DOI: 10.5433/1679-0359.2012v33n5p1877

Antagonistic activity against *Listeria monocytogenes* and *Escherichia coli* from lactic acid bacteria isolated from raw milk

Atividade antagonista contra *Listeria monocytogenes* e *Escherichia coli* de bactérias ácido láticas isoladas de leite cru

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Abstract

Lactic Acid Bacteria (LAB) are naturally detected in several foods and can be important on pathogens control. Through production of numerous antimicrobial substances, LAB can inhibit microorganisms such as *Listeria monocytogenes* and *Escherichia coli*. Aiming to verify the presence of *L. monocytogenes,* enumerate *E. coli* and LAB, and verify the antagonistic activity of isolated LAB against *L. monocytogenes* and *E. coli,* samples of raw milk were collected from 45 dairy farms from Agreste region of Pernambuco. For *L. monocytogenes* detection VIDAS Listeria (bioMeriex) system was used while *E. coli* was enumerated by Petrifilm™ EC (3M) after incubation at 35ºC for 48 hours. For LAB enumeration, samples were diluted in MRS broth, distributed in Petrifilm™ AC (3M) plates and incubated at 30°C for 72 hours in microaerophilic conditions. Antagonism of 671 LAB isolates was determined through spot-on-the-lawn modified methodology using *L. monocytogenes* ATCC 7644 and *E. coli* ATCC 25922 as targets. *L. monocytogenes* was not detected in any milk sample. *E. coli* counts varied from $< 10^3$ to 1.3 x 10^5 CFU/mL. LAB counts varied from 11.1 x 10^4 to 9.9 x 10^6 CFU/mL. When LAB isolates were tested for antagonistic activity against *L. monocytogenes*, 549 (81.8%) samples were positive, from which 410 (61.1%) showed total inhibition and 139 (20.7%) partial inhibition. Concerning *E.coli*, 258 (38.5%) isolates showed antagonistic activity, all with partial inhibition. High counts of *E. coli* and total coliforms found indicate poor hygienic conditions during obtainment of milk. In addition the elevated frequency of LAB inhibitory to *L. monocytogenes* could explain the absence of this pathogen in milk samples studied.

Key words: Dairy microbiology, lactic acid bacteria, *Listeria monocytogenes*, *Escherichia coli*, antagonism

Resumo

Bactérias Ácido Láticas (BAL) são naturalmente encontradas em vários alimentos e podem ser importantes no controle de patógenos. Por meio da produção de diversas substâncias antimicrobianas, BAL podem inibir microrganismos como *Listeria monocytogenes* e *Escherichia coli*. Com o objetivo de verificar a presença de *L. monocytogenes*, enumerar *E. coli* e BAL, e verificar a atividade antagonista das BAL isoladas em relação a *L. monocytogenes* e *E. coli* foram coletadas amostras de leite cru de

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Recebido para publicação 18/01/12 Aprovado em 19/07/12

45 propriedades leiteiras da região agreste de Pernambuco. Para detecção de *L. monocytogenes* foi utilizado o sistema VIDAS Listeria (bioMeriex) enquanto *E. coli* foi enumerada pelo Petrifilm™ EC (3M), após incubação a 35°C por 48 horas. Para contagem de BAL as amostras foram diluídas em caldo MRS, semeadas em placas de Petrifilm™ AC (3M) e incubadas a 30°C por 72 horas em microaerofilia. O antagonismo dos 671 isolados de BAL foi determinado através da metodologia spot-on-the-lawn modificada, utilizando a cepa *L. monocytogenes* ATCC 7644 e *E. coli* ATCC 25922 como alvos. Em nenhuma amostra de leite foi detectada a presença de *L. monocytogenes*. As contagens de *E. coli* variaram de < 1, 0 x 103 UFC/mL a 1,3 x 105 UFC/mL. As contagens de BAL variaram de 11,1 x 104 UFC/mL a 9,9 x 106 UFC/mL. Quando os isolados de BAL foram testados para capacidade antagonista contra *L. monocytogenes*, 549 (81,8%) foram positivos, sendo que 410 (61,2%) com inibição total e 139 (20,7%) com inibição parcial. Quanto à *E. coli*, 258 (38,5%) dos isolados apresentaram atividade antagonista, sendo todas com inibição parcial. As altas contagens de *E. coli* e coliformes totais encontradas indicam condições higiênicas inadequadas durante a obtenção do leite. Além disso, a elevada freqüência de BAL com capacidade antagonista a *L. monocytogenes*, poderia explicar a ausência deste patógeno nas amostras de leite estudadas.
Palavras-chave: Microbiologia do leite, bactérias ácido láticas, *Listeria monocytogenes*, *Escherichia coli*, antagonismo

Introduction

In general, milk produced in Brazil is obtained under deficient hygienic conditions, resulting in high counts of microorganisms such as mesophiles aerobes, psychrotrophs, coliforms, and also pathogens. (GOMES et al., 2011; SILVA et al., 2011; MATTOS et al., 2010; SILVA et al., 2010; ALVES et al., 2009; CITADIN et al., 2009; ARCURI et al., 2008; TEBALDI et al., 2008; CAMPOS et al., 2006; MORAES et al., 2005; NERO et al., 2004; CATÃO; CEBALLOS, 2001; PADILHA et al., 2001; BELOTI et al., 1999).

In some researches, poor microbiological quality of food, that is, elevated levels of indicator microorganisms, has been associated with low incidence of pathogens. In the same way, when an improvement to the microbiological quality is observed, the incidence of pathogens tends to increase (RAMIREZ et al., 2011; GUERRA; MCLAUCHLIN; BERNARDO, 2001; JAY, 1995). In milk, this could be explained mainly by the presence of a natural microbiota that presents a great inhibitory potential against pathogens. Lactic acid bacteria (LAB), which are naturally present in milk, are probably responsible for the inhibition of pathogens, as some studies demonstrate (RAMIREZ et al., 2011).

LAB are potentially important in the control of unwanted microorganisms in food as they can interfere with multiplication of spoilage and pathogenic bacteria through several mechanisms: competition for oxygen, competition for linking sites and production of antagonist substances (VÁSQUEZ; SUAREZ; ZAPATA, 2009; DE MARTINIS; ALVES; FRANCO, 2002). The production of lactic acid associated with other acids, like acetic acid and propionic acid contributes to microorganism control by creating a small decrease in the pH of food, which is unfavorable to multiplication and survival of Gram positive and negative bacteria, as well as molds and yeasts (ROSS; MAROGAN; HILL, 2002). Several antagonistic substances with antibiotic potential are widely described, like hydrogen peroxide, diacetyl and bacteriocins (MATAMOROS et al., 2009; VÁSQUEZ; SUAREZ; ZAPATA, 2009; LEWUS; MONTVILLE, 1991).

Bacteriocins are biological active proteins characterized for their activity against a specific group of susceptible microorganisms (OLIVEIRA; SIQUEIRA JUNIOR; SILVA, 2012; NASCIMENTO; MORENO; KUAYE, 2008; CARR; CHILL; MAIDA, 2002; LEWUS, MOTVILLE, 1991). Traditionally, bacteriocins

have been defined as protein compounds, produced by bacteria with bacteriostatic or bactericide effect (OLIVEIRA; SIQUEIRA JUNIOR; SILVA, 2012; CARR; CHILL; MAIDA, 2002; RILEY; WERTS, 2002).

The present paper aimed to research *Listeria monocytogenes,* enummerate total coliforms, *Escherichia coli* in milk, estimate lactic acid bacteria (LAB) counts which are present in raw milk and also identify the presence of LAB with antagonistic activity against *L. monocytogenes and E. coli.*

Material and Methods

Samples of raw milk from 45 dairy farms were collected. These dairy farms were selected with help from the Office for Rural Production and Agrarian Reform and from a cooperative of dairy producers both located in Pernambuco, Brazil. The criterion adopted for the selection of the dairy farms was that they would represent the predominant characteristics of production of the Agreste Region of Pernambuco (MONTEIRO et al., 2007), where most of milk production of the state occurs. The selected farms were small, had a small number of animals and presented low productivity and low technification.

Samples were collected directly from bulk tanks or milk cans after homogenization in sterile bags (Nasco®, California, United States of America). When collected from milk cans, samples were formed by a pool of the cans found. After collection, samples were immediately refrigerated and transported in styrofoam boxes with ice packs to the Laboratory of Experimentation and Food Analysis at Pernambuco Federal University, where the analysis were performed.

VIDAS Listeria system (bioMeriex®, Craponne, France) was used to search for the presence of *Listeria monocytogenes* in milk samples. The strain *L. monocytogenes* ATCC 7644 was used as positive control.

To enumerate total coliforms and E*scherichia coli,* samples were diluted in sterile saline solution 0,85%, inoculated in Petrifilm™ EC plates (3M, St. Paul, MN, United States of America) that were incubated at 35±1°C for 24 hours for total coliforms and for 48 hours for *E. coli* enumeration, as recommended by the manufacturer.

To enumerate lactic acid bacteria (LAB), samples were diluted in MRS broth, inoculated on Petrifilm™ AC plates (3M, St. Paul, MN, United States of America) and then incubated at 30ºC for 72 hours in microerophilic conditions (NERO et al., 2006).

The antagonism of LAB against *L. monocytogenes* and *E. coli* was carried out at the Laboratory of Inspection of Animal Origin Products at Londrina State University through the methodology spot-on-the-lawn modified (LEWUS; MONTVILLE, 1991), using strains of *L. monocytogenes* ATCC 7644 and *E. coli* ATCC 25922 as targets.

The LAB isolates were obtained from 45 samples of raw milk. All 671 LAB isolates that could be recovered from the Petrifilm AC™ plates were tested for antagonistic activity. Each isolate was inoculated punctually in four PCA plates (Acumedia ®, United States of America) and incubated at 30ºC for 48 hours. After growth, two PCA plates were used to evaluate antagonistic activity against *L. monocytogenes* and other two against *E. coli.*

An inoculum of each pathogen was prepared in TSB-YM. The prepared inoculums were diluted in saline solution (0.85%) until a concentration close to 1.5 x 10^8 CFU/mL (0.5 on the McFarland scale) and then were added to semi-solid TSA – prepared with half the proportion indicated by manufacturer. A layer of approximately 7 mL of the inoculums in semi-solid TSA were poured on the PCA plates containing LAB isolates forming a upper-layer. Next the plates were re-incubated at 30ºC for *L. monocytogenes* and at 35º C for *E. coli,* during 48 hours.

Colonies presenting a translucent halo in the TSA upper-layer were considered antagonistic. This halo is caused by growth inhibition of the pathogen inoculated in the upper-layer. Well-defined halos were classified as a result of complete inhibition while diffused halos were classified as partial inhibition (NERO et al., 2009).

Results and Discussion

L. monocytogenes was not detected in any of the 45 raw milk samples. Studies conducted by other researchers indicate that the frequency of *L. monocytogenes* isolation is highly variable. In Brazil, Camargo (2010) obtained the same result while searching for *L. monocytogenes* in 287 raw milk samples produced in São Paulo, Brazil. Nero et al. (2004) also did not detect *L. monocytogenes* in a study involving 210 samples of raw milk produced in four states (Rio Grande do Sul, Paraná, São Paulo and Minas Gerais). Arcuri et al. (2006) were unable to isolate *L. monocytogenes* in a research conducted in 14 dairy farms located at Southeast of Minas Gerais and North of Rio de Janeiro. Padilha et al. (2001) also could not isolate this pathogen in 50 samples of raw milk produced in the metropolitan region of Recife, Pernambuco. However, Catão and Ceballos (2001) detected *Listeria spp.* in 33 (73.3%) of 45 samples of raw milk from the states of Paraíba and Pernambuco, but in only 17 (37.9%) of them *L. monocytogenes* was present.

The absence of this pathogen in raw milk, as most of the studies presented above demonstrate, could be related to the microorganisms naturally present in this product. Raw milk microbiota includes Lactic Acid Bacteria, which are known for its antagonistic effects against pathogens, such as *L. monocytogenes*, by different mechanisms (JAY, 1995; DE MARTINIS; ALVES; FRANCO, 2002).

In a research performed in Turkey, Aygun and Pehlivanlar (2006) did not detect *L. monocytogenes* in 47 samples of raw milk, but they isolated *L. ivanovii* and *L. grayi* in 1 sample (2.2%). In India, *L. monocytogenes* was also not detected in 35 analyzed raw milk samples; however *L. innocua* was detected in 1 sample (DHANASHREE et al., 2003). Still in India, from 2060 raw milk samples analyzed, 105 (5.1%) were positive for *L. monocytogenes* (KALOREY et al., 2008). In Mexico, 1300 raw milk samples were analyzed and *Listeria* spp. was detected in 300 (23.0%) of them, while 162 (13.0%) were positive to *L. monocytogenes*. This research also detected *L. inanovii* (6.0%), *L. seeligeri* (4.0%) and *L. innocua* (1.0%) (CARLOS; OSCAR; IRMA, 2001). In Sweden, Waak, Than and Danielsson-Than (2002) isolated *Listeria* spp. in 10 (3,4%) out of 294 samples of raw milk, from which *L. monocytogenes* was detected in 3 (1.0%) and *L. innocua* in 7 (2.3%). In the United States of America, a research with 861 samples of raw milk from bulk tanks isolated *L. monocytogenes* in 56 samples (VAN KESSEL et al., 2004).

This great variation among frequencies of *L. monocytogenes* detection could still be related to the microbiota present in raw milk. Different levels of contamination would correspond to different levels of pathogens. As new approaches to reduce pathogens in several foods are applied throughout the world, harmless background microbiota is also reduced, since the treatments applied are not specific to pathogens. As a result, food with reduced microbiota becomes a favorable environment to pathogens multiplication (JAY, 1997).

The counts obtained for total coliforms (Table 1) varied from 1.5×10^3 CFU/mL to 2.4×10^6 CFU/mL. As demonstrated in table 1, the majority of samples 31(68.9%) presented levels of total coliforms above 10^5 CFU/mL. This result indicates that hygienic conditions in milking process were unsatisfactory, since literature recommends that total coliforms counts should not exceed 100 CFU/mL (CHAMBERS, 2002). Silva et al. (2011) studied six dairy farms from Agreste region of Pernambuco and found in all of the farms counts

above this recommendation, resulting on a mean of 2.5 x 10^5 CFU/mL for total coliforms. Citadin et al. (2009) analyzed 31 dairy farms located at Marechal Cândido Rondon, Paraná, Brazil and found in 19 (61.3%) samples of milk total coliforms counts above recommended by literature. Nero et al. (2004) found total coliforms counts above this pattern in 80.4% of the studied milk samples.

Table 1. Distribution of raw milk samples from the Agreste Region of Pernambuco, according to counts of total coliforms, *Escherichia coli* and lactic acid bacteria.

	Total Coliforms	*Escherichia coli*	Lactic Acid Bacteria
	n (%)	n (%)	n (%)
Range of the CFU/ml count			
< 1000	0 (0.0)	19 (42.2)	0 (0.0)
1.000 ├ 10.000	1 (2.2)	14 (31.1)	0 (0.0)
10.000 ├ 100.000	13 (28.9)	10 (22.2)	13 (28.9)
100.000 ├ 1.000.000	24 (53.3)	2 (4.5)	13 (28.9)
≥ 1.000.000	7 (15.6)	0 (0.0)	19 (42.2)
Total	45 (100.0)	45 (100.0)	45 (100.0)

Source: Elaboration of the authors.

Arcuri et al. (2006) analyzed samples from 24 dairy farms located in the Southeast of Minas Gerais and North of Rio de Janeiro, Brazil and found total coliform counts between 5 CFU/mL and 2.3 x 10^3 CFU/mL, and counts higher than 100 CFU/mL were found in 19 (79.2%) farms. Moraes et al. (2005) analyzed samples of milk from 42 dairy farms, located in Rio Grande do Sul, Brazil and found total coliforms counts between 2.3 x 10^3 CFU/mL and 3.0 x 10^5 CFU/mL. In another study conducted in Malasia, almost 90% of 930 samples were contaminated by total coliforms, with an average count of 1.7 x 10^5 CFU/mL (CHYE; ABDULLAH; AYOB, 2004).

The presence of *E. coli* can be concerning, since some of its lineages are pathogenic to man and animals, and it could also indicate possible contamination by entheropathogens. More often, *E. coli* is a reliable indicator of fecal contamination of food (FRANCO; LANDGRAF, 2008; JAY, 2005). In this study, the counts obtained for *E. coli* varied from <10^3 CFU/mL to 1.3 x 10^5 CFU/mL and 26 (57.8%) samples presented rages of *E. coli* contamination above 10^3 CFU/mL (Table 1).

Nero et al. (2004) found *E. coli* in 36.8% of the samples of raw milk, in which 29.4% showed counts higher than 100 CFU/mL. Moraes et al. (2005) isolated *E. coli* in 19.0% of the samples of raw milk. Campos et al. (2006) analyzed 24 samples of raw milk collected in Goiás and found *E. coli* in 19 (79.2 %). In a research performed in Saudi Arabia, *E. coli* was found in 8 (72.7%) of the 11 samples studied (ALTALHI; HASSAN, 2009). In Malaysia, *E. coli* was isolated from 600 (64.5%) of the 930 tested samples of raw milk (CHYE; ABDULLAH; AYOB, 2004). In Pakistan, Soomro et al. (2002) verified that 9 (45.0%) of the 20 raw milk samples collected from farms were contaminated with this microorganism.

The LAB are of great importance in milk quality control, due to the fact that they produce several substances with antimicrobial potential, such as organic acids and bacteriocins, which affect spoilage and patogenic microorganisms (RAMIREZ

et al., 2011; DE MARTINIS; ALVES; FRANCO, 2002). The LAB counts of the 45 samples of milk analyzed (Table 1) varied from 1,1 x 10^4 CFU/mL to 9.9 x 10^6 CFU/mL, most of samples were placed at ranges higher than 10^6 CFU/mL. Afif, Faid and Najimi (2007) investigating two milk cooperatives of Morocco during two different time periods found LAB counts similar to the ones found by this study. From July to August LAB counts were 0.9 x 10^6 and from, November to January, counts were of 0.2 x 10^6 CFU/mL in cooperative 1. Cooperative 2 showed counts of 176.9 x 10^6 and 2.7 x 10^6 CFU/mL in the same time periods.

The results found in this study for total coliforms, *E. coli* and LAB enumeration show that raw milk produced in Pernambuco carried a large microbiota. These observations reinforce the results obtained for *L. monocytogenes* that might have been inhibited by this elevated number of microorganisms in raw milk (JAY, 1997). Table 2 shows the results of antagonism tests performed with LAB isolates from raw milk samples, against *L. monocytogenes* and *E. coli.* Colonies presenting a translucent halo , caused by inhibition growth of the inoculated pathogen on the upper-layer, were considered antagonistic. When antagonistic activity against *L. monocytogenes* was considered, from 671 LAB isolates tested, 549 (81.8%) presented such activity, from which 410 (61.1%) presented complete inhibition and 139 (20.7%) partial inhibition. Regarding *E. coli* inhibition, 258 (38.5%) LAB cultures were positive, although all of them were considered as partial inhibition.

Table 2. Frequency of Lactic Acid Bacteria (LAB) with complete and partial antagonism and non-antagonistic activity to *Listeria monocytogenes* and *Escherichia coli* isolated from 45 samples of raw milk from the Agreste region of Pernambuco.

	Antagonistic Activity of LAB			
	Listeria monocytogenes		*Escherichia coli*	
	N	%	n	%
Complete antagonism	410	61.1	0	0
Parcial antagonism	139	20.7	258	38.5
Total of Antagonistics Cultures	549	81.8	258	38.5
Non-antagonistic Cultures	122	18.2	413	61.5
Total	671	100	671	100

Source: Elaboration of the authors.

It was observed that LAB cultures inhibitory frequency and intensity against *L. monocytogenes* was superior to the one against *E. coli.* Antagonism assessment resulted in 186 (27.7%) antagonistic cultures against both *L. monocytogenes* and *E. coli*, 363 (54.1%) cultures only antagonistic against *L. monocytogenes,* 72 (10.7%) were antagonistic only against *E. coli* and 50 colonies (7.5%) did not present inhibitory effects.

Many studies also demonstrated inhibitory activity of LAB present in cheese against *L. monocytogenes*, but there are still few studies of this type in milk. Nero et al. (2009) found a smaller frequency of LAB isolated from raw milk inhibitory to *L. monocytogenes*. The researchers isolated 360 cultures from 15 samples of raw milk, collected in the reception of a dairy industry. Antagonistic activity was detected in 91 (25,3%) cultures, from which 11 (3.1%) were classified as complete inhibition and 80 (22,2%) as partial inhibition.

Guerra and Bernardo (2001) used cheese-isolated LAB to verify the antagonistic effect on *L. monocytogenes*, and from 531 tested cultures, 208 (39.2%) were positive. Alexandre et al. (2002) evaluated inhibitory capacity of LAB isolated from cheese "Queijo Minas do Serro" against *L. monocytogenes* and observed that 29 (15.1%) of the 192 tested strains were inhibitory. Ramírez et al. (2005) analyzing antagonistic activity of LAB isolated from different types of cheese, found only 8 (2.4%) out of 330 cultures with inhibitory capacity against *L. monocytogenes*.

Regarding antagonistic activity against *E. coli*, studies demonstrate a low frequency of antagonistic LAB. Ramírez et al. (2005) found only 1 (0,3%) culture with inhibitory effect against *E. coli.* Alexandre et al. (2002) discovered that none of the tested cultures were inhibitory to *E. coli* growth. The lower frequencies for LAB with antagonistic activity against *E. coli* reported by different studies are in agreement with the present study. This may be explained by the fact that gram-negative bacteria are not affected by bacteriocins (LEWUS; MONTVILLE, 1991).

Conclusions

Listeria. monocytogenes was not detected in raw milk samples from the Agreste region of Pernambuco, Brazil. However microbiological quality of milk was considered poor due to high counts of total coliforms and *Escherichia coli* detected, which indicate that hygienic conditions during milking process in this region are unsatisfactory. In addition, high counts of *E. coli* may constitute a health risk, since it indicates potential contamination by other entheropathogens.

An elevated percentage of lactic acid bacteria (LAB) with antagonistic activity against *L. monocytogenes* was detected in this study, which may be responsible for the absence of this pathogen in the studied milk samples. LAB naturally present in milk can play an important role in the microbiological safety of this product.

Acknowledgments

This work was financed by FINEP **(**Studies and Projects Financier) and MDS (Ministry of Social Development and Agrarian Reform) and was part of the Project "Implementation of Good Practices for the production of quality and safe milk in Agreste region of Recife, Pernambuco".

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