Characterization of lactic acid bacteria in coalho cheese and antagonism to some pathogenic food-related bacteria

Caracterização de bactérias láticas em queijo de coalho e antagonismo a alguns patógenos

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RESUMO
O queijo de coalho é um produto tradicional do nordeste brasileiro e o conhecimento da sua microbiota autóctone é de fundamental importância. Neste trabalho foi caracterizada a microbiota do queijo de coalho de três propriedades rurais (A, B e C), situadas respectivamente nas mesorregiões do Agreste, Borborema e Sertão paraibanos, com o intuito de avaliar a importância do perfil das bactérias láticas encontradas neste tipo de queijo e verificar a atividade antimicrobiana destas bactérias frente a agentes patogênicos. As contagens de bactérias láticas do queijo de coalho dos produtores A e B foram na ordem de 10^6 e as maiores contagens (10^9 UFC/g) foram observadas no queijo do produtor C. Foram selecionadas 49 bactérias láticas típicas das três propriedades e os gêneros predominantes foram: *Enterococcus*, *Lactococcus, Streptococcus e Leuconostoc*. Dos 20 isolados, 19 apresentaram halos de inibição sobre as três bactérias patogênicas, com zonas de inibição de 2 a15 mm, e os maiores halos foram formados por *Lactococcus lactis* ssp lactis sobre *S. enterica* e *S. aureus*. A atividade antimicrobiana revelada por alguns isolados de bactérias láticas sugerem a possibilidade de seu uso contra agentes patogênicos e podem atuar como barreira no desenvolvimento destes micro-organismos e como conservantes biológicos.
Palavras-chave. queijo, *Lactococcus*, *Staphylococcus aureus*.

ABSTRACT
The coalho or curdled cheese is a traditional product of the Brazilian Northeast region, but the knowledge on its autochthonous microbiota is critical. This survey aimed at characterizing the microbiota of coalho cheese produced in three rural properties (A, B and C), located in Agreste, Borborema and Sertão regions of State of Paraíba - Brazil, respectively. The relevance of the profile of lactic acid bacteria found in this type of cheese was assessed, and the antimicrobial activity of these identified bacteria against *Salmonella enterica*, *S. aureus* and *L. monocytogenes* was analyzed. The lactic acid bacteria counts of coalho cheese from producers A and B were 10^6 and the highest counts (10^9 UFC/g) were found in cheese samples from producer C. Forty-nine lactic acid bacteria from three rural properties were selected and predominant genera was *Enterococcus*, *Lactococcus*, *Streptococcus* and *Leuconostoc*. Of 20 bacteria isolated, 19 showed inhibition halos on the three pathogenic bacteria with diameter of 2 to 15 mm, and the largest halos were formed by *Lactococcus lactis* ssp lactis on *S. enterica* and *S. aureus*. The antimicrobial activity displayed by some lactic acid bacteria isolates suggests the possibility of its use against pathogens**,** and they might be effective as a barrier in these microorganisms development and as biological preservatives in coalho cheese.
Keywords. cheese, *Lactococcus*, *Staphylococcus aureus*.

INTRODUTION

Cheese are important sources of nutrients and have great diversity of flavors, textures and shapes due to natural variability of milk, local of production, and processing techniques by which were produced the following basic principles the more than a thousand varieties of existing cheeses, basically involving the combination four ingredients: milk, curds, salt and micro-organisms, for its production[1].

The Coalho cheese is a cheese made with crud or semi-cooked dough, produced for over a century from raw and/or pasteurized cow's milk[2]. It is part of alimentary habits, and source of income and employment for a portion of small and medium producers. This cheese is one of the traditional milk products produced in Northeastern Brazil and has its origin related to artisanal manufacturing, a fact that persists today[3].

The handicraft cheeses present a typical and different microbial population, which is related with the region of origin of the raw material and its manufacturing technology[4]. In general, these milk products have a variety of bacteria, including lactic acid bacteria that produce enzymes and are used for the production and preservation of foods, having the ability to promote changes beneficial to animal and human health. These micro-organisms are essential in all varieties of natural cheese and play important roles during manufacture and maturation[1].

Lactic bacteria are used for the production and preservation of foods. The fermentation of milk, besides increasing its conservation life, provides greater availability of their nutrients. Some of these micro-organisms also have the ability to alter the intestinal microbiota and promote beneficial changes to human and animal health. Thus, the acidity developed by these micro-organisms used in the manufacture of fermented dairy products aids in the absorption and utilization of nutrients by the body of the consumer, which can be used in industry as a way to provide products with increased nutritional values, and prevent possible food borne illness.

The Coalho cheese is a typically Brazilian product and yet there are few studies that can bring important scientific contributions, particularly to improve the quality of these products. This study aimed to characterize the microbiota of the Coalho cheese, produced in the region of Paraiba - PB, Brazil, in order to assess the importance of profile of the lactic acid bacteria found in this type of cheese.

MATERIAL AND METHODS

Count, isolation, characterization and identification of lactic acid bacteria

Randomly we selected three farms that produced and traded artisanal Coalho cheeses, located in the regions of Paraiba: Agreste of Paraiba (in the City of Bananeiras), Boa Vista (micro region of the Cariri) and of the region of the Sertão (city of Patos).

Were collected from each property on the same date, samples indicative of Coalho cheese (1 kg), every fortnight, in triplicate, in the period from March to July 2009. These properties adopted systems of cheese production based on traditional techniques, without registration and without adopting good manufacturing practices, leaving the responsibility for the preparation and sale of products made to the owner. All these producers of the not used to pasteurize to the milk and did not add yeast in the production of Coalho cheese. Each processed on average 50 liters of raw milk, obtaining about five units of 1 kg of Coalho cheese per day. A piece of Coalho cheese was wrapped in plastic bags, using, in the collection of materials previously sterilized and aseptic collection procedures. The samples identified were transported in cool boxes containing ice, kept refrigerated and taken to the laboratories of Food Microbiology - CT-UFPB, Campus I-João Pessoa – PB, for the analyzes. In the laboratory, portions of 25 grams of Coalho cheese, taken alternately, were homogenized in a blender during 2 minutes at low speed. For the dilution of samples, we used 0.1 % peptone saline[5].

For the count of lactic acid bacteria from curd cheese samples, were inoculated 0.1 mL of the dilutions of the samples, on surfaces, in MRS agar (1.5 % agar) (Himedia) modified by the addition of cysteine-HCl (0.05 %), calcium carbonate (5 g/litro) and bromcresol purple (0.04 g/liter). After seeding samples made by the addition of a cover layer, consisting of the own medium containing 0.75 % of agar (semisolid medium), which together with cysteine-HCl allowed incubation of lactic acid bacteria under anaerobic conditions (37 °C). The purple and calcium carbonate have been used to allow viewing of yellow halos around the colonies, indicators of acid production.

After incubation at 37 °C during 48 hours, were counted plates containing 15-150 colonies surrounded by yellow haloes, considered typical of lactic acid bacteria. Of these plates it was isolated, in medium similar to that used in counting, randomly, a number of typical

colonies equivalent to the square root of the count, using the technique of grooves on quadrant. After incubation at 37 °C for 24 hours, tests were conducted Gram and catalase. The Gram-positive and catalase negative were streaked on inclined MRS agar, incubated at 37 ºC for 48 hours, and then kept in refrigeration until the testing and confirmation of identification. Purification was performed by repeating of previously isolated strains, maintained in inclined MRS agar incubated at 37 °C for 48 hours.

Initially, the isolated cultivated in MRS broth at 37 °C for 48 hours, were subjected to the following characterization tests: gram stain, catalase, gas production, growth at 45 °C temperature and increased concentration of 6.5 % of sodium chloride. These tests have been used in the presumptive identification of isolates at the genus level, according to the scheme proposed by Cogan et al[6].

Subsequently, the isolated of the samples of Coalho cheese were analyzed for biochemical profile from the fermentation of 49 carbohydrates, using the API 50 CHL system (BioMérieux, Marcy-IÈtoile - France) and identified to the species level by using a database (V5.1) provided by the system itself (API software WEB TM), making it according to the manufacturer's instructions. The results of this test were compared, in terms of gender, with those obtained by the scheme proposed by Cogan et al[6].

Detection of antimicrobial activity

The antagonistic activity of lactic acid bacteria was tested on *Salmonella enterica* (ATCC 6017), *Listeria monocytogenes* (ATCC 7664) and *Staphylococcus aureus* (ATCC 6538). The pathogens were cultured in BHI (Himedia) at 36 °C during 24 hours. The deferred method was used for detection of antagonistic activity of lactic acid bacteria. The identified lactic cultures, maintained on MRS agar were grown in MRS broth, incubated at 36 °C for 16 hours. After incubation, it was read the optical density of the bacterial suspension at 530nm in a spectrophotometer. When necessary, dilutions were made of lactic culture using the culture broth as a diluent in order to standardize the inoculum to an optical density of 0.4, equivalent to the McFarland 2. Subsequently, 10 uL of the lactic culture was inoculated onto the surface of plates containing 15 mL of solid medium (1.5 % agar) MRS, three bacteria being added per plate.

The plates with MRS medium was incubated at 37 °C for 24 hours in an anaerobic jar at 37 °C for 24 hours. Simultaneously, pathogenic bacteria were grown in BHI broth at 37 °C during 24 hours. After incubation during 24 hours, the plates were covered with 5 mL of MRS semisolid agar (0.75 % agar), according to the media contained in the base plate, 300 µL added to the suspension of the pathogen grown in BHI. These plates were again incubated under aerobic conditions at 37 °C during 24 hours and examined for formation of inhibition zones. For the purpose of recording, the external diameter of inhibition halo of the colony subtracted from the diameter of colony corresponded to the zone of inhibition[7]. The results were analyzed using conventional descriptive statistics, with determinations of average, minimum and maximum values and their respective standard deviations.

RESULTS AND DISCUSSION

Lactic bacteria count

In the Table 1 we see the lactic acid bacteria counts of samples of Coalho cheese; it was observed in the samples A and B properties, average values in the order of 10^6 CFU/g, being observed for the producer C higher counts ranging from 1.1 x 10^7 to 3.4 x 10^9 CFU/g. Possibly, the largest number of lactic acid bacteria in milk, combined with a lower contamination of the raw material (Table 1) was the factors that contributed most to the greatest number of lactic acid bacteria in cheese samples from producer C.

Table 1. Counts of lactic acid bacteria in Coalho cheese from producers A, B and C

Repetition	Produtor A	Produtor B	Produtor C
I	$3{,}0x10^5$	$7{,}2x10^6$	$3{,}4x10^9$
II	$7{,}1x10^6$	$3{,}5x10^6$	$1{,}1x10^7$
III	$1{,}0x10^7$	$5{,}4x10^5$	$1{,}2x10^7$
Average	$5{,}8x10^6$	$3{,}7x10^6$	$1{,}1x10^9$

PA- from farm A; PB- from farm B; PC- from farm C

According to Nascimento[8], analyzing samples of frescal cheese from Minas Gerais, found lactic acid bacteria counts above 10^5 CFU/g. Ouadghiri et al[9], studying the biodiversity of these bacteria in Moroccan white cheese Jben, found lactic acid bacteria counts between 10^8-10^9 CFU/g.

The main function of lactic acid bacteria in food of the these products is acidification at pH around four, which prevents the development of undesirable bacteria (by the production of organic acids, predominantly lactic

acid). This allows that the conservation time of fermented products is much greater than the product in which the feedstock has not been fermented. Another function of lactic acid bacteria is to develop the organoleptic properties of fermented products[9].

Isolation of lactic acid bacteria

We isolated 49 colonies typical of lactic acid bacteria from the samples of Coalho cheese analyzed, which were grouped into four different genres, with different levels of occurrence between samples. Of the total of 20 isolates tested for species identification using API, 12 were possible of identification as twelve strains of *Lactococcus lactis* ssp *cremoris*, one of *Lactococcus lactis* ssp *lactis*, one of *Leuconostoc mesenteroides* ssp *cremoris*, one of *Pediococcus damnosus* and three of *Pediococcus* spp. Still, five strains were identified as *Lactobacillus* and three unidentified (Table 2). Of the six isolates that were not identified to the species level, four were identified according to the traditional method of Cogan et al[6] as possible *Enterococcus*, and as the API is not kit the for the identification of the kind, such isolates obtained from unacceptable profile identification (Table 2).

Some species of the genus *Enterococcus* (*E. faecium* and *E. faecalis*) are the most common in foods. This genus has the capacity to grow over a wide temperature range of 10 to 60 °C. In artisanal cheeses made from raw milk, these microorganisms can come from raw materials or the environment and can develop sensory characteristics to the cheese through biochemical reactions during cure[10].

The identification of bacteria belonging to the genus *Lactococcus* was that better matched between the two methods mentioned, with the best similarity indices ranging between 55.0 % and 98.0 %. Among the species of *Lactococcus*, *L. lactis* is the most important commercially and is commonly used as a simple yeast, mixed or multiple for manufacturing different types of dairy products[8]. The *Lactococcus* are predominant in fresh cheese that does not undergo a baking of dough and its presence is reduced during the cure process and the species *Leuconostoc mesenteroides* subsp *cremoris* spp. and subsp. *lactis* are associated with dairy products, are used as flavoring micro-organisms[11]. The genus *Pediococcus* produces diacetyl from glucose, thereby improving the flavor of cheeses during maturation[12].

The identification of isolates using the methods of Cogan et al[6] and identification by API coincided by 25 %, diverging for most identifications (75 %) performed. In general, the classification was given as "low discrimination", demonstrating the difficulty of establishing a relationship between the two methodologies.

Although the API 50 CHL system have allowed the identification of some lactic acid bacteria at species level, with a higher power of discrimination, some lactic acid bacteria have been identified so inconclusive as to the isolates (Table 2), which were identified as the Lactobacillus when their morphology corresponded to form of cocci. This discrepancy may be related to the fact that bacteria of the genus Lactobacillus have a physiology similar to the genera Leuconostoc and Pediococcus, these genres being considered phylogenetically interrelated[12].

According to Morsi el soda et al[13] isolating and identifying lactic acid bacteria of some Egyptians cheeses (Ras, Domiatti, Kareish Zabady and Laban), using the API system, achieved a satisfactory identification, identifying about 51 % of the isolates obtained, indicating that some commercial identification systems, can often produce good results on identification of genres, but they were not totally appropriate to the species level.

Abd El Gawad et al[14], identifying lactic acid bacteria in traditional Egyptian milk, using the API 50CHL commercial system and API 20 Strep and identified 38 isolates (23 %) and 22 (13 %), respectively. The growing use of rapid identification techniques has promoted significant contributions to the knowledge of the microbiota of certain foods, including cheeses, but other factors must be taken into account as: taxonomic changes, changes in nomenclature used and additional tests such as PCR, RAPD.

Antagonist activity

Of the twenty strains of lactic acid bacteria tested for production of antimicrobial substances, nineteen were positive for inhibition of the three strains of pathogenic bacteria tested as indicator (*S. enterica*, *L. monocytogenes* and *S. aureus*), so the isolated AP16 was effective in inhibition of *S. enterica* and *L. monocytogenes* (Table 3).

The inhibition halos formed ranged between two and 15 mm, with the largest inhibition halo formed by strain PC17 (identified as suggestive of the genus *Enterococcus* according to traditional methodology) against *S. aureus*. The *Enterococcus* are producers of enterocins, with already established activity against *L. monocytogenes* and *S. aureus*.

Table 2. Distribution of species of lactic acid bacteria isolated from samples of Coalho cheese and identified by API 50 CHL test

Isolated	Identification at genus level according Cogan et al[6]	Identification at species level according to the API	% Similarity Index
PA1	*Streptococcus*	*Leuconostoc mesenteroides* ssp. *cremoris*	78,4 %
PA7	*Lactococcus*	*Lactococcus lactis* ssp. *cremoris*	55,8 %
PA8	*Lactococcus*	*Lactococcus lactis* ssp. *cremoris*	55,8 %
PA13	*Lactococcus*	*Pediococcus* spp.	61,7 %
PA15	*Streptococcus*	*Lactobacillus*	68,4 %
PA16	*Leuconostoc*	*Pediococcus* ssp.	14,0 %
PB5	*Enterococcus*	Unacceptable	-
PB6	*Enterococcus*	*Lactobacillus*	90,0 %
PB10	*Lactococcus*	*Lactobacillus*	88,0 %
PB11	*Enterococcus*	*Lactococcus lactis* ssp. *cremoris*	90,0 %
PB13	*Enterococcus*	*Lactococcus lactis* ssp. *cremoris*	87,3 %
PB14	*Lactococcus*	*Lactococcus lactis* ssp. *cremoris*	75,5 %
PC4	*Streptococcus*	Unacceptable	-
PC7	*Leuconostoc*	*Pediococcus damnosus*	94,0 %
PC10	*Lactococcus*	*Lactococcus lactis* ssp. *lactis*	55,0 %
PC11	*Enterococcus*	Unacceptable	-
PC13	*Lactococcus*	*Lactococcus lactis* ssp. *cremoris*	98,0 %
PC14	*Lactococcus*	*Pediococcus* spp.	56,0 %
PC17	*Enterococcus*	*Lactobacillus*	67,9 %
PC18	*Lactococcus*	*Lactobacillus*	66,0 %

PA – producer A, PB - producer B and PC - producer C.

The largest inhibition zones (14 mm) formed by a same lactic bacterium was found for the species *L. lactis* ssp *lactis* (PB13 and PC10) on *S. enterica* and *S. aureus*. The strain identified as *L. mesenteroides* ssp. *cremoris* (PA1) showed greater activity against *S. enterica* (8mm) and *S. aureus* (7 mm). Lactic acid bacteria identified as *Pediococcus* spp. (PA13, PA16, PC7 and PC14) showed broad spectrum of antibacterial activity against bacteria *S. aureus* and *S. enterica*. Bacteria of the genus *Pediococcus* can produce bacteriocins, called pediocins such as *Pediococcus pentosaceus* (pediocin A) and *Pediococcus damnosus* (pediocin PD-1), which have an antagonistic effect on spoilage and pathogenic bacteria in foods[8].

The species of the genus *Lactococcus* showed those identified most frequently in the cheese samples analyzed, and the isolated *L. lactis* ssp. *lactis* and *L. lactis* spp. *cremoris* showed broad spectrum of activity against the bacterial strains tested as revealing. Nascimento[8] evaluating the antimicrobial activity of bacteriocinogenic cultures, found that strains of *L. lactis* subsp. *lactis* showed antimicrobial effect on 80 % (8/10) of the strains of *L. monocytogenes* and 40 % (2/5) of strains of *S. aureus* evaluated. *Lactococcus lactis* ssp *lactis* produce nisin, bacteriocin already used as preservatives in foods, has a broad antimicrobial spectrum being active against Gram positive bacteria and spores, besides being used as a food preservative[15].

S. aureus was found as the most sensitive microorganism to antagonistic substances produced by the lactic acid bacteria tested. Some authors report that the higher the rate of multiplication of lactic acid bacteria in growth substrate and subsequent production of lactic acid, the stronger inhibition of *S. aureus*. The rapid reduction in pH associated with the characteristics of biological competition of lactic acid bacteria are factors inhibiting the growth inhibition of *Staphylococcus* species[16]. It is worth noting also that the inhibitory effect established by lactic acid bacteria against strains of pathogenic and deleterious bacteria related to food also occurs due to the production of bacteriocins and other antimicrobial substances such as diacetyl and hydrogen peroxide[17].

Changes in measures of inhibition zones, and consequently the sensitivity of the revealing strains tested, observed when the action of lactic acid bacteria strains of the same species or different species may also be related to the existence of different mechanisms of inhibition and/or nature inhibitory chemical substance that influences its diffusion in the culture medium[18].

Table 3. Zones of inhibition (mm) produced by lactic acid bacteria isolated on *Salmonella enterica, Listeria monocytogenes and Staphylococcus aureus* in MRS

Isolated	Lactic acid bacteria Identified by API	*S. enterica*	*L. monocytogenes*	*S.aureus*
PA1	*Leuconostoc mesenteroides* ssp. *cremoris*	8	5	7
PA7	*Lactococcus lactis* ssp. *cremoris*	8	4	3
PA8	*Lactococcus lactis* ssp. *cremoris*	4	6	3
PA13	*Pediococcus* spp.	8	7	11
PA15	NI	8	9	14
PA16	*Pediococcus damnosus*	3	0	7
PB5	NI	8	9	14
PB6	NI	7	9	8
PB10	NI	12	10	9
PB11	*Lactococcus lactis* ssp. *cremoris*	8	4	8
PB13	*Lactococcus lactis* ssp. *cremoris*	8	3	14
PB14	*Lactococcus lactis* ssp. *cremoris*	8	4	8
PC4	NI	14	6	9
PC7	*Pediococcus damnosus*	10	3	6
PC10	*Lactococcus lactis* ssp. *lactis*	14	6	14
PC11	NI	7	8	6
PC13	*Lactococcus lactis* ssp. *cremoris*	5	5	5
PC14	*Pediococcus* spp.	5	3	5
PC17	NI	8	9	15
PC18	NI	5	9	6
	Average (DP)	7,9 (2,84)	5,9 (2,68)	8,6 (3,73)

PA- producer A, PB- producer B and PC- producer C; NI – unidentified; Halo inhibition in millimeters (mm)

It is also worth mentioning that many bacteriocins are active against pathogenic micro-organisms of interest in food, so that is growing interest in the food industry about the potential use of these compounds in foods to replace chemical preservatives[19].

L. monocytogenes is a pathogen commonly inhibited by bacteriocins produced by lactic acid bacteria. According to Alexandre[20], from 48 strains isolated from cheese, 25 % were able to inhibit the *S. aureus* and *L. monocytogenes*. In contrast, other authors evaluating the microbiota of raw milk, mentioned that the bacteriocins produced by lactic acid bacteria may not have much influence on the possible pathogens, since *S.* Typhimurium and *L. monocytogenes* show good match with regard to micro-organisms present in the raw material[21].

The lactic acid bacteria strains tested showed greater inhibition capacity of the strains of Gram-positive bacteria (*L. monocytogenes and S. aureus*), shown by the formation of larger zones of inhibition of growth. Other studies also reported greater inhibition of strains of Gram-positive bacteria by bacteriocinogenic lactic cultures[16, 22, 23, 24]. Specifically against Gram-negative bacteria, the double lipid layer present on its outer cell structure prevents interaction of specific antagonists substances with wall and bacterial plasma membrane[9]. The antimicrobial activity of bacteriocins produced by lactic cultures can act as a barrier in the development of these micro-organisms, acting as biological preservatives in Coalho cheese[4].

Guedes Neto et al[17] demonstrated functional properties, such as in vitro antagonism against undesirable microorganisms and antibiotic resistance in strains of lactic acid bacteria isolated from curd cheese produced in the state of Pernambuco (Brazil). The presence of lactic acid bacteria antagonists of endogenous micro flora as part of samples of Coalho cheese can act as an agent of controlling the population of pathogenic microorganisms in these substrates by inhibiting their multiplication in different stages of processing as well as during its storage[9, 19].

The results show that there are a variety of autochthonous lactic acid bacteria in Coalho cheese made by hand with the ability to produce different antimicrobial substances. The selection of these bacteria can be used as cultures in the preparation of the Coalho cheese type and may thus enhance the consumption of these products.

CONCLUSION

From a total of twenty (20) isolates, twelve (60 %) were identified by API KIT and identification of the isolates between the two methodologies coincided 25 % compared to the genus *Lactococcus*, diverging identifications with most (75 %) performed by methodology according Cogan et al[6].

The antimicrobial activity displayed by some strains of lactic acid bacteria suggest the possibility of its use against pathogens such as: *Salmonella enterica*, *Listeria monocytogenes*, *S. aureus*, and can act as a barrier in the development of microorganisms and as biological preservative in Coalho cheese.

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