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ORIGINAL RESEARCH

Microbiological quality of Brazilian UHT milk: Identification and spoilage potential of spore-forming bacteria

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Counts of aerobic mesophilic micro-organisms and aerobic mesophilic spore-forming bacteria were determined in 91 ultra-high-temperature (UHT) commercial Brazilian samples. Forty-six spore-forming bacteria were identified and characterised in terms of their spoilage potential. Among the 20 brands evaluated, 45% had counts of aerobic mesophilic micro-organisms higher than 1.0 × 102 cfu/mL. The sporulated bacteria were identified as Bacillus subtilis/amyloliquefaciens, Bacillus licheniformis, Bacillus pumilus *and* Bacillus megaterium, *and 31% and 33% showed proteolytic and lipolytic activity, respectively. Our findings indicate that there is a potential risk of UHT milk samples becoming spoiled during their commercial shelf life.*

Keywords UHT milk, Spore-forming bacteria, Quality.

INTRODUCTION

UHT milk in Brazil is processed between 130 °C and 150 °C for 2–4 seconds by the continuous thermal flow process, and the product is immediately cooled to a temperature lower than 32 °C, bottled in aseptic conditions in a previously sterilised packaging and then hermetically sealed (Brazil Ministry of Agriculture, Livestock and Supply (MAPA) 1997).

UHT milk is consumed extensively throughout the world (Pujol *et al.* 2013), although the market share of UHT milk varies considerably by country: Australia 9%, France 88%, Spain 83%, Germany 63%, Italy 55% and the United Kingdom 5% to 13% (Chavan *et al.* 2011). The increase in consumption in Brazil has evolved greatly over the last 20 years. The volume of milk produced was about 450 million litres in 1992 but reached more than 6 billion litres in 2013, with a total market of 10.5 billion litres of milk. UHT processing is responsible for 78% of the total fluid milk consumed in Brazil and this product was worth R$ 14 billion in 2012 (Brazilian Association of Long Life Milk Industry (ABLV) 2013). From bacteriological and food safety standpoints, UHT milk must be stable at room temperature (~25 °C) for about 4–6 months.

The quality of UHT milk is highly influenced by the microbiological characteristics of the raw milk, the heat treatment applied, storage conditions and aseptic packaging. The biochemical processes that occur during the shelf life of UHT milk are related to residual enzymatic activities of proteinases, lipases and phosphatases (either from the milk or from bacterial origin), among other factors (*Topçu et al.* 2006; Gaucher *et al.* 2008). UHT milk contamination by spore-forming bacteria also represents one of the main problems in the milk producing chain (Te Giffel *et al.* 2002). If the conditions for obtaining the raw material or the processing conditions are inadequate, sporulated

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micro-organisms may be present as contaminants, although UHT treatment inactivates any vegetative cells of micro-organisms present in the milk. Sporulated bacteria pertaining to the genera *Bacillus* and *Paenibacillus* are the main micro-organisms identified as being limiting factors for fluid milk quality, due to inadequate hygienic conditions (Huck *et al.* 2007; Ivy *et al.* 2012). Therefore, the detection and identification of micro-organisms resistant to heat treatments are of great importance for food industries, in order to adequately handle microbiological risks (Postollec *et al.* 2010).

Bacillus species, which are aerobic, Gram-positive and spore-forming, are widely dispersed in nature, with soil and pasture as habitats (Te Giffel *et al.* 2002; Carlin 2011). *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus coagulans*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus subtilis* and *Geobacillus stearothermophilus* are sporulated bacteria that have been isolated from dairy processing environments (Banykó and Vyletelová 2009; Postollec *et al.* 2010; Ghellai and Moussaboudjemaa 2013). These authors pointed out that such bacteria are the cause of reduced sterility in processed milk (UHT), associated with failures during thermal treatment, packaging failures and poor microbiological quality of the raw milk.

Therefore, monitoring of hygienic procedures, the time/temperature used in the process and the implementation of microbiological standards for the raw milk destined for UHT processing is of fundamental importance. The present study aimed to evaluate the microbiological quality of Brazilian UHT milk, in order to identify and evaluate the spoilage potential of spore-forming bacteria.

MATERIALS AND METHODS

UHT milk sample collection

A total of 91 samples belonging to 20 commercial brands of UHT whole milk (1-litre packages) produced in different Brazilian regions (south-east, south, north-east and Midwest areas) were randomly acquired from supermarket chains in the city of Juiz de Fora, Minas Gerais, Brazil. The brands originated from 20 production plants, which represented almost the total of brands sold in this region. All brands had the seal of the Federal Inspection Service (SIF) on the label, which enables the product to be marketed throughout the Brazilian territory. The collections were carried out at five different times (once in the summer, autumn and winter, and twice in the spring), along the duration of the study (2013 and 2014), with the objective of ensuring seasonal coverage. The brands were collected in duplicate when available at the point of sale. Not all of the producers were sampled an equal number of times. The sampling approach chosen can be considered to be convenience sampling (Felicio *et al.* 2013), for which the main purpose was to obtain a representative sample of the UHT milk commercially available in Brazilian market. It is important to note that the most of Brazilian UHT dairy factories are located in south-east, from where most of the samples in this study were obtained.

Microbiological analyses

The mesophilic aerobic plate count was conducted using classical methodology (Morton 2001), as well as the count of mesophilic aerobic spore formers (Stevenson and Segner 2001) with results expressed as colony-forming units (cfu/mL). Briefly, the UHT milk samples were pre-incubated at 36 °C for 7 days to check for any changes in product characteristics such as acidification, coagulation and bloating. After pre-incubation, visually unchanged samples were shaken 25 times and an 11 mL sample was aseptically homogenised in 99 mL of 0.1% peptone water in dilution bottles. We adopted this procedure due to the possibility that visually altered samples had been contaminated with spoilage micro-organisms that had accessed the milk via micro-holes in the packaging, flaws in welds, exchange of coil or tape, among other critical points in the production process. Serial dilutions were made up to 10^{-2}. The mesophilic aerobic plate count were determined by the pour plate technique using 1 mL of the dilutions 10^{0}, 10^{-1} and 10^{-2}, using Standard Methods Agar containing enzymatic digest of casein 0.5%, yeast extract 0.25%, glucose 0.1% and agar 1.5%, pH 7.0 $\pm$ 0.2 (Acumedia, Lansing, Michigan, USA).

To determine the bacterial count of mesophilic aerobic spore formers, 200 mL UHT milk samples were kept for 12 minutes in a water bath at 80 °C. The flasks were occasionally agitated gently to assist heat distribution. After the heat treatment, the samples were immediately cooled in an ice bath. Then, 11 mL samples were aseptically transferred to 99 mL of 0.1% peptone water in dilution bottles. Serial dilutions were made up to 10^{-2}, and pour plates were prepared using 1 mL of the dilutions 10^{0}, 10^{-1} and 10^{-2}, using Standard Methods Agar (Acumedia) supplemented with 0.1% soluble starch (Stevenson and Segner 2001). The plates were incubated at 32 °C for 48 hours.

Three to five colonies were then isolated from Petri dishes of higher dilutions. The isolates obtained were frozen at −80 °C in brain–heart infusion broth (BHI; Himedia, Mumbai, India) with added 20% glycerol, to establish a culture collection.

Identification of the isolates and spoilage potential

Prior to the completion of the identification experiments, the cultures were passaged twice in BHI broth, with incubation at 32 °C for 48 hours. Afterwards, 46 isolates with distinct colony characteristics were incubated in brain–heart infusion agar (BHI, Himedia) at 32 °C, for identification and evaluation of their spoilage potential. These isolates were identified (Entis *et al.* 2001) by the API50CH system (bioMérieux, Marcy L'Etoile, France) in accordance with the manufacturer's guidelines.

To determine spoilage potential, the isolates were evaluated with regard to their capacity to produce proteases in Standard Methods Caseinate Agar (Marcy and Pruett 2001) as well as lipases in tributyrin agar (Haas 2001). The inoculation of the isolates on the surface of the respective culture media was performed with the aid of a platinum needle. The Petri dishes were then incubated at 6.5 °C for 10 days and at 21 °C and at 35 °C for 72 hours. After incubation, the presence of any clarification halo indicating proteolysis and lipolysis was recorded.

Statistical analysis

The results were reported for the analysed microbial group and for each product brand in a manner similar to Brooks *et al.* (2012), who analysed the microbiological quality of raw cheeses. Additionally, for each commercial brand of UHT milk, the relative frequency over the limits imposed by the Brazilian legislation (1.0×10^2 cfu/mL) was calculated for mesophilic aerobic micro-organisms (Brazil Ministry of Agriculture, Livestock and Supply (MAPA) 1997). Finally, the relative frequency of isolates in each commercial brand was noted.

RESULTS AND DISCUSSION

General microbiological quality

Table 1 reports the microbiological quality of the UHT milk. Among the 20 brands of UHT milk evaluated, 45% contained aerobic mesophilic micro-organism counts higher than 1.0×10^2 cfu/mL (Table 1), exceeding the microbiological standard required by the Brazilian Ministry of Agriculture, Livestock and Supply (Brazil Ministry of Agriculture, Livestock and Supply (MAPA) 1997). In addition, 45% of the brands contained counts of sporulating bacteria. Among all the samples analysed, 22% and 18.7% of milk samples contained counts of aerobic mesophilic micro-organisms and sporulating bacteria higher than 1.0×10^2 cfu/mL, respectively. Another recent study that tested UHT milk samples in Brazil, reported 60% had aerobic mesophilic counts above the limits established in current legislation. The aerobic mesophilic counts ranged from <1.0 to 6.7×10^3 cfu/mL and were clearly influenced by storage time (Longhi *et al.* 2012). In disagreement with the latter research, all UHT milk samples commercialised in Viçosa, Brazil, had low counts of mesophilic aerobes and coliforms, while *Listeria monocytogenes* and *Salmonella* spp. were absent, which highlighted the suitability of pasteurised and UHT milk for human consumption (Pieri *et al.* 2014).

Several reports indicate the need for microbiological control strategies for raw milk processing, including the assessment of sanitary hygienic conditions of the equipment and the appropriate thermal treatment for obtaining UHT milk. According to Postollec *et al.* (2010), the lack of a fast and efficient routine diagnostic method to detect and identify spore-forming contaminants represents a major hurdle for better understanding and controlling these bacterial communities in food and feedstuff. In addition, according to Lücking *et al.* (2013), information concerning the origin and food quality-related properties of highly heat-resistant spore formers is still limited. To overcome some of the known analytical limitations, Martínez-Blanch *et al.* (2009) developed a highly sensitive real-time PCR (RT-PCR) procedure, targeting the phosphatidylcholine-specific phospholipase C gene. They verified that the RT-PCR assay was suitable for detecting and quantifying strains of *B. cereus* strains in food samples without any enrichment step. In the same way, Fernández-No *et al.* (2011) developed a protocol for real-time PCR to quantify *B. cereus*, *B. licheniformis* and *B. subtilis* in food products, with the aim of preventing the presence of these undesirable species in the food chain.

Overall, preventing contamination by sporulating bacteria in the UHT milk chain is of paramount importance, considering that these bacteria produce proteolytic and heat-resistant lipolytic enzymes that cause off-flavours, sweet coagulation and bitter flavour in milk. Moreover, some species, such as *B. cereus*, may form biofilms on poorly sanitised surfaces in the dairy industry (Peña *et al.* 2014). Attention should be given to the presence of this bacterium in dairy products as it is pathogenic and represents a risk to the consumer's health. Additionally, recent research work reported the isolation of *B. cereus* from dairy products, including UHT milk, marketed in Brazil. This bacterium was positive for the *npr* gene, which is considered a reference for molecular detection of proteolytic activity; in addition, the proteolytic activity of *B. cereus* increased with the incubation temperature (Montanhini *et al.* 2013). This can be a potential problem due to the particular conditions used in the storage of UHT milk in Brazilian establishments (>25 °C).

Spore-forming bacteria detection and identification

Table 1 also reports the incidence and identification of spore-forming bacteria present in the UHT commercial brands. A total of 46 (100%) isolates were identified and were characterised as rod-shaped and Gram-positive. Interestingly, all these samples were processed in the south-east region of the country. Among them, 68% were identified as *B. subtilis/amyloliquefaciens*, 6% as *B. licheniformis*, 4% as *B. pumilus* and 2% as *B. megaterium*. Different findings were observed for aerobic spore-forming bacteria present in UHT milk marketed in Tunisia (Aouadhi *et al.* 2014); in this study, *B. sporothermodurans* was the predominant species followed by *B. cereus*, *B. licheniformis* and *B. sphaericus* (15.5, 2.5 and 2.5%, respectively). The difference in the data is caused mainly by the intrinsic and natural microbial load of the raw milk present in these areas and by subsequent transfer of spores in the course of milk processing. This can explain the presence of *B. licheniformis,*

Table 1 Plate counts of mesophilic aerobic and mesophilic aerobic spore formers, and identification, incidence and spoilage potential of mesophilic aerobic spore formers bacteria of UHT milk samples

Commercial brand	*Location*	*Number of samples*	*Mesophilic aerobic (cfu/mL)*	*Mesophilic aerobic spore formers (cfu/mL)*	*Number of spore formers isolates identified*	*Identification and Incidence of the bacterium in the samples*	*Spoilage*	
							Proteolytic	*Lipolytic*
A	Southeast	5	$1.8 \times 10^4 \pm 1.1 \times 10^4$	$3.7 \times 10^3 \pm 4.8 \times 10^3$	4	*Bacillus subtilis /amyloliquefaciens* (25%)	−	−
						Bacillus pumilus (25%)	−	−
						Bacillus firmus (25%)	−	+
						Brevibacillus (25%)	−	+
B	Southeast	8	$3.7 \times 10^3 \pm 1.1 \times 10^4$	$<1.0 \times 10^0 \pm 0.0$	4	*Bacillus subtilis/amyloliquefaciens* (100%)	−	−
							−	−
							−	−
							+	+
C	Southeast	7	$1.6 \times 10^4 \pm 2.8 \times 10^4$	$1.9 \times 10^2 \pm 4.1 \times 10^2$	7	*Bacillus subtilis/amyloliquefaciens* (100%)	−	−
							−	−
							−	−
							+	+
							+	+
							−	−
							+	+
D	Southeast	7	$<1.0 \times 10^0 \pm 0.0$	$<1.0 \times 10^0 \pm 0.0$	0			
E	Southeast	9	$9.6 \times 10^1 \pm 2.1 \times 10^2$	$1.0 \times 10^2 \pm 2.2 \times 10^2$	5	*Bacillus mycoides* (20%)	+	+
						Bacillus licheniformis (60%)	+	+
						Bacillus pumilus (20%)	−	−
F	Southeast	1	3.2×10^2	3.2×10^2	3	*Brevibacillus* (33.3%)	−	−
						Bacillus subtilis/amyloliquefaciens (33.3%)	−	−
						Bacillus lentus (33.3%)	−	−
G	Southeast	6	$<1.0 \times 10^0 \pm 0.0$	$<1.0 \times 10^0 \pm 0.0$	0			
H	Southeast	7	$<1.0 \times 10^0 \pm 0.0$	$<1.0 \times 10^0 \pm 0.0$	0			
I	Southeast	7	$1.2 \times 10^4 \pm 2.3 \times 10^4$	$1.6 \times 10^3 \pm 2.8 \times 10^3$	5	*Bacillus subtilis/amyloliquefaciens* (60%)	+	−
						Brevibacillus (20%)	−	−
						Bacillus megaterium (20%)	−	−
J	Southeast	4	$<1.0 \times 10^0 \pm 0.0$	$<1.0 \times 10^0 \pm 0.0$	0			
K	Southeast	8	$<1.0 \times 10^0 \pm 0.0$	$<1.0 \times 10^0 \pm 0.0$	0			
L	Southeast	4	$9.3 \times 10^3 \pm 1.6 \times 10^4$	$3.2 \times 10^2 \pm 6.5 \times 10^2$	6	*Bacillus subtilis/amyloliquefaciens* (100%)	+	+
M	Southeast	2	$1.3 \times 10^4 \pm 1.2 \times 10^4$	$4.3 \times 10^3 \pm 5.4 \times 10^3$	5	*Bacillus subtilis/amyloliquefaciens* (100%)	+	+
N	Midwest	2	$<1.0 \times 10^0 \pm 0.0$	$<1.0 \times 10^0 \pm 0.0$	0			
O	South	2	$<1.0 \times 10^0 \pm 0.0$	$<1.0 \times 10^0 \pm 0.0$	0			
P	Southeast	4	$3.0 \times 10^0 \pm 5.0 \times 10^0$	$3.0 \times 10^0 \pm 5.0 \times 10^0$	2	*Bacillus mycoides* (50%)	−	−
						Bacillus subtilis/amyloliquefaciens (50%)	+	+
Q	Southeast	2	$<1.0 \times 10^0 \pm 0.0$	$<1.0 \times 10^0 \pm 0.0$				

(continued)

Table 1 (Continued)

Commercial brand	Location	Number of samples	Mesophilic aerobic (cfu/mL)	Mesophilic aerobic spore formers (cfu/mL)	Number of spore formers isolates identified	Identification and Incidence of the bacterium in the samples	Spoilage	
							Proteolytic	Lipolytic
R	Northeast	2	$<1.0 \times 10^0 \pm 0.0$	$<1.0 \times 10^0 \pm 0.0$				
S	Southeast	2	$1.3 \times 10^4 \pm 1.4 \times 10^3$	$7.7 \times 10^3 \pm 4.7 \times 10^3$	5	*Bacillus subtilis/amyloliquefaciens* (80%)	+	+
						Bacillus mycoides (20%)	+	+
U	South	2	$<1.0 \times 10^0 \pm 0.0$	$<1.0 \times 10^0 \pm 0.0$	0			

B. subtilis and B. pumilus, which, although not believed to be highly heat tolerant, were isolated in this research.

According to Vaerewijck *et al.* (2001), *B. licheniformis* and *B. subtilis* are species that may, when in high concentrations, cause sensorial deterioration in UHT milk, decreasing its quality during storage. The same authors isolated heat-resistant spores from silages offered to the dairy cattle and identified *B. subtilis, B. amyloliquefaciens* and *B. pumilus,* clearly indicating possible contamination of feedstock and animal feed by soil fragments. It is also noteworthy that reprocessing UHT milk lots is also a way to contaminate the milk, as the exchange of coils and the processing stops may be considered to be additional sources of contamination with bacterial spores. Indeed, *Bacillus* species found in milk and dairy products do not originate exclusively in either raw or pasteurised milk but from postpasteurisation contamination and propagation during the manufacturing process (Banykó and Vyletelová 2009).

UHT milk present multifactorial drawbacks related to contamination of spores as the local microbial ecology of the soil, the type of animal feeding, the farming practices, the weather and the hygienic practices during processing contribute significantly to the contamination of milk. Along the milk processing chain, milking equipment and packaging material can also be a detrimental route of spoilage (Carlin 2011). Heat-resistant spores have already been highlighted as a problem in the dairy industry. It can be assumed that these spores are initially introduced due to bad practices in dairy farming as the change from land-own crops to the extensive use of new feeds and feed ingredients; ingredients such as manioc, coconut meal and citrus pulp can be a source of unknown spore-forming species (Scheldeman *et al.* 2006; Quigley *et al.* 2013). In addition, seasonal changes also deserve attention by farmers as recent research reports an effect of this on the bacteria present in the raw milk: in the summer, a higher incidence of thermophiles at dairy farms in US Midwest area was detected (Buehner *et al.* 2014).

Finally, it should be considered that the raw milk used to manufacture UHT milk with a high preprocessing microbial count is more susceptible to gel formation than milk with a low count. Micro-organisms that produce heat-stable enzymes cause the most serious gelation problems. Longer refrigeration times prior to sterilisation allow increased growth of psychrotropic micro-organisms and concomitant production of heat-stable enzymes, especially proteinases and lipases (Chavan *et al.* 2011).

Spoilage potential

Table 1 also reports the spoilage potential of the isolates obtained from UHT milk. A total of 26.1% of isolates showed proteolytic activity at 21 °C and at 35 °C, while 28.3% showed lipolytic activity at these temperatures. However, not even one isolate grew at 6.5 °C in other words,

the isolates were not psychrotrophic micro-organisms. It is relevant to mention that the isolates belong to UHT milk processed in south-east region, which suggest a specific problem in the handling step of the raw milk in this area. UHT milk available in Brazilian market is kept at room temperature (~25 °C) but the temperature can reach higher values depending on the region. Thus our findings suggest Brazilian UHT milk contains a considerable aerobic mesophilic and spore-forming micro-organism load, suggesting a high risk of deterioration during the commercial shelf life.

A recent study reported that the spoilage potential of mesophilic spore-forming bacteria that produce thermoresistant spores is much higher than the spoilage potential of thermoresistant spores isolates that belong to strictly thermophilic species (Lücking *et al.* 2013). According to these authors, although the latter microbial group is frequently found in foods, they are not associated with spoilage. Overall, it is assumed that highly heat-resistant aerobic spore-forming bacteria are a problem related to food quality rather than a food safety issue.

Overall, our findings reinforce the need for strict adoption of measures concerning raw milk collection and handling by producers, demonstrating that there is a real risk of spoilage of UHT milk during the commercial shelf life by spore-forming bacteria. In this sense, the principles of food safety systems based on Hazard Analysis and Critical Point Control (HACCP) are needed and recommended in order to prevent contamination of the raw milk (Cusato *et al.* 2013, 2014), because these bacteria produce not only spores that remain in the product after UHT processing, but also enzymes associated with technological problems such as off-flavours, sweet clotting and bitter flavour (Baur *et al.* 2015; von Neubeck *et al.* 2015). Indeed, dairy processing plants showed very poor hygiene practices and HACCP compliance as well as limited food safety training for permanent workers and did not extend such training to management, technical staff or temporary workers (Karaman 2012)

Further studies should also evaluate the behaviour of these spore-forming bacteria during storage of the milk, with the aim of developing predictive models to better understand the problems and more effectively formulate decisions (Lobacz and Kowalik 2015).

CONCLUSIONS

This study revealed that Brazilian UHT commercial milk samples are contaminated with aerobic mesophilic and spore-forming micro-organisms, which consequently affect the commercial shelf life of this food. This finding can have important economic consequences, as such contamination can increase the probability of consumers purchasing a product that may have problems related to appearance and flavour. Therefore, the poor microbiological quality of UHT milk reinforces the importance of implementing good hygienic practices during the production, storage, transport and processing steps along the milk processing chain as a way of preventing contamination and consequent loss of final product quality.

Moreover, the implementation of bactofugation or microfiltration during raw milk processing in Brazil in order to remove these micro-organisms prior to UHT treatment is important. Overall, there is a need for periodic monitoring with regard to the microbiological quality of UHT Brazilian milk in order to detect any spoilage risk during the shelf life.

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