Identificação de micro-organismos contaminantes e susceptibilidade a agentes antimicrobianos em amostras de leite de tanques de expansão

Identification of contaminants microorganisms and susceptibility to antimicrobial agents in milk samples from expansion tanks

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RESUMO

O objetivo do presente estudo foi avaliar a qualidade de nove amostras de leites coletadas de tanque de expansão na região de Maringá -PR, tendo em vista os padrões estabelecidos pela Instrução Normativa 62. Foram isolados micro-organismos Gram negativos e determinado o perfil de resistência a antimicrobianos de 29 cepas de *Staphylococcus* spp. e de 21 cepas de *Escherichia coli*. Os resultados mostraram que três amostras estavam acima dos padrões exigidos pela legislação com relação à contagem total bacteriana. Para algumas amostras, as contagens de mesófilos proteolíticos e de psicrotróficos foram consideradas altas. No teste de lactofermentação, três amostras apresentaram formas visuais não desejáveis; no teste de resistência térmica todas as mantiveram-se estáveis. As bactérias Gram negativas foram identificadas como *E. coli*, *Enterobacter agglomerans*, *Serratia liquefaciens*, *Proteus miriabili* e *Enterobacter sakazakii.* A resistência a antimicrobianos observada entre os isolados foi considerada baixa. As amostras de leite analisadas possuem micro-organismos que comprometem sua qualidade.

Palavras chave: antibiograma; identificação bacteriana; leite cru

ABSTRACT

The purpose of this present study was to evaluate the quality of nine samples of milk collected from the expansion tank in the region of Maringá-PR, in view of the standards established by the Normative Instruction 62. The Gram negative microorganisms have been isolated and determined the profile of antimicrobial resistance of 29 strains from *Staphylococcus* spp. and of 21 strains from *Escherichia coli*. The results showed that three samples were beyond the standards required by the legislation in relation to the total bacterial countings. For some samples, the counting of proteolytic mesophilic and psychrotrophic were considered high. In the lacto-fermentation test, three samples presented undesirable visual forms, in the thermal resistance testing all remained stable. The Gram negative bacteria have been identified as *E. coli*, *Enterobacter agglomerans, Serratia liquefaciens, Proteus miriabili* and *Enterobacter sakazakii.* The antimicrobial resistance observed among the isolates was considered low. The milk samples analyzed have microorganisms that compromise their quality.

Key words: antibiogram, bacterial identification, raw milk

INTRODUCTION

The raw milk quality in Brazil is considered in general unsatisfactory associated with high counts of aerobic mesophilic microorganisms, coliforms and psychrotrophic (NERO et al., 2005; MARTINS et al., 2008, PINTO et al 2006). The criteria used to define the quality have been modified to meet the regulatory officials demands, from the industry and consumers and look foward to assist, primarily, the requirements of food safety and better industrial yield (BRESSAN & MARTINS, 2004).

The predominant microbial in the raw milk includes species of lactic acid bacteria (*Lactococcus*, *Lactobacillus* spp., *Leuconostoc*, *Enterococcus* or *Streptococcus* spp.),

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Pseudomonas spp., bacteria belonging to the family Micrococcaceae (*Micrococcus* and *Staphylococcus* spp.) and yeast. Other microbial groups present in raw milk include *Bacillus, Clostridium, Listeria* spp. and enterobacteria. There is also species such as *Acinetobacter, Alcaligenes, Flavobacteriumand Aeromonas, Arthrobacter, Corynebacterium, Brevibacterium* and *Propionibacterium* (LAFARGE et al., 2004).

Many of these microorganisms are psychrotrophic, which, at low temperatures imposed by the storage in expansion tank, can multiply and produce enzymes, proteases and lipase able of deteriorate the milk under refrigeration, not exceeding five days of validity. According to the Regulation of Industrial and Sanitary Inspection of Products of Animal Origin (Brazil -Riispoa, 1980), the milk must have no more than 10% of psychrotrophic microorganisms in relation to the total counting of aerobic mesophilic.

The objective of this work was to determine the counts of mesophilic aerobic, mesophilic proteolytic, *Staphylococcus* and psychrotrophic in milk samples collected from expansion tanks, isolating and identifying the main genders of bacteria belonging to the family *Enterobacteriaceae* and to evaluate the susceptibility of strains from *staphylococci* and *E. coli* to antimicrobial agents.

MATERIAL AND METHODS

It were collected nine samples of refrigerated raw milk, from primiparous and pluriparous cows, from different degrees of blood in different stages of lactation, belonging to nine rural properties of dairy farming located in the region of Maringá, with mechanical milking type, between the months of July and September 2012.

The milk was collected directly from the cooling tank with the aid of a stainless steel shell. The samples were stored in isothermal containers containing recyclable ice, and then stored under refrigeration.

The samples were brought to the Microbiology laboratory (Food Engineering Course), in the Universidade Estadual de Maringá for the microbiological analyzes. For the counting of aerobic mesophilic (default count), appropriate dilutions were inoculated on main agar for counting (Standard Methods Agar), and the plates were incubated at 32 ± 1°C during 48 ± 3 hours according to standard techniques preconized American Public Association (APHA, 2001) and 7°C for 10 days for the evaluation of psychrotrophic count.

For the mesophilic proteolytic bacteria counts we used 20 ml of main agar for counting (PCA) supplemented with 10.0% of skimmed and reconstituted milk powder (RDM) and sterilized. After the homogenization the plates were incubated at 21°C for 72 hours (SILVA et al. 1997).

The milk samples were submitted to proof of lactofermentation the type of microbial mesofilic predominant in the milk based on the aspect, odor and coagulum type formed. Aliquots of 10 ml of milk were incubated at 37°C for 24 to 48 h, for posterior evaluation of the coagulum type formed and for the thermical resistance test. In the test of thermical resistance, the previously sterilized tube containing 10mL of each sample were subjected to a water bath at 75ºC per 1 minute. At the end of this period the readings were performed by observing the aspect of the pipes internal contents (BRAMLEY & MCKINNON, 1990).

For the enterobacteria and *Escherichia coli* counting were utilized Petrifilm plates. The enterobacteria colonies were identified by biochemical kit for enterobacteria (triptofano desaminase, lactose test, H_2S, glucose fermentation, gas production, lysine descarboxilation, indol production, ornithine descarboxilation, motility, citrate utilization, rhamonose fermentation).

For the count of staphylococci was sowed the material in Agar Baird Parker (BP) at 35°C for 24 hours being selected the typical colonies after the Gram test, catalase and coagulase test.

It was used the Kirby & Bauer method (Bauer et al., 1966) to evaluate the profile of in vitro susceptibility of isolates bacteria to the antimicrobial agents: ampicillin (10 mcg), cephalothin (30 mcg), ciprofloxacin (5 mcg) , gentamicin (10 mcg), trimethoprim (5 micrograms) and tetracycline (30 mcg) in concentrations recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2011).

According to this method, isolated colonies of *Staphylococcus* spp. (BP) and *Escherichia coli* (previously isolated in Petrifilm plates and after 24 hours at 44.5° C in EC broth, striated for purification on EMB agar, Eosine Methylene Blue incubated at 35° C for 24 hours) were touched with bacteriological grip and inoculated in 0.9% physiological saline until the turbidity was obtained similar to the scale 0.5 of Mac Farland. The suspension was then inoculated into Mueller-Hinton Agar with the help from a sterilized swab soaked in the suspension. After drying the suspension in agar, the antimicrobial discs were

distributed in equidistantly way on the plate, without exceeding the time of 15 minutes. The plates were then incubated at 37°C for 18-24 hours. After this period, the reading was performed by visualizing and measurement of the diameters of the halo formed and for the interpretation, was used the default table for the interpretation of the inhibition halo, according to the NCCLS (2011).

RESULTS AND DISCUSSION

In the thermal resistance, it was observed that all of the samples were stable to the thermal treatment at 75°C, indicating that the collected samples maintain a pH range which would support the current processes for treatment of milk which the industries perform to ensure the microbiological quality without changing its physical and chemical characteristics.

As for the lacto-fermentation test, three samples presented coagulum type digestaste, floculoso, cheesy or sulcate (bubbles formation), which indicates the predominance of proteolytic microbial, lactic and coliforms microorganisms, giving unpleasant taste in milk and butter and being able to cause early bloating on cheeses.

The counts of staphylococci (Table 1) in the present study were below those found by Tebaldi et al. (2008) and, according to this author, besides reflecting the health conditions of the herd, large numbers of staphylococci coagulase positive, greater than 10^5 CFU/mL, increase the risk of staphylococcal toxins production that are resistant to the pasteurization process. Since the milk is kept for about 24 hours in the cooling tank at temperatures above 4°C, this fact is likely to occur.

The proteolytic microorganisms produce extracellular enzymes with proteolytic activity that will result in changes such as taste, reduce the nutritional quality and reduced shelf life. The results in this survey show that the values varied between 10^3 and 10^6 CFU/ml (Tab. 1). PINTO et al. (2006) obtained average scores in log of 4.65.

In accordance with the an IN62, the limit of aerobics mesophiles in cooling raw milk is of 3.00 x 10^5 UFC/mL and therefore 66.67% from the analyzed samples were within the standards (Table 1). CITADIN et al. (2009) have found that 48.57% of the samples were in discordance to the legislation.

The IN51/62 determines the refrigeration of milk after milking (Brazil, 2002), however, does not establish standards for psychrotrophic counting, ideal indicator for evaluating the microbiological quality of refrigerated milk. SILVA et al. (2011) found psychrotrophic average values 3.40 x 10^7 CFU/ml and ARCURI et al. (2008) between 10^2 and 10^7 CFU/ml.

Although there are no standards in the legislation for psychrotrophic counting, such microorganisms in the amount of 1.00 x 10^4 CFU/ml may produce thermostable enzymes responsible for unpleasant smell and taste and coagulation of the product, shortening the useful life there, responsible for the development of "off flavor" as well as loss of income and the appearance of defects in the production of cheese, being found counts above this amount in four samples.

The raw milk samples from six producers showed high incidence of *Escherichia coli* showing high contamination of raw material (Table 1).

The Gram negative microorganisms isolated by Petrifilm and identified by the Enterokit system were *E. coli* (ten strains), *Proteus miriabilis* (two strains), *Serratia liquefaciens* (three strains), *Enterobacter aglomerans* (four strains), *Enterobacter sakazaki* (one strain), *Citrobacter freundii/ Enterobacter cloacae* (two strains).

TEBALDI et al. (2008) assessing samples of cooling tank found that pathogenic microorganisms such as *Escherichia coli*, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*, *Klebsiella pneumoniae i*n most investigated properties.

In this study there was a higher incidence of *E. coli* (10) in the evaluated samples. ÁVILLA & GALLO (1996) evaluated the microbiota of raw milk and found that of 30 bacterial isolates analyzed, 14 of them (46.68%) were identified as *Enterobacter* spp., 10 (33.33%) as *Pseudomonas* spp., 4 (13.33%) as *E. coli*, 1 (3.33%) as *Citrobacter* spp. and 1 (3.33%) as *Hafnia alvei*.

ARCURI et al. (2008) found *Pseudomonas* (11), *Acinetobacter* (39), *Pantoea* (17), *Aeromonas* (five), *Moraxella* (four), *Serratia* (three), *Yersinia* (two), *Klebsiella* (one), *Enterobacter* (one) and *Methylobacterium* (one) among the Gram psychrotrophic bacteria negative isolated.

In the Table 2 can be visualized the data obtained in tests of susceptibility from *Staphylococcus* spp. isolated. From the six antimicrobials tested in 29 isolates it was observed resistance to five of them, the ampicillin had the highest frequency in ten isolates.

Table 1- Averages of the counts of aerobic mesophiles, mesophilic proteolytic in psychrotrophic and *staphylococci* (CFU/ml) in milk samples collected directly from the expansion tanks.

Sample	AM	MP	ET	EC	P	BP
1	9.90×10^4	1.94×10^5	5.20×10^3	aus	3.40×10^4	2.99×10^4
2	1.02×10^7	5.48×10^6	2.20×10^5	8.00×10^2	2.29×10^3	2.01×10^3
3	3.10×10^3	1.13×10^4	Aus	aus	7.10×10^2	1.21×10^3
4	1.50×10^4	8.00×10^3	9.00×10^2	1.10×10^1	4.00×10^2	7.00×10^2
5	2.83×10^5	2.60×10^5	3.50×10^3	aus	4.30×10^4	9.50×10^2
6	4.04×10^6	1.85×10^6	1.12×10^5	2.50×10^3	2.20×10^4	2.20×10^4
7	3.86×10^6	2.50×10^5	4.30×10^3	2.60×10^3	1.78×10^5	2.90×10^2
8	2.80×10^4	4.10×10^4	2.40×10^3	1.70×10^3	2.78×10^3	2.50×10^2
9	$< 10^3$	$< 10^3$	2.30×10^3	1.10×10^3	4.72×10^3	2.70×10^2

AM = aerobic mesophiles; MP = mesophilic proteolytic; ET = enterococcus, EC= *E. coli;* P= psychrotrophic; BP = Agar Baird Parker

Table 2- Profile of antimicrobial susceptibility from *Staphylococcus* spp. isolated from samples of refrigeration tank milk.

Antimicrobial	Total nº of microorganisms/ total number of samples/(%)		
	S	I	R
Gentamicin	27/29 (93,10)	1/29 (3,45)	1/29 (3,45)
Trimethoprim	26/29 (89,65)	1/29 (3,45)	1/29 (3,45)
Cephalothin	28/29 (96,55)	0/29	1/29 (3,45)
Tetracycline	24/29 (82,76)	3/29 (10,34)	2/29 (6,89)
Ampicillin	19/29 (65,52)	0/29	10/29 (34,48)
Ciprofloxacin	28/29 (96,55)	1/29 (3,45)	0/29

S = Sensitive, I = Intermediate and R = Resistant

Table 3- Profile of antimicrobial susceptibility of *E. coli* isolated from samples of refrigeration tank milk.

Antimicrobial	Total nº of microorganisms/ total number of samples/(%)		
	Sensitive	Intermediate	Resistant
Gentamicin	21/21(100)	0/21	0/21
Trimethoprim	21/21(100)	0/21	0/21
Cephalothin	18/21 (85,71)	1/21 (4,76)	2/21(9,52)
Tetracycline	21/21 (100)	0/21	0/21
Ampicilin	17/21 (80,95)	1/21 (4,76)	2/21(9,52)
Ciprofloxacin	21/21(100)	0/21	0/21

S = Sensitive, I = Intermediate and R = Resistant

Researchers have reported resistance of *Staphylococcus* spp. isolated from milk to antimicrobials such as gentamicin, enrofloxacin and penicillin (Zafalon et al., 2008, Medeiros et al., 2009, Ribeiro et al., 2009).

Regarding the antibiograms strains of *E. coli* (Table 3), the majority of the isolates were sensitive to tested antibiotics.

Campos et al. (2006) evaluating the strains susceptibility of *E. coli* isolated from raw milk samples, to antimicrobials cephalothin, ampicillin, ciprofloxacin, gentamicin, sulfametazol and tetracycline verified that the highest resistance (18.2%) were found for tetracycline, and no isolate showed resistance to ciprofloxacin and gentamicin.

RIBEIRO et al. (2009) observed that penicillin (53.5%), ampicillin (41.6%) and neomycin (38.6%) were the antimicrobials that had the highest rates of resistance compared to strains isolated from bovine milk. The presence of lineages resistant to three or more antimicrobials was observed in 40 (39.6%) among 101 isolates.

It was verified the presence of satellite colonies in various sowing, in other words, in a sensitive population some resistant individuals both for strains of *E. coli* as well as for those of staphylococci.

The knowledge of the antimicrobial resistance patterns that present microorganisms is of fundamental importance in the development of preventive methods that are effective, as well as

the elaboration of treatment strategies when they are necessary (ZAFALON et al., 2008).

CONCLUSIONS

The samples of milk analyzed showed unsatisfactory microbiological quality in relation to the microorganisms studied. The majority of the evaluated strains were sensitive to the tested antimicrobials.

REFERENCES

ÁVILA; C. R.; GALLO, C. R. Pesquisa de *Salmonella* spp. em leite cru, leite Pasteurizado Tipo C e queijo "minas frescal" comercializados no município de Piracicaba- SP. **Scientia agricola,** vol. 53, n. 1, Jan./Apr. 1996.

ARCURI, E.F.; SILVA; P.D.L.; BRITO, M.A.V. et al. Contagem, isolamento e caracterização de bactérias psicrotróficas contaminantes de leite cru refrigerado. **Ciência Rural,** v.38, n.8, nov, 2008.

AMERICAN PUBLIC HEALTH ASSOCIATION – APHA. **Compendium of methods for the microbiological examination of foods**. 4.ed. Washington: APHA, 2001. 676p.

BAUER A.W., KIRBY, W.M.M., SHERRIS J.C. et al. Antibiotic susceptibility testing by a standardized single disk method. **American Journal of Clinical Pathology,** v. 45, n.4, p. 493-496. 1966.

BRAMLEY, A.J., MCKINNON, C.H. **The microbiology of raw milk.** In: Dairy microbiology. 2. ed. England Elsevier Science, 1990. v. 1, p. 163-208.

BRESSAN, M.; MARTINS, M.C. Segurança alimentar na cadeia produtiva do leite e alguns de seus desafios. **Revista de Política Agrícola**. Ano XIII - Nº 3 -Jul./Ago./Set., 2004.

BRASIL. Ministério da Agricultura. R.I.I.S.P.O.A. **Regulamento de Inspeção Insdustrial e Santária de Produtos de Origem Animal** aprovado pelo decreto nº 30690, de 20.03.52, alterado pelo decreto nº 1255 de 25.03.52. Brasília, 66p.1980.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Departamento de Inspeção de Produtos de Origem Animal. Instrução Normativa nº 51, de 18 de setembro de 2002. Aprova e oficializa o regulamento técnico de identidade e qualidade de leite cru refrigerado. **Diário Oficial da União**, n. 172, p.13-22, 2002. Seção I.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Departamento de Inspeção de Produtos de Origem Animal. Instrução Normativa no 62, de 27 de dezembro de 2011. **Diário Oficial da República Federativa do Brasil**, 30 de dezembro de 2011. Seção I.

CAMPOS, M.R.H.; KIPNIS, A.; ANDRÉ, M.C.D. et al. Caracterização fenotípica pelo antibiograma de cepas de *Escherichia coli* isoladas de manipuladores, de leite cru e de queijo "Minas Frescal" em um laticínio de Goiás, Brasil. **Ciência Rural,** v.36, n.4, jul-ago, 2006.

CITADIN, A.S.; POZZA, M. S.S.; POZZA, P.C. et al. Qualidade microbiológica de leite cru refrigerado e fatores associados. **Revista Brasileira de Saúde Produção Animal,** v.10, n.1, p.52-59, jan/mar, 2009.

LAFARGE, V.; OGIER, J. C.; GIRARD, MALADIN, V. et al. Raw cow milk bacterial population shifts attributable to refrigeration. **Applied Environmental Microbiology**, v.70, n. 9, p. 5644-5650, 2004.

MARTINS, M.E.P.; NICOLAU, E.S.; MESQUITA, A.J. et al. Leite cru de quatro regiões leiteiras brasileiras: perspectivas de atendimento dos requisitos microbiológicos estabelecidos pela Instrução Normativa nº51. **Ciência e Tecnologia de Alimentos**, v. 25, n. 1, p. 191-195, jan.-mar. 2005.

Medeiros, E.F. et al. Perfil de sensibilidade microbiana *in vitro* de linhagens de *Staphylococcus* spp. isoladas de vacas com mastite subclínica. **Pesquisa Veterinária Brasileira,** v. 29, n. 7, p. 569-574. 2009.

NATIONAL COMMITTEE FOR CLINICAL LABORATORY STANDARDS. Performance standardfor antimicrobial susceptibility testing. Document M100-S20. **National Committee for Clinical Laboratory Standards,** Wayne, Pa. EEUU. 2011.

RIBEIRO, M. G.; GERALDO, J. S.; LANGONI, H.; LARA, G. H. B. et al. Microrganismos patogênicos, celularidade e resíduos de antimicrobianos no leite bovino produzido no sistema orgânico. **Pesquisa Veterinária Brasileira,** v. 29, n. 1. 2009.

SILVA, N.; JUNQUEIRA, V. C. A; SILVEIRA, N. F.A. **Manual de Métodos de Análise**

Microbiológica de Alimentos. São Paulo: Varela, 1997. 295p.

SILVA, L.V.; BELOTI, V.; TAMANINI, R.; D'OVIDIO, L. et al. Rastreamento de fontes da contaminação microbiológica do leite cru durante a ordenha em propriedades leiteiras do Agreste Pernambucano. **Semina: Ciências Agrárias**, v. 32, n. 1, p. 267-276, jan./mar. 2011.

TEBALDI, V.M.R.; OLIVERIA, T.L.C.; BOARI, C.A. et al. Isolamento de coliformes, estafilococos e enterococos de leite cru provenientes de tanques de refrigeração por expansão comunitários: identificação, ação lipolítica e proteolítica. **Ciência e Tecnologia de Alimentos,** v. 28, n.3, p. 753-760, jul-set 2008.

PINTO, C.L.O.; MARTINS, M.L.; VANETTI, M .C. D. et al. Qualidade microbiológica do leite cru refrigerado e isolamento de bactérias psicrotróficas proteolíticas. **Ciência e Tecnologia de Alimentos,** v. 26, n. 3, p. 645-651, jul/set 2006.

WEHR, H.M. Standard Methods for Examination of Dairy Products. 17 [a] . ed. **Washington: American Public Health Association**, 2004. 570 p.

ZAFALON, L. F. ARCARO J.R.P., NADER FILHO A. et al. Investigação de perfis de resistência aos antimicrobianos em *Staphylococcus aureus* isolados na ordenha de vacas em lactação. **Revista do Instituto Adolfo Lutz**, São Paulo, v. 67, n. 2, p. 118-125. 2008.