various buffalo milk proteins have been previously studied, and their post-translational modifications, genetic variants, native and non-native disulphide bonds, and non-disulphide cross-links have been identified using proteomic techniques (Chevalier & Kelly, 2010; Holland et al. 2011). Some studies have discussed the protein content from several varieties of River water buffalo, such as M and N that originated in India and Pakistan, and Mediterranean water buffalo (Aggarwal et al. 2007; Bonfatti et al. 2012). However, few studies have focused on the differences in milk protein composition between swamp water buffalo and their crossbreeds.

Notably, the resulting prolific crossbreed offspring yielded a great improvement in milk production (Nanda & Nakao, 2003) and total milk protein content (Han et al. 2007). Microbiological evidence, compositional information, genetic polymorphisms of the milk proteins and breed detection have all been reported (Han et al. 2007; Cottenet et al. 2011; Ren et al. 2011), but there is limited information on the influence of crossbreeding on the content and distribution of milk protein components.

The objective of this study is, therefore, to investigate the effects of crossbreeding on the protein composition, content and distribution among the four types of buffalo milk. The results obtained in this study will be useful in future research that directs breeding to produce high-quality raw buffalo milk for specific purposes, such as cheese-making.

Material and methods

Materials

Milk samples were obtained from the Guangxi Buffalo Institute Experiment Farm in Nanning, Guangxi Province of China. A total of 156 individual milk samples (500 ml) were obtained from pure river buffalo (M, $n = 41$; and N, $n = 34$), crossbreeds between M and N (M-N, $n = 36$), and crossbreeds of river buffalo and local swamp buffalo (C, $n = 45$). All of the milk samples were taken from animals during mid-lactation that were fed the same diet (Table 1). Buffalos were housed in indoor tie stalls and fed individually with free access to feed and water. The diets were offered as roughage first then the concentrate was fed. The buffalos were milked twice a day. All of the buffalo were 493 ± 30 kg in weight, 2~5 parity, and 3~6 years old. All of the selected crossbreeds derived from a third-generation breeding or after, where the M-N crossbreed was obtained from an M father and an N mother and the C crossbreed was obtained from a local swamp buffalo father with either a 25 M or 20 N mothers. The fresh milk was collected, and the protein content was analysed in triplicate samples by infrared spectroscopy using a Milko Scan FT120 (Foss Electric, Denmark). The milk samples were stored at −20 °C until the protein composition was tested and two-dimensional gel electrophoresis was performed.

Table 1. Ingredients and chemical compositions of experimental diets (DM basis)

Items	Content
Ingredient composition, % DM	
Corn	15·58
Soybean meal	3·54
Wheat bran	12·21
$CaHPO_4$	1·06
Limestone	0·89
NaCl	0·71
$NaHCO_3$	1·06
Premix†	0·35
Elephant grass	9·58
Cassava pulp	34·48
Brewer's grain	20·54
Nutrient levels, % DM	
CP	15·88
NDF	34·75
ADF	22·80
GE (MJ/kg)	16·30

CP, Crude protein; NDF, Neutral detergent fibre; ADF, Acid detergent fibre; GE, Gross energy
†One kilogram of premix contained 3 000 IU VE, 150 000 IU VD, 500 000 IU VA, 1·3 g Cu, 4·0 g Fe, 3·0 g Mn, 80 mg I, 6·0 g Zn, 80 mg Co, 50 mg Se

Protein composition analysis

The milk samples were mixed 1:1 with a buffer solution (pH 7, containing 0·1 M BisTris buffer, 6 M guanidine hydrochloride, 5·37 mM sodium citrate, and 19·5 mM dl-dithiothreitol), shaken for 10 s, incubated in the 1·5 ml tube for 1 h at room temperature, centrifuged (16 000 ***g***) for 10 min and then diluted 1:3 in a solvent consisting of acetonitrile and water according to Bonfatti et al. (2008). An Agilent 1100 Series (Agilent Technologies, Santa Clara, CA, USA) HPLC equipped with an analytical C8 column (Zorbax 300SB-C8 RP, 3·5 µm, 300 Å, 150 × 4·6 I.D., Agilent Technologies) preceded by a Security Guard Cartridge System (ZorbaxC4 4 × 3·0 mm^2, Agilent Technologies) was used for separation. The gradient elution conditions according to Bonfatti et al. (2008) included a flow rate of 0·5 ml/min. The total analysis time per sample was 45 min, with a column temperature maintained at 45 °C, a detection wavelength of 214 nm, and an injection volume of 10 µl. The protein composition was identified by comparing the elution times of the various chromatographic peaks with reported cow and buffalo proteins (Bobe et al. 1998; Bonfatti et al. 2008, 2013). The chemicals used in this experiment were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Two-dimensional gel electrophoresis separation

Three random individual samples from each breed (a total of 12 milk samples) were used for two-dimensional gel electrophoresis (2-DE). The milk samples were centrifuged at 3000 ***g*** for 15 min at 4 °C, and the top fat layer and precipitate were removed (Yang et al. 2013). The concentration of

https://doi.org/10.1017/S0022029916000455 Published online by Cambridge University Press

buffalo milk protein was determined by the Bradford protein assay kit (Bio-Rad Laboratories, Hercules, USA) with bovine serum albumin as standard according to the manufacturer's protocol. Buffalo milk protein samples (approximately 280 μg) were mixed with 350 μl of solubilisation buffer consisting of 8 M urea (GE HealthCare), 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulphonate (CHAPS) (GE HealthCare), 0·2% pH 4-7 carrier ampholytes (GE HealthCare), 6·5 mM dithiothreitol (Bio-Rad) and 0·001% bromophenol blue (Songon Biotech, Shanghai, China). The sample was placed on a 17 cm, pH 4-7, immobilised pH gradient (IPG) strip (Bio-Rad) for 12 h of passive rehydration at room temperature and subjected to isoelectric focusing using an Ettan IPG phor 3 (GE Healthcare). The programme including six steps was as following: 100 V, 1 h; 500 V, 1 h; 1000 V, 1 h; 4000 V, 1 h; 8000 V, 1 h; and 8000 V, 65 000 Vhr.

Focused strips were embedded with 0·5% agarose (GE HealthCare) on top of 12·5% polyacrylamide gels in an Ettan DALT II system (GE HealthCare). The gels were stained with Colloidal Coomassie Brilliant Blue G-250 (GE HealthCare) and destained in acetic acid. For the technical replication, three gels were run for an individual buffalo in each breed, and all sample gels were performed under identical conditions. Images were captured using an ImageScanner (GS800, Bio-Rad) and ImageMaster platinum 6·0 (GE HealthCare) to determine the protein spots of interest as reported by Goncalves Lda et al. (2010). Briefly, the 2D gels of triplicate biological were compared each other to obtained a master gel. Every protein spot position, shape and optical density was averaged in each master gel. At least 5 well-defined landmarks were chose to match gels. The spots in other studied breeds exhibited a greater than two fold change compared to breed C which was considered as differentially expressed. Then the different expressed spots would be sent to a commercial company for MS analysis.

In-gel digestetion

The twenty-three spots were removed manually from the gel using pipette tips then placed into a 1·5 ml tube. After washed with Milli-Q water for 15 min, the gel fragments were destained with 100 mM ammonium bicarbonate (Sigma) in 30% acetonitrile (Merck, Darmstadt, Germany). Subsequently, the spots were digested with 5 μl of 10 ng/μl trypsin solution ((Promega, modified sequencing grade) and incubated overnight at 37 °C, and then stopped reaction with 100 μl of 60% acetonitrile in 0·1% trifluoroacetic acid (Merck). After sonication for 15 min, the supernatants were concentrated to near dryness.

Mass spectrometry analysis

Mass spectrometry (MS) experiments were carried out on a 4800 Plus MALDI TOF/TOF™ Analyser (Applied Biosystems, USA) and data acquisition in the positive ion mode with a selected mass range of 800 to 4000 Da. The resuspended sample in 20% acetonitrile was placed on the ABI 4800 target plate (384 Opti-TOF 123×81 mm^2 ss, Applied Biosystems) and then 0·5 μl of the supersaturation CHCA matrix solution in 50% acetonitrile/0·1% trifluoroacetic acid was added. Eight of the most intense ions (a signal to noise ratio above 50) were performed for MS/MS analysis. Protein was identified of using the MASCOT software to search the NCBI database (http://www.ncbi.nlm.nih.gov, 2012-2-10). The following search parameters were used with trypsin as the enzyme, carbamidomethyl (C) as a fixed modification, mammalia as taxonomy, monoisotopic mass values, unrestricted protein mass, ±100 ppm peptide mass tolerance, ±0·4 Da fragment mass tolerance, and max missed cleavages 1.

Statistical analysis

The relative intensity of protein spots in the buffalo milk of different breeds were captured using the Quantity One software (v4·6·2, Bio-Rad). Statistical analysis was conducted by GLM procedure of SAS (SAS, V9·3) with Duncan's multiple range tests. All differences were considered statistically significant at $P < 0·05$.

Results and discussion

In this study, both reverse-phase high performance liquid chromatography (RP-HPLC) and a gel-based proteomic approach were used to investigate the content, composition and distribution of milk protein from the N, M, Nili-Ravi-Murrah crossbreeds (M-N) and crossbreeds of river buffalo with local swamp buffalo (C). A total of 23 proteins were identified. Some previous studies have reported milk proteomes similar to the buffalo in Holstein, Jersey and other bovine species (Hinz et al. 2012; Yang et al. 2015). In a previous work, a quantitative proteomic method performed on the Holstein, Jersey and buffalo milk fat globule membrane fractions revealed abundant κ-casein in the Jersey bovine milk and abundant β-casein and α_{s1}-casein in the buffalo milk (Yang et al. 2015). In another study, a higher abundance of κ-casein was found in the Bangladeshi buffalo than in cows' milk (Islam et al. 2014). In our study, high levels of κ-casein were discovered in buffalo breed C and confirmed by two analytical methods, indicating that through specific crossbreeding, buffalo breed C can yield milk with more κ-casein content that is high enough for improving cheese-making yield than other studied breeds. Thus, this finding suggests that this crossbreed may modify the pathway of milk protein synthesis in the buffalo mammary glands through activation of mammalian target of rapamycin (mTOR) signalling pathway and janus kinase 2 - signal transducer and activator of transcription 5 (Jak2-Stat5) signalling pathway (Bionaz & Loor, 2011; Shimobayashi & Hall, 2014). However, further studies are needed to explore the mechanism.

Nanda & Nakao (2003) reported that the milk nutrient content in crossbred buffalo were higher than the content

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Table 2. Protein profiles of milk from four breeds of buffalo

	Buffalo breed					
	M ($n = 41$)	N ($n = 34$)	M-N ($n = 36$)	C ($n = 45$)	SEM	P
Total protein (%)†	4·35^{ab}	4·06^{c}	4·16^{b}	4·46^{a}	0·14	0·005
α_{s1}-casein (%)	27·98	28·04	27·48	28·48	0·39	0·920
α_{s2}-casein (%)	12·55	12·68	12·72	12·12	0·32	0·354
β-casein (%)	32·81	32·46	33·54	32·86	0·50	0·619
κ-casein (%)	10·03^{b}	10·04^{b}	9·86^{b}	11·14^{a}	0·32	0·025
β-lactoglobulin (%)	9·32	8·97	9·04	8·58	0·29	0·573
α-lactalbumin (%)	7·10^{ab}	7·60^{a}	6·79^{ab}	6·23^{b}	0·21	0·014

Means with different superscripts in the same row differ significantly ($P < 0·05$)
M, Murrah; N, Nili-Ravi; M-N, Crossbreeds from Murrah and Nili-Ravi; C, Crossbreeds of river buffalo (Murrah and Nili-Ravi) with local swamp buffalo
†Total protein (%) was determined by infrared spectroscopy using a Milko Scan FT120, and protein composition (%) was determined by RP-HPLC

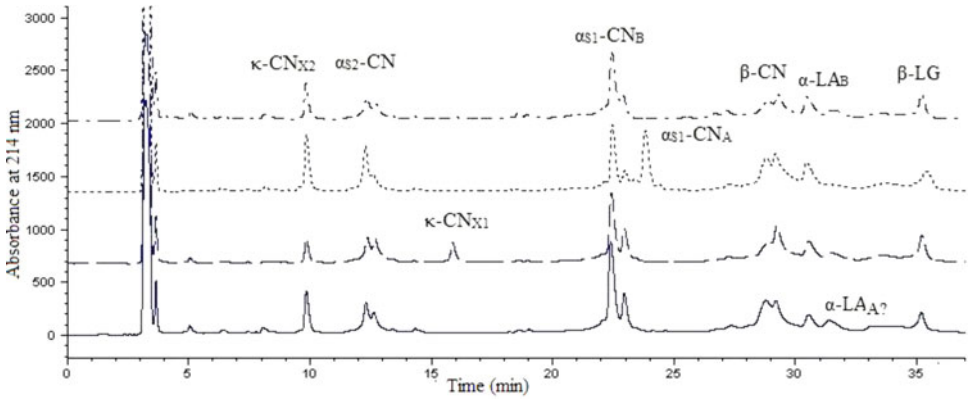

Fig. 1. RP-HPLC chromatograms of individual samples of the four types of buffalo milk with different α_{S1}- and κ-casein genetic variants (α_{S1}-casein A or B, and κ-casein X1 or X2) obtained using the optimised elution conditions reported by Bonfatti et al. (2008).

measured in purebred river buffalo, and therefore, crossbreeding may improve milk production in addition to providing a higher nutrient content. In addition to the type of breeds, many other factors, such as forage, feeding systems, milking frequency and method, seasonal changes and lactation period, can affect the physicochemical parameters of buffalo milk (Khedkar et al. 2016). In this study, all of the milk samples came from the same farm at the same time to avoid factors involving seasonality. However, future research into the differences in genetics and the translational efficiency of transcripts are still needed to provide methods for developing buffalo breeds with optimised milk protein production.

Protein composition

The protein composition of individual buffalo milk samples obtained by RP-HPLC is shown in Table 2 and Fig. 1. Significant differences ($P < 0·05$) in the total protein content were found among the four groups, with the crossbred group (C) having the highest protein percentage and N containing the lowest which was agreed with Ren et al. (2015). The total protein content did not have significant difference between C and M buffalo. The results in this study were consistent with the protein contents for purebred water buffalo, M (4·27%) and N (4·16%), and for the native swamp crossbred buffalo, C (4·75–5·23%), previously reported by Han et al. (2007). Han et al. (2007) showed that the protein level in the milk with each buffalo crossbreed and multi-generation crossbreed in their study were similar to the levels observed among the multi-generation crossbreeds in this study (4·75 ± 0·53% and 4·46 ± 0·48%, respectively), suggesting that crossbreeds have great potential to produce more milk protein than other buffalo.

The differences ($P < 0·05$) were also observed in the α-lactalbumin and κ-casein content of the milk. The lowest α-lactalbumin content was found in the milk from the crossbred C buffalo (6·23%) and was significantly lower than the levels found in the milk from the N (7·60%) buffalo, while the α-lactalbumin content in M, N, M-N buffalo were similar. The amount of κ-casein in the milk was significantly higher in the crossbred buffalo C (11·14%) than in the other buffalo. The result that crossbred buffalo C had higher κ-casein content than M buffalo was consistent with Ren et al. (2015). However, Ren et al. (2015) found that there was no significant difference in κ-casein content between

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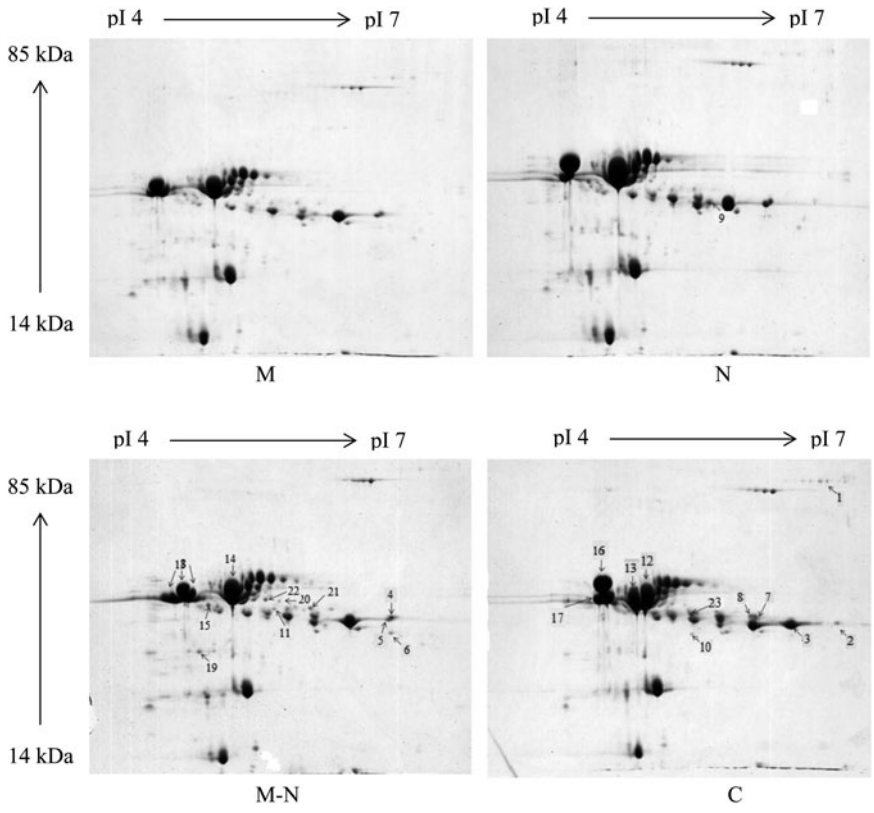

Fig. 2. Two-dimensional electrophoresis of the four types of buffalo milk. C, crossbreeds of river buffalo (Murrah and Nili-Ravi) with local swamp buffalo; M-N ,crossbreeds from Murrah and Nili-Ravi; M, Murrah; N, Nili-Ravi.

crossbred buffalo C and N buffalo which may be due to the more amount of buffalo milk used in this study. The content of κ-casein is an important factor in cheese making, as it can influence the milk coagulation ability, cheese yield and quality (Comin et al. 2008; Ren et al. 2013; Sanchez-Macias et al. 2013). In the future, it may be possible to produce high quality cheese by specifically crossbreeding to yield milk with high κ-casein content.

As shown in Fig. 1, κ-casein consisted of several partially co-eluting peaks at 10 and 16 min that can be ascribed to the presence of different glycosylated and phosphorylation forms of this protein (Bonfatti et al. 2013). The separation of buffalo protein has been achieved by several studies, but only a few of them detected the buffalo κ-casein polymorphism (Chianese et al. 2009; Feligini et al. 2009; Bonfatti et al. 2013). Similarly, in the case of α_{s1}-casein, two alternative forms were detected, and all of the samples gave rise to a double peak, which can be ascribed to the varying degrees of post-translational phosphorylation (Addeo et al. 1977).

The expression profile of buffalo milk protein

The protein expression profiles of the four types of milk are shown in Fig. 2, and the changes in the protein spot quantity values are summarised in Fig. 3. The identification of the proteins contained in the spots is listed in Table 3. As shown in Fig. 2, a total of 23 differentially expressed spots were performed for MS analysis.

Polymeric immunoglobulin receptor. Protein spot 1 is a polymeric immunoglobulin receptor (PIGR), with a molecular weight of 83·71 kDa that was found only in the C buffalo (Table 3; Fig. 3). PIGR is an important member of the major histocompatibility complex family and immunoglobulin superfamily (Kaetzel, 2005), which can be expressed in mammary gland cells (De Groot et al. 2000) and in human and macaque milk (Beck et al. 2015). PIGR plays an important role in mucosal immunity by translocating IgA and IgM into external body fluids (Kaetzel, 2005). Furthermore, the amount of PIGR produced is a limiting factor in the transport of IgA into the milk under normal non-inflammatory circumstances (De Groot et al. 2000). So PIGR could play a part in the humoral immunity through the transport of IgA and IgM then in turn preventing microorganisms and foreign proteins from penetrating the mucosal surfaces (Monteiro & Van De Winkel, 2003). Therefore, the relatively high abundance of PIGR in C buffalo milk may indicate that this could provide more immunological protection and even the development of immune function in humans.

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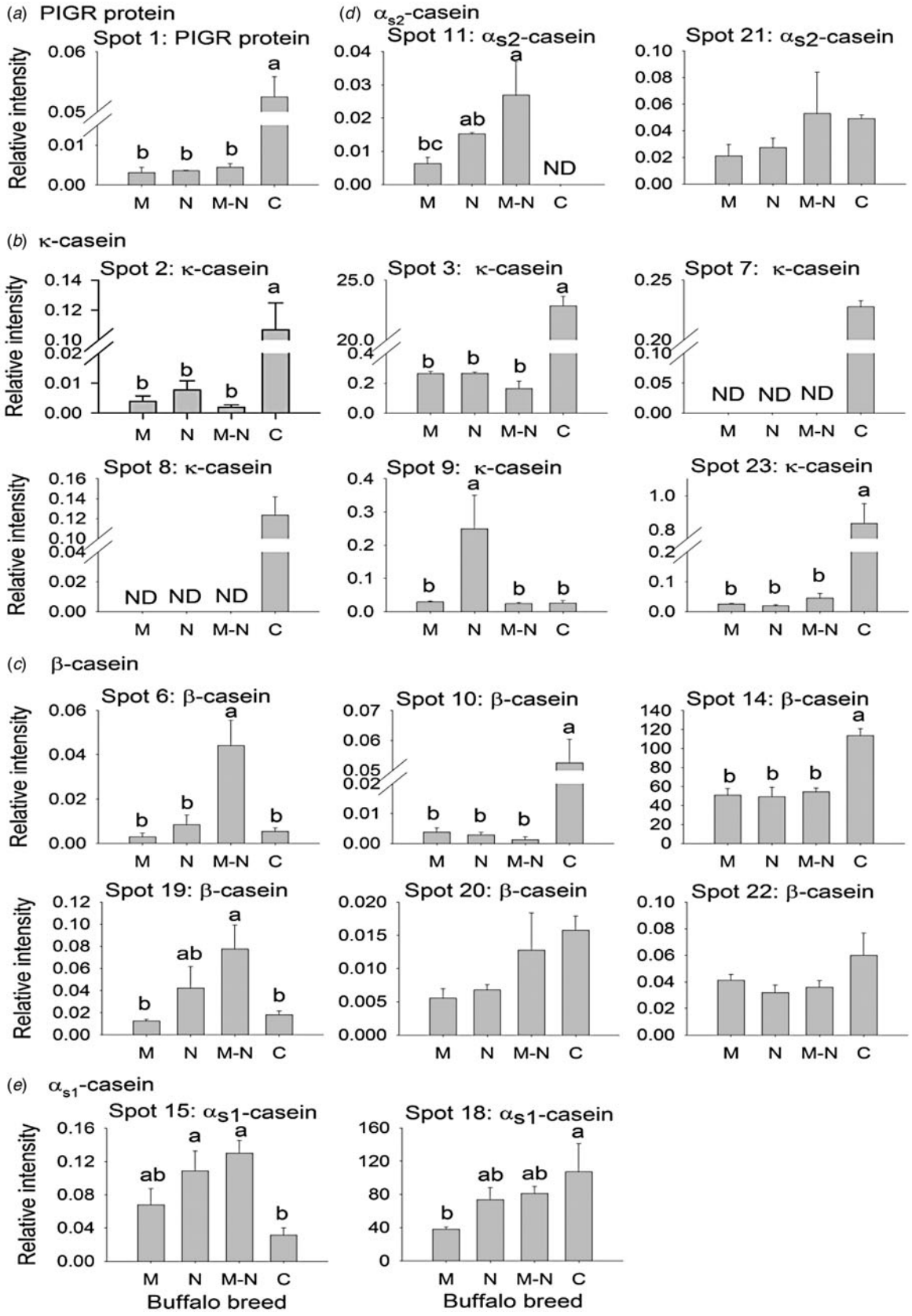

Fig. 3. Densitometric values of protein spots (numbered in Fig. 2) detected in the milk of the four studied breeds of buffalo. The bars indicate the standard error mean ($n = 3$). Means with different letters differ significantly ($P < 0{\cdot}05$). ND, Non-detectable; PIGR, polymeric immunoglobulin receptor; C, crossbreeds of river buffalo (Murrah and Nili-Ravi) with local swamp buffalo; M-N, crossbreeds from Murrah and Nili-Ravi; M, Murrah; N, Nili-Ravi.

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Table 3. List of selected milk protein spots identified in Fig. 2 from two-dimensional electrophoresis gels and identified using MALDI-TOF-MS/MS

Spot	Protein name	Accession no.	Molecular weight (kDa)	Isoelectric point	Sequence coverage (%)
1	PIGR protein	gi:3914346	83·71	7·08	17
2	κ-casein	gi:1168778	21·51	6·84	35
3	κ-casein	gi:1168778	21·51	6·84	25
4	κ-casein	gi:1168778	21·51	6·84	35
5	κ-casein	gi:1168778	21·51	6·84	35
7	κ-casein	gi:1168778	21·51	6·84	35
8	κ-casein	gi:315143016	15·92	6·32	24
9	κ-casein	gi:1168778	21·51	6·84	35
23	κ-casein	gi:1168778	21·51	6·84	31
6	β-casein	gi:76364007	17·29	8·20	53
10	β-casein	gi:3776019	25·12	5·26	24
12	β-casein	gi:76364007	17·29	8·20	48
13	β-casein	gi:76364007	17·28	8·20	16
19	β-casein	gi:76364007	17·29	8·20	48
14	β-casein	gi:76364007	17·28	8·20	16
20	β-casein	gi:3776019	25·12	5·26	44
22	β-casein	gi:3776019	25·12	5·26	44
11	α_{S2}-casein	gi:211919054	25·22	8·54	38
21	α_{S2}-casein	gi:211919054	25·22	8·54	53
15	α_{S1}-casein	gi:75038951	24·37	4·85	47
17	α_{S1}-casein	gi:75038951	24·39	4·85	46
18	α_{S1}-casein	gi:75038951	24·44	4·85	46
16	α_{S1}-casein	gi:211954417	23·49	4·89	21

PIGR, Polymeric immunoglobulin receptor;
gi, GenInfo Identifier

κ-casein. The eight protein spots shown in Table 3 correspond to κ-casein, and five of these spots—2, 3 (which is in a different configuration with 4 and 5), 7, 8 and 23—were observed in high abundance in buffalo breed C and there were no significant difference between other buffalos. Although the κ-casein in spot 9 was upregulated in breed N compared with other breeds, this upregulation amount was not enough to affect the total content of κ-casein in the milk of breed C. This may be partly consistent with the high content of κ-casein in breed C (Table 2), suggesting that the crossbreed may modify the pathway of milk protein synthesis in the buffalo mammary glands. Many spots corresponding to the phosphorylated, dephosphorylated, phosphorylated-glycosylated, phosphorylated-diglycosylated and dephosphorylated-diglycosylated forms of κ-casein were found in the buffalo milk (D'Ambrosio et al. 2008). Additionally, amino acid replacements may be another reason for the two variants of κ-casein that were found in the buffalo, as shown in Fig. 1. Similar results were observed in Mediterranean water buffalo, where κ-casein X1 and X2 were identified with the amino acid Ile^{135} changed to Thr^{135} in the mature polypeptide chain (Bonfatti et al. 2013). Casein composition and structure, especially κ-casein content and protein phosphorylation, are important to cheese making (Ageitos et al. 2006; Ren et al. 2013) and the antioxidant activity of yogurts (Perna et al. 2013), primarily because of the role of κ-casein in milk aggregation and the relationship of Ca^{2+}-sensitive proteins in the formation of casein micelles. These properties imply that modulating the casein content in varieties of milk will have an effect on any resulting dairy product, including cheese or yogurt.

β-casein. Additionally, eight spots related to β-casein were identified (spots 6, 10, 12, 13, 14, 19, 20 and 22) (Table 3; Fig. 3). The β-casein identified in spot 14 for buffalo breeds M-N, N and M, however, appeared in different configurations for breed C, corresponding to spots 12 and 13. This difference may be attributed to milk protein polymorphism. Bonfatti et al. (2013) showed that analytical methods employing RP-HPLC could not separate the β-casein polymorphisms. The reason for this inability to separate the proteins may be due to their similar molecular weights and isoelectric points, which we also observed in the β-casein variants contained within spots 12 and 13. The different breeds and geographic locations may influence this as well. The expression of β-casein in spots 6 and 19 was most abundant in breed M-N, whereas the levels of β-casein expression in spots 10 and 14 were the most abundant in breed C buffalo than other buffaloes. This finding may explain the no effect of the breed on total β-casein (Table 2). The expression of β-casein may have a compensation effect between the variants.

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Some researchers reported that more than one genetic variant of β-casein has been identified in Italian buffalo (Ferranti et al. 1998), but only one variant was found in Venezuelan and Mediterranean water buffalo (Ferranti et al. 1998; Bonfatti et al. 2013). Two-dimensional electrophoresis is a powerful tool that may aid the identification of β-casein polymorphisms, where four allele genes (A1, A2, B and I) and 10 variants of β-casein were found in Holstein cow milk by 2-DE (Jensen et al. 2012). Compared to κ-casein, few spots corresponding to β-casein were identified during the course of this study. This difference may be attributed to the lack of a putative phosphorylation site in the β-casein sequence, which leads to a reduced degree of phosphorylation in buffalo β-casein compared with other ruminants (Mamone et al. 2003; Farrell et al. 2004), thereby resulting in fewer variants of the protein to detect.

α_{s1}-casein. The four spots for α_{s1}-casein are shown in Fig. 2. The spot containing α_{s1}-casein in M-N (spot 18) corresponded to different spots (spot 16 and 17) in crossbreed C. The differences in the proteins were also evident from the RP-HPLC analysis, as shown in Fig. 1, and may also be due to protein polymorphism. These results are similar to those reported by Bonfatti et al. (2013), who discovered two variants of α_{s1}-casein. This polymorphism is the result of the substitution of amino acid Leu^{178} in variant A to Ser^{178} in variant B of the mature polypeptide chain. This has also been previously reported in Mediterranean water buffalo and river buffalo, which are similar to breeds M and N (Ferranti et al. 1998; Chianese et al. 2009). Compared to other ruminants, the α_{s1}-casein protein in buffalo exhibits reduced phosphorylation activity because it lacks a series of phosphorylation sites that are present in bovine and ovine proteins (Farrell et al. 2004). In this study, α_{s1}-casein of breed C in spot 15 has the lowest expression, while α_{s1}-casein in spot 18 has the highest expression. No significant difference was found between breeds M, N and M-N buffalo. Likewise, no significant difference was found in the expression of total α_{s1}-casein, which may be the result of a compensation effect between the variants (spot 15 and 18).

α_{s2}-casein. Only two spots corresponding to α_{s2}-casein (spot 11 and 21) were found, which are shown in Fig. 2, and both of them were identified as α_{s2}-casein. No polymorphism has previously been described for α_{s2}-casein in the Mediterranean water buffalo as analysed by RP-HPLC (Ferranti et al. 1998; Feligini et al. 2009). The expression of α_{s2}-casein in spot 11 was more abundant in breed M-N than M and C, which is consistent with the relatively high content of α_{s2}-casein in M-N (Table 2). Little is known about the relatively high expression of α_{s2}-casein in buffalo breed M-N, and further studies are needed to explore this.

β-lactoglobulin and α-lactalbumin. As for α_{s2}-casein, unique peaks for β-lactoglobulin were expected (Fig. 1). Similar results have been shown in the analysis of milk from Mediterranean water buffalo; however, two variants of β-lactoglobulin have been detected in Indian and Egyptian buffalo populations (Patel et al. 2007). Similar to previous research studies (Chianese et al. 2004), we also found two variants of α-lactalbumin (B: Asn^{45} changed to A: Asp^{45}).

In conclusion, this study found that the C buffalo has higher milk protein content, κ-casein content, and lower α-lactalbumin content than the other three breeds studied. And the distribution differences of milk casein are attributed to milk protein polymorphism in the various crossbreeds. Therefore, understanding how crossbreeding can influence the protein content and composition will provide insight into breeding buffalo with higher total protein and κ-casein contents in their milk and potentially provide a method for controlling quality in cheese production applications.

This work was supported by the National Science Foundation of China (Grant No. 31260384 and 31501504), the Opening Project of Guangxi Key Laboratory of Buffalo Genetics, the Reproduction and Breeding (SNKF-2014-02) and the Fundamental Research Funds for the Central Universities (2016FZA6016). The authors thank Dr Moushumi Paul, the Dairy and Functional Foods Research Unit, the Eastern Regional Research Center, the ARS and the USDA for their useful advice and linguistic help.

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