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Composition, somatic cell count and casein fractions of ethanol unstable milks

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ABSTRACT. This study determined the composition, somatic cell count (SCC) and relative percentage of α -S₁, α -S₂, β and κ caseins in ethanol-unstable (UNA) and stable bulk milk samples. The sampling plan involved farms that supplied milk to one dairy plant located in the northeast region of São Paulo, Brazil, in May (fall), July (winter), and September (spring) 2007. Three hundred thirty-four bulk milk samples within an acceptable range of pH and acidity were included in the study and divided into two groups: a) stable milk, when protein precipitation only occurred with 78% ethanol (v/v); and b) unstable (UNA) milk, when the precipitation occurred with 72% ethanol (v/v). From the total samples analyzed, 77 (23%) were unstable in the 72% ethanol (v/v), although they have shown normal pH and acidity, and 41 (12.3%) were stable in 78% ethanol (v/v). No differences were found between relative percentages of α and β -casein in UNA or stable milks. However, UNA samples showed higher SCC, as well as lower casein and lactose contents. Results indicated that the high SCC may be involved as a causal factor for the high incidence of UNA milks in the herds studied.

Keywords: ethanol stability, milk SCC, milk quality.

Composição, contagem de células somáticas e frações de caseína em leites instáveis ao etanol

RESUMO. O objetivo deste estudo foi determinar a composição, contagem de células somáticas (CCS) e percentual de α -S₁, α -S₂, β e κ caseínas em amostras de leite estável e de leite instável ao etanol (LINA). A amostragem envolveu fazendas fornecedoras de um laticínio localizado em São Paulo, Brasil, nos meses de maio (outono), julho (inverno) e setembro (primavera) de 2007. Trezentas e trinta e quatro amostras de leite de conjunto encontradas dentro da faixa aceitável de pH e acidez foram incluídas e divididas em dois grupos: a) leite estável, quando a precipitação de proteínas ocorreu apenas com 78% de etanol (v/v) e b) LINA, quando ocorreu com 72% de etanol (v/v). Do total de amostras analisadas, 77 (23%) eram instáveis ao etanol 72% (v/v), embora apresentassem pH e acidez normais, e 41 (12,3%) mantiveram-se estáveis ao etanol a 78% (v/v). Não foram encontradas diferenças nos percentuais de α e β caseínas dos leites estáveis e identificados como LINA. Entretanto, as amostras de LINA apresentaram CCS maior que as de leite estável, e menores teores de caseína e lactose. Os resultados indicam que alta CCS nas amostras de leite pode estar envolvida como fator causal da elevada incidência de LINA no estudo.

Palavras-chave: estabilidade ao etanol, CCS, qualidade do leite.

Introduction

Milk ethanol stability is defined as the minimum concentration of added aqueous ethanol that causes milk coagulation (HORNE; PARKER, 1979). This test is used worldwide in reception platforms of dairy plants to predict heat stability of raw milk (CHAVEZ et al., 2004). In Brazil, the Ministry of Agriculture recommends the use of 68-72% (v/v) ethanol in the ethanol stability test (BRASIL, 1981).

However, several dairy factories employ even higher ethanol concentrations, especially manufacturers of ultra-high temperature milk

(OLIVEIRA; TIMM, 2006). Although ethanol stability is considered a simple, reliable test for detecting milk of poor microbiological quality, false-positive results in milks with normal pH have been reported in Brazil, mainly in the southern states of the country (ZANELA et al., 2009).

The causal factors for the occurrence of UNA milks are not well understood. Previous studies have pointed out multifactorial causes associated with metabolic and/or nutritional disorders that affect milk synthesis and secretion of milk components, especially ions (CHAVEZ et al., 2004).

In relation to the composition, UNA milk showed greater fat content and lower lactose levels than stable milk (MARQUES et al., 2007; OLIVEIRA; TIMM, 2006). These changes in the concentrations of the main constituents of fluid milk are common consequences of mastitis and generally observed together with increased somatic cell counts (SCC). SCC has also been associated with changes in milk composition (AULDIST; HUBBLE, 1998), but no association between SCC and UNA milks has been found.

Studies on the differences of protein concentrations between UNA and stable milks have reported contradictory results. While some authors showed that there were no differences (OLIVEIRA; TIMM, 2006; SOBHANI et al., 1998), others observed greater protein concentrations in UNA milks (BARROS et al., 1999; MARQUES et al., 2007). Furthermore, there is no information on the variation of casein fractions in UNA milks. The objective of this study was to determine the composition, SCC and relative percentage of α -s₁, α -s₂, β and κ caseins in UNA and in ethanol-stable bulk milk samples.

Material and methods

Three hundred thirty-four bulk milk samples were collected from farms that supplied milk to a dairy plant located in the northeast of the State of São Paulo, Brazil. Samples were collected in only one day in May (fall), July (winter) and September (spring) 2007, from all suppliers delivering milk to the plant on that day.

Milk samples were collected in each farm from the bulk tank immediately after the end of the evening milking. Bulk milk samples were collected in labeled 200 mL-flasks containing 2-bromo-2-nitropropane-1,3-diol (0.05%, m v⁻¹) to prevent bacterial growth until the moment of analysis. All samples received in the laboratory and within the acceptable range of pH (6.6-6.8) and acidity (14-18°D) were included in the study.

Ethanol stability test was carried out by adding equal volumes of milk and ethanol aqueous solutions at different concentrations into Petri dishes, and stirring slightly. Results were read immediately, and samples were classified in one of two groups: a) stable milk, when precipitation occurred with 78% (v/v) ethanol; and b) unstable (UNA) milk, when precipitation occurred with 72% (v/v) ethanol (HORNE; MUIR, 1990).

One day after collecting the samples, somatic cell counts were determined by means of flow cytometry in an electronic counter (Bentley, Chaska,

Minnesota, USA), and milk composition (total solids, fat, protein and lactose content) was determined using a Bentley mid-infrared analyzer (Chaska, Minnesota, USA).

Concentration of milk casein fractions was determined in stable and UNA milk samples by reversed-phase high performance liquid chromatography (HPLC), according to Bobe et al. (1998). Briefly, aliquots containing 500 μ L of milk were frozen at -20°C. A solution with 0.1 M Bis Tris buffer (pH 6.8), 6 M guanidine hydrochloride, 5.37 mM sodium citrate, and 19.5 mM dithiothreitol (pH 7.0) was added directly to the frozen aliquots in a 1:1 ratio (v:v) at room temperature. After thawing, each sample was shaken for 10 s, incubated for 1h at room temperature, and centrifuged for 5 min. at 16,000 g. The fat layer was then removed with a spatula, and the remaining soluble sample was diluted 1:3 (v/v) with a solution containing 4.5 M guanidine hydrochloride and solvent A (100:900:1; acetonitrile: water: trifluoroacetic acid; v/v/v; pH 2). Separation and identification of the proteins were performed at 220 nm in a HPLC system (Shimadzu, Japan) equipped with an UV detector and a Jupiter C18 column (4 μ m, 4.6 X 150 mm) (Phenomenex, Torrance, USA). Chromatographic run was carried out at room temperature using the following mobile phases: solvent A (acetonitrile: water: trifluoroacetic acid, 100:900:1) and solvent B (acetonitrile: water: trifluoroacetic acid; 900:100:1). The gradient program started with 25% solvent B, and the proportion of the solvent was gradually increased after the injection of the sample [34% (4 min.), 48% (11 min.), 50% (13 min.), 10% (17 min.)] and returned to the initial conditions after 2 min. Flow rate was adjusted at 1.0 mL min.⁻¹ The following retention times were obtained for α -s₁-casein, α -s₂-casein, β -casein and κ -casein, respectively: 10.2, 8.3, 10.9 and 7.6 min.

Quantification of casein fractions (α -s₁, α -s₂, β and κ) was performed by measuring peak areas of the samples, and plotting them against the calibration curves of each casein fraction. Purified α s, β and κ casein standards (Sigma, Saint Louis, MO, USA) were diluted in distilled water, and aliquots were frozen at -20°C. Individual casein standards were prepared in the same way described for milk samples, at the following concentrations: α s-casein: 0.5, 1.0, 2.0 and 4.0 mg mL⁻¹; β -casein: 0.375, 0.75, 1.50 e 3.0 mg mL⁻¹; κ -casein: 0.187, 0.375, 0.75 and 1.50 mg mL⁻¹, and α s₁: α s₂ casein ratio was assumed to be 4:1 (w/w).

Results from physical and chemical analyses, log SCC, and concentrations of casein fractions in stable and UNA milks were submitted to ANOVA using

the SAS® General Linear Model (SAS INSTITUTE, 2004). Means showing significant differences in the ANOVA were compared by the Tukey's test. Significance level was set at 0.05.

Results and discussion

Distribution of the percentages of milk samples stable and unstable (UNA) in the ethanol stability test is shown in Table 1.

Table 1. Frequency of ethanol-stable and unstable milk samples in São Paulo, Brazil.

Season when milk collected	Number of samples analyzed	Ethanol-stable (78%, v/v) samples		Ethanol-unstable (72%, v/v) samples	
		N	%	N	%
Fall	100	15	15.0	30	30.0
Winter	125	16	12.8	23	18.4
Spring	109	10	9.2	24	22.0
Total	334	41	12.3	77	23.0

Of the 334 milk samples analyzed, 77 (23%) were unstable in 72% (v/v) ethanol, although they have presented normal pH (6.6-6.8) and acidity (14-18oD). Forty-one samples (12.3%) were stable in 78% (v/v) ethanol. These results showed that a large percentage of milk samples may be misclassified as sour milk, leading to economic losses to the whole production chain (CHAVEZ et al., 2004).

Greater percentages (> 50%) of UNA milks were reported by Zanela et al. (2009) in southern Rio Grande do Sul State. Frequencies of UNA milk samples obtained in the present study were higher than those reported by Roma Jr. et al. (2009), who observed unstable milks in 7.4% of the samples tested with 78% (v/v) ethanol and collected in the States of Minas Gerais, Rio de Janeiro and São Paulo. As the causative factors for the occurrence of UNA have not been determined and because the percentage of UNA milk samples reported in different studies was highly variable, it is difficult to compare the results of the present study with those of other authors.

Results on the composition of stable and UNA milk samples are listed in Table 2. No differences ($p > 0.05$) were found for pH, acidity, total solids, total protein and relative percentages of casein fractions in UNA and stable milks. Mean SCC in UNA milk samples was higher ($p < 0.05$) than that of stable milks, although both SCC means obtained were below the tolerance limit adopted by Brazilian laws (BRASIL, 2002) for SCC in refrigerated raw milk (750,000 cells mL⁻¹, or 5.88 Log cells mL⁻¹). The United States of America also established the maximum level of 750,000 cells mL⁻¹ as a criterion for milk acceptability by the industry

(FONSECA; SANTOS, 2000). However, only ethanol stable milk samples had a mean SCC that could be considered in accordance with the regulations for SCC in milk (400,000 cells mL⁻¹, or 5.60 Log cells mL⁻¹) adopted in the European Union, New Zealand and Australia (FONSECA; SANTOS, 2000).

Marques et al. (2007) also observed significant differences between SCC in stable (401,000 cells mL⁻¹) and UNA milk samples (463,000 cells mL⁻¹). However, Zanela et al. (2009) did not find any correlation between high SCC and positive results in the ethanol stability test. High SCC are related to changes in milk composition, reduced calcium, lactose and casein levels, and increased sodium, chloride, and serum protein concentrations (MUIR, 1996).

Table 2. Composition of bulk ethanol-stable and unstable milk samples¹.

Composition parameters	Ethanol-stable samples (N = 41)	Ethanol unstable samples (N = 77)
SCC (Log cells mL ⁻¹)	5.48 ± 4.95 ^b	5.69 ± 4.71 ^a
pH	6.72 ± 0.02	6.67 ± 0.02
Acidity (°D)	16.8 ± 0.1	16.9 ± 0.2
Total solids (g L ⁻¹)	118.4 ± 2.0	120.1 ± 1.4
Fat (g L ⁻¹)	34.6 ± 1.9 ^b	37.3 ± 1.3 ^a
Lactose (g L ⁻¹)	44.1 ± 0.5 ^a	43.5 ± 0.4 ^b
Total protein (g L ⁻¹)	31.0 ± 0.7	30.6 ± 0.4
Casein (g L ⁻¹)	24.4 ± 1.5 ^a	23.1 ± 1.1 ^b
α _{s1} -casein (%) ²	58.8 ± 2.1	56.7 ± 1.5
α _{s2} -casein (%)	4.6 ± 0.5	4.2 ± 0.3
β-casein (%)	25.6 ± 1.6	24.4 ± 1.1
κ-casein (%)	12.3 ± 1.4	13.1 ± 1.0

¹Values expressed as mean ± standard deviation. ²Percentage of each casein fraction in relation to the total casein content of milk. ^{a,b}Means within a row with no common superscript are statistically different ($p < 0.05$).

Accordingly, in the present study, UNA milk samples showed lower ($p < 0.05$) lactose and casein contents when compared with stable milks. Fat concentration was greater ($p < 0.05$) in UNA milks, which was unexpected because high SCC is generally associated to a decrease in fat synthesis by the epithelial cells of the mammary gland (SCHULTZ, 1977).

Nevertheless, previous studies have indicated that the correlation between SCC and fat percentage may be negative, positive or null (MUNRO et al., 1984; SCHULTZ, 1977). Similar to the findings of the present study, Barros et al. (1999), Oliveira and Timm (2006), Marques et al. (2007) and Zanela et al. (2009) also reported greater fat contents in UNA milks. All these previous studies also observed lower lactose and casein content in UNA milks.

High SCC in milk is also related to the degradation of casein fractions, particularly α_s- and β-casein, as a consequence of proteolytic and lipolytic enzymes released by somatic cells (FERNANDES et al., 2008). Furthermore, changes

in the proportion of casein fractions may be associated with the occurrence of UNA milks. Guo et al. (1998) reported that the ethanol stability of goat milk was highly dependent on the casein composition of milk. In the present study, UNA milk samples showed lower, non-significant ($p > 0.05$) relative percentages of α and β -casein than the stable milk. However, it remains to be determined if the changes in casein ratio of higher SCC milks could be associated with the occurrence of UNA milk.

Conclusion

The results obtained in this study showed a high incidence of UNA milks in the dairy herds that supplied milk to one dairy plant in São Paulo, Brazil. UNA milks had higher SCC, which is consistent with the lower lactose and casein levels found. Although there were no significant differences in the relative percentages of casein fractions in stable and unstable milk samples, further studies should be carried out in order to assess a possible association between SCC, α and β -casein ratios and the occurrence of UNA milk.

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