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Comparative Analysis of Yak Milk and Holstein Cow Milk in Composition of Protein, Amino Acid and Fatty Acid

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Abstract A total of 124 individual yak milk samples and 400 individual Holstein cow milk samples were collected from Tibet Autonomous Region and Inner Mongolia Autonomous Region, China. In order to better understand the difference between yak and Holstein cow milk. The composition of two milks, protein composition, β-casein gene sequence, amino acid composition, fatty acid composition and other parameters were analyzed and compared by using techniques such as capillary electrophoresis, gene sequence sequencing, amino acid analysis and gas chromatography. Yak milk has a higher total solids content than Holstein cow milk (18.99 g/100g and 12.44 g/100g). The casein composition of yak milk is suitable for the nutritional needs of infants. The proportion of β-Lactoglobulin content in yak milk whey protein is significantly higher than that in Holstein cow milk and all β-casein in yak milk is A2β-casein. Yak milk contains more amino acids and essential amino acids than Holstein cow milk, especially for phenylalanine (0.267 g/100g) and leucine (0.747 g/100g). Yak milk has higher n-3 and n-6 unsaturated fatty acids, especially for DHA (1.848 mg/100g) and EPA (3.542 mg/100g), which are not found in Holstein cow milk. So far, little attention has been paid to the role of yak milk. This paper will provide data to support subsequent studies.

Keywords: *yak milk, A2β-casein, DNA sequencing, yak milk amino acid, yak milk fatty acid*

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1. Introduction

Yaks are widely distributed in the Qinghai-Tibetan plateau at altitudes above 3000 m where the natural environment is harsh, with thin and dry air, low oxygen content, low are pressure, low temperature and strong ultraviolet radiation. The unique living environment and species isolation give yak milk its unique nutritional value. At present, the total number of yaks in the world is about 16 million, of which about 15 million are in the Qinghai-Tibetan plateau region of China, and yaks are an important production tool for Tibetans. Tibetan nomads use yaks for various purposes as they provide milk, meat, transportation, and fur and hide for clothing and shelter, as well as dried dung for fuel [1]. Generally, yak milk has higher total solids, fat 5.5-7.5%, protein 4.0-5.9%, lactose 4.0-5.9% [2,3]. For protein composition, like the other mammalian milk, yak milk mainly consists of 4 individual caseins (αs1-casein,αs2-casein, β-casein, κ-casein). But two milks have difference in casein proportion, the proportion of β-casein (β-CN) in total casein is higher than Holstein cow milk over 45% [2]. Although the composition and nutrients of yak milk have been studied in the past and more recently, very little literature is available, and results are often contradictory due to limitations in sample size and sampling methods. In this study, 128 samples of yak milk and 400 samples of Holstein cow milk were obtained using standard sampling methods. This result should be an important contribution for future research.

This paper compares in detail the differences in milk composition, β-CN, amino acids, fatty acids and other parameters between yak milk and Holstein milk (the most populated cow in the world). Significant differences were found between the two types of milk in terms of composition and nutrient content. β-casein constitutes up to 45% of the casein, the most abundant protein in cow's milk, and it is reported to be the most polymorphic milk protein gene. A2 milk is an emerging area of research in the dairy industry due to its ability to reduce the discomfort caused by casein during digestion and reduce the risk of some diseases [4]. After the mutation of A2 gene, the crossbreeding development through many generations makes the current Holstein cows exist many different kinds of β-casein. And because yaks live on the Qinghai-Tibetan plateau and there is natural reproductive isolation, the β-CN in yak milk theoretically should be pure A2β-CN. In this study, a method was proposed for the rapid qualitative and quantitative detection of A2β casein in milk using CE technology. The genetic sequencing analysis technique was also used to confirm that, unlike Holstein cow, which have multiple β-CN genotypes, only the A2 genotype exists in yaks. Amino acids and fatty acids are both important nutrients in milk. Amino acids are the building blocks of protein synthesis in the body, and can also be converted into acids, hormones, antibodies and other substances. They can also provide structural components needed for a number of critical body functions, such as strong musculoskeletal systems, oxygen delivery to muscles, metabolism of other nutrients, and healthy immune function. The body can only synthesize a limited variety of fatty acids on its own, so the intake of fatty acids from food is crucial for the body [5]. Especially for infants, such as DHA and EPA have been proved to promote neurological development and improve memory and learning ability [6,7]. Amino acid analysis techniques and GC techniques were used to analyze and compare amino acids and fatty acids in the two milk types. The two nutrients vary in content and ratio between counties.

2. Materials and Methods

2.1. Milk Samples

400 milk samples from Holstein-Friesian cows in their first lactation, located in 2000 herds, were collected as described in Schennink [8]. The samples were preserved with 0.03% (w/w) sodium azide, transported refrigerated and stored frozen at -40°C within 1 day. Holstein cow samples were collected in Modern Animal Husbandry pasture, Hohhot, China. 128 Yak samples were collected with same method in Yak breeding field of Linzhou county, Lhasa, China.

2.2. Blood Samples

Same 400 Holstein cow samples and 128 yak samples were collected. All samples were collected from tail hair follicles into dry, sterile tubes, stored in -40°C and prepared for experiment.

2.3. Content of Protein, Fat, Lactose

Protein, fat, lactose were determined according the method of AOAC (920.105, 989.04, 984.15)

2.3.1. CGE Sample Preparation

The method of sample preparation is according to Recio and Olieman reported [9]. 335 mL homogenous milk sample was dissolved by 500 mL buffer solution. Then the sample was left at room temperature for 1 h and centrifuged to remove remaining fat (5000 g 5 min). The sample buffer (pH 8.0±0.1) consisted of 167 mM hydroxymethyl-aminomethane (TRIS; Sigma, St. Louis, MO, USA), 42 mM 3-morpholinopropanesulphonic acid (MOPS; Sigma), 67 mM ethylene-diamine-tetraacetic acid disodium salt dihydrate (EDTA; Merck, Darmstadt, Germany), 17 mM D,L-dithiothreitol (DTT; Sigma), 6 M urea (Merck) and 0.05% (w/w) methylhydroxyethylcellulose 30000 (MHEC; Serva, Heidelberg, Germany). The run buffer (pH 3.0) consisted of 0.19 M citric acid (Merck), 20 mM sodium citrate (Merck), 6 M urea and 0.05% (w/w) MHEC.

2.3.2. CGE Analyses

Samples were carried out with a Beckman P/ACE MDQ Plus Capillary Electrophoresis system controlled by 32 KaratTM Software, version 7.0 (Beckman Instruments, Fullerton, CA, USA). Separations were performed using a fused-silica OV-1701-OH deactivated capillary (TSP-050375-P-10; BGB Analytik, Boeckten, Switzerland) with dimensions 57 cm * 50 mm I.D and a slit opening of 100 * 800 mm. Separations were carried out at 45 1C and a linear voltage gradient from 0 to 25 kV in 3 min was used, followed by constant voltage at 25 kV. Sample solutions were injected at the anode by pressure injection at 0.5 psi for 20 s. One series consisted of approximately 20 milk samples, six standard samples prepared with known amounts of whey or casein, and two reference milk samples. Identification was done by spiking with milk protein standards: α-La, β-LG (Sigma). Genetic variants of the main milk proteins were determined by comparison of the electropherograms with literature electropherograms and analyses with milk samples containing known genetic variants.

2.4. DNA Sequence Analysis

The DNA extraction and PCR amplification method was according to Dai [10]. DNA was extracted from somatic cells in hair follicles using the direct lysis method (TransDirect Animal Tissue PCR Kit, Trangen Biotech, BeiJing).

The PCR amplifications were using 4 μl tissue extract in a reaction volume of 20 μl containing 10 μl 2 × TransDirectTM PCR SuperMix(+dye), 10 μM forward primer and reverse primer. PCR was carried out in a thermal cycler by following cycling programme: 95 °C for 5 min, then followed by 35 cycles at 95 °C for 30 s, 60–66 °C for 30 s and 72 °C for 1 min with a final extension at 72 °C for 7 min. The amplified products were loaded on 1.5% agarose gel at 120 V for 20 min using ethidium bromide staining. The purification was carried out using AxyPrepTM DNA Gel Extraction kit (Corning, NY, USA).

The purified PCR products sequences were carried out using a BigDye Terminator cycle sequencing kit (Applied Biosystems, CA, USA) on an automated Genetic Analyzer ABI 3730 (Applied Biosystems, CA, USA). The results were analysed and compared with the genomic GenBank sequence NM_181008.

2.5. Amino Acid Analysis

10 mg milk sample was mixed with 10 mL of 6 M HCl then hydrolyzed under nitrogen protected at 110°C for 22h. The volume of mixture adjusted to 50 mL with distilled water. 1 mL mixture was dried in a rotary evaporator at 40 C under vacuum to remove excess acid. The dry residues were dissolved by citrate buffer (pH 2.2) and filtered 0.2 μm membrane. Then amino acid composition was measured using amino acid analyzer. (L-8800, Hitachi, Japan)

2.6. Fatty Acid Analysis

About 50 mL milk sample was centrifuged at 15000 g for 15 min at 4 C. 100 mg cream cake, 10 mL distilled water and 5 mL ammonium hydroxide were mixed in a flask, then hydrolyzed for 20 min at 70 C using water-bath heating.

10 mL 95% ethanol and 50 mL petroleum ether were added to hydrolyzed solution then shaking 5 min. Mixture solution was concentrated using a rotary evaporator. Fat extract and 8 mL 2% sodium hydroxide-methanol solution were adding to a reflux condenser then refluxing at 80 C until oil droplet disappeared, then adding 7 mL 15% boron trifluoride-methanol solution refluxing for 2 min.

Separation and identification of fatty acid were performed using GC with a flame ionization detector (Agilent 7890 , Agilent Tech.). SP-2560 capillary GC column (100 m ×0.25 mm × 0.2 µm, Sigma-Aldrich Co.) were used for detection. GC-FID analysis was performed under the following instrumental conditions: injection volume of 1 µl and N_2 carrier gas flow with a split ratio 100:1 and constant flow control. Injector and detector temperatures were set at 270 and 280°C, respectively. The oven programme was as follows: 100°C for the first 13 min, increased by 10 °C/min until 180°C, maintained for 6 min, increased by 1°C/min until 200 °C and maintained for 20 min, increased by 4°C/min until 230 °C and maintained for 10.5 min. The FA methyl esters were identified by comparison of their relative retention times with authentic standards, and the mass distribution was calculated electronically by quantification of the peak areas.

3. Result

3.1. Basic Composition of Yak Milk and Holstein Cow Milk

Compositions of yak milk and Holstein cow milk are different (Table 1). In general, yaks live in highland grassland at altitudes of over 3000 meters and the average temperature of Tibetan highland is far below than plain area. Low-altitude cattle and cross-fertile cattle like Holstein cow suffer from severe pulmonary hypertension when reared in the Qinghai-Tibetan plateau [11]. Because of the harsh living conditions, yak needs strong foraging ability and high energy metabolism [12,13]. Fat can provide more calories than other nutrient contents, so the fat in yak milk is 126.5% higher than Holstein cow milk. Holstein cows have longer lactation usually over 10 months, but the lactation of yaks is only 5 months, yak milk needs higher energy density. The major nutrients and total solids in yak milk are higher. The protein in yak milk is 71.4% higher than Holstein cow milk and total solid is 52.7%.

3.2. Composition of Proteins

The protein content and distribution in the two milks is also different. Especially for casein proteins, for yak milk, the proportion of β-casein in total casein is over 45% while the proportion of Holstein cow milk is about 35%, and the proportion of αs-casein is lower than 40%, the value of Holstein cow milk higher than 48%. For κ-casein in two milks are similar (yak milk is about 15% and Holstein cow milk is about 12%). The β-casein proportion of yak milk is closer to that of human milk [14], the high level of β-casein can be easily digested by the enzymes of the intestinal for infant. Yak milk was always used to feed babies by Tibetans as the complement of breast milk [15]. Compared with the other milk proteins, β-casein is reported to be the most polymorphic milk protein gene. For Holstein cow, over 12 different variants of β-caseins have been reported including A1, A2, A3, B, C, D, E, F, H1, H2, I, G are the common variants [16,17,18]. A1β-casein can produce β-casomorphin-7 (BCM-7) during digestion, and BCM-7 is an exorphin that interacts with a variety of systems in the body [19]. Studies have shown an association between A1β-casein or BCM-7 and increased risk of type 1 diabetes, immune response, digestive dysfunction, autism and respiratory dysfunction in some infants [20,21,22].

The capillary electrophoresis (CE) technique has many advantages (detection speed, excellent resolution, simplicity) in milk protein detection, separation, purification and it can be used in many different kinds of milk detection [23,24,25]. A characteristic CE profile of Holstein cow milk proteins is presented in Figure 1. All proteins were separated and their peaks were sharp. Four major including α- Lactalbumin, β- Lactoglobulin, A1β-casein and A2β-casein characteristic absorption peaks were identified by adding standard protein samples under the same experimental conditions. Yak and Holstein cow from the same genus (Bos), the peaks identified from Holstein cow milk can also be used in yak milk.

Table 1. Comparison of basic nutrients in yak and Holstein cow milks

	Total solid(g/100g)	Protein(g/100g)	Fat(g/100g)	Lactose(g/100g)
	Individual yak milk simples, n=128，*P<0.05			
Mean	18.99±1.52*	5.45±0.48*	9.06±1.14*	4.66±0.26*
	Individual Holstein cow milk simples, n=400，*P<0.05			
	Holstein cow milk			
Mean	12.44±0.34*	3.18±0.09*	4.00±0.18*	4.86±0.10*

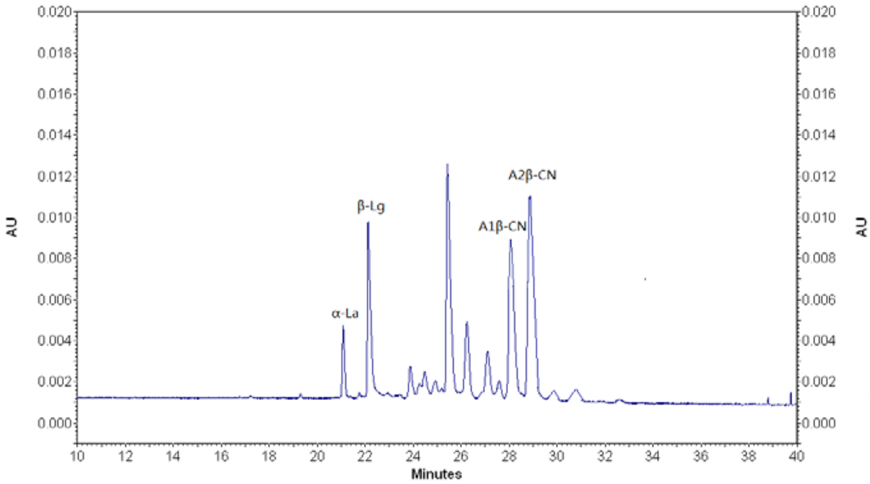

Figure 1. Electropherograms of Holstein cow milk

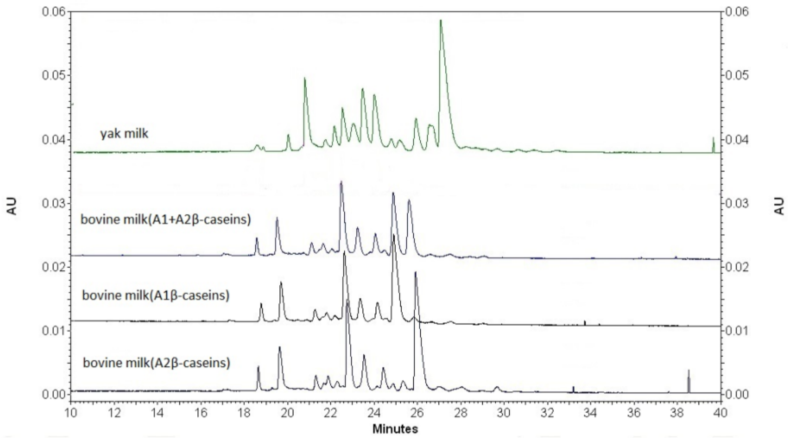

Figure 2. Electropherograms of different β-caseins of yak milk and Holstein cow milk

In this study, Holstein cow milk samples and yak milk samples were detected by the CE method using the same experimental conditions. The result shows in Figure 2. After data analysis, there are 3 combination types of β-casein that were found in Holstein cow milk including A1β-casein type, A2β-casein type, A1+A2β-casein type. For yak milk only A2β-casein type of β-casein was detected. The distribution of whey in two milk is different. The ratio of β-Lg and α-La in Holstein cow milk is approximately 2.5-3 but for yak milk the ratio is about 8.7, which means the major component of yak whey protein is β-Lg. Only few literature reported the data of yak whey protein, result in this study is consistent with the conclusion in Li's [2] literature.

The CE technique has many advantages, but it cannot identify all types of β-caseins. The technique of sequence analysis was successfully used for the detection of different point mutations of milk β-caseins gene in genomic DNA from samples. Table S1 shows the 6 bovine β-caseins and 1 yak β-casein variants were found in our research, and corresponding amino acid differences deduced from the sequence analysis. In cow's milk, the original β-casein is A2 type, the gene and amino acid sequence results validate the fact that after the initial genetic mutations, the β-casein of bovine gradually developed multiple variants from pure β-caseins with cross breeding and gene exchange. On the Tibetan Plateau, natural geological conditions produce biotic isolation such that there is little genetic exchange between the yak and other species of cow. Only A2β-casein was found in yak milk. One or two amino acids change produced different alleles, for instance the A2 allele differ from A1 by the amino acid change in position 67 (Pro/His). The differences between A2, B, and F involve two amino acid changes at positions 67, 122, and 152.

Frequencies for all detected variants and genotypes of the β-casein in two milks shows in table S2, for yak milk only 1 homozygote genotype A2A2 was found. For bovine, 2 homozygote A1A1 and A2A2 were found with percentage 0.117 and 0.28. The remaining 9 genotypes are all heterozygote genotype including A1A2, A1A3, A1B, A1F, A1I, A2A3, A2B, A2F, A2I, A1A2 is the most common genotype over 39%. For Chinese Holstein cow only 28% of cows can lactate pure A2β-casein. The most common forms of β-casein in this population are A1 and A2 with relative frequencies of 0.353 and 0.543, the sum of these two types is 89.6%, F is rare only observed 0.5%.

3.3. Composition of Amino Acid

The amino acid composition of yak milk and Holstein cow milk are shown in table S3. Both kinds of milk have similar amino acid profiles. They were all rich in Glutamic acid (0.893 g/100g and 0.515 g/100g), leucine (0.747

g/100g and 0.31 g/100g), valine (0.453 g/100g and 0.217 g/100g), aspartic acid (0.4 g/100g and 0.197 g/100g), but low in glycine (0.094 g/100g and 0.048 g/100g) and methionine (0.116 g/100g and 0.055 g/100g), and rare in cysteine (0.016 g/100g and 0.01 g/100g).

Amino acid is an important nutrient in milk, according the detection results, all kinds of amino acids in yak milk are higher, but the average protein content is also higher in yak milk. The ratio of protein content in two milks is 1.71:1. Essential amino acids cannot be made by the body and they must come from diet. The Table 2 shows the ratio of the 7 essential amino acids of the two milks. All 7 kinds of essential amino acids ratio are higher than 1.71 means the proportion of essential amino acids in yak milk is higher than that in bovine milk. Especially for phenylalanine and leucine. In the body, most phenylalanine is oxidized to tyrosine by the enzyme phenylalanine hydroxylase, and together with tyrosine, it synthesizes important neurotransmitters and hormones, and participates in the body's glucose and fat metabolism [26,27]. Leucine's functions include promoting skin, wound and bone healing, controlling blood sugar, regulating protein metabolism, and repairing muscle [28].

Table 2. Essential amino acids ratio of yak and Holstein cow milk

Amino acid	Yak milk(g/100g)	Holstein cow milk(g/100g)	yak amino acid/Holstein cow amino acid
Phenylalanine	0.27	0.11	2.35
Methionine	0.12	0.06	2.13
Valine	0.45	0.22	2.09
Leucine	0.75	0.31	2.41
Threonine	0.24	0.11	2.09
Isoleucine	0.29	0.14	2.19
Valine	0.23	0.13	1.79

3.4. Composition of Fatty Acid

Fat is an essential nutrient in milk, but there are still many diseases associated with it, such as cardiovascular disease, cancer, obesity and diabetes. The amounts and types of dietary lipids consumed are believed to be of critical importance [29,30]. In general, milk fatty is composed of saturated fatty acid, monounsaturated fatty acid, and polyunsaturated fatty acid, and each fatty acid has unique nutritional properties. In this study, 33 fatty acids were detected for all yak milk and bovine milk, and the result shows in Table S4 and Table 3.

Table 3. Composition of saturated FA, MUFA, PUFA in yak milk and Holstein cow milk

Fatty acid	Yak milk(mg/100g)	Holstein milk(mg/100g)
Saturated fatty acids	4525.69	2030.22
Unsaturated fatty acids	2525.67	878.41
Monounsaturated fatty acids	2344.15	775.38
Polyunsaturated fatty acids	181.52	103.03

The content of fatty acid in yak milk is higher than that in bovine milk (yak milk is 7051.36 mg/100g and bovine milk is 2908.63 mg/100g), because yak milk has higher fat (yak milk is 9.06% and bovine is 4.0%). FA in milk are mainly composed of FA between 14-18 carbon atoms, accounting for more than 85% of total FA content. Palmitic acid (C16:0) and oleic acid (C18:1) are the most abundant FA. The proportion of unsaturated fatty acids in yak milk is as high as 35.8% of the total fatty acids (bovine milk is 30.2%), the majority of which are monounsaturated.

n-3 fatty acids are essential fatty acids that are needed throughout life from pregnancy, and they cannot be synthesized by the body itself [31]. n-3 FAs are not only an important nutrient but also been shown to modulate many diseases, for instance atherosclerosis, coronary artery disease, inflammatory diseases, and possibly even behavioral disorders [6,7,31]. n-6 FAs is another essential FA family for humans, intake more n-6 fatty acids can lower the death of coronary heart disease and myocardial infarction [32,33,34]. The data of n-3 and n-6 FAs in two milks show in Table 4.

Yak milk contains approximately three times as much n-3 PUFA as milk from Holstein cows. It is important to note that no DHA was detected in the milk of Holstein cows, while the amount of DHA in yak milk was 1.848 mg / 100g. DHA has an extremely important role for humans, especially for infants in brain and nervous system development. Some literature reports that the ratio of n-6 PUFAs to n-3 PUFAs can modulate inflammation, n-3 PUFAs can reduce inflammation while n-6 PUFAs enhance inflammatory manifestations. The ratio for these two milks is different, the ratio in yak milk is 3.71 and Holstein cow milk is 7.21. Erucic acid (C22:1) and nervonic acid (C24:1) were only detected in yak milk, and they all from n-9 PUFA family.

Table 4. Composition of n-3 FAs and n-6 FAs in yak milk and Holstein cow milk

Fatty acid		Yak milk(mg/100g)	Holstein milk(mg/100g)
n-3	C18:3(n-3)	32.54	10.79
	C20:5(EPA)	3.54	1.59
	C22:6(DHA)	1.85	/
	sum	37.93	12.39
n-6	C18:2	90.81	67.79
	trans-C18:2	37.24	15.63
	C20:3	1.43	3.77
	C20:4	11.25	2.20
	sum	140.74	89.38

4. Conclusion

Yaks have been domesticated for more than 4500 years and more than 16 million yaks remain an important productive resource for Tibetans, but there is a huge lack of knowledge of composition and nutritional function of yak milk. In this study, some biochemical parameters for two milks (yak milk and Holstein cow milk) i.e. fat, protein, amino acid, genotype, allel, fatty acid were compared. For the same volume, yak milk has higher total solid that it can provide more energy and nutrients. Two techniques (CE and DNA sequence analysis) were used to analyze the β-Casein of two milks, only A2β-Casein (A2A2 genotype) was found in yak milk and 11 different genotypes of β-Casein were found in Holstein cow milk. Higher protein content means yak milk has higher amino acids content, and yak milks has a higher ratio of essential amino acids, especially for phenylalanine and leucine. Compared with Holstein cow milk, yak milk has more n-3 FAs and n-6 FAs, including DHA and EPA, which were important fatty acids for infant growth and development. Yak milk has the advantages of high energy density, rich nutritional value, reduced risk of some diseases, making it worthy of future research and utilization. These findings contribute in several ways to our understanding of yak milk value and provide some basic data for future research.

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