

Original Research Article

<https://doi.org/10.20546/ijcmas.2019.806.035>

Profile of Susceptibility to Antimicrobials and Antagonist Activity of Lactic Acid Bacteria with Probiotic Potential Isolated from Artisanal Coalho Cheese of the Sertão Region of the State of Paraíba

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ABSTRACT

The study was carried out with the objective of evaluating the antimicrobial susceptibility profile and antagonist activity of lactic acid bacteria of the genus *Streptococcus* and *Enterococcus* isolated from artisanal coalho cheese produced in the Sertão region of the State of Paraíba. 29 strains were analyzed, distributed among: *E. faecium*, *E. faecalis*, *E. durans*, *E. casseliflavus* and *S. infantarius subsp. infantarius*. All the strains were activated in broth and De Man, Rogosa and Sharpe Agar, evaluated for their susceptibility to antibiotics by the disk diffusion technique and antagonism on pathogenic strains *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Escherichia coli*. The strains of the genus *Enterococcus spp.* were sensitive to ampicillin, chloramphenicol, vancomycin and amoxicillin and resistant to colistin, aztreonam and cefoxitin, while the strains of *Streptococcus spp.* were sensitive to the same antibiotics and resistant to aztreonam and colistin. As for the antagonist activity of the strains *Enterococcus spp.* inhibited about 61.1%, 72.2% and 28.8 % of the strains of *P. aeruginosa*, *K. pneumoniae* and *E. coli*, respectively. Similar results were observed for the strains of *Streptococcus spp.* that inhibited about 63.6 %, 72.7 % and 36.4 % of the strains of *P. aeruginosa*, *K. pneumoniae* and *E. coli*, respectively. All the strains, of both genera, did not inhibit *S. aureus*. Due to the results of elevated sensitivity to the antibiotics and antagonist activity on pathogenic strains, the lactic acid bacteria isolated from artisanal coalho cheese from Paraíba presented favorable preliminary characteristics of probiotic profile.

Keywords

Coalho cheese;
Lactic acid bacteria;
Antimicrobialsuscep-
tibility;
Antagonism;
Probiotic

Article Info

Accepted:
04 May 2019
Available Online:
10 June 2019

Introduction

The presence of lactic acid bacteria (LAB) in cheese is very important for its production, as they accelerate the development of milk coagulation (Awad *et al.*, 2007), and also, they produce a great number of enzymes (glycolytic, lipolytic and proteolytic) which contribute to the desirable sensorial properties (Lima *et al.*, 2009). With reference to the State of Paraíba, the Sertão region is considered to be the greatest producer of coalhocheese (Luíz, 2014), and this product is characterized as an excellent source of LAB (Medeiros *et al.*, 2016).

Lactic acid bacteria (LAB) form a morphologically heterogeneous group, belonging to the Gram-positive, non-spore forming, catalase-negative, facultative anaerobe, fermentative in anaerobiosis and aerobiosis, with the form of cocci and bacillus (Silva, 2011). An important attribute is the capacity of synthesizing antimicrobial compounds, the bacteriocins (Soomro *et al.*, 2002), as well as other substances which may also be considered antagonistic, such as the diacetyl and hydrogen peroxide (Tamanini *et al.*, 2012).

This antimicrobial effect is mainly because of the production of lactic acid, one of the essential metabolites produced by the LAB, which may reduce the hydrogenionic potential (pH) of the medium, a sufficient factor to have an inhibitory effect on many microorganisms (Poppi *et al.*, 2008).

In contrast, researchers speculate that the commensal bacteria, such as the LAB, may also act as reservoirs of resistance genes to certain antibiotics commonly used against human pathogenic agents, becoming a potential risk to health, due to the transfer of resistant genetic material to the bacteria of the microflora of the human gastrointestinal tract (Marthur e Singh, 2005).

The lactic acid group of bacteria are the most used microorganisms as probiotics, and for this must survive the passage through the gastrointestinal tract (GIT) (Domingo, 2017), in addition to presenting sensitivity to the main synthetic antibiotics and antagonistic effect against bacteria present in the GIT. As they are live-organism probiotics, when administered in the correct quantity, attribute a series of benefits to the hosts (FAO/WHO, 2001).

According to Domingo (2017), the probiotics participate in the control of intestinal infection, the levels of serum cholesterol, support to the immunological system, influences influentially the intestinal microbiota, helps in the reduction of toxic compounds, favors the anti-cancer activity and in the use of lactose, among others.

Given the beneficial health-promoting effects provided by the LAB to their hosts, as well as their importance in nutrition, this work had as an objective to evaluate the sensitivity and resistance profile to the synthetic antibiotics, and the antagonist activity of lactic bacteria of the genera *Streptococcus* and *Enterococcus* isolated from artisanal coalho cheese produced in the Sertão region of the State of Paraíba.

Materials and Methods

Obtention of the strains

The bacterial strains were acquired from the collection of the Microbiology Laboratory of the Academic Unit of Biological Sciences (UACB) of the Health and Rural Technology Center (CSTR) of the Federal University of (UFCG), Patos-PB Campus.

These lactic strains were isolated from samples of artisanal coalho cheese from the Sertão region of the State of Paraíba, from the

micro-region of the municipality of Catolé do Rocha-PB (Figure 1).

Activation of the selected species

The selected species of lactic acid bacteria were activated using Harrigan's method (1998). The bacterial strains were preserved in *ependorf* tubes with glycerol and stored at -20 °C. The activation was initiated with the inoculation of 500 µL of the inoculum in test tubes containing 5 mL De Man, Rogosa and Sharpe – MRS broth (Himedia), and incubated at 37 °C for 24 hours. Then the samples were transferred to Petri dishes containing MRS agar (Himedia) using a platinum strap and incubated at 37 °C for 48 hours. When this period was completed, the colonies were transferred again to test tubes containing 5 mL of MRS broth and incubated at the same temperatures for 24 hours. After this process, each sample was transferred to tubes with Ágar Plate Cout – PCA agar (Kasvi) inclined for the isolation, growth and preservation of the colonies and incubated at 37 °C for 24 hours.

Sensitivity to antibiotics test

After the activation, the bacterial isolates were submitted to the process of susceptibility to antibiotics by the standard disk diffusion method, using Mueller Hinton – MHA agar (Kasvi). Each tested strain was transferred and inoculated in tubes containing 1 mL of 0.9% physiological solution, with the aid of a flamed platinum strap, in a sufficient quantity for the observation of the turbidity similar to the 0.5 McFarland standard, where each inoculum contains 10^8 UFC/mL, this method was also used by Guimarães et al (2012).

Subsequently, with the aid of a sterile swab, the cultures were sown in Petri dishes containing MHA until a uniform smear was

obtained. After this stage, were distributed in the different dishes of antibiotics, grouped according to the following classes: Penicillins (Amoxicillin - AMO 10 µg; Ampicillin- AMP 10 µg); Macrolide (Azithromycin- AZI 15 µg); Anfenicol (Chloramphenicol - CLO 30 µg); Second generation cephalosporin (Cefoxitin - CFO 30 µg); Glycopeptide (Vancomocin - VAN 30 µg); monocyclic β-lactamic (Aztreonam - ATM 30 µg) and Lipopeptide (Colistin - COL 10 µg) (Sensidisc - DME), lastly, were incubated at 37°C for 24 h.

The antimicrobial susceptibility profile was submitted to a qualitative classification as recommended in the interpretation tables of the Clinical Laboratory Standards Institute (CLSI), version M100-S27 of 2017.

However, in the inexistence of a reference to a certain antibiotic for this group specific were used values expressed as resistant (≤ 15 mm), intermediary (16-20mm) or sensitive (≥ 21 mm) (Sehn, 2015; Halder and Mandal, 2016).

***In vitro* antagonism**

The antagonism test of the producer lactic acid bacteria was performed according to the adaptations of Guedes Neto *et al.*, (2005) against strains of indicator pathogenic microorganisms such as *Pseudomonas aeruginosa* (ATCC 9027), *Klebsiella pneumoniae* (ATCC 13883), *Staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 3539).

The LAB were cultivated in MRS broth and incubated at 37 °C, during 24 hours, under aerobiosis, after the growth, the strains were standardized observing the degree of turbidity similar to the standard 0.5 of the MacFarland Scale (10^8 UFC/mL), then, 15µL of the inoculum were pipetted in filter paper discs

sized 6 mm in the surfaces of the Petri dishes containing MRS agar and these dishes were incubated in aerobiosis, 37 °C for 24 hours.

The pathogenic indicator bacterial strains were incubated in Brain Heart Infusion –BHI broth (Himedia), at 37 °C, during 24 hours, under aerobiosis. After this incubation period, the strains were standardized according to the MacFarland scale, and therefore, an inoculum was transferred to the BHI broth, where it had approximately 10⁷ CFU/ml of pathogenic bacteria.

In such a way that formed an over layer, the medium containing the strains of indicator bacteria was poured over the Petri dishes already stored with the LAB. After 24 hours of incubation at 37 °C, in aerobiosis, the diameters of the halos were measured (Guedes Neto *et al.*, 2005).

Results and Discussion

Were analyzed 29 strains registered in the GenBank, from which 18 belong to the genus *Enterococcus*, such as *Enterococcus faecium* KT990017, *E. faecium* KT990009, *E. faecium* KT990013, *E. faecium* KT990024, *E. faecium* KT990030, *E. faecium* KT990021, *E. faecium* KT990029, *E. faecium* KT990020, *E. faecium* KT990022, *E. faecium* KT990023, *E. faecium* KT990025, *E. faecium* KT990027, *E. faecium* KT990026, *E. faecium* KT990019, *E. faecium* KT990028, *E. faecalis* KT990001, *E. durans* KT989997, *E. casseliflavus* KT989994, and 11 strains to the genus *Streptococcus*, being *Streptococcus infantarius subsp. infantarius* KT990067, *S. infantarius subsp. infantarius* KT990068, *S. infantarius subsp. infantarius* sKT990072, *S. infantarius subsp. infantarius* KT990069, *S. infantarius subsp. infantarius* KT990070, *S. infantarius subsp. infantarius* KT990071, *S. infantarius subsp. infantarius* KT990073, *S. infantarius subsp. infantarius* KT990074, *S. infantarius subsp. infantarius*

KT990075, *S. infantarius subsp. infantarius* KT990076, *S. infantarius subsp. infantarius* KT990077.

According to the references, a great proportion of the isolates of the genus *Enterococcus* spp. Proved to be sensitive to the majority of the antibiotics. All the samples of *E. faecium* proved to be sensitive to ampicillin, chloranphenicol, vancomycin and amoxicillin. As for the Cefoxitin and the azithromycin, these proved to be variable; the results for these antibiotics are present in chart 1. All the strains of *E. faecium* presented total resistance to the antibiotics aztreonam and colistin.

Fernandes (2014) when testing the antibiotics against isolates of *E. faecium* observed that about 78% of their strains proved to be resistant to at least one of the antibiotics tested, and sensitive to ampicillin and vancomycin.

Triveldi *et al.*, (2011) evaluating the susceptibility of the antibiotics to the enterococcus of lactic products found, in them, sensitivity to vancomycin. Just as Brandalize (2013) did not find resistance to vancomycin in *E. faecium* isolated from cheese.

All the other species of *Enterococcus* spp. analyzed proved to be 100% susceptible to ampicillin, chloramphenicol, vancomycin and amoxicillin. On the other hand, presented 100% of resistance to aztreonam, colistin and cefoxitin. The isolates of *E. durans* and *E. casseliflavus* showed intermediary profiles against the drug azithromycin, while *E. faecalis* proved to be sensitive to this same drug.

Fracalanza *et al.*, (2007) when carrying out susceptibility tests with antimicrobials against lactic acid bacteria, observed that the strains of *E. faecalis* presented resistance to at least

two antibiotics, ampicillin and vancomycin, and the isolates of *Enterococcus durans* and *Enterococcus casseliflavus* showed susceptibility to at least three antibiotics, chloramphenicol, vancomycin and ampicillin.

E. faecalis showed to have a great sensitivity spectrum in face of the tested antimicrobials, similar to the results of (2014), which revealed that strains of this same species presented susceptibility to ampicillin, vancomycin, gentamicin and teicoplanin and resistance to one of the antibiotics tested.

For *Streptococcus infantarius subsp. infantarius* the same types of antibiotics were tested on 11 strains. A broad number of isolates presented total sensitivity to ampicillin, chloramphenicol, vancomycin and amoxicillin, and in a contrary manner expressed resistant profiles against aztreonam and colistin, similar results to the tests of the strains of *Enterococcus* spp. In reference to the cefoxitin and azithromycin, they also proved to be variable, the values for these antibiotics are present in Chart 2.

In a study carried out by Neves (2008) using the complex “*Streptococcus bovis/ Streptococcus equinus*”, a group related to *S. infantarius subsp. infantarius*, revealed that all the analyzed samples were susceptible to antibiotics such as vancomycin and ampicillin, a similar result to the one found in this study.

In general, the strains of LAB presented resistance to at least two antibiotics, aztreonam and colistin.

These results were similar to those observed by (2017), where, the strains isolated from artisanal coalho cheese from the State of Paraíba also presented resistance to at least two types of antibiotics.

Charteris *et al.*, (1998) explains that the resistance to the beta-lactams, in this case the antibiotic aztreonam, may be explained by the production and action of beta-lactamases or by the possible impermeability that the bacteria's cell wall possesses.

The sensitivity to the antimicrobials expressed by lactic acid bacteria is considered one of the key factors so that these may be used as probiotics. On the other hand, as specified by Botelho *et al.*, (2015) the resistance to the antimicrobials expressed by lactic acid bacteria is a cause for concern, seen as the genes for antimicrobial resistance may be transferred by conjugation, or other mechanism, to pathogenic bacteria.

As for the antagonist profile, the strains of *Enterococcus* spp. and *Streptococcus* spp. were assessed according to the presence or absence of the inhibition halo, indicating antagonist activity against the indicator pathogenic bacteria which are also common in GIT of human beings.

The species of *E. faecalis* and *E. durans* demonstrated complete antagonist action against the Gram-negative microorganisms (*P. Aeruginosa*, *K. pneumoniae* and *E. coli*), being divergent only the species of *E. casseliflavus* which did not present antagonistic activity against these indicator species. In relation to the activity of *E. faecium*, the results are presented in Chart 3.

In reference to the inhibition of the Gram-positive indicator species *Staphylococcus aureus* ATCC 29213, all the strains of the genus *Enterococcus* spp. did not demonstrate antagonist activity against this strain.

When assessing the probiotic profile of the lactic acid bacteria isolated from bovine transition milk, Guimarães *et al.*, (2018) reported that the strains of the LAB isolated

from this product were capable of inhibiting indicator bacteria of *E.coli*, with medium inhibition halos. Tamanini *et al.*, (2012) observed that the lactic acid bacteria isolated from raw milk of dairy milk farms from the Agreste region of Pernambuco were capable of partially inhibiting *E. coli* and *Listeria monocytogenes* isolated from the same product.

A study developed with the genus *Enterococcus* spp. From sheep's milk in the State of Minas Gerais found, as in this work, antagonism against indicator pathogens such as *E. coli*, in addition to *L. monocytogenes*, *P. aeruginosa* and *S. aureus*, being the later, divergent from the results, that is, the strains of *Enterococcus* spp. did not inhibit *S. aureus* (Acurcio *et al.*, 2014).

Sánchez and Tromps (2014) when assessing the probiotic profile of lactic acid bacteria isolated from the milk of Venezuelan cows observed that about 72.7% of their sampling was capable of inhibiting the growth of pathogens such as *P. aeruginosa*, an approximate percentage to the one found in this study.

Galo and Valencia (2013) observed the inhibition of pathogens such as *E. coli* and *K. pneumoniae* by a lactic strain isolated from female bovine, corroborating with the results reached in this research concerning these indicator species.

The results of the antagonist activity of the lactic acid bacteria in the face of the indicator microorganism of the Gram-positive group found in this study are not consistent with the reports found in the scientific literature, which report a satisfactory inhibition of pathogenic microorganisms such as *Staphylococcus aureus* (Acurcio *et al.*, 2014; Koch *et al.*, 2014; Souza, 2015).

In reference to the samples of the genus *Streptococcus* spp., the percentage of antagonist activity on the tested pathogens is represented in the Chart 4.

Similarly to the genus *Enterococcus* spp., all the strains of *Streptococcus* spp. also did not inhibit the indicator species of *Staphylococcus aureus* ATCC 29213, belonging to the Gram-positive group.

Marino et al (2003) reported that the genus *Streptococcus* is commonly found in products of lactic fermentation, being the *Streptococcus thermophilus*, until years ago, the only species which remained viable in reasonable quantities from the raw material to the ripening stage of the cheese.

However, recent studies such as those of Nobrega (2012); Ferreira *et al.*, (2015) and Medeiros (2016) after the analysis of the lactic microbiota of Brazilian artisanal cheeses identified a greater diversity of LAB species such as the *Streptococcus infantarius* subsp. *infantarius*.

Cabral et al (2016) observed, when assessing the technological aspects of the strains isolated from coalho cheese produced in the Agreste region of the State of Pernambuco, that the strains of *Streptococcus* sp. presented the largest inhibition halos against *Klebsiella pneumoniae* and *Staphylococcus aureus*.

Similar results were also described by Duarte et al (2013), which demonstrated the antagonist action of lactic acid bacteria of the genus *Lactobacillus* spp. and *Streptococcus* spp. on the growth of a pathogenic strain of *E. coli*.

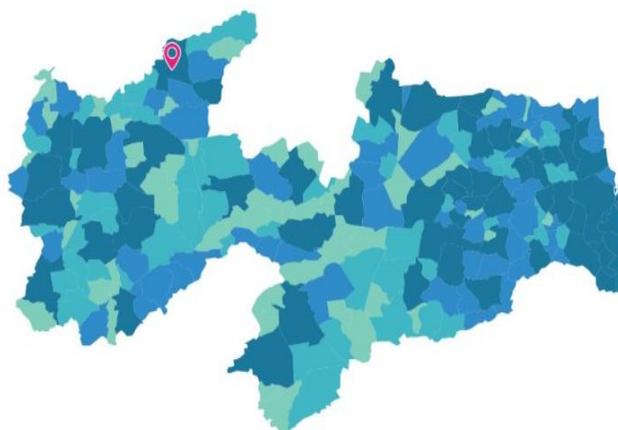
A relevant factor was that five (27.8%) of the strains of *Enterococcus* spp. and four (36.4%) of the strains of *Streptococcus* spp. tested presented antagonist activity to three

pathogenic species indicators of the Gram-negative group and did not present antagonistic effect against the Gram-positive species.

According to Stevens (1991) the target of the bacteriocins produced by the lactic acid bacteria is the cytoplasmic membrane, however, due to the protective barrier supplied by the lipopolysaccharides (LPS) of the external membrane of the Gram-negative bacteria, these bacteriocins generally are only

active against Gram-positive strains. However, mutant strains of Gram-negative bacteria have become sensitive to the bacteriocins after the exposure to sub-lethal stress such as, heating, freezing or unfreezing, which may cause the rupture of the external membrane and permit the access of this substance to the plasmatic membrane, leading to an increase of the sensitivity (Stevens *et al.*, 1991; Schved *et al.*, 1994; Hauben *et al.*, 1996).

Fig.1 Location of the study area



Source: IBGE, 2018

Chart.1 Standards of sensitivity to antibiotics of *Enterococcus faecium*, isolated from artisanal coalho cheese produced in the Sertão region of the State of Paraíba

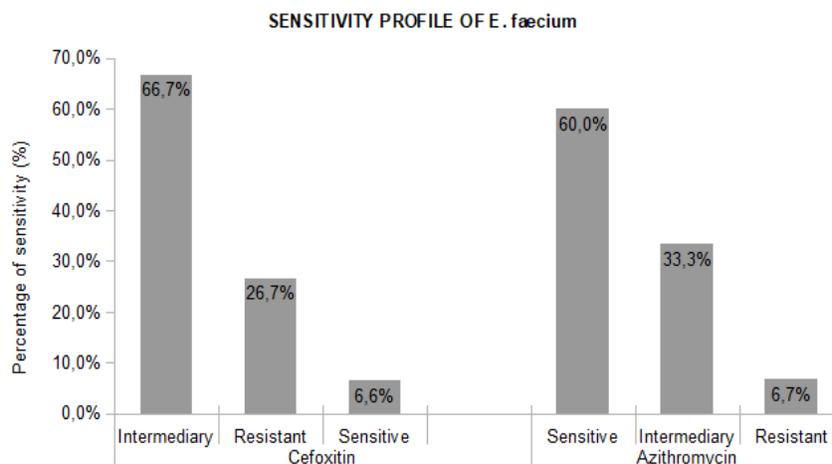


Chart.2 Sensitivity to antibiotics rates of strains of *Streptococcus infantarius subsp. infantarius* isolated from artisanal coalho cheese produced in the Sertão region of the State of Paraíba

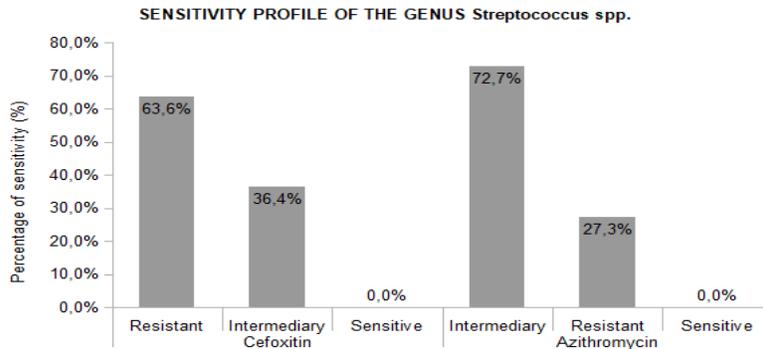
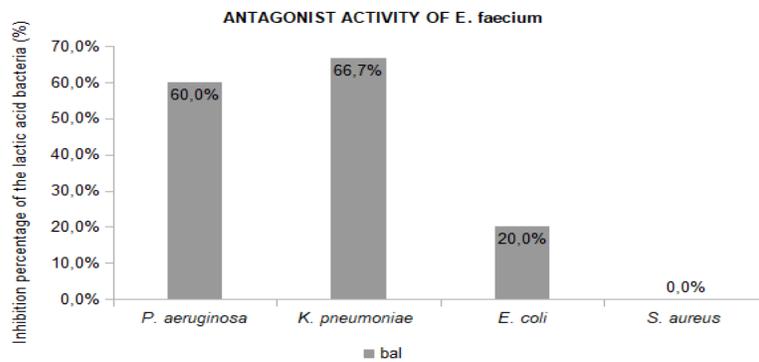
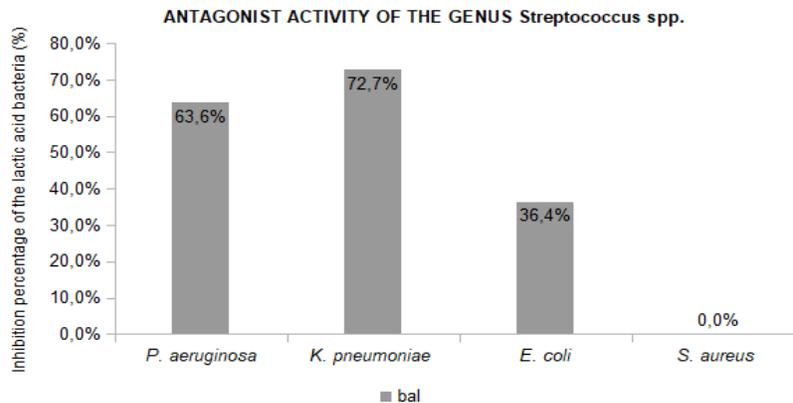


Chart.3 Antagonist percentage of the strains *E. faecium* isolated from artisanal coalho cheese produced in the Sertão region of the State of Paraíba on the pathogenic strains



Graph.4 Antagonist percentage of the genus *Streptococcus* spp. Isolated from artisanal coalho cheese produced in the Sertão region of the State of Paraíba on pathogenic strains



In conclusion, the lactic acid bacteria *E. faecium*, *E. faecalis*, *E. duran* and *S. infantarius subsp. infantarius* analyzed in this study were sensitive to a series of synthetic antibiotics (ampicillin, chloramphenicol, vancomycin, amoxicillin and azithromycin) and presented an antagonist profile against Gram-negative strains present in the GIT (*P. aeruginosa*, *K. pneumoniae* and *E. coli*), as for *E. casseliflavus*, it presented sensitivity to these antibiotics, however, did not exhibit antagonism to the tested pathogens.

In the light of these results, the acid lactic bacteria isolated from the artisanal coalho cheese from the State of Paraíba, from the microrregion of Catolé do Rocha-PB, presented results which were indicative of probiotic activity, however, for this confirmation, further tests which aim at the capacity of the LAB of enduring the GIT conditions and transfer their benefits to the hosts must be carried out.

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How to cite this article:

Leandro Paes de Brito, Rosália Severo de Medeiros, Abrahão Alves de Oliveira Filho, Onaldo Guedes Rodrigues, Julia Laurindo Pereira and Emmily Vieira de Oliveira Brito. 2019. Profile of Susceptibility to Antimicrobials and Antagonist Activity of Lactic Acid Bacteria with Probiotic Potential Isolated from Artisanal Coalho Cheese of the Sertão Region of the State of Paraíba. *Int.J.Curr.Microbiol.App.Sci.* 8(06): 306-316. doi: <https://doi.org/10.20546/ijcmas.2019.806.035>