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Unsatisfactory microbiological aspects of UHT goat milk, soymilk and dairy beverage of goat milk and soy protein: A public health issue

Taís Ramalho dos ANJOS[1], Valéria Quintana CAVICCHIOLI[2], Joyce Aparecida Santos LIMA[1], Alenia Naliato VASCONCELLOS[3], Andréia Cristina Nakashima VAZ[3], Gabriel Augusto Marques ROSSI[1], Maria Emilene Martino CAMPOS-GALVÃO[2], Svetoslav Dimitrov TODOROV[2], Luis Antonio MATHIAS[1], Rubén Pablo SCHOCKEN-ITURRINO[1], Luís Augusto NERO[2], Ana Maria Centola VIDAL[3]*

Abstract

Spore forming bacteria can play an important role in food quality and safety as spoilage and pathogenic microorganisms due to resistance to heat-treatment. However, there are limited number of studies focused on evaluate the microbiological quality and the occurrence of these bacteria in UHT goat milk, soymilk and dairy beverage of goat milk and soy protein. In this context, 75 samples of these beverages were evaluated regarding heterotrophic mesophilic microorganisms by conventional plate count and selective methods to detect microorganisms from *Bacillus cereus* group and *Clostridium perfringens*. Population counts greater than 10^4 CFU.ml^{-1} of heterotrophic mesophilic microorganisms were observed in 80% of the lots of goat milk and 100% of the lots of soymilk and dairy beverage of goat milk and soy protein. The presence of bacteria belonging to *B. cereus* group was observed in 16%, 52% and 44% of goat milk, soymilk and dairy beverage of goat milk and soy protein, respectively. *C. perfringens* was isolated from 8% samples of UHT soymilk. The frequency of genes *hblA, hblB, hblC, nheA, nheB, nheC* in 29 isolates obtained from these products was 62%, 48.2%, 96.5%, 79.3%, 68.9% and 79.3%, respectively. The microbiological quality of the evaluated products was unsatisfactory.

Keywords: foodborne pathogens; microbiology; PCR; spores.

Practical Application: Presence of spore-forming bacteria in UHT products whose microbiological quality is unknown.

1 Introduction

Spore forming microorganisms can be present in dairy products, and they are usually associated with spoilage of milk and dairy products, especially those processed under high temperature, including powder milk, canned dairy products, goat milk, cow milk and some cheeses (Eijlander et al., 2019; Jindal & Anand, 2018; Pinto et al., 2018; Oliveira et al., 2016c; Vidal et al., 2016; Reindl et al., 2014). These microorganisms can also be isolated in non-dairy products, such as soymilk, that has been increasingly consumed by lactose-intolerant people and children's formulas. For this reason, and based on the beneficial aspects these products promote, several consumers are including these alternatives on their regular diet (Rezende et al., 2015; Kwok et al., 2002).

Goat milk, for example, is considered an option for several consumers, due to low alergenic properties, characterizing it as an alternative to substitute cow milk (Mituniewicz-Małek et al., 2019; Clark & Mora García, 2017; Kuchtík et al., 2015). The goat milk has high dietary value and nutritional quality, and it has been highly recommended to feed children, adults and elderly people suffering from cow milk allergies, and I is also being used as a substitute for dairy products by consumers with dietary restrictions (Fangmeier et al., 2019; Mituniewicz-Małek et al., 2019; Pradeep Prasanna & Charalampopoulos, 2019; Beltrán et al., 2018; Nakajima et al., 2010). In this sense, goat milk products have high added values with a growing marketing demand (Mituniewicz-Małek et al., 2019; Fonseca et al., 2013).

Another option for lactose intolerants and people suffering from milk allergy are soymilk and soy beverages, due to their higher digestibility and low fat content compared to cow milk. However, some of these products are not subjected to efficient thermal treatments, capable to eliminate spores (Karaçali et al., 2018; Blum et al., 2016).

Thermal treatments are employed to milk and dairy products in order to reduce or to eliminate vegetative cells, however, spore forming bacteria are heat resistant and they may remain stable after pasteurization or even Ultra Hight Temperature (UHT) processes, being classified as "*highly heat resistant spores*" – HHRS (Eijlander et al., 2019; Jindal & Anand, 2018; Pinto et al., 2018; Kmiha, et al. 2017; Schoken-Iturrino et al., 1996). Thermal resistance is also related to several factors, such as strain type, growth temperature, age of the spores and environmental features (Oliveira et al., 2016c).

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[1] *Faculdade de Ciências Agrárias e Veterinárias – FCAV, Universidade Estadual Paulista – UNESP, Jaboticabal, SP, Brasil*
[2] *Universidade Federal de Viçosa – UFV, Viçosa, MG, Brasil*
[3] *Faculdade de Zootecnia e Engenharia de Alimentos – FZEA, Universidade de São Paulo – USP, Pirassununga, SP, Brasil*
*Corresponding author: anavidal@usp.br

Ohmic heating has been considered an alternative to eliminate spores in milk, by using temperatures around 105°C for 60 seconds, resulting in an effective way to protect food products due to structural damages caused to the spore structure (Ryang et al., 2016). Promote a rapid and homogeneous heating guaranteeing a greater amount of nutrients and sensory attributes to the producer (Ferreira et al., 2019a; Ferreira et al., 2019b; Cappato et al., 2018a; Cappato et al., 2018b).

The main species of spore forming bacteria considered as microbiological risks to food safe and quality are *Bacillus* spp. and *Clostridium perfringens*, and these microorganisms are usually present in milk, once they are widespread in soil, silage, digestive tract of animals and grains as soy, possibly contaminating soy products (Eijlander et al., 2019; Garcia et al., 2018; Oliveira et al., 2018d; Oliveira et al., 2016c; Ryang et al., 2016; Quigley et al. 2013). Then, if the raw material is obtained in poor hygienic conditions and if the thermal treatment is ineffective, there are chances to find spore forming bacteria contaminating end products (Pinto et al., 2018; Schoken-Iturrino et al., 1996).

The microbiological control of these products is required to provide safe food, improve their shelf life and avoid the transmission of foodborne diseases, mainly to susceptible individuals. However, there are few studies available in the literature focused on evaluating the microbiological quality of goat milk, soymilk and dairy beverage of goat milk and soy protein.

Thus, this study focused on count populations of heterotrophic mesophilic microorganisms, the bacteria belonging to *Bacillus cereus* group and *C. perfringens* in UHT treated goat milk, soymilk and dairy beverage based on combination of goat milk and soy proteins, and posteriorly verify the diarrheagenic potential of the isolates belonging to *B. cereus* group.

2 Materials and methods

2.1 Sample collection and microbiological analyses

A set of 75 samples of goat milk, soymilk and dairy beverage of goat milk and soy protein from different brands were obtained from supermarkets located in the state of São Paulo, Brazil. Selection criteria used were UHT processing, collecting 5 different lots, and 5 samples per lot, in a total of 25 samples of each kind of product. Goat milk from the same brand, soymilk from different 3 brands, and dairy beverage of goat milk and soy protein from the same brand were collected. The convenience sampling had the purpose to obtain a representative sample of UHT products commercially available in Brazil. Samples were collected from April to December 2015, and they were incubated at 37 °C during seven days (Brasil, 2001). Posteriorly serial dilutions were prepared on 0.1% peptone water and plated on recommended agar mediums. To perform heterotrophic mesophilic microorganisms counts, samples were seeded on Plate Count Agar (Difco, Detroit, MI, USA) and incubated at 35 °C (American Public Health Association, 2001).

To evaluate the presence of vegetative cells of bacteria belonging to the *B. cereus* group the samples were incubated at 30°C for 24-30 hours (Stadhouders, 1992). For the selective plating, an aliquot of 0.1 ml of selective enrichment culture was inoculated in Petri dishes containing mannitol egg yolk polymyxin B agar (MYP) (Mossel et al., 1967) and incubated at 30°C for 18-40 hours. Characteristic colonies described by Mossel et al. (1967) and Stadhouders (1992) and gram-positive bacilli were considered as possible representatives of *B. cereus* group.

To evaluate the presence vegetative cells or spores from *C. perfringens*, the samples were seeded on plates containing tryptose-sulfite-cycloserine agar (TSC) with egg yolk with a TSC overlay, incubated 35 °C during two days under anaerobic conditions (American Public Health Association, 2001). Gram staining, catalase test, lactose fermentation, indol, motility, gelatinase test, nitrate reduction and *C. perfringens* confirmation were also performed (American Public Health Association, 2001).

2.2 Identification of virulence factors in isolates included in B. cereus group

DNA extraction was performed using Wizard Genomic DNA Purification Kit Protocol (Promega, USA), according to manufacturer's instruction. DNA quantification was performed in NanoDrop 2000 (Thermo Scientific Inc., Waltham, MA, USA). The detection of genes *hblA, hblB, hblC, nheA, nheB* and *nheC* in the selected isolates was performed through PCR targeting mentioned genes on chromosomal DNA in order to verify the potential risk to cause diarrheal disease. Primers and conditions are specified in Table 1.

3 Statistical analyses

The quantitative data was initially subjected to Shapiro-Wilk test in order to verify normality and then transformed into log 10x+1 and x+1 square root and subject again to normality test when normality was not observed. Thus, the data were subjected to non-parametric tests in order to compare the three types of UHT products. Kruskal-Wallis test ($p < 0.05$) was used initially for multiple comparisons using "pgirmess" package in Software R®. Mann-Whitney test ($p < 0.05$) was used when a significant statistical difference was observed in the initial analysis in order to compare groups with the highest difference. For the comparison regarding the qualitative data was used the Fisher's exact test ($p < 0.05$). All analyses were performed in Software R®, v. 3.3.0.

4 Results

Heterotrophic mesophilic microorganisms counts of UHT goat milk, soymilk and dairy beverage of goat milk and soy protein were <1.0 to $3.5x10^4$ UFC.ml^{-1}, <1.0 to $3.7x10^4$ UFC.ml^{-1} and <1.0 to $5.5x10^4$ UFC.ml^{-1}, respectively. A significant statistically difference ($p=0.04$) was observed in the detection of heterotrophic mesophilic microorganisms counts among samples of goat milk (80%) and goat milk with soy dairy beverage (100%).

In this study, the presence of bacteria belonging to *B. cereus* group, that comprises *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus thuringiensis*, *Bacillus cereus*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis* and *Bacillus cytotoxicus*, was detected in 28 of 75 (37.33%) samples and 10 of 15 (66.66%) lots evaluated, with 52%, 44% and 16% of the samples of soymilk, dairy beverage of goat milk and soy protein and goat milk, respectively.

B. cereus was found in 16% of UHT goat milk samples and in 52% samples of dairy beverage of goat milk and soy protein, exhibiting statistical difference (p=0,023).

High numbers of strains belonging to *B. cereus* group were found to carry genes *hblC* (96.5%), *nheA* (79.3%), *nheC* (79.3%), *nheB* (68.9%), *hblA* (62%) and *hblB* (48.2%), as presented in Table 2. Twenty-nine isolates of *B. cereus* were recovered from goat milk (n = 4), soymilk (n = 13) and dairy beverage of goat milk and soy protein (n = 12). From these isolates, all of them (100%) presented amplification for *hblA*, *hblB*, *nheA*, *nheB* and *nheC* genes, and 75% amplified products corresponding to *hblC* gene.

Microorganisms from *B. cereus* group isolated from UHT goat milk samples (n = 4), 100% presented *hblA*, *hblB*, *nheA*, *nheB* and *nheC genes while* 75% amplified the fragment corresponding to *hblC* gene.

Considering those isolates belonging to *B. cereus* group obtained from soymilk (n = 13), 100% amplified *hblC fragment*, 76.9% amplified *nheA*, 61.5% were positive to *nheC*, 53.8% were positive to *hblA* and *nheB*, while 46.1% amplified the fragment corresponding to *hblB gene*.

Finally, the isolates from *B. cereus* (n = 12) obtained from dairy beverage of goat milk and soy protein, all of them (100%) amplified the fragment corresponding to *hblC gene*, while different percentages were observed for nheC, nheA and nheB, hblA and hblB, representing 91.6%, 75%, 58.3% and 33.3%, respectively.

C. perfringens was only detected in two samples of UHT soymilk. In UHT goat milk and dairy beverage of goat milk and soy protein this specie was not detected and there was no significant difference among UHT products evaluated (p=0,1317).

5 Discussion

Heterotrophic mesophilic microorganisms count in foods is useful to evaluate the microbiological quality and hygienic conditions of manufacturing. Kondyli et al. (2012) evaluated the microbiological quality of raw goat milk and found values of heterotrophic mesophilic microorganisms ranging from 6.05 to 6.14 log CFU.ml^{-1} and >10x10^5 CFU.ml^{-1}, respectively. Oliveira et al. (2005a) evaluated 16 samples of UHT goat milk and did not isolated heterotrophic mesophilic microorganisms, differing from our study. The results showed here, allows us to suspect that the addition of soy can contribute to increase the presence of microorganisms in dairy products, such as dairy beverage, requiring further studies to prove it.

The Brazilian legislation (Brasil, 2001) establishes the absence of pathogenic microorganisms in UHT goat milk. The presence of high counts of *B. cereus* (>10^6 organisms/ g^{-1}) is an indicative of a potential risk for human health (Food and Drug Administration, 2012). Considering the increase in the consumption of UHT products, and from a bacteriological and food safety point of view, the UHT products must be stable at room temperature from four to six months (Pinto et al., 2018).

A significant statistical difference (p=0.023) was observed among bacteria belonging to *B. cereus* group in goat milk and soymilk. These results highlight the need of improvements during milk and soy obtaining in farms, storage and processing industries in order to improve their microbiological quality.

Table 1. Toxin, gene name, primer, amplicon size (bp), and annealing temperature, according to Guinebretière et al. (2002), used for the detection of virulence factors.

Toxin	Gene	Primer	Amplicon Size (bp)	Annealing temperature (ºC)
Hemolysin BL	*hblA*	F 5'-AAGCAATGGAATACAATGGG-3'	1154	55
		R 5'-AGAATCTAAATCATGCCACTGC-3'		
	hblB	F 5'-AAGCAATGGAATACAATGGG -3'	2684	55
		R 5'-AATATGTCCCAGTACACCCG-3'		
	hblC	F 5'-GATACYAATGTGGCAACTGC -3'	740	52
		R 5'-TTGAGACTGCTCGYTAGTTG -3'		
Nonhemolytic enterotoxin	*nheA*	F 5'-GTTAGGATCACAATCACCGC -3'	755	53
		R 5'-ACGAATGTAATTTGAGTCGC -3'		
	nheB	F5'-TTTAGTGGATCTGTACGC -3'	743	48
		R 5'-TTAATGTTCGTTAATCCTGC -3'		
	nheC	F 5'-TGGATTCCAAGATGTAACG -3'	683	54
		R 5'-ATTACGACTTCTGCTTGTGC -3'		

Table 2. Prevalence of *hblA*, *hblB*, *hblC*, *nheA*, *nheB* and *nheC* in 29 isolates.

Product	Number of isolates	Genes					
		hblA	*hblB*	*hblC*	*nheA*	*nheB*	*nheC*
UHT goat milk	**4**	4(100%)	4(100%)	3 (75%)	4(100%)	4(100%)	4(100%)
UHT soymilk	**13**	7(53.8%)	6(46.1%)	13(100%)	10(76.9%)	7(53.8%)	8(61.5%)
UHT dairy beverage of goat milk and soy protein	**12**	7(58.3%)	4(33.3%)	12(100%)	9(75%)	9(75%)	11(91.6%)
Total	**29**	18(62%)	14(48.2%)	28(96.5%)	23(79.3%)	20(68.9%)	23(79.3%)

Nakajima et al. (2010) report that the consumption of soymilk is done mostly by people concerned about their health, aiming the substitution of cow milk, reduction of hypercholesterolemia and the risk of osteoporosis and diabetes. The mean age of the interviewed consumers in the cited study was 32.5 years, reaching the age of 78 years, considered more susceptible to opportunistic infections (Nakajima et al., 2010).

Detection of *nheA, nheB, nheC, hblA, hblB* and *hblC* genes is useful to verify the potential to cause diarrheal illness in strains belonging to *B. cereus* group (Ehling-Schulz et al., 2004). According to Lee et al. (2017), from 90% to 100% from *B. cereus* group isolated from food samples can carry the *hblACD* and *nheABC* genes. Chaves et al. (2011) evaluated 97 strains of *B. cereus sensu stricto* collected over three years and observed that 84.5% and 62.9% of the strains were positive for NHE and HBL complex, respectively.

A high number of isolates containing toxigenic genes were found in this study, highlighting the potential for production of hemolytic and nonhemolytic toxins. Amplicons for *hblC gene* were detected in 100% of the isolates obtained from dairy beverage of goat milk and soy protein. The members of *B. cereus* group are commonly present contaminants of fresh and heat-treated milk (Bartoszewicz et al., 2008). In a study performed by Bartoszewicz et al. (2008), the authors established a prevalence of 80 and 55% for *nheA and hblA* in isolates obtained from dairy farms and dairies in Poland.

Zhou et al. (2008) evaluated 100 isolates obtained from samples of pasteurized whole milk sold in China. These authors revealed that the enterotoxin genes *hbl*A, *hbl*C, *hbl*D, *nhe*A, *nhe*B and *nhe*C occurred in *B. cereus* isolates with frequencies of 37.0%, 66.3%, 71.7%, 71.7%, 62.0% and 71.7%, respectively.

There are few studies available in literature regarding the presence of toxigenic *B. cereus* group in soy products. Yim et al. (2015) evaluated the toxigenic profile of *B. cereus sensu stricto* in Korean soybean fermented products, detecting *hblACD* and *nheABC* genes in 34.5% and 98.9% of the strains, respectively. Park et al. (2016) reported high number of strains positive for diarrheal toxin genes in Doenjang, a Korean fermented soybean past, demonstrating the importance of potentially pathogenic strains in soy products.

In the work of Lee et al. (2017), only 8.6-23% of the isolates containing *hbl* encoded the enterotoxin HBL. The production of hemolytic and nonhemolytic enterotoxins is complex and involves transcriptional regulator proteins, posttranscriptional and posttranslational regulatory mechanisms and environmental conditions (Jeßberger et al., 2015).

The genes *nheA*, *nheB* and *nheC* show more toxicity for the epithelial cells. *In vitro* tests, under high concentrations of the toxin nhe, the target cells suffer quick cellular apoptosis, concomitant with a necrotic condition (Liu et al., 2017). The genes of the complex HBL are also associated with a strong degenerative effect on the cell membrane (Berthold-Pluta et al., 2015), which shows the degree of pathogenicity of the strains of *B. cereus* group.

Clostridium perfringens strains are widely prevalent in feces of lactating cows and play an important role in diarrheal diseases (Food and Drug Administration, 2012). Meat and poultry dishes are the most important source of *C. perfringens* infection for humans during foodborne outbreaks; however, an outbreak due its presence in milk was already reported (Bennett et al., 2013).

C. perfringens type A food poisoning occurs due to the production of the enterotoxin after the ingestion of $>10^7$ cells of *C. perfringens*. Contaminated food is almost always heat-treated which kills competing bacteria while spores survive and is the dominating, as shown in this study. Besides *C. perfringens* detection in this study, its presence cannot be considered as a risk for foodborne disease if adequate conditions of storage are observed. Complementary studies are required to verify the presence of *C. perfringens* virulence factors, due to its ability to cause foodborne disease.

Obtained data indicate the unsatisfactory microbiological quality of the analyzed UHT products, suggesting the implementation of a rigorous quality assurance system for food safety using Good Manufacturing Practices (GMP) and Analysis and Critical Control Point (HACCP) (Cusato et al., 2013; Oliveira et al., 2016b). Using this strategy, it is possible to reduce the prevalence of foodborne diseases (Carrascosa et al., 2016).

Knowing that *B. cereus* can be present in raw, pasteurized and UHT milk (Vidal-Martins et al., 2006), spores of this microorganism are able to resist to heat treatments and that enterotoxigenic strains were detected in this study, improvements in milking and storage conditions should be implemented.

6 Conclusion

The observed microbiological quality of UHT goat milk, soymilk and dairy beverage of goat milk and soy protein evaluated was unsatisfactory. Pathogenic microorganisms such as *C. perfringens* and potentially diarrheagenic strains belonging to *B. cereus* group were detected, highlighting the needs of improvements on adoption of hygienic practices during obtaining, manufacturing and storage of these products in order to improve food safety, mainly because they are consumed by elderly people and consumer suffering with allergies, being a potential hazard for this population.

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