

## Full Length Research Paper

# ***Escherichia coli* O157, *Staphylococcus aureus* and coliforms in crude and processed bovine milk marketed**

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Fifty-five (55) samples from five municipalities in the Recôncavo from Bahia region were analyzed between May and July 2015 to evaluate the sanitary quality and the presence of pathogens of crude and processed bovine milk samples. Psychrotrophic, mesophilic and thermophile rates and the count of *Escherichia coli*, *E. coli* O157: H7 *Staphylococcus aureus* and *Listeria monocytogenes* were done by microbiological methods rapid detection. There was a greater contamination and presence of pathogens in the raw milk when compared to processed milk. However, total coliforms were detected in 14.28%, *E. coli* in 7.14% and *E. coli* O157: H7 in 2.04% of processed milk. The enforcement by authorities against the illegal sale of raw milk and the monitoring of steps in milk production up to marketing should be mandatory.

**Key words:** Sanitary conditions, dairy industry, food safety.

## INTRODUCTION

Although Brazilian law forbids selling of milk *in natura*, it is still common practice in Brazil, associated with cultural, regional and social factors (Bersot et al., 2010; Menezes et al., 2015).

Dairies use thermal processes that reduce initial microbial load of raw milk, such as pasteurization and Ultra High Temperature (UHT), to commercialize safer food with regard to sanitary hygiene and shelf time increase, but deficient hygiene conditions during milking, handling and conservation are the main factors for decrease in milk quality produced in Brazil (Menezes et al., 2015), damaging the country's economic development in milk production.

Such practice may be harmful to consumer's health

since milk is a good vector of pathogenic microorganisms which cause Food-Transmitted Diseases (FTD) (Claeys et al., 2013).

Pathogenic bacteria in food is a matter of global food security. The risk of human illness associated with raw products can best be predicted by monitoring potential sources of microbial contamination in the area during the collection, during processing and distribution or retail. Thus, rapid and accurate identification of pathogenic bacteria in food samples are important for food quality assurance and to find outbreaks of these bacteria (Bersot et al., 2010; Tamanini et al., 2011; Weschenfelder et al., 2016).

Further, several studies have revealed the bad quality

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of milk produced in Brazil (Tamanini et al., 2011; Pereira et al., 2013; Weschenfelder et al., 2016), affected not merely by prime matter used but by lack of post-processing health control, especially during bottling and packaging. Prevention against milk contamination during milking and storing is mandatory to reduce microbial multiplication and to produce quality milk products (Salvador, 2012).

Since milk is a high economic asset in Brazil (USDA, 2015; IBGE, 2016) and its intake may be a health risk for the population due to microbial multiplication, the aim of the current paper is to assesses raw and UHT milk quality sold in the towns of the Recôncavo from Bahia region. The study is a warning for authorities for drastic control on milk producing farms and in the region's market.

## MATERIALS AND METHODS

### Sampling

Fifty-five bovine milk samples were retrieved and analyzed between May and June 2015: four samples of untreated milk and seven whole UHT milk samples of different trademarks, from each municipality. Samples were purchased in five municipalities in the Recôncavo da Bahia region, namely, Cruz das Almas, Sapeaçu, Governador Mangabeira, Muritiba and São Felipe. Physical integrity, airtightness, packaging and lots verified during UHT milk sample collection were the selection criteria.

Samples were maintained in isothermal boxes with recyclable ice and immediately transported to the Laboratory for Food and Water Analytic Investigation of Agrarian, Environmental and Biological Sciences of the Universidade Federal do Recôncavo da Bahia, Cruz das Almas BA Brazil.

Packages for raw and UHT milk were initially washed with water and neutral detergent, dried with white disposable paper, hygienized with ethanol 70% and homogenized (shaken 25 times). UHT milk samples were incubated at 35-37°C for seven days, following Brazilian legislation (1997, 2001).

### Microbiological analysis

Psychrotrophic, mesophyll and thermophile microorganisms were counted by pour plate technique in Plate Count Agar (PCA) medium (APHA 1.05463.0500). Initially it was used 1 ml of each milk sample and transferred into a sterile tube containing 9 ml of 0.1% peptone water to give a dilution of  $10^{-1}$ . From this dilution serial dilutions were performed to obtain the dilution  $10^{-5}$ . Further, 1 mL of each dilution was transferred to sterilized Petri plates with 25 mL agar previously combined and warmed at 43-45°C. After homogenization and solidification, the plates were incubated in a at 7°C for 10 days; 35°C for 48 h; 50°C for 48 h for psychrotrophic, mesophyll and thermophile microorganisms, respectively.

Coliforms were counted in medium HiCrome ECC Selective Agar Base-M1294 Himedia® (HiMedia Laboratories Pvt. Ltd., Mumbai-India.) (ISO 9001:2008). Methodology complied to manufacturer's instructions. Colonies ranging between salmon and red color were typical colonies of total coliforms, while dark blue to violet revealed *Escherichia coli* colonies.

*Staphylococcus aureus* populations were calculated by fast method on plates (3M Company) Petrifilm™ (AOAC 2003.11), following manufacturer's instructions. Colonies of a red-violet color were typical of *S. aureus*.

*E. coli* O157:H7 analysis initially comprised a warming stage; the samples were then added to the selective enrichment broth MTSB Novobiocin, MERCK™, and incubated at 35°C-37°C for 18-24 h (Ahmed and Shimamoto, 2014).

Species of *E. coli* O157:H7 was identified by fast immunological scanning Singlepath®- *E. coli* O157, MERCK™ (Merck KGaA, Darmstadt - German) (AOAC 010407), following manufacturer's instructions. Test apparatus was incubated at room temperature and result was given 20 min after the sample was applied to the apparatus. A negative result for strain *E. coli* O157:H7 occurred when only a red line appeared within the control zone (C); result was positive when red line appeared in tests (T) and control (C).

*Listeria monocytogenes* was identified by fast kit Singlepath® L'mono, MERCK™ (1.04148.0001). Milk samples were previously added to Brain and Heart Infusion (BHI) broth (Merck KGaA, Darmstadt - German) and incubated at 29-30°C for 24 h, for selective enrichment. The presence of *L. monocytogenes* in kits with red line in the test zone (T) and in the control zone (C) of the apparatus was positive, but negative when no line occurred in the test zone (T), although it appeared clearly in the control zone (C).

Mean number of colonies in all plates was multiplied by the respective dilution factor and results were given in log CFU/ml (Brasil, 2003). Moreover, statistical analysis was undertaken by SPSS 17. Averages and standard deviation of the microorganisms were calculated with descriptive analysis.

### Statistical analysis

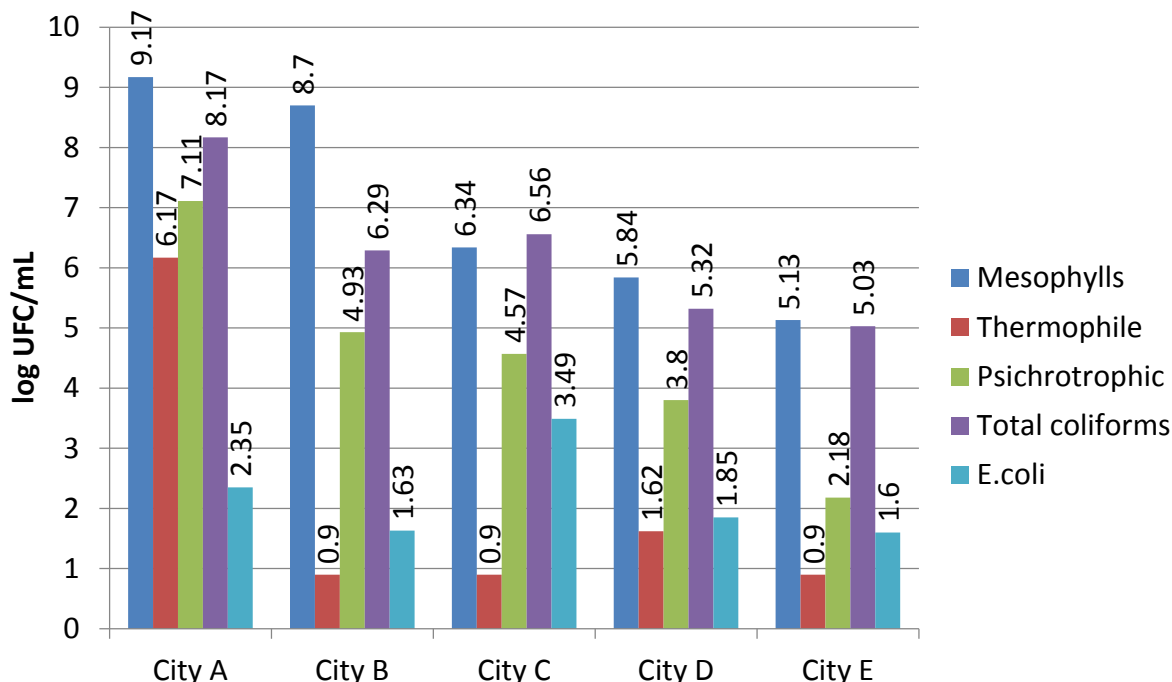
Means were compared by Student's *t* test for independent samples and evaluated whether there were any differences in the quantity of microorganisms according to type of milk. Pearson  $\chi^2$  test was employed to analyze qualitative categories, whilst rates  $p \leq 0.05$  were significant.

## RESULTS AND DISCUSSION

Since the commercialization of raw milk is illegal, Brazilian legislation has not provided parameters for the product. When Norm 62 published on 29 December 2011 (Brasil, 2011) on refrigerated raw milk for processing is applied, it has been found that 80.95% of samples revealed mesophyll microorganisms, of which 76.47% were above the rate allowed by current legislation, or rather, 5.87 log CFU/mL (Brasil, 2011). Samples from three out of the five towns averaged above the acceptable rate (Figure 1).

High population of mesophyll microorganisms in the raw milk analyzed may be attributed to inadequate sanitary conditions during milking and mainly to lack of refrigeration in transport, storage and commercialization of the product. It was perceived during collection of samples that most samples lacked refrigeration and directly contributed towards the proliferation of mesophyll microorganisms since best temperature for growth was that of room temperature.

Silva et al. (2010) evaluated the physical, chemical and microbiological quality of raw milk of a dairy farm in the state of Rio Grande do Sul and reported that three out of the six milk samples analyzed were above legal rates (Brasil, 2011) for mesophyll microorganisms. Similarly to current study, Silveira and Bertagnolli (2014) analyzed



**Figure 1.** Means of microbial populations in milk samples in natura traded informally in the 2015 Bahia Reconcavo cities.

the quality of raw milk commercialized illegally in the street fairs of Santa Maria-RS Brazil and detected that three out of ten samples failed to comply with Norm 51 of 20/09/2002 for mesophyll organisms in refrigerated milk.

On the other hand, samples of UHT milk complied with Rule 146 of 07/03/1996 with rates up to 2 log CFU/mL for mesophylls. Studies by Frata et al. (2014) provided similar results, or rather samples were within the maximum mesophyll limit for UHT milk. However, Domareski et al. (2010) assessed UHT milk commercialized in three Mercosur countries (Brazil, Argentina and Paraguay), respectively reported 37.5, 62.5 and 12.5% mesophyll aerobic bacteria in milk, and thus failing to comply with microbiological criteria for UHT milk.

Storage temperature and type and initial microbial load are parameters that contribute towards the proliferation of bacteria during the storage of raw milk even when submitted to low temperatures. Raw milk kept for long periods at low temperatures may reveal psychrotrophic microorganisms and their concentration in milk may be associated to conditions in which the milk was obtained.

Although Brazilian legislation on the subject (Brasil 2001, 2011) does not establish a maximum limit for psychrotrophic and thermophile microorganisms, the Rules for Industrial and Sanitary Inspection of Animal-derived Products (RIISPOA) (Brasil, 1980) determine that milk should have a maximum of 10% psychrotrophic and thermophile microorganisms with total mesophyll counts.

According to the above recommendation, only 9.52% of

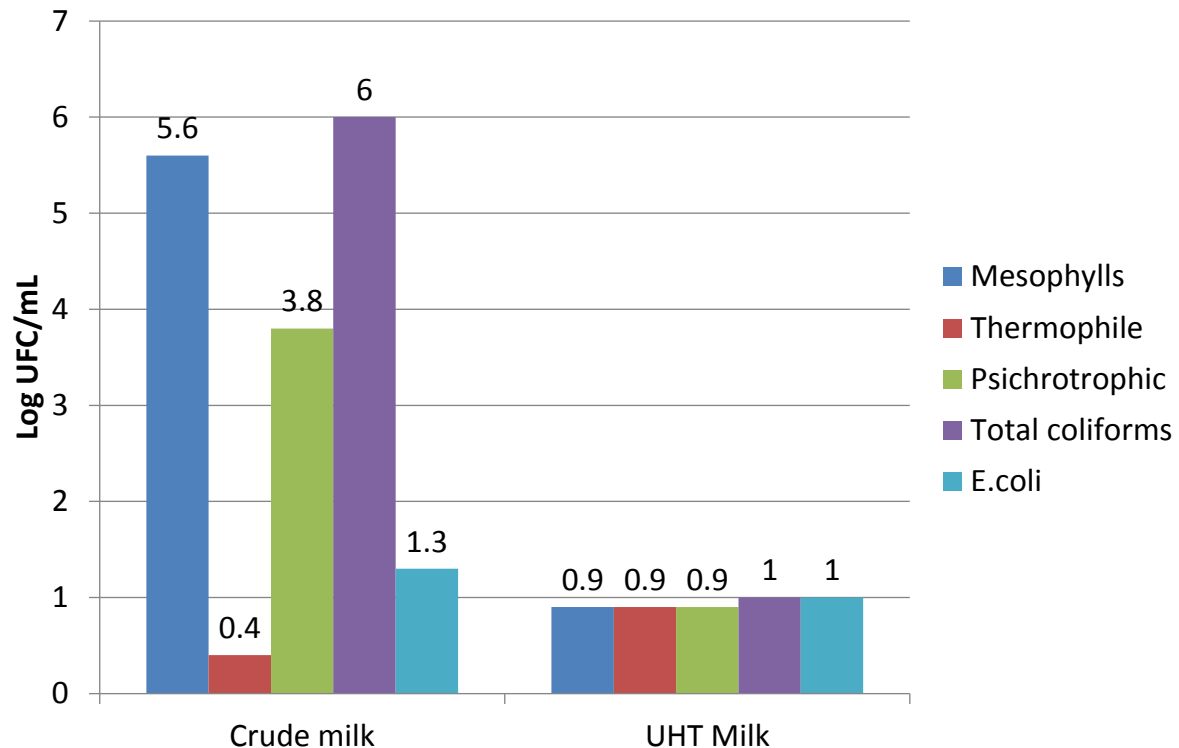
raw milk samples in current analysis had the best pattern for psychrotrophic microorganisms. Zeni et al. (2013) verified the occurrence of psychrotrophic and mesophyll microorganisms in raw milk in the production of UHT milk. Samples of refrigerated raw milk had mesophyll and psychrotrophic counts above 6 log CFU/mL, which will surely interfere in the quality and shelf life of the final product.

Although raw milk does not normally have high rates of thermophile microorganisms, current study reveals averages between 6.17 and 1.62 log CFU/mL respectively for municipalities A and D, with 14.28% of samples above 10% of mesophyll microorganisms, blatantly not complying with current legislation (Brasil, 1980). There was a microbial multiplication of <1 log CFU/mL in all UHT milk samples.

Further, 90.47% of samples had over 5 log CFU/mL of total coliforms in raw milk. Although there is no maximum limit for total coliforms in raw milk when current sanitary legislation is taken into account, the microbial load is high in the samples. In fact, microorganisms reveal flaws in hygiene control and the possible presence of pathogenic microorganisms.

Corroborating results in current analysis, Dias et al. (2015) assessed the hygiene and sanitary conditions of raw milk commercialized in outdoor markets in the north of the state of Piauí, Brazil, and found that the 16 samples analyzed were positive for total and thermotolerant coliforms.

In a study in Solânea in the state of Paraíba, Brazil,



**Figure 2.** Compare the means of populations of microorganisms found in the types of milk.

Amaral and Santos (2011) reported that raw milk sold by street vendors had more than 1.100 most probable number (MPN/mL) of total and thermotolerant coliforms. Another study with similar results was performed by Maciel et al. (2008). The authors analyzed 30 samples of raw milk and detected total coliforms in all samples.

Samples from municipalities A and B showed average of 0.87 and 2.25 log CFU/mL of total coliforms in UHT milk, respectively, and thus not adequate for consumption. In fact, Resolution 370 of 4/9/1997 rules that UHT milk should not contain microorganisms which are able to proliferate at normal storage and distribution conditions after the incubation of the closed package at 25-37°C for seven days.

According to Menezes et al. (2015), hygienic milking procedures, cleansing and disinfection of utensils and equipments are basic to avoid milk contamination by coliforms. Training of milk producers with regard to hygiene at all stages in the provision and commercialization of raw milk is mandatory to guarantee the safeness of the product for industrialization.

*E. coli* was detected in samples of raw milk from all the municipalities and varied between <1 and 1.64 log CFU/mL, whereas it was detected in UHT milk from one municipality only, averaging 1.46 log CFU/mL. Its occurrence and its high population in milk are highly relevant for public health since, besides the existence of pathogenic strains, it is the main indicator for feces-

caused contamination.

Microbiological analysis of raw milk revealed *S. aureus* in 76.19% of all samples analyzed, varying between <1 and 9.0 log CFU/mL. UHT milk samples had rates lower than <1 log CFU/mL. Student's *t* test showed a statistical difference between raw and UHT milk, with higher contamination in the former ( $p < 0.05$ ).

Although there is no maximum limit for *S. aureus* in raw milk, its occurrence is associated with lack of hygiene of handlers' hands, utensils used in milking, bad storage, transport and commercialization conditions. The above contamination situation was observed during sampling. In fact, milk could be found in inadequate containers, without any identification, featuring dirt rates. Others lacked refrigeration and this fact may have contributed towards the multiplication of the microorganism in most samples.

One may conclude that average counts for *S. aureus* populations in the milk from the municipalities were similar and showed that the presence and high population rates of *S. aureus* is normal in raw milk (Figure 2)

Research evaluating the microbiological quality of raw milk informally commercialized in Areia PB Brazil showed 80% of samples were contaminated by *Staphylococcus* sp (30% were presumably *S. aureus*) corroborating results in current study (Souza et al., 2011). Alves et al. (2009) assessed the microbiological quality of raw milk

commercialized in São Luís MA Brazil, and detected strains of *Staphylococcus* positive coagulase in 31.0% of samples.

Similar results were reported for UHT milk by Nascentes and Araújo (2012) who compared the microbiological quality of raw, pasteurized and UHT milk commercialized in Patos de Minas MG Brazil. The authors reported that all samples of raw milk were contaminated by *S. aureus*, whereas no growth of the microorganism was detected in UHT milk sample.

*E. coli* O157:H7 occurred in 6.12 and 2.04% of raw and UHT milk samples respectively, or rather, a potential risk for consumers since it is a pathogenic strain. Further, Pearson's  $\chi^2$  test showed no significant difference between the types of milk studied with regard to *E. coli* O157:H7 ( $p > 0.05$ ). Since the microorganism is highly susceptible to heat, it may be easily destroyed during milk processing. However, post-processing contamination may occur due to its ability to form biofilms on utensils and equipments. According to Pillai and Jesudhasan (2006), the ability of *E. coli* O157:H7 in forming biofilms may be attributed to the self-induction-2 signal involving the regulation of genes in chemotaxis, flagellar synthesis and motility.

Batista et al. (2014) evaluated the hygiene and sanitary parameters of raw milk on 26 farms in the region of the Recôncavo da Bahia BA Brazil and reported one positive sample of *E. coli* O157:H7.

Above data show the importance of permanent studies on raw and processed milk, underscoring the need for the non-commercialization of raw milk directly to the consumer and the hygiene conditions during the post-processing stage.

It should be emphasized that *E. coli* O157:H7 in UHT milk is a serious factor due to the fact that milk had undergone commercial sterilization process and the consumer is liable to be contaminated by the pathogenic microorganism by purchasing an unsafe product.

In the case of *L. monocytogenes*, the result in all milk samples proved to be negative. In a study on *Listeria* spp. in raw and pasteurized milk, 10% of raw milk samples were contaminated by the microorganism, with no contamination of any pasteurized milk sample (Almeida et al., 2013).

Although current analysis did not reveal *L. monocytogenes*, its presence in milk is of great concern due to the microorganism's high pathogenicity which causes listeriosis worldwide. Lack of detection of the pathogenic microorganism may also be related to its low capacity of competition by nutrients and thus undetectable, albeit present.

When all the aspects evaluated and results for microorganisms which indicate sanitary and pathogenic quality in raw milk in the municipalities under analysis are taken into account, the samples do not comply with current Brazilian legislation for refrigerated raw milk (Brasil, 2011) with regard to microbiological aspects. In

fact, it is risk for public health and may be a FTD vector.

Good Practices in handling should be practiced by producers to reduce diseases caused by the incorrect handling of raw milk on the farms and in storage, transport and commercialization of the final product after a correct processing. Efficient measures for the eradication of the sale of raw milk should be aimed at by the health authorities since no commercialization of such a product should occur.

When the microbiological quality of UHT milk samples commercialized in the municipalities evaluated is assessed, several samples were not fit for consumption, possibly by post-processing microbial contamination by pathogens *E. coli* and *E. coli* O157:H7.

Since the microorganisms analyzed were not resistant to extreme temperatures such as in UHT milk processing, it may be suggested that samples with pathogenic microorganisms were contaminated after thermal processing either during packaging or due to biofilms on the equipments or deficient processing. According to Vittori et al. (2008), post-processing contamination may be due to deficiency in packaging sterilization or to recontamination by handlers. The emergence of biofilms in milk processing environments contributes towards an increase in the probability of microbial contamination of processed milk products with deteriorating and pathogenic microorganisms (Marchand et al., 2012).

Bad quality of raw milk used as prime matter and preservation problems during commercialization may also cause microbial contamination in processed milk with the possibility of milk deterioration prior to the recommended preservation period (Dey and Karim, 2013).

Dairy industries should guarantee the quality of the product by sanitary control during the whole processing period, from the acquisition of prime matter to sale.

## Conclusions

- 1) The commercialization of raw milk is highly dangerous for consumers due to rates of pathogenic microorganisms found in it.
- 2) Contamination of UHT milk may have been due to flaws in post-processing sanitary control that favored strains of total coliforms, *E. coli* and *E. coli* O157:H7 in the commercialized milk.
- 3) Negative results occurred for *L. monocytogenes* in all samples under analysis.

## Conflict of Interests

The authors have not declared any conflict of interests.

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