Journal of Microbiology and Antimicrobials Vol. 3(8), pp. 206-212, August 2011
Available online http://www.academicjournals.org/JMA
ISSN 2141-2308 ©2011 Academic Journals

Full Length Research Paper

Aerobic and anaerobic bacteria and *Candida* species in crude milk

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Accepted 24 May, 2011

Since milk is a high nutrition food, it is extremely liable to microbiological contamination. Maximum hygiene in mechanical milking and in health practices management throughout the production process is necessary so that the quality of crude milk is guaranteed. Current research comprises the collection of milk in milk-producing farms during April, May and June 2009 so that the microbiological safety of milk produced in the municipality of Amargosa BA Brazil may be verified. Results show that crude milk samples are unsatisfactory due to the presence of 9.68 and 35.48% of clinical and sub-clinical mastitis, respectively; 12.74% of *Staphylococcus aureus*, 18.26% of entero-bacteria, 12.5% of *Candida albicans*, 7.5% of *Candida krusei*, 10% of *Candida tropicalis* and 42.5% of other types of *Candida*. It is mandatory that sanitary education should be provided to milk-producing farmers so that the microbiological quality of milk could be improved.

Key words: Crude milk, microbiology, entero-bacteria, fungi.

INTRODUCTION

Due to the number and type of microorganisms, undesirable alterations occur in the milk's appearance, taste and smell, or in its derivatives. Since certain microorganisms may jeopardize the consumer's health (Fonseca and Santos, 2000), the milk's microbiological quality may be seen from two different aspects: Industrial quality and risks to public health (Fonseca and Santos, 2000). In the case of industry, several researchers have listed the main risks provided by high microbial load with special reference to problems with acidification and coagulation, gas production, jellification, sour taste, coagulation without acidification, increase in viscosity, change of color, production of several tastes and smells, among others. Such changes cause a decrease in shelf-life and in industrial yield. Besides the industries' quality problems, inadequate conditions of milking management, storage and transport may jeopardize public health due to the activities of pathogenic bacteria, especially when crude milk is consumed. *Listeria, Salmonella, Staphylococcus aureus, Brucella, Mycobacterium* etc. (Brito et al., 1997) are the main pathogenic bacteria found in milk, which may be characterized as mastitis milk.

Mastitis-causing microbiota has been conveniently classified with regard to its origin and transmission mode, namely, (1) contagious or transmissible microorganisms and (2) environmental microorganisms. Contagious microorganisms are mainly transmitted during milking. They may be called "cow-dependent" since they are extant in the cow with or without mastitis. The all-present environmental microorganisms are extant in the air, water, feces and in the cow-shed. The first group

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comprises *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Corynebacterium bovis*, *S. aureus* and other staphylococci. Contagious microorganisms in milk samples originating from the refrigerating tank are consequently a good indicator of intra-mammalian infection by such agents in herd animals. *Streptococcus uberis* and other streptococci (with the exception of those previously cited), bacteria of the family Enterobacteriaceae, filamentous fungi and yeasts, algae of the genus *Prototheca*, *Arcanobacterium pyogenes*, among others, may be found in the second group (Cruz et al., 1994).

Cryptococcus sp., *Rhodotorula* sp., *Aspergillus* sp., *Pichia* sp., *Candida kruse*, *Candida tropicalis*, *Candida albicans*, *Trichosporon cutaneum* and others are the main microorganisms involved in mycosis mastitis. The contamination of milking equipments and utensils by the aforementioned microorganisms may infect a great number of animals even though cases of mycosis mastitis do not normally occur with great frequency. Fungi may occur as contaminating factors in drugs and antibiotics diluting agents, in milking equipment washing water and on milkmen's hands (Cruz et al., 1994).

So that milk quality could be improved, changes are occurring in the milking sector with regard to its refrigeration on the farm and during bulk transport. Milk refrigeration between 4 and 5°C controls the multiplication of mesophyl microorganisms. This alteration has been introduced under the aegis of the Brazilian Normative Instruction 51 (Brasil, 2002) which has contributed towards the modernization of the milk market through a more attractive product within Brazil and abroad. Interesting programs for producers such as the National Program for the Control and Eradication of Brucellosis and Tuberculosis aiming at decreasing the incidence of pathogenic microorganisms in milk and thus guaranteeing its quality are worth mentioning. Current research, therefore, undertaken on six milk-producing farms in the municipality of Amargosa BA Brazil, verifies the incidence of aerobic and anaerobic bacteria of the species *Candida* in crude milk.

MATERIALS AND METHODS

Current research was undertaken on six small milk-producing farms in the municipality of Amargosa BA Brazil during April, May and June 2009; whereas on three farms milking was produced manually, in the others milking production was mechanical.

Collection of milk samples was undertaken in 10% of milk-producing females during the first milking session of the day. Hygienization of the cows' udders was done with water and udders were dried with paper towels; teat canals were then cleansed with ethylic alcohol 70%. Four samples, one from each teat, were retrieved from each animal and the material was conditioned aseptically in 500 ml-glass flasks, placed in isothermal boxes with ice and sent to the Milk Technology Laboratory of the Universidade Federal do Recôncavo da Bahia, where analysis were undertaken on the same day of collection.

Milk samples collected for the isolation of aerobic and anaerobic bacteria were inoculated in blood and MacConkey agar and incubated at 37°C for 24 h. Plates were then examined for bacterial growth and when negative they were re-incubated for 48 to 96 h at 37°C. Bacterial isolates were characterized by macro- and micro-morphology, Gram staining and bio-chemical tests with different types of sugars. Other tests include motility, coagulase, catalase and oxydase reactions so that the different bacterial isolated could be identified. Special tests for suspected organisms, such as CAMP test for *S. agalactiae*, methylene blue staining for *Corynebacterium* and Ziehl-Neelsen (ZN) staining for *Nocardia*, were done when deemed necessary.

Milk aliquots were also inoculated with Sabouraud dextrose agar and incubated at 28ºC and at 37ºC for 48 to 72 h for fungi isolation. Fungus isolates were classified according to colony characteristics, hyphae's size and shape, microscope exam and formation of germinal appendixes. Other tests, such as carbohydrate fermentation tests, nitrate and urease-carbohydrate tests for the detection of *Candida* and other species were also undertaken.

RESULTS AND DISCUSSION

Milk microbiological analyses provide useful information which shows the conditions in which the former was obtained, processed and stored (Pietrowski et al*.,* 2008). Bacteria counts which indicate the herd's health, sanitation measures taken by the plantation management, milk manipulation and storing temperatures, were obtained (Hayes et al., 2001).

One hundred and twenty-four samples were obtained from 31 milking cows on the six farms under analysis, from all samples submitted to CMT. Table 1 shows the results. Whereas 17 out of the 31 milking cows were negative to mastitis, only 3 showed clinical mastitis and eleven revealed sub-clinical mastitis, one of the main causes of economical losses worldwide associated with bovine mastitis.

The milk-producer's economical losses may be attributed to a decline in milk production (70%), costs of treatment, laboratory exams and veterinary expenses (8%), decrease in milk quality and in price paid, loss in production from milk waste during the infection and treatment period (8%), decrease in production potential for the remaining lactation period, increasing risk in subsequent infections, increase in early animal waste and risks in the animal's death (14%) (Wescor, 1995).

Results shown in Table 1 corroborate the fact that mastitis prevention is a key factor in the reduction of losses in this entrepreneurship. According to Fonseca and Santos (2000), frequently serious faults in the milking system may be found which jeopardize the most elementary task of a milk-producing farm, which comprises the simple job of retrieving milk from the mammalian gland. Brito et al. (1997) consider CMT's regular use as an important tool for the improvement of the herd's health state which gives orientation on the adoption of means for mastitis control or associated with

Table 1. California mastitis test (CMT) test results from six milk-producing farms in the municipality of Amargosa BA Brazil.

Farm	Milking cows	Animals*	CMT results
1	63	2	Negative
		3	Sub-clinical mastitis
		1	Clinical mastitis
2	50	1	Negative
		3	Sub-clinical mastitis
		1	Clinical mastitis
3	30	2	Negative
		1	Clinical mastitis
4	100	9	Negative
		1	Sub-clinical mastitis
5	40	2	Negative
		2	Sub-clinical mastitis
6	30	1	Negative
	30	2	Sub-clinical mastitis

*Number of animals undergoing test (10% of milking cows).

proper management and hygiene practices. According to Dingwell et al. (2003), CMT has an important effective role in milking programs such as sampling tests for detecting cows with sub-clinical mastitis.

Investigating milk-producing farms in the state of Rio Grande do Sul, Brazil, Ribeiro et al. (2003) co-related milk samples derived from cows with clinical mastitis, sub-clinic infectious and non-infectious mastitis, directly and indirectly, with CMT and microbiological examination. Results from the aforementioned research with regard to the identification of sub-clinical mastitis by CMT suggested great caution when it is used to indicate intra-mammalian infection owing to the lack of bacteriological growth in a great number of samples.

Mastitis, an inflammation in the cow's udder, is caused by microorganisms freely encountered in the environment. Fungi are opportunistic microorganisms which parasite the animal's weakened immunological system and develop by causing disease and impairing the normal flow of milk, with heavy loss for the farm owner. *Candida* sp. normally lives in saprobiosis, although in favorable circumstances, it may develop its pathogenic potential. As a rule, *Candida* sp. occurs in milk without any associated pathogens, although it may cause mastitis in the sub-clinical, clinical or chronic modes. Decrease in milk production may be fast, with macroscopic changes in the milk, coupled to the formation of flakes and grains, in proportion to the infection's severity (Wawron and Szczubial, 2001).

Table 2 shows results with regard to research on fungi of the species *C. albicans*, *C. tropicalis*, *Candida krusei* and others. Identification test for *Candida* was done, in duplicate, in twenty samples; forty samples in all. There would have been 24 samples, in duplicate, for 48 samples. However, only bio-chemical tests with 6 samples were undertaken on Farm 4 since 90% were negative for mycosis mastitis. Table 2 shows that only 2.5% out of the 20 samples analyzed for biochemical tests failed to showed the presence of *Candida*, even though Table 1 revealed a 50% negative stance for mastitis.

Table 2 shows that the filamentous fungi of the genus *Candida* sp.: *C. krusei, C. albicans, C. tropicalis* and other non-identified types were found in the crude milk directly derived from the cows' teats. Fungi were found in all milk samples, with the exception of a repetition of a sample on Farm 4. High contamination index by *Candida* sp. may be related to lack of hygiene during the milking process.

Filamentous fungi in the samples analyzed included 12.5% *C. albicans*, 7.5% *C. krusei*, 10% *C. tropicalis* and 42.5% other types of *Candida*. Further, more than one filamentous fungus in a single striation was found in several samples (Table 2), or rather, 15% *C. albicans*

Table 2. Results on the isolation and differentiation of fungi of the genera *C. albicans*, *C. tropicalis*, *C. krusei* and other types of *Candida*, isolated from cow's milk from milk-producing farms in Amargosa BA Brazil.

Farm	Isolates	Sample	Presence of *Candida*
1	2	1	Other types of *Candida* *C. albicans*
	2	2	*C. krusei* Other types of *Candida*
	2	3	*C. albicans* and *Candida krusei* *C. krusei*
	2	4	Other types of *Candida* *C. albicans* and *C. krusei*
	2	5	*C. tropicalis* *C. albicans*
	2	6	*C. albicans* *C. albicans* and *C. krusei*
2	2	1	*C. albicans* and *C. krusei* *C. tropicalis* and *C. krusei*
	2	2	*C. tropicalis* *C. tropicalis*
	2	3	*C. albicans* and *C. krusei* *C. tropicalis* and *C. krusei*
	2	4	*C. tropicalis* Other types of *Candida*
	2	5	Other types of *Candida* Other types of *Candida*
3	2	1	Other types of *Candida* Other types of *Candida*
	2	2	Other types of *Candida* Other types of *Candida*
	2	3	Other types of *Candida* *C. albicans*
4	2	1	*C. albicans* and *C. tropicalis* Other types of *Candida*
	2	2	Other types of *Candida* *C. albicans*

Table 2. Contd.

2	3	*C. albicans* and other types of *Candida* Other types of *Candida*
2	4	*C. krusei* *C. albicans* and *C. krusei*
2	5	Absence of *Candida* Other types of *Candida*
2	6	Other types of *Candida* Other types of *Candida*

and *C. krusei*, 5% *C. tropicalis* and *C. krusei*, 2.5% *C. albicans* and other types of *Candida* and 2.5% *C. albicans* and *C. tropicalis*, respectively in some samples. Whereas fungi may be isolated from milk by microbiological tests, in current research, filamentous fungi of the genus *Candida* were isolated. Although 50% of analyzed milk did not present mycosis mastitis (Table 1), 97.5% of biochemical tests were positive to *Candida* (Table 2). In the state of São Paulo, Brazil, *C. albicans* was isolated in 8.9% of the 260 milk samples from mastitis-infected cows (Santos and Marin, 2005). In the state of Rio Grande do Sul, Brazil, Ferreiro et al. (1985) detected *Candida* sp. in 1.3% of samples from 896 teat milk samples and found that 0.9% were *C. albicans*. Moreover, another study in the same state failed to detect *C. albicans* in the milk of animals with clinical and sub-clinical mastitis, even though the species of the genus *Candida* were 37.9% of fungus isolates (Spanamberg, 2008).

Results show that although fungi may peacefully live with the host, they may develop their pathogenic power and cause infections when they encounter favorable conditions. Opportunist mycoses may be associated with factors which are intrinsic (neoplasia, diabetes and others, coupled to diseases that change the cell immunity) or which are extrinsic (antibiotic-therapy, corticoid-therapy and others) to the host. As a rule, these microorganisms are associated with pathogenic issues such as allergies, otomycoses, keratomycoses, lesions of the hard palate, meningitis, endocarditis, systemic, gastro-intestine, pulmonary, genital-urinary, cutaneous, subcutaneous and bone diseases (Trabulsi et al., 1999).

The consumption of contaminated milk by fungi of the genus *Candida* sp., or by toxins produced by them may be harmful to human health. Risks are always present since the product did not undergo any pasteurization and thus people, especially sick ones, may receive opportunistic mycoses. Adequate management procedures, especially concerning the milking process, and hygiene methods employed at this stage of milk production, may lessen the occurrence of fungus-produced mastitis and decrease its contamination during the milking process.

Samples from three milk-producing farms in Amargosa BA Brazil were employed for the isolation of aerobic and anaerobic bacteria (Table 3). When analyzed, 96 bacterial colonies were isolated. *S. aureus* was identified in two colonies with 9.30 and 3.44%, respectively on Farms 1 and 3.

Pathogenic bacteria in crude milk are a public health issue due to the potential risk to people who consume it directly or its derivates and to those who handle it.

According to results shown in Table 3, great quantities of the family Enterobacteriaceae were found, with 2.32% of the species *Pragia fontium, Xenorhabdus nematophilus* and *Phtorhabdus asymbiotica* (Farm 1); 12.5% of the species *P. asymbiotica*, *Photorhabdus luminescens* and *Yersinia aldovae* (Farm 2) and 3.44% of *X. nematophilus, P. asymbiotica* and *Shigella dysenteriae* (Farm 3).

Streptococcus sp., which occurred in all farms, was identified micro-morphologically by Gram staining. Within the context of Gram-negative isolates submitted to oxydase biochemical test, 85.71% featured negative oxydase, and thus facultative anaerobic bacteria.

High contamination in crude milk samples may be associated with inadequate hygienization procedures in the production system in which milking utensils and equipments are a relevant source in milk contamination. In fact, milk residues on the surface of the utensils used are nutrients for the growth of bacteria which contaminate the product and slowly multiply themselves. Further, contact of the milk with the animals and an unclean environment directly affect the microbial contamination rate (Guerreiro et al., 2005).

The occurrence of *S. aureus*, a subspecies of the entero-bacteria genus, and *Estreptococcus* sp. are related to positive results for sub-clinical and clinical mastitis identified by CMT (Table 1), which was applied to 10% of the milking cows in each farm in Amargosa. This

Table 3. Aerobic and anaerobic microorganisms identified in crude milk samples from farms in Amargosa BA Brazil.

Farm	Isolated species	Number of isolate	Percentage (%)
1	*S. aureus*	4	9.30
	P. fontium	1	2.32
	X. nematophilus	1	2.32
	P. asymbiotica	1	2.32
	S. sp.	10	23.25
2	*P. asymbiotica*	3	12.5
	P. luminescens	3	12.5
	Y. aldovae	3	12.5
	Streptococccus sp.	6	20.68
3	*X. nematophilus*	1	3.44
	P. asymbiotica	1	3.44
	S. dysenteriae	1	3.44
	S. aureus	1	3.44

fact suggests mammalian gland infection caused by these microbial agents.

In their studies on the quality of crude milk, Fagliari et al. (1990) detected *S. aureus* in 54.0% milk samples of animals with clinical mastitis and in 40.7% of samples in animals with sub-clinical mastitis in Ilha Solteira SP, Brazil.

The microorganisms of the genus *Staphylococcus* are also harmful to the population which consumes mastitis-infected milk, since certain strains may produce thermo-stable toxins and cause food intoxication, with severe processes of gastroenteritis especially in children and in the elderly.

The most common contamination sources by the entero-bacteria group are feces (human or animal), milkmen and water. Santos and Marin (2005) isolated individually 2.44% of entero-bacteria in 82 milk samples (2/82) from cows with mastitis in Uberlândia MG Brazil.

Although more than 80 different microorganisms have been identified as bovine mastitis-causing agents, the species which are more frequently isolated are *S. aureus*, *S. agalactiae, S. dysgalactiae*, *S. uberis* and *Escherichia coli.*

Kapronezai et al. (2005) evaluated 467 milk samples from 119 buffalo cows in India through several indirect tests to compare the efficiency of modified CMT in the detection of sub-clinical mastitis. The authors reported a 26.77% rate in positive mammalian quarters by the microbiological exam, in which *Staphylococcus* spp. (38, 99%), *Bacillus* spp. (18, 24%), *E. coli* (18, 24%), *Klebsiella* spp. (11, 3%), *Corynebacterium* spp. (6, 29%) and *Streptococcus* spp. (3, 78%) were isolated. The genus *Streptococcus* may cause several infections in humans, such as meningitis, pneumonia, endometriosis, pyelonephritis and septic arthritis.

Only 2.5% of the 20 samples analyzed registered *Candida*, even though there was a negative 50% for mastitis. *C. krusei, C. albicans* and *C. tropicalis* were present in milk. High diversity of representatives of the family Enterobacteriaceae occured with 2.32% of the species *P. fontium, X. nematophilus* and *P. asymbiotica* (Farm 1); 12.5% of the species *P. asymbiotica*, *P. luminescens* and *Y. aldovae* (Farm 2) and 3.44% *X. nematophilus, P. asymbiotica* and *S. dysenteriae* (Farm 3). *Streptococcus* sp. occurred in all farms, whereas *S. aureus* was identified only on two farms, with 9.30 and 3.44%, respectively, on Farms 1 and 3.

Identification of aerobic and anaerobic bacteria and of *Candida* species in the milk produced in the Bahia Reconcavo region demonstrate the conditions by which milk is produced and processed by small and medium-sized farmers for whom milk is the main source of income. Milk is obtained under unsatisfactory hygienic and sanitary schemes. Frequently, neither technical assistance nor investments in new technologies and animal improvement are available, with the subsequent low quality of the final product.

Conclusions

Since there is a strict relationship between the occurrences of microbial agents and clinical and sub-clinical mastitis detected in the analyzed samples, public policies directed towards the solution of the problem and the improvement of milk quality are urgent. The revision

of milking procedures, hygienization of utensils, refrigeration tanks for milk storage and adequate management is mandatory. These factors may contribute towards an improvement of milk quality. Finally, a greater financial investment and personnel's capacitation, education and training of farm owners of the analyzed area are recommended owing to the features in the milk-producing farms in Amargosa BA Brazil, aiming at the application of IN 51 of MAPA.

ACKNOWLEDGEMENTS

The authors would like to thank the Research Foundation of the state of Bahia (FAPESB) for granting a Master's scholarship which proved to be highly useful for the execution of the project.

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