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Effect of beta-lactoglobulin polymorphism and seasonality on bovine milk composition

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The objective was to evaluate the effect of β-lactoglobulin (β-lg) polymorphism and seasonality on milk composition (fat, lactose, total solids, milk urea nitrogen, total protein, true protein, casein and somatic cell counts) of Holstein and Girolando cows. Milk and blood samples from 278 Holsteins cows and 156 Girolando cows were taken during two dry seasons and two rainy seasons, for milk composition analysis and to determine β-lg genotypes, respectively. BB genotype was the most frequent for both breeds, followed by AA genotype for Holstein (BB>AA>AB) and by AB for Girolando cows (BB>AB>AA). No differences were found in milk compositional characteristics among genetic variants of β-lg (AA, AB and BB) either between Holstein or Girolando cows. No association between milk composition and β-lg genetic polymorphism was observed. During the dry season, independently of the breed considered, higher contents of lactose, true protein, casein and casein : true protein ratio were found.

Keywords: Milk protein, β-lactoglobulin, genetic variants, seasonality.

The discovery of electrophoretically distinct types of β-lactoglobulin (β-lg) by Aschaffenburg & Drewry (1955) initiated the beginning of extensive investigations of the different genotypes of this whey protein (Hambling et al. 1992). The two main genetic forms of β-lg differ in the substitution of a Gly and an Ala in the B variant, for an Asp and a Val in the A variant (Ghashghaei, 2003) and this slight molecular difference has been widely associated with significant differences in milk composition in dairy cows (Dove, 2000).

Several studies have reported the association of significantly higher fat content (Ng Kwai-Hang et al. 1986; Aleandri et al. 1990; Tsiaras et al. 2005), protein (Litwinczuk et al. 2006), casein (Robitaille et al. 2002), true protein (Bobe et al. 1999) and total solids (Celik, 2003) with BB variant, while others have found higher milk component concentrations due to the AA variant (Ng Kwai-Hang et al. 2002b; Robitaille et al. 2002; Molina et al. 2006). Nonetheless, some authors have found no relationship between β-lg polymorphism and milk composition, and attributed the differences observed in fat and protein contents to other factors (Ng Kwai-Hang et al. 2002a).

Factors other than genetic polymorphism of milk proteins, e.g., seasonal variations (Paquin & Lacroix, 1994; Carlsson et al. 1995; Lacroix et al. 1996; Allore et al. 1997; Auldist et al. 1998; Sharma et al. 2002; Lindmark-Mänsson et al. 2003; Teixeira et al. 2003; Amenu et al. 2006) and breed (Auldist et al. 1998; Arunvipas et al. 2003) all affect milk composition but their interaction with β-lg genetic variants has not been studied. Moreover, data on the association between β-lg genetic variants of Girolando cows and milk composition of this breed are still rare. The Girolando breed is basically the result of crossing between the Holstein and Gir (indigenous Zebu breed) in standard proportions of 3/8 Zebu and 5/8 Taurine. This crossbred breed is very common in Brazil because it is claimed to have both the high resistance to heat and humidity characteristic of Indian cattle and the high productivity characteristic of European cattle (Bicalho et al. 2006). The objective of this study was to evaluate the

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effect of genetic polymorphism of β-lg on milk composition (fat, lactose, total solids, milk urea nitrogen, total protein, true protein, casein and somatic cell counts) in Holstein and Girolando cows, during dry and rainy seasons.

Material and Methods

Selection of herds and cows for sampling

The study was carried out in eleven commercial dairy herds in the state of São Paulo, Brazil; among them six were composed of Girolando (crossbred Holstein-Zebu) and five of Holstein cows. Milk and blood samples were taken from 278 lactating Holstein cows and 156 lactating Girolando cows during two dry seasons and two rainy seasons (Sampling period 1, September and October, 2003; Sampling period 2, June and July, 2004; Sampling period 3, November and December, 2004; and Sampling period 4, January and February, 2005). In each dairy herd, the sampled cows had to fulfil the following criteria: first–third lactation and 30–250 d of lactation. Cows with clinical mastitis, or submitted to mastitis treatment 2 weeks before milk sampling, were excluded from this study. In addition, at the time of milk sampling, information such as age, days in lactation and type of feeding system (pasture, supplemented pasture or total mixed ration) for each selected dairy cow was registered.

Milk samples were collected during morning milking in order to represent the whole milking of each animal. The individual samples were collected directly from the collection bucket immediately after milking, or during milking, using flux measurement devices connected to the milk line. Collected samples were split into two flasks: one treated with Bronopol (Boots Microcheck, Nottingham, UK) was used for fat, lactose, total solids, milk urea nitrogen and somatic cell determinations; and the other was used for analysis of milk protein fractions.

Methods of analysis

Milk composition: Milk samples were analysed for fat, lactose and total solids by infrared absorption methodology (Bentley 2000, Bentley, Chasca MN, USA). Milk urea nitrogen (MUN) was determined by the colorimetric-enzymic method (Chemspec 150, Bentley Instruments Inc., Chasca MN, USA). Somatic cells were electronically counted (Somacount 300, Bentley Instruments Inc.) and converted to log scale (LogSCC). Milk protein fractions were determined for total nitrogen (AOAC, 1990; method 33.2.11; 991·20), non-casein nitrogen (NCN) (Lynch et al. 1998) and non-protein nitrogen (NPN) (AOAC, 1995; method 33.2.12; 991·21). In order to express the results as total protein (TP), total nitrogen values found were multiplied by 6·38 (Barbano et al. 1990). Milk true protein (MTP) and casein concentrations were obtained by difference according to: TP−(NPN × 6·38)=milk true protein, and MTP−(NNC × 6·38)=casein, respectively.

β-lg genetic polymorphism analysis: After DNA extraction from blood samples according to Ciulla et al. (1988), extracted DNA samples were submitted to PCR amplification as described by Schlee & Rottmann (1992). The oligonucleotides primers used for DNA amplification were synthesized by Invitrogen Custom Primers (Inivitrogen Corp., Carlsbad CA 92008, USA), according to the sequence described by Faria et al. (2000):

Forward: 5′ACCTGGAGATCCTGCTGCAGAAATG3′
Reverse: 5′CATCGATCTTGAACACCGCAGGGAT3′

Each amplification reaction consisted of PCR buffer 1X (500 mM-KCl, 100 mM-Tris–Cl pH 8·3), 0·1 µl of the described primers, 2·0 µl dNTP (0·125 mM), 0·1 µl Taq polymerase (Cenbiot/RS, PHN/MG), 0·75 µl $MgCl_2$ (Cenbiot/RS, PHN/MG), 5·0 µl of DNA and mili-Q water qsp 25 µl. In all amplified reactions, a control (blank/no-DNA) was used to confirm the absence of contamination during the analysis. The amplification process was carried on a PTC 100-MJ Research thermocycler (MJ Research Inc., Watertown MA 02472, USA).

After DNA amplification of the targetted fragment, a 961-bp region of the β-lg gene, the PCR product (20 µl) was submitted to RFLP in a 2% agarose gel electrophoresis, and digested by the restriction enzyme Hph-I (Invitrogen Corp.) according to Wilkins & Kuys (1992) to show the presence of either AA, AB or BB genotypes. Hph-I cleaved amplified fragments in 741 and 220 bp (AA genotype), 741, 166 and 54 bp (BB genotype), or a combination of both (AB genotype), with 741, 220, 166 and 54 bp (Wilkins & Kuys, 1992). After β-lg gene identification, the genotype and allele frequencies for the studied herd were obtained.

Statistical analysis

Effects of β-lg, breed and seasonality were analysed in a randomized block design with number of lactation (1, 2 and ⩾3) and feeding system (pasture, supplemented pasture or total mixed ration) as blocks. For statistical analysis, selected cows were distributed according to the β-lg genetic variant (AA, AB or BB), breed (Holstein or Girolando) and seasonality (dry or rainy season). Descriptive statistics of the given results were stored (arithmetic mean and SEM) and analysis of variance was performed, evaluating the effects of breed, seasonality and genetic variation of β-lg on the response variables, by the SAS PROC GLM procedure (Statistical Analysis System, Version 8.02; SAS Institute Inc., Cary NC 2001, USA).

In the adopted model, dependent variables used were milk composition (MC) characteristics and fixed factors were β-lg polymorphism, breed and seasonality. The

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Table 1. β-lactoglobulin gene and allele frequencies for Holstein and Girolando cows

Breed	Genotypes	Genotype frequencies $n_{observed}$	%	Allele frequencies ± SEM†
Holstein	AA	91	32·73	
	AB	79	28·42	A = 0·4694 ± 0·0211
	BB	108	38·85	B = 0·5305 ± 0·0211
	Total	278		
Girolando	AA	33	21·15	
	AB	53	33·97	A = 0·3815 ± 0·0278
	BB	70	44·87	B = 0·6185 ± 0·0278
	Total	156		

† SEM according to Oner & Elmaci (2006)

model is described as follows:

$$MC_{ilm} = \mu + L_i + F_j + B_l + P_m + S_n + B_l * P_m + B_l * Sn + P_m * S_n + B_l * P_m * S_n + e_{ijlmn}$$

MC is the variable considered for milk composition; μ = mean value for the characteristic; L_i = lactation number (i = 1, 2 and ⩾3), F_j = feeding system (j = pasture, supplemented pasture or total mixed ration), B_l = Breed (l = Holstein and Girolando); P_m = genotypes (m = AA, AB and BB); S_n = seasonality (n = rainy and dry season); e_{ijlmnp} = error.

Results

Genetic polymorphism of β-lg in Holstein and Girolando cows

A total of 278 Holstein and 156 Girolando cows were tested for genetic polymorphism of β-lg. The distribution of genotype and allele frequencies for the β-lg gene of Holstein and Girolando cows, obtained by PCR-RFLP, is summarized in Table 1.

All three β-lg genotypes (AA, AB and BB) were present in both breeds. The allele B was present in a higher frequency both for Holstein and Girolando breed (0·5305 ± 0·021 and 0·6185 ± 0·027, respectively). The frequency of appearance of genotypes differed between Holstein and Girolando cows. BB genotype was the most frequent (38·85% and 44·87%) among studied breeds. AA and AB genotypes had frequencies of 32·73% and 28·42% for Holstein, but 21·15% and 33·97% for Girolando cows.

Effect of β-lg genetic polymorphism and breed on milk composition

Milk composition mean values and SEM for β-lg genotypes in Holstein and Girolando cows are shown in Table 2.

No statistical differences in milk composition of both breeds were observed among β-lg genotypes for any of the studied variables except for milk urea nitrogen concentration, which was higher (16·64 mg/dl) for Holstein than for Girolando cows (14·38 mg/dl). No interaction between β-lg and breed was found for milk composition.

The average of milk fat and total solids content for the AA, AB and BB genotypes did not differ in any of the breeds. For Holstein cows, milk fat averages were 3·305, 3·267 and 3·359% for the AA, AB and BB genotypes, respectively, while for Girolando, the averages were 3·143, 3·274 and 3·166% for the same genotypes. Total solids averages 11·824, 11·823 and 11·876% among Holstein cows of AA, AB and BB genotypes, respectively, and 11·595, 11·808 and 11·755%, respectively to the cited genotypes for Girolando animals.

Concentrations of total protein, true protein, casein, MUN as well the casein : true protein ratio were not influenced by the β-lg genotypes in this study. Milk from Holstein and Girolando BB cows, respectively, had total protein of 3·125 and 3·091%, true protein of 2·892 and 2·859%, and casein of 2·095 and 2·070%. Averages for the casein : true protein ratio among BB genotype cows were 0·722 for the Holstein breed and 0·721 for Girolando.

Effect of β-lg genetic polymorphism and seasonality on milk composition

Effects of seasonality and β-lg polymorphism on milk composition are shown on Table 3.

Concentrations of lactose, MTP, casein and casein : MTP ratio were significantly higher during the dry season than in the rainy season. Average concentrations for the dry season were 4·501, 2·918 and 2·132 g/100 g of milk, respectively for lactose, TP, MTP and casein, while rainy season corresponding means were 4·349, 2·846 and 1·994 g/100 g of milk. No interaction between seasonality and β-lg polymorphism was observed.

When non-significant interactions were removed from the statistical model, casein concentrations observed during the rainy season were different among β-lg genetic variants (AA > BB) with higher averages for AA (2·067 g/100 g of milk) compared with BB cows (1·949 g/100 g of milk). But no differences were observed for milk composition variables among β-lg variants within the dry season.

Discussion

Ng Kwai-Hang et al. (1986), Van Eenennaam & Medrano, (1991), Celik (2003), Oner & Elmaci (2006) reported higher frequencies for the B allele for Holstein cows. In the present study, the frequency of genotype appearance differed between Holstein and Girolando cows. Although the BB genotype was the most frequent for both breeds (38·85% and 44·87%), AA genotype was the second higher in frequency level in Holstein breed (BB > AA > AB), while the AB was more frequent than AA genotype in

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Table 2. Effect of β-lactoglobulin polymorphism in Holstein and Girolando cows on milk composition

Breed	Girolando			Holstein			*P*		
β-lg polymorphism	AA	AB	BB	AA	AB	BB	Breed	β-lg	B∗β-lg
Fat, g/100 g	3·143	3·274	3·165	3·305	3·267	3·359	0·877	0·850	0·083
SEM	0·108	0·088	0·060	0·054	0·054	0·047			
Lactose, g/100 g	4·447	4·460	4·458	4·444	4·429	4·443	0·465	0·857	0·697
SEM	0·031	0·024	0·022	0·017	0·018	0·016			
Total Solids, g/100 g	11·595	11·808	11·755	11·824	11·823	11·876	0·497	0·782	0·534
SEM	0·126	0·103	0·081	0·064	0·067	0·057			
Total protein, g/100 g	3·150	3·066	3·091	3·146	3·144	3·125	0·914	0·213	0·716
SEM	0·039	0·031	0·025	0·020	0·020	0·018			
True protein, g/100 g	2·912	2·853	2·859	2·927	2·915	2·892	0·795	0·204	0·774
SEM	0·044	0·031	0·027	0·020	0·020	0·018			
Casein, g/100 g	2·123	2·065	2·070	2·129	2·111	2·095	0·449	0·123	0·804
SEM	0·048	0·032	0·030	0·023	0·023	0·020			
Casein:true protein	0·731	0·720	0·721	0·725	0·722	0·722	0·165	0·114	0·894
SEM	0·007	0·004	0·005	0·003	0·004	0·003			
Log (SCC $\times 10^{-3}$ cells/ml)	2·501	2·275	2·445	2·300	2·404	2·413	0·278	0·271	0·107
SEM	0·088	0·065	0·056	0·049	0·046	0·038			
Urea nitrogen, mg/100 ml	14·008	14·628	14·517	16·571	16·823	16·527	0·001	0·439	0·720
SEM	0·580	0·472	0·430	0·298	0·314	0·225			

Table 3. Effect of seasonality and β-lactoglobulin polymorphism on milk composition

Seasonality	Rainy season			Dry season			*P*		
β-lg polymorphism	AA	AB	BB	AA	AB	BB	Season	β-lg	S∗β-lg
Fat, g/100 g	3·208	3·252	3·292	3·308	3·279	3·296	0·995	0·8501	0·988
SEM	0·080	0·082	0·063	0·060	0·057	0·046			
Lactose, g/100 g	4·354	4·353	4·339	4·500	4·488	4·514	0·001	0·857	0·272
SEM	0·023	0·023	0·019	0·019	0·017	0·016			
Total Solids, g/100 g	11·591	11·714	11·663	11·883	11·876	11·937	0·124	0·782	0·599
SEM	0·093	0·099	0·082	0·072	0·068	0·055			
Total protein, g/100 g	3·131	3·093	3·048	3·155	3·129	3·149	0·052	0·213	0·348
SEM	0·027	0·035	0·024	0·023	0·019	0·018			
True protein, g/100 g	2·920	2·842	2·775	2·934	2·901	2·922	0·019	0·204	0·218
SEM	0·032	0·037	0·027	0·023	0·019	0·018			
Casein, g/100 g	2·062	2·008	1·912	2·148	2·111	2·139	0·001	0·123	0·168
SEM	0·039	0·043	0·031	0·024	0·020	0·019			
Casein:Milk true protein	0·716	0·709	0·699	0·729	0·725	0·730	0·001	0·114	0·188
SEM	0·007	0·007	0·005	0·003	0·003	0·003			
Log (SCC $\times 10^{-3}$ cells/ml)	2·449	2·405	2·468	2·287	2·333	2·398	0·073	0·271	0·773
SEM	0·064	0·063	0·047	0·056	0·047	0·041			
Urea nitrogen, mg/100 ml	16·877	16·159	15·727	15·438	16·014	15·937	0·747	0·439	0·703
SEM	0·556	0·524	0·403	0·282	0·301	0·244			

Girolando cows (BB>AB>AA). Higher levels of BB genotype frequencies among Holstein cows were also observed by Celik (2003) but not by Hill (1993) and Oner & Elmaci (2006), who found a higher number of the heterozygous AB variants compared with homozygous cows.

Nevertheless, no report on β-lg genetic variants in Girolando animals was found in the literature, and data on β-lg allele frequency among *Bos indicus* breeds show a higher prevalence for the B gene (Silva & Del Lama, 1997; Neves et al. 1998; Faria et al. 2000) and a higher frequency for the BB genotype.

The present results differ from those of Ng Kwai-Hang et al. (1986), Aleandri et al. (1990), Bovenhuis et al. (1992) and Hill (1993). These authors all reported higher milk fat concentration in milk of Holstein cows comprising B variants of β-lg, and Hill (1993) concurred for significantly lower concentrations of fat and total solids in AA Holstein milk.

Milk nitrogen fractions were not influenced by β-lg genetic polymorphism in any of the breeds, although TP, MTP, casein means and casein : MTP ratio for AA cows were numerically higher for both breeds. Similarly, Molina

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et al. (2006) and Bobe et al. (1999) found an increase in TP concentrations of milk when B allele was substituted for A. According to Bobe et al. (1999) such increment is due to the higher percentage of casein in β-lg A milk, while Molina et al. (2006) attributed this increase to the higher content of whey protein in milk with A variants. Mean values for casein and MTP concentrations in the present study, however, seem to be in disagreement with Robitaille et al. (2002), who found a tendency for higher casein concentrations in BB β-lg milk in Holsteins, and with a report of increased MTP when B allele was more frequent in the Holstein breed (Ng Kwai-Hang, Monardes & Hayes, 1990).

Although not statistically significant, the casein: MTP ratios for AA milk of both breeds were slightly higher than those observed for either AB or BB genotypes, and this is particularly relevant as MTP, especially β-lg concentrations in milk true protein, plays an important role in milk stability during heat treatment, since this protein strongly interacts with other milk molecules, particularly κ-casein (Karatzas & Turner, 1997; Fox & McSweeney, 1998; Singh, 2004).

MUN concentrations the present study ranged from 12 to 18 mg/dl within the normal minimum and maximum limits for lactating Holstein cows (Meyer et al. 2004). Higher MUN was observed for Holstein cows, but no effect of season and β-lg polymorphism was found. According to Arunvipas et al. (2003), effects of non-nutritional factors on MUN, such as breed, stage of lactation and cow pregnancy, production and milk composition explain 13·3 % of the variability of MUN concentrations.

Based on the results of this study, there was no association between milk composition (fat, lactose, total solids, TP, MTP, casein, casein:MTP ratio, somatic cell count and MUN) and β-lg genetic variant itself. During the dry season, independently of breed considered, higher contents of lactose, TP, MTP, casein and casein: MTP ratio were found.

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