Buffalo milk protein polymorphism analysis and research of its effect on milk processing characteristics and mozzarella cheese quality

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Abstract: In this study, milk protein composition and phenotypic variation from 260 individuals of buffalo was analyzed by reverse phase high performance liquid chromatography (RP-HPLC). The study found that the polymorphisms were existed on k-casein, αs2-casein, αs1-casein and β-casein but β-lactoglobulin or α- lactalbumin of buffalo milk from china. The k-casein had three variants(BB, AB and AC), αs2-casein and αs1-casein had AA, AB type and AB, BB type respectively. For β-casein gene, mainly AA and AB type were found, β-lactoglobulin and α- lactalbumin just were found BB type. The milk samples were divided into three groups according to difference of κ-CN variants.The milk and milk protein composition, as well as processing properties, including coagulation properties, anti-oxidant, emulsibility and hydrophobicity were compared in each group, besides, Mozzarella cheese was manufactured from each group and been measured the parameters of cheese yield, fat, protein, moisture and texture. Milk samples with AB type κ-CN variant showed lower milk total solid ($P>0.05$), fat ($P<0.05$) and protein ($P>0.05$) than BB type, while milk with BC type of κ-CN has higher κ-CN and whey protein ($P<0.05$) and much lower β-CN content than other two kinds of milk. The milk with BB and BC type of κ-CN variants had shorter rennet coagulation time and higher curd firmness than AB type ($P<0.05$).The Mozzarella cheese production ($P>0.05$), hardness ($P<0.05$) and springiness ($P<0.05$) of milk with AB type of κ-CN were lower than BB and BC. Milk with AB type κ-CN variants had higher emulsifying stability, than the other two kinds of milk, while BB type of milk showed higher antioxidant ability than the other two types of milk. In conclusion, κ-CN variants determined by RH-HPLC influenced buffalo milk protein processing properties, the milk with A type κ-CN variants showed lower coagulation ability and mozzarella cheese production. Results showed milk samples with AA type αs1-CN variant showed higher milk freezing point, non-fat milk solids content (SNP) and lactose content ($P<0.05$) than BB type, while lower protein, fat and total milk solids ($P<0.05$) than BB type, milk with two types of αs1-CN had higher titratable acidity and SNP ($P<0.05$) and much lower fat and total milk solids than MMS. The milk with AA type of αs1-CN variant had shorter rennet coagulation time and pH than AB type. The Mozzarella cheese pH, melt ability ($P<0.05$) of milk with AA type of αs1-CN were higher and much lower cheese production and free-oil than AB. The Mozzarella cheese had lower hardness, springiness, adhesiveness, chewiness (P＞0.05) but higher cohesiveness ($P<0.05$) of milk with AA type of αs1-CN were lower than AB. The cheese made from type AA milk showed dense protein structure and many holes. In conclusion, the αs1-CN had a significant influence on milk traits and mozzarella cheese quality.
Keywords: Chinese buffalo, Polymorphism, Mozzarella cheese, Milk quality.

Introduction: The influence of the polymorphisms of caseins (aS1, aS2, β and k) and whey proteins (α-lactalbumin and β-lactoglobulin) on milk curd formation and cheese-making properties is well established. Buffalo milk is popular in many Asia and Europe countries due to it can be used for making different dairy products, such as butter, yogurt and cheeses Hussain et al. (2011). It is particularly suitable for Mozzarella manufacture due to its good milk coagulation properties (MCP), white color and high total solids Jana et al. (2011), Li et al. (2012). It has been reported that the milk coagulation ability was tightly related with buffalo κ-CN genotype, as κ-CN is a protein which played an essential role in milk coagulation process Ikonen et al. (2004).Most results showed BB genotype of κ-CN is good for milk rennet coagulation when producing cheese Ren et al. (2013). Analysis of buffalo milk protein by phase high performance liquid chromatography (RP-HPLC) indicated there were at least two κ-CN phenotypes existing in buffalo milk. Feligini et al. (2009). found three κ-CN variants in buffalo milk protein can be detected by RP-HPLC and mass spectrometry (MS). Therefore, research into the relationship between κ-CN phenotypic variants and milk processing properties or cheese production has attracted much attention in recent years Jensen et al. (2012). Up to now, many studies have researched effects of milk protein gene polymorphism on milk processing properties and cheese yield Ren et al. (2013), Bonfatti et al. (2011). while the effects of milk protein phenotypic variants on milk processing properties or cheese production still few reported, especially for buffalo milk and its main product Mozzarella cheese. Besides, due to variation of κ-CN not only changes structure of κ-CN itself, but also influences total milk protein composition or properties Bonfatti et al. (2011), it can be predicted that the milk protein processing properties, such as coagulation, antioxidant, emulsifying and surface hydrophobic, would also be influenced by κ-CN variation, these changes would lead to the change of yield or quality of resulting cheese. In this study, milk samples were separated into different groups according to difference of κ-CN variants identified by RP-HPLC, the milk processing properties and Mozzarella cheese quality and yield were compared in each group, aimed to research the effects of κ-CN variants on the buffalo milk processing characteristics and Mozzarella cheese production.

***Materials and Methods*：** Milk sampling and process: A total of 260 individual milk samples of water buffaloes was collected. Milk composition was measured by infrared analysis with a 4-channel spectrophotometer (MilkoScan, Foss Electric, Hillerd, Denmark). The reserved milk samples were aliquot, skimmed (centrifuged for 30 min at 2000g at 4°C) and preserved at -40°C until RP-HPLC analysis.After the milk samples were assessed by HPLC, herds were separated into different groups according to the κ-CN variants identified from HPLC. Six buffalos in each group were selected to supply milk for processing properties analysis and Mozzarella cheese production. After the milk were taken, somatic cell count (Fossomatic 5000, Foss Electric) and titratable acid were determined immediately. RP-HPLC assessment of milk protein: The method used for pretreatment of buffalo milk samples and separation of buffalo milk protein fractions by RP-HPLC was the same as method of Bonfatti et al. (2013). Determination of rennet coagulation properties: Rennet coagulation time (RCT) of individual milk samples were measured by rotational viscometer following the method of Jacob et al. Jacob et al. (2011). Determination of emulsifying activity: Emulsifying activity index (EAI) and emulsifying stability index (ESI) were measured following Pearce et al. (1978). Determination of surface hydrophobicity: Surface hydrophobicity (SH) of skimmed milk samples was determined by the SDS-binding method. Determination of antioxidant ability: An antioxidant property of skimmed milk was measured by ABTS method according to Re et al. Re et al. (1999). Manufacturing and measurement of Mozzarella cheese: Technological procedure of mozzarella cheese manufacture was reported by Jana et al. (2011). One-way ANOVA procedure of SAS software 18 was used to compare the data of milk composition, protein compositions, and milk processing properties, including RCT and A30. The means of parameters of cheese manufacturing experiment were obtained on three different (independent) determinations of every parameters, ANOVA was used to compare the means. Statistical signicance was considered to exist if $P < 0.05$.

***Results and discussion*：**

Milk protein and kappa-casein variants determination by RP-HPLC: In this study, three RP-HPLC chromatographic peaks were regarded to correspond with three variants of κ-CN, named as A,B and C (Fig. 1), although the three variants composited together formed can form 6 different types of κ-CN, while just three types, BB, BC and AB, were indentified in all the samples, the frequencies of each type was 76.5%, 6.1% and 17.3%, respectively. The B and A variant of κ-CN identified here corresponded well with X1 and X2 variants in results of Bonfatti et al. Bonfatti et al. (2013). The C variant of κ-CN was judged from result of Feligini et al. (2009). in their results, three variants of κ-CN also been separated from buffalo milk, and one variant corresponded chromatographic peak located before αs2-CN, the other two variant peaks located between αs2-CN and αs1-CN, which were well consisted with our results. Milk and milk protein composition in samples with different κ-casein variants: In this study, milk samples with AB type of κ-CN variant showed lower milk total solid ($P>0.05$), fat ($P<0.05$) and protein ($P>0.05$) than BB type, there were no differences in AB and BC type of milk samples (Table 1). The higher milk solid, fat and protein in BB type of milk indicated this type of buffalo milk would be fitted for cheese production better than AB. In the previous studies, relationship between κ-CN genotype and milk composition was widely researched, and most of studies showed cows with the κ-CN BB genotype can produce more fat and protein than cows with the κ-CN AA genotype Pearce et al. (1978), Bonfatti et al. (1978), Michalcová et al. (2007). Until now on, studies on relationship between κ-CN phenotype and milk composition were quite limited. Jensen et al. (2012) reported that B type κ-CN variant which indentified by isoelectric focusing electrophoresis was found to be favorable for both protein and casein percentages. Somatic cell count (SCC) and titratable acid are two important milk hygienic indexes which tightly related with MCP, lower SCC or higher titratable acid were good for increase of MCP Ikonen et al. (2004). In this study, no differences was observed in somatic cell count among 3 groups ($p<0.05$) indicated that the buffalos used to supply milk were maintained in a similar health condition, therefore, MCP was not influenced by somatic cell count. The titratable acid in AB type milk samples was lowest among the three types of milk ($p<0.05$) and indicated AB type of milk was harder to coagulate than other kinds of milk, while the BC type of milk with highest titrate acid ($p<0.05$) was more prong to coagulate, because titrate acid usually positively correlated with milk coagulation ability De et al. (2009). As for the milk protein composition, the BC types of milk has higher κ-CN, β-LG and much higher α-LA ($P<0.05$), while lower αS2-CN and much lower β-CN ($P<0.05$) content than the other two types of milk. These results indicated A and B type of κ-CN variants have no influence on milk protein composition, while C type of κ-CN variants tend to decrease κ-CN and increase whey protein content. Processing properties of buffalo milk protein: Milk coagulation property (MCP) is an important processing property of milk influencing cheese yield and cheese quality Johnson et al. (2007). Commonly, the MCP traits can be expressed by milk rennet coagulation time (RCT, min) and curd firmness, RCT is the time from the addition of coagulant to milk until the beginning of coagulation, and curd firmness is measured at 30 min after coagulant addition (A30) Pretto et al. (2011). In this study, results showed RCT of buffalo milk with BB and BC type of κ-CN variants were shorter than milk with AB type ($P<0.05$, Table 2), and curd firmness were higher than AB type milk ($P>0.05$), the BB type of milk has the shortest RCT compared with other types of milk ($P<0.05$), and curd firmness of BB type milk was higher than AB type of milk ($P<0.05$). These results indicated MCP of AB type milk was lowest among all the three types of milk, while BB type of milk has the best MCP among the three types of milk. Our results further confirmed the conclusion of other studies that the κ-CN protein variation also influenced milk coagulation ability Jensen et al. (2012). Except MCP which is a property tightly related with cheese producing, processing properties of milk protein also including emulsifying stability, surface hydrophobicity and anti-oxidant ability Augustin et al. (2007), Hiller et al. (2008) while influence of κ-CN genetic or phenotypic variation on these properties were not so extensively researched as MCP. In this study, no differences were observed in emulsifying activity index (EAI) among all the three kinds of milks ($P>0.05$, Table 2), while AB type κ-CN variants has the highest emulsifying stability index (ESI) in all the three kinds of milk, especially much higher than milk with BB type of variants (P<0.05), and milk with BB type of variants has the lowest ESI, suggested A type of κ-CN variants is good for increase of milk protein ESI. The reason for difference of ESI in milk with different κ-CN variants was unclear, probably due to change of κ-CN structure influenced the surface charge or the particle size of casein, which resulted in change of total milk protein stability Ye et al. (2011). The surface hydrophobicity doesn't have difference in all the three types of milk in this study (Table 2, P>0.05), suggested the variation of κ-CN was not due to difference in extent of glycosylation on κ-CN and stability of milk protein structure was not influenced by κ-CN variation, because glycosylation is a key factor determining protein hydrophilicity O'Connell et al. (2000), while hydrophobicity plays an important role in keeping the stability of milk protein Giovambattista et al. (2008). The BB type of milk showed higher antioxidant ability than the other two types of milk (P<0.05), it indicated B type of κ-CN variant is beneficial for Mozzarella cheese storage, because Mozzarella is a fresh cheese, the increased antioxidant ability of milk protein will prolong its shelf life. Our study would be the first time on effects of different κ-CN variants indentified by HPLC on other milk processing properties in buffalo, and indicated this is a potential area for further research.Mozzarella cheese yield, composition and texture: Result of our study showed cheese yield(CY) was higher in cheeses made from milk with BB and BC than AB type of κ-CN (P>0.05, Table 3), this result can be explained by the total solids and protein, especially fat of milk with AB type κ-CN were lower than milk with BB and BC. Another reason would be due to higher milk coagulation ability of these two types of milk than AB type of milk, because high milk coagulation ability will lead to higher cheese production De et al. (2008). Bonfatti et al. (2011) found that influence of genetic variants A and B of κ-CN on cheese yield is small when milks of similar protein composition and contents are processed. Our study also found CY of the three different types of milk doesn't have much difference, which indicated influence of κ-CN protein variants on milk yield was also small. There were no compositional differences observed among the three kinds of Mozzarella cheeses (Table 3, P>0.05) which indicated cheese composition was not influenced by κ-CN phenotypic variants. Compared with chemical composition, texture is more related with quality of cheese. In this study, cheese produced from AB type milk has lower hardness, gumminess and chewiness compared with BB and BC (table 3, P<0.05), and cheese produced from BB type milk has the highest springiness and gumminess in the three groups (P<0.05). No differences were observed in cohesiveness between every groups (P>0.05). These results were consistent with other reported cheese textures, hardness, springiness and chewiness were positively related with MCP Ren et al. (2013).

Tables and Figures:

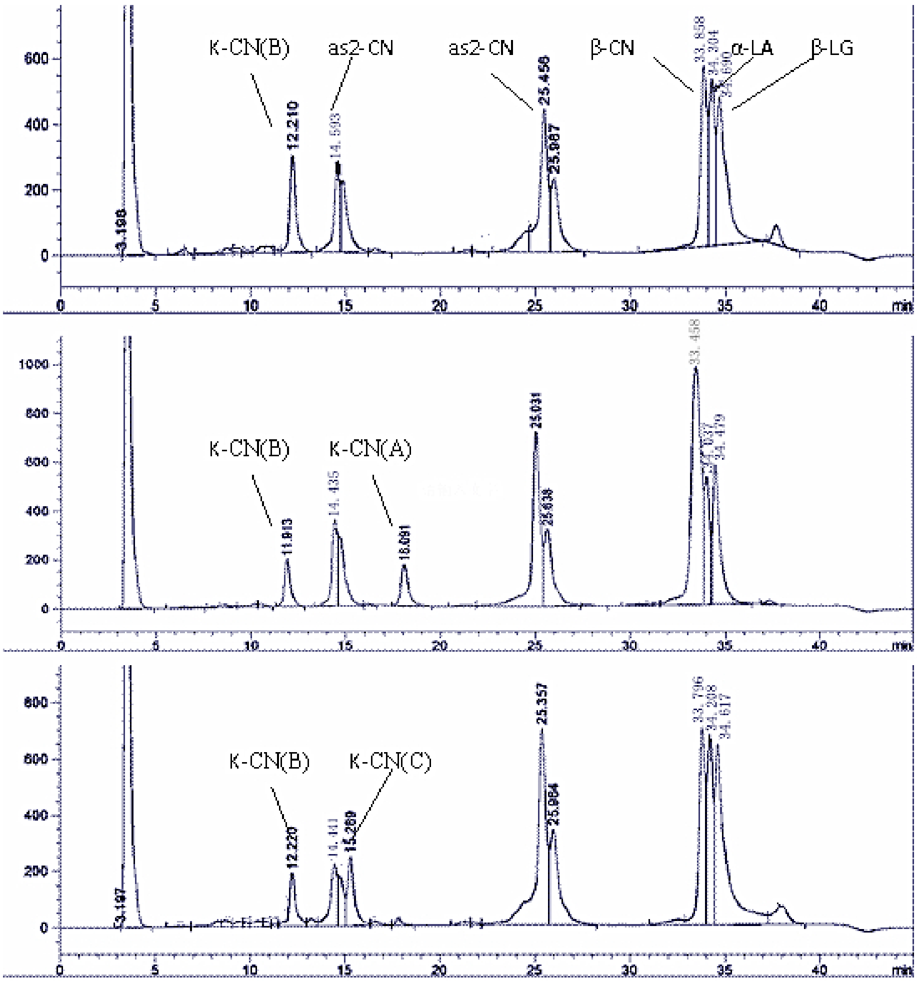

Figure 1. RP-HPLC chromatograms of buffalo milk with different κ-CN genetic variants (κ-CN A, B or C)

Table 1. Milk composition and milk protein composition of samples with different κ-casein variants.

Item	κ-CN variants [1]			S.E.M
	BB	AB	BC	
Milk composition				
Total solid, %	19.5	18.6	20.0	0.93
Fat, %	9.81[a]	7.92[b]	9.46[ab]	0.36
Protein, %	4.71	4.50	5.00	0.12
Milk quality				
Somatic cell (10 thousand/ml)	24.5	23.8	24.2	1.80
Titrate acid (°T)	16.3[b]	15.1[c]	17.8[a]	0.19
Protein composition, % [2]				0.00
κ-CN	9.3[b]	9.0[b]	10.7[a]	0.18
αS2-CN	7.8[a]	7.7[a]	6.5[b]	0.23
αS1-CN	21.3	21.3	21.6	0.47
β-CN	28.7[a]	29.5[a]	19.7[b]	1.93
α-LA	14.1[b]	13.3[b]	19.3[a]	0.87
β-LG	19.0[b]	18.8[b]	21.8[a]	0.63

1 Experimental milks with different type of κ−CN variants; 2 protein composition was calculated by peak area normalization method (without the use of correction factors).

Table 2. Milk protein processing properties of milk with different κ-casein variants.

Item	κ-CN variants [1]			S.E.M
	BB	AB	BC	
Milk protein coagulation properties [2]				
RCT, min	25.3[b]	30.7[a]	28.0[c]	0.29
Curd firmness	0.11[a]	0.09[b]	0.10[ab]	0.002
Emulsifying activity				
Emulsifying activity index (m^2/g)	115.7	120.1	118.8	3.01
Emulsifying stability index (min)	23.4[b]	37.7[a]	28.8[b]	2.21
Surface hydrophobicity	9.9	11.0	9.6	0.95
Anti-oxidant ability	80.8[a]	75.0[b]	74.8[b]	1.63

1 Experimental milks with different type of κ -CN variants; 2 Milk protein coagulation properties.

Table 3. Yield, composition and texture of Mozzarella cheese manufactured from milk different κ-casein variants.

Item	κ-CN variants [1]			S.E.M
	BB	AB	BC	
Cheese yield (DM/kg milk)	6.55	6.40	6.55	
Cheese composition				
DM %	46.8	46.8	46.9	2.82
Protein, % DM	40.3	38.4	39.4	2.40
Fat, % DM	45.5	45.8	44.9	3.15
Cheese texture				
Hardness, N	9.2a	7.2b	9.8a	0.00
Cohesiveness, Ratio	0.71	0.72	0.69	0.004
Springiness, mm	8.3[a]	6.7[b]	7.0[b]	0.23
Gumminess, N	7.3[a]	5.1[b]	7.7[a]	0.15
Chewiness, mJ	60.9[a]	39.9[b]	55.2[a]	1.73

1 Experimental milks with different type of κ -CN variants.

Conclusions**:** Our results showed three kinds of κ-CN, named as AB, BB and BC, were identified by RH-HPLC from buffalo milk. They are comprised by three types of variants, A, B and C, while B type of variant was the most prevent variant and beneficial for milk coagulation compared with A type. The C type of variants, which was least prevented variants, changed milk protein composition by greatly decreased β-CN and increased α-LA, but didn't influence milk coagulation and Mozzarella cheese yield due to κ-CN was increased. The A type of κ-CN variants showed a negative effect on milk fat content, coagulation and cheese textures, but have no influence on cheese yield. Except coagulation ability, other process properties such as emulsifying stability, antioxidant and coagulating ability were influenced by κ-CN variation. Therefore, it can be concluded that buffalo κ-CN protein can be separated into different variants despite most studies reported κ-CN gene was monomorphic, and these κ-CN variants cased influences on buffalo milk processing properties and Mozzarella cheese manufacturing, which endowed this topic with great value to further research and clarify the reason behind the variation of κ-CN.

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