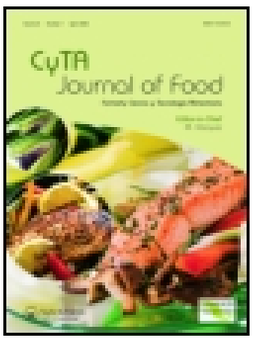

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Identification of lactic acid bacteria isolated from artisanal Coalho cheese produced in the Brazilian Northeast

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ABSTRACT

Coalho cheese is a traditional dairy product from the northeast of Brazil, which is currently commercialized in other regions of the country and even abroad. The pasteurization process eliminates most of the lactic acid bacteria (LAB), which are responsible for the specific characteristics of the cheese such as taste or aroma. This work aimed to identify the LAB present in different artisanal Coalho cheeses produced in the 'Sertão' region of the State of Paraíba, northeast of Brazil. The LAB populations showed some diversity comprehending species of the genus *Lactococcus, Enterococcus, Streptococcus, Lactobacillus, Leuconostoc* and *Weissella*. Different prevailing LAB species were found in different micro-regions of the Sertão region of the State of Paraíba, indicating that local environmental conditions, animal genetics and cheese production characteristics may influence the milk and the cheese microbial populations.

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KEYWORDS
Coalho cheese; lactic acid bacteria; prevalence

PALABRAS CLAVE
queso de Coalho; bacterias de acido láctic; prevalencia

Identificación de bacterias lácticas aislado de queso Coalho artesanal producidos en el Nordeste Brasileño

RESUMEN

Queso Coalho es un producto lácteo tradicional del Nordeste de Brasil, que se comercializa actualmente en otras regiones del país y incluso del extranjero. El proceso de producción utiliza la leche no pasteurizada, que puede ser una fuente de microorganismos patógenos en contraste con el proceso de producción industrial que favorece el uso de la leche pasteurizada. El proceso de pasteurización elimina la mayor parte de las bacterias de ácido láctico (LAB), que son responsables por las características específicas del queso como el sabor o el aroma. Este estudio tuvo como objetivo identificar las LAB presente en diferentes quesos Coalho artesanales producidos en la región Sertão del Estado de Paraíba, Nordeste de Brasil. Las poblaciones de LAB mostraron una cierta comprensión de la diversidad del género *Lactococcus, Enterococcus, Streptococcus, Lactobacillus, Leuconostoc* y *Weissella*. Las diferentes especies predominantes de LAB fueron encontradas en diferentes microrregiones del Sertão de Paraíba, lo que indica que las condiciones ambientales locales, la genética de los animales y las características de producción de queso pueden influir en la leche y las poblaciones microbianas del queso.

1. Introduction

Coalho cheese is traditionally produced in the northeastern states of Brazil, through the coagulation of milk with rennet or other appropriate coagulating enzymes, complemented or not by the action of selected lactic acid bacteria (LAB) (Brasil, 2001). This cheese is usually commercialized fresh or with a maturation period of up to 10 days. In the northeastern region, artisanal cheese production is an important economic activity of the local populations, often representing an additional source of income for families with limited resources (Menezes, Cruz, & Menasche, 2010). Regarding the economic, social and political organizations, the State of Paraíba is divided in four meso-regions, which comprise the regions of Zona da Mata, Agreste, Borborema and Sertão. The Sertão region has the greatest concentration of cattle farming of the State of Paraíba and the milk produced in this region is supplied to most of the cheese dairies responsible for the production of Coalho cheese and butter, in the State of Paraíba (Luíz, 2014). The Sertão region is divided in seven micro-regions: Catolé do Rocha, Cajazeiras, Sousa, Patos, Itaporanga, Piancó and Serra do Teixeira, each one with specific edaphoclimatic characteristics.

Despite the economic importance and popularity of Coalho cheese, its production with both artisanal and industrial processes is not optimized, and cheese quality could be improved using appropriate technologies (Lima, Telles, Macedo, & Benevides, 1998). In particular, different organoleptic properties are found in the cheeses produced in the various states of the northeast. Moreover, the use of conventional lactic yeast in the manufacturing of Coalho cheese may cause unwanted alterations of their typical characteristics (Franciosi, Settanni, Cavazza, & Poznanski, 2009; Macedo, Tavares, & Malcata, 2004).

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The LAB naturally present in raw milk or intentionally added during the cheese manufacturing process are associated with properties such as taste, texture and aroma of dairy products, being largely used as starter cultures in various products of this industry (Carr, Chill, & Maida, 2002).

The LAB group comprehends 16 genera (Ferreira, 2003; Jay, 2005), among which the ones more commonly found in cheeses are *Lactococcus, Lactobacillus, Streptococcus, Leuconostoc* and *Enterococcus* (Fox, Guinee, Cogan, & Mcsweeney, 2000). The interest in the microbiota of raw milk cheese and other traditional dairy products results from the need of characterization of their complex populations and namely the identification of new strains of LAB (Wouters, Ayad, Hugenholtz, & Smit, 2002). Traditional dairy products host an enormous pool of microbial genetic diversity, which has a high biotechnological potential and is of great importance to the food industry (Alegría et al., 2009).

The molecular techniques are nowadays commonly used for the identification of microorganisms. In particular, the comparison of the sequences of the gene 16S of the ribosomal RNA (rRNA) is one of the most powerful and efficient techniques for the determination of the phylogenetic degree of relatedness among microorganisms (Woese, 1987). Therefore, the sequencing of the gene that codifies the 16S rRNA and the sequencing of the intergenic region is a technique that has been increasingly used to characterize the microbial diversity of food, including dairy products (Guedes Neto, 2008; Tilsala-Timisjärvi & Alatossava, 1997).

The present study aimed to identify the microbial diversity of the LAB present in the artisanal Coalho cheese produced in Sertão of the State of Paraíba, northeast of Brazil, in order to evaluate the prevalent LAB species and identify correlations between the cheese's geographical origin and its characteristic LAB composition. Moreover, the present work intends to contribute to the definition of a mix of starter cultures adequate for the production of Coalho cheese with reproducible and desirable organoleptic characteristics.

2. Material and methods

2.1. Sampling

The 28 samples of Coalho cheese produced with non-pasteurized milk were obtained from cheese dairies or local markets located in the seven micro-regions of the Sertão region (Paraíba state): Catolé do Rocha, Cajazeiras, Souza, Patos, Piancó, Itaporanga and Serra do Teixeira. Sample collection occurred in the period from June 2013 to October 2014 and the samples were transported in isothermal ice boxes to the Laboratory of Agricultural Raw Materials, of the Federal University of Campina Grande (UFCG), Patos, Paraíba, Brazil.

2.2. Sample preparation and isolation of the LAB

The cheese samples (25 g) were weighed in a semi-analytical balance Shimadzu® (São Paulo, Brazil) and homogenized with 225 mL of 2% sodium citrate solution (Vetec® – Rio de Janeiro, Brazil), for a period of 3 min using a stomacher Seward, 400® (West Sussex, United Kingdom). The homogenized samples were diluted (1:10) with a 0.1% sterile peptone solution (Merck® – São Paulo, Brazil), (Harrigan, 1998). An aliquot (1.0 mL) of the diluted samples was placed in Petri dishes (six replicates for each sample), and 20 mL of the appropriate culture medium was added to each replicate; Man, Rogosa, and Sharp culture medium (MRS) was used for two of the replicates, whereas M17 culture medium (Himedia® – Mumbai, India) was used for the other four replicates. An overtone layer was then added to each Petri dish using the corresponding culture media, but with the following modifications: to the M17 agar was added bromocresol purple (Merck®) (0.04 g L^{-1}) and glucose monohydrate PA (Vetec® – Rio de Janeiro, Brazil) (10%); the MRS agar was supplemented with bromocresol purple (0.04 g L^{-1}) and calcium carbonate (Vetec® – Rio de Janeiro, Brazil) (5.0 g L^{-1}). These modifications were introduced in order to facilitate the visualization of the yellow halos around the colonies, indicators of the acid production (APHA, 2001). The two Petri dishes with MRS medium and the two Petri dishes with M17 medium were incubated at the temperature of 30°C (mesophilic LAB), whereas the other two M17 agar dishes were incubated at 42°C (thermophilic LAB), in all cases for 48 h in an anaerobic jar (Carvalho, 2007).

2.3. Purification of LAB colonies

After incubation, the colonies were counted with the aid of a colony counter Phoenix® (São Paulo, Brazil), and the dishes that presented 25–250 colony forming units (CFU) were selected for isolation and purification. Ten colonies were selected from each culture medium (MRS and M17) and each incubation temperature. The colonies were transferred to flasks containing 10 mL of the corresponding media broth (MRS or M17) and incubated at the temperatures of 30°C and 42°C, respectively, for a period of 24 h (Silva et al., 2007).

After the growth in broth, the cultures were transferred to dishes containing MRS and M17 agar, using the striation method, and were incubated at 30°C and 42°C, respectively, for a period of 48 h in an anaerobic jar. The viable colonies were submitted to the catalase test and Gram staining, and were visualized under an optical microscope Olympus® (São Paulo, Brazil) under oil immersion at a 100-fold magnification. The colonies that reacted to the Gram test (Gram positive) in the cocci, bacilli or coccobacilli and with a negative catalase result were included in the LAB group.

Ten colonies from each medium were transferred to the M17 and MRS broths and incubated at 30°C and 42°C, respectively, for 24 h. After this period, 800 µL of each broth was transferred to *Eppendorf* microtubes, 200 µL of glycerol (Amresco® – Ohio, USA) was added, and the cultures were cryopreserved at −20°C (Acurcio, 2011; Silva et al., 2007). In parallel, 1.0 mL of the broths was transferred to *Eppendorf* microtubes and sent to the Laboratory of Molecular Biology of the Semi-arid of the UFCG, Patos, Paraíba, Brazil, for further analysis.

2.4. Molecular analysis

The 28 Coalho cheese samples collected in the meso-region of Sertão from the State of Paraíba yielded 609 distinct colonies with LAB characteristics. These colonies were submitted to DNA extraction, 16S rRNA gene amplification by the polymerase chain reaction (PCR), purification and sequencing of the PCR product.

2.5. DNA extraction

For DNA extraction, 1.0 mL of the broths was transferred to 2.0 mL *Eppendorf* microtubes and centrifuged at 12.000 rpm for 10 min (Centrifuge HT®, Essex, United Kingdom). The supernatant was discarded and the DNA was extracted from the culture pellets using QIAzol, Qiagen® (Hilden, Germany) following the manufacturer's instructions. The extracted DNA was dried at room temperature and solubilized in 100 µL of ultrapure water (Himedia® – Mumbai, India) and maintained at rest for 1 h. Subsequently, its concentration and degree of purity were tested using the spectrophotometer BioPhotometer plus, Eppendorf® (Hamburg, Germany).

2.6. PCR and purification of the PCR product

PCR was performed with the pair of *primers* plb16 (5′-AGAGTTTGATCCTGGCTCAG-3′) and mlb16 (5′-GGCTGCTGGCACGTAGTTAG-3′), (Invitrogen, Life technologies® – São Paulo, Brazil). The reaction media with a final volume of 20 µL was constituted of 5 UI One *Taq* DNA Polymerase BioLabs® (Shiga, Japan), 10X buffer, 1.5 mM magnesium chloride, 10 mM of dNTP, 10 µmol of each primer, 5 µL of DNA and ultra-pure water. The amplification was performed using a thermal cycler BIOCYCLER® (Foster, California, USA) under the following conditions: initial denaturation at 94°C for 5 min; 30 denaturation cycles at 94°C, for 30 s each; hybridization at 55°C for 45 s; extension at 72°C for 1 min and 30 s; and a final extension at 72°C for 7 min and 4°C for maintenance (Kullen, Sanozky-Dawes, Crowell, & Klaenhammer, 2000). Agarose gel was applied to the amplified material at 1.2% and it was submitted to electrophoresis for 40 min at U = 080 V (80), I = 400mA (47) and P = 065 W (3). The amplified material was stained with Safer-dye non-mutagenic fluorescent reagent KASVI® (Curitiba, Brazil), and the gel was observed under ultraviolet light and photographed.

The PCR product of the amplified samples was purified with the Invisorb Clean-Up kit Invitrogen Life technologies® (São Paulo, Brazil) according to the manufacturer's recommendations and frozen at −20°C for posterior sequencing. All disposable products used in this work were purchased from Axigen (New York, USA)

2.7. Sequencing

Sequencing was carried out in the Sequencing Platform of the Genetic Department of the Federal University of Pernambuco (UFPE). The readings were performed using the ABI 31001 sequencer AB Applied Biosystems/HITACHI® (Foster, California, USA), composed of 16.50-cm-long capillaries. The obtained sequences were analysed and aligned in the Mega 6.0 program and compared to the data stored in the GenBank (National Center for Biotechnology Information – NCBI) (http://www.ncbi.nlm.nih.gov/blast) using BLAST (Basic Local Alignment Search Tool).

3. Results

3.1. Microbiological parameters of LAB

The microbial population of LAB cultivated in specific media and at the temperatures of 30°C (mesophilic) and 42°C (thermophilic) corresponded to total counts varying from 10^8 to 10^{11} CFU g^{-1}, evidencing the microbial richness of the cheese samples (Table 1). The micro-region of Itaporanga was the one with the highest number of mesophilic colonies, both in the MRS medium (4.6×10^{10} CFU g^{-1} of Coalho cheese) and in the M17 medium (2.3×10^{11} CFU g^{-1} of Coalho cheese); this region was also the one with the highest number of thermophilic colonies (4.3×10^{10} CFU g^{-1} of Coalho cheese). Moreover the adjacent regions of Piancó and Serra do Teixeira also present a tendency for high numbers of CFUs, both for mesophilic and for thermophilic bacteria (Table 1, Figure 1).

Table 1. Culture medium and incubation temperature of lactic acid bacteria (CFU g^{-1}) isolated in artisanal Coalho cheese produced in Sertão of the State of Paraíba, northeast Brazil.

Tabla 1. Medio de cultivo y la temperatura de incubación de las bacterias lácticas (CFU g^{-1}) aisladas en el queso Coalho artesanal producido en Sertão del Estado de Paraíba, Nordeste de Brasil.

	Culture medium and temperature		
MICRO-REGION	MRS (30°C)	M17 (30°C)	M17 (42°C)
Catolé do Rocha-R1	2.7×10^{10}	3.3×10^{9}	1.9×10^{10}
Cajazeiras-R2	5.5×10^{8}	5.1×10^{8}	2.8×10^{8}
Sousa-R3	4.5×10^{8}	4.9×10^{10}	3.4×10^{8}
Patos-R4	4.2×10^{8}	4.0×10^{9}	3.4×10^{8}
Piancó-R5	4.8×10^{9}	4.5×10^{9}	3.6×10^{9}
Itaporanga-R6	4.6×10^{10}	2.3×10^{11}	4.3×10^{10}
Serra do Teixeira-R7	2.8×10^{9}	3.8×10^{9}	3.0×10^{10}

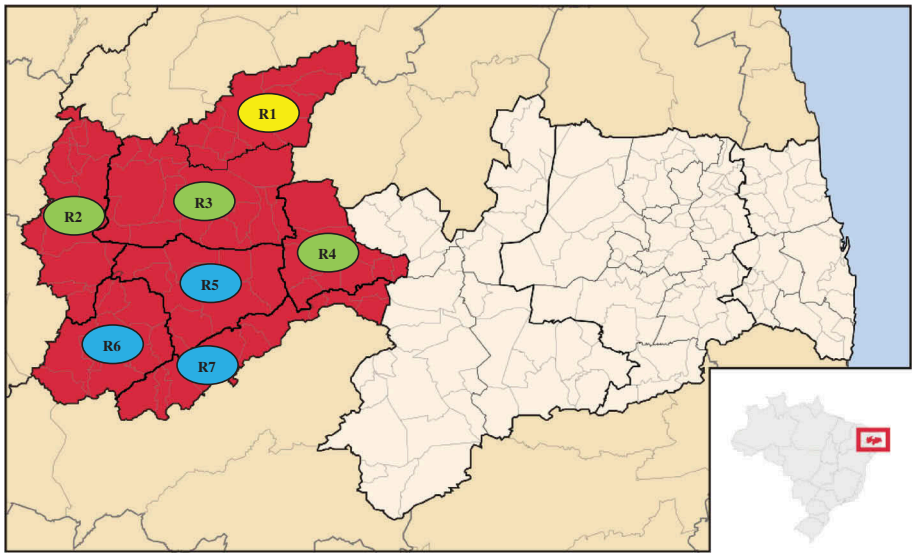
On the other hand, the micro-regions of Cajazeiras, Sousa and Patos presented the lowest numbers of colonies grown in the MRS medium (30°C) and the M17 medium (42°C), again suggesting the influence of regional conditions in the microbiology of the cheese since these are also adjacent micro-regions (Table 1, Figure 1).

The morphological characteristics of the LAB colonies were observed before the DNA extraction. The Gram-positive colonies with negative catalase reaction were classified as cocci, bacilli or coccobacilli. The M17 culture medium showed an elevated selectivity for the cocci form (91.9%) of the isolated colonies, followed by bacilli (7.2%) and coccobacilli (0.9%). In the MRS medium, bacilli colonies were 81.9% of the total colonies whereas coccobacilli represented 18.1% of all isolated colonies; no growth of cocci colonies was registered in this medium. Figure 2 shows the distribution of the different morphological types found in the microbial populations. The cocci mesophilic colonies were significantly higher in samples from regions R4–R7 when compared with the remaining regions and the bacilli colonies were more abundant in the samples from regions R2 and R3; these results suggest that not only the size of the microbial populations but also their morphological types distribution is influenced by environmental and genetic parameters that are different for the various micro-regions considered.

3.2. PCR and sequencing

From the 28 samples of Coalho cheese, 609 colonies with morphological characteristics suggestive of LAB, i.e. Gram-positive rod-shaped, cocci or coccobacilli and catalase-negative, were selected. A total of 456 colonies (74.9%) were identified by the sequencing as being LAB, of which 93.3% were cocci and 6.7% were bacilli. Again, 140 colonies were classified as non-lactic bacteria (22.9%) and 13 colonies did not amplify.

The lactic acid microbiota isolated from Coalho cheese from the State of Paraíba presented an elevated diversity.



Legend: Microregions R1 – Catolé do Rocha; R2 – Cajazeiras; R3 – Sousa; R4 – Patos; R5 – Piancó; R6 – Itaporanga; R7 – Serra do Teixeira.

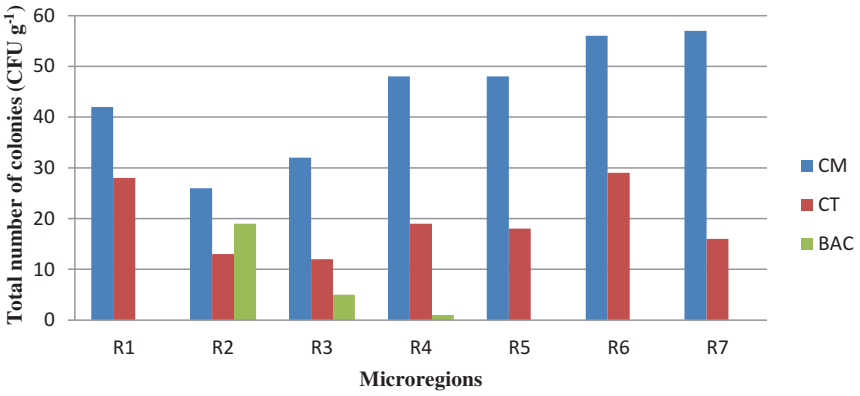
Leyenda: Microregiones R1 – Catolé do Rocha; R2 – Cajazeiras; R3 – Sousa; R4 – Patos; R5 – Piancó; R6 – Itaporanga; R7 – Serra do Teixeira.

Streptococcus *Enterococcus* *Lactococcus*

Figure 1. Prevalence of LAB genus isolated from Coalho cheese by regions from Paraíba, northeast Brazil.

Figura 1. La prevalencia de los géneros LAB aislados de queso Coalho por las regiones de Paraíba, Nordeste, Brasil.

Legend: Micro-regions: R1, Catolé do Rocha; R2, Cajazeiras; R3, Sousa; R4, Patos; R5, Piancó; R6, Itaporanga; R7, Serra do Teixeira.Leyenda: Microregiones R1 – Catolé do Rocha; R2 – Cajazeiras; R3 – Sousa; R4 – Patos; R5 – Piancó; R6 – Itaporanga; R7 – Serra do Teixeira.



Legend: Microregions: R1-Catolé do Rocha; R2-Cajazeiras; R3-Sousa; R4-Patos; R5-Piancó; R6-Itaporanga; R7-Serra do Teixeira. MC-**mesophilic** cocci; TC-thermophilic cocci; BAC-bacilli.

Leyenda: Microregiones: R1-Catolé do Rocha; R2-Cajazeiras; R3-Sousa; R4-Patos; R5-Piancó; R6-Itaporanga; R7-Serra do Teixeira. CM- cocos mesófilos CT- cocos termófilos; BAC- bacilos

Figure 2. Distribution of cocci and rods isolated from Coalho cheese expressed in total number of colonies by regions from Paraíba, northeast Brazil.

Figura 2. Distribución de cocos y bacilos aislados de queso de Coalho expresado en número total de colônias por las regiones de Paraíba, Nordeste, Brasil.

Legend: Micro-regions: R1, Catolé do Rocha; R2, Cajazeiras; R3, Sousa; R4, Patos; R5, Piancó; R6, Itaporanga; R7, Serra do Teixeira. MC, **mesophilic** cocci; TC, thermophilic cocci; BAC, bacilli.Leyenda: Microregiones: R1-Catolé do Rocha; R2-Cajazeiras; R3-Sousa; R4-Patos; R5-Piancó; R6-Itaporanga; R7-Serra do Teixeira. CM- cocos mesófilos CT- cocos termófilos; BAC- bacilos

Table 2 presents the distribution of LAB species identified in the seven micro-regions based on the score identity of the gene 16S rRNA region. Within the 456 LAB colonies sequenced, six genera were identified: *Lactococcus* (40.1%), *Enterococcus* (35.3%), *Streptococcus* (18.6%), *Lactobacillus* (5.5%), *Leuconostoc* (0.4%) and *Weissella* (0.2%). The level of identity in the *score* of the sequences varied from 100 to 96%, when compared to the GenBank NCBI database.

In this research the greatest prevalence per species was of *E. faecium* (26.9%), followed by *L. lactis* subsp. *lactis* (20.3%), *L. garvieae* (15.1%) and *Streptococcus infantarius* subsp. *infantarius*, representing 9.4%.

The greatest diversity of LAB genera was verified in the micro-region of Patos (R4), where six different genera were found in accordance with the following order: *Enterococcus* (52.2%), *Lactococcus* (39.1%), *Streptococcus* (5.8%), *Lactobacillus, Leuconostoc* and *Weissella* (1.4%) each. *E. faecium* presented the highest prevalence amongst the bacteria of the *Enterococcus* genus (46.4%). This was also the genus that presented the greatest diversity, including species such

Table 2. Lactic acid bacterial species isolated from artisanal Coalho cheese produced in the micro-regions of the Sertão region of the State of Paraíba, northeast Brazil.

Tabla 2. Especies de bacterias lácticas aisladas de queso Coalho artesanal producido en la region Sertao del Estado de Paraíba, Nordeste de Brasil.

Identified species based on the elevated identity in the 16S region *score* (grouped strains)	Gen Bank accession number	COLONIES OF NUMBER BY MICRO-REGION							TOTAL
		R1	R2	R3	R4	R5	R6	R7	
Enterococcus casseliflavus 99%	KT989994/KT989995/KT989996	1	0	0	0	0	2	5	08
Enterococcus durans 99-96%	KT989997/KT989998/KT989999/KT990000	1	0	3	0	1	0	3	08
Enterococcus faecalis 100 –99%	KT990001/KT990002/KT990003/KT990004/KT990005/KT990006/ KT990007/KT990008	2	1	3	2	0	2	3	13
Enterococcus faecium 100 –96%	KT990009/KT990010/KT990011/KT990012/KT990013/KT990014/ 990015/KT990016/KT990017/KT990018/KT990019/KT990020/ KT990021/KT990022/KT990023/KT990024/KT990025/KT990026/ KT990027/KT990028/KT990029/KT990030	17	25	11	32	10	20	11	126
Enterococcus gallinarum 100-99%	KT990031/KT990032	0	0	0	0	0	2	0	02
Enterococcus italicus 100%	KT990033/KT990034	0	0	0	2	0	0	2	04
Lactobacillus fermentum 99%	KT990035/KT990036/KT990037	0	5	2	0	0	0	0	07
Lactobacillus plantarum 99 – 98%	KT990038/KT990039/KT990040/KT990041/KT990042	0	8	0	0	0	0	0	08
Lactobacillus plantarum subsp. *plantarum* 100 – 99%	KT990043/KT990044/KT990045/KT990046/KT990047KT990048	0	6	0	0	0	0	0	06
Lactobacillus rhamnosus 99%	KT990049/KT990050/KT990051/KT990052	0	0	3	1	0	0	0	04
Lactococcus garvieae 100 – 99%	KT990053/KT990054/KT990055/KT990056/KT990057/KT990058/ KT990059/KT990060/KT990061	0	16	5	9	12	5	24	71
Lactococcus lactis 100 – 99%	KT990062/KT990063/KT990064/KT990065	0	0	1	2	2	9	3	17
Lactococcus lactis subsp. *lactis* 100 – 99%	KT990066	10	3	2	12	20	28	20	95
Leuconostoc mesenteroides subsp. *mesenteroides* 99%	KT990092/KT990093	1	0	0	1	0	0	0	02
Streptococcus infantarius subsp. *infantarius* 100% – 99%	KT990067/KT990068/KT990069/KT990070/KT990071/KT990072/ KT990073/KT990074/KT990075/KT990076/KT990077	20	0	10	0	14	0	0	44
Streptococcus lutetiensis 99%	KT990079/KT990080/KT990081/KT990082/KT990083/KT990084/ KT990085/KT990086/KT990087/KT990088/KT990089	3	0	10	4	2	19	0	38
Streptococcus macedonicus 99%	KT990090	0	0	0	0	0	0	1	01
Streptococcus waiu 99%	KT990091	0	0	0	0	0	0	1	01
Weissella paramesenteroides 99%	KT990094	0	0	0	1	0	0	0	01
Total		**55**	**64**	**50**	**66**	**61**	**87**	**73**	**456**

Micro-regions: R1, Catolé do Rocha; R2, Cajazeiras; R3, Sousa; R4, Patos; R5, Piancó; R6, Itaporanga; R7, Serra do Teixeira.
Micro-regions: R1, Catolé do Rocha; R2, Cajazeiras; R3, Sousa; R4, Patos; R5, Piancó; R6, Itaporanga; R7, Serra do Teixeira.

as *E. casseliflavus, E. durans, E. faecalis, E. faecium*, *E. gallinarum* and *E. italicus*.

Analysing the geographical distribution of LAB genera as shown in Figure 1, it is possible to conclude that the genus *Streptococcus* is predominant in the micro-region of Catolé do Rocha (R1), the genus *Enterococcus* prevails in the micro-regions of Cajazeiras (R2), Sousa (R3) and Patos (R4), and the genus *Lactococcus* was more frequently found in the micro-regions of Itaporanga (R5), Piancó (R6) and Serra do Teixeira (R7).

4. Discussion

The results found in this research revealed a high and significant diversity of LAB identified in the samples of Coalho cheese produced in the State of Paraíba. This diversity is due to the fact that this is an artisanal cheese, i.e. processed with non-pasteurized milk. Raw milk is rich in lactic and non-lactic bacteria, which may originate from the mammary gland, the environment, water or other materials involved in the milk production process, and therefore is the main source of the Coalho cheese microbiota. A similar result was found for the Coalho cheese produced in Pernambuco, which also presented an elevated number of mesophilic colonies, as a consequence of the traditional manufacturing process, which does not include the cooking of the cheese mass (Guedes Neto, 2008).

The LAB isolated from Coalho cheese in the M17 medium confirmed its specificity for cocci. The elevated number of colonies with a coccoid morphology that grew in M17 in both temperatures was responsible for 91.9% of all isolated colonies. Similar results were obtained by Albuquerque for Coalho cheese produced in the State of Pernambuco, Brazil, which also presented approximately 86% of the cocci among the LAB groups (Albuquerque, 2010). The majority of the LAB isolated in M17 agar, from samples of Bryndza cheese, an artisanal European cheese produced with non-pasteurized milk, also belonged to the *Lactococcus* group (Pangallo et al., 2014).

The lactic microbiota of raw milk and traditional dairy products still stimulates interest due to the necessity of identifying the microorganisms responsible for their organoleptic characteristics and, in particular, new strains of LAB, typical of a given product or region. This study used molecular techniques to provide a more detailed knowledge of the diversity of lactic microbiota present in Coalho cheese produced in the State of Paraíba and could identify some specific trends in different micro-regions.

The genus *Enterococcus* was more represented in the samples from the micro-regions of Cajazeiras (R2), Sousa (R3) and Patos (R4), and the species *E. faecium* was dominant in this group, corresponding to the high percentages of the total isolated colonies, namely 39.1% in Cajazeiras (R2), 22.0% in Sousa (R3) and 48.5% in Patos (R4). Several authors associate the presence of bacteria from the *Enterococcus* genus and their participation in the cheese's maturation process with the organoleptic characteristics of the artisanal dairy products such as the São Jorge cheese, produced in Portugal (Kongo, Ho, Malcata, & Wiedmann, 2007), the Raschera cheese (Dolci, Alessandria, Zeppa, Rantsiou, & Cocolin, 2008) and the Fontina cheese (Giannino, Marzotto, Dellaglio, & Feligini, 2009) both produced in Italy, some

artisanal cheeses made from goat's milk, in Spain (Martín-Platero, Maqueda, Valdivia, Purswani, & Martínez-Bueno, 2009), or the Istriano cheese, from Croatia (Fuka, Engel, Skelin, Redžepović, & Schloter, 2010).

Albuquerque (2010) also found the *Enterococcus* genus as being prevalent in the LAB isolated from Coalho cheese produced in State of Pernambuco, Brazil. Several species of the *Enterococcus* genus such as *E. faecium, E. faecalis, E. italicus* and *E. durans* have already been isolated from cheeses. The most prevalent species found in this research was *Enterococcus faecium* (26.9%). A similar result was found by Carvalho (2007) in Coalho cheese produced in State of Ceará, Brazil. Acurcio and co-workers also isolated 56.3% of *Enterococcus faecium* from LAB bacteria of sheep's milk (Acurcio et al., 2014). Other species from the *Enterococcus* genus (*E. faecalis, E. durans, E. italicus, E. casseliflavus* and *E. gallinarum*) were also identified in the Coalho cheese samples studied in this work. The species *Enterococcus italicus* was isolated in Tunisia (Gaaloul et al., 2014) from raw milk, and the identification was made by sequencing the 16S rRNA gene. In Brazil, this is the first record of *E. gallinarum* and *E. casseliflavus* isolated in artisanal Coalho cheese.

In the micro-regions of Piancó (R5), Itaporanga (R6) and Sierra de Teixeira (R7), the genus *Lactococcus* was the most prevalent, followed by the genera *Enterococcus* and *Streptococcus*. The species *L. lactis* subsp. *lactis* was the most abundant *Lactococcus* species in the micro-regions of Piancó (R5) and Itaporanga (R6), with 32.8% and 32.2%, respectively, whereas the bacteria from the species *L. garvieae* accounted for 32.8% of the total LAB in the micro-region of Serra do Teixeira (R7).

Considering all the cheese samples analysed, the bacteria from the *Lactococcus* genus were dominant, corresponding to 39.9% of the total LAB and comprehending the species *L. garvieae, L. lactis* and *L. lactis* supsp. *lactis*. The isolation of *Lactococcus sp*. in artisanal Coalho cheese in the northeast region has been reported by different authors (Carvalho, 2007; Guedes Neto, Souza, Nunes, Nicoli, & Santos, 2005; Silva et al., 2012). There are no reports in the literature about the isolation and identification of *L. garvieae* in industrial or artisanal Brazilian cheeses, but the species has been found in cheeses and cow's milk in other countries (Alegría et al., 2009; Alomar, Loubiere, Delbes, Nouaille, & Montel, 2008; Fortina, Ricci, & Borgo, 2009). *L. garvieae* was identified in the samples of almost all the micro-regions evaluated in this work, exception made to the micro-region of Catole do Rocha (R1).

The microbial diversity of LAB found in the micro-region of Itaporanga is represented by the following genera: *Lactcoccus* (46.5%), *Enterococcus* (30.2%) and *Streptococcus* (23.3%). The greatest prevalence verified was of the *L. lactis* subsp. *lactis* species (31.8%), followed by *E. faecium* (22.7%) and *S. lutetiensis* (21.6%).

The micro-region of Catolé do Rocha (R1) presented a higher prevalence of the genus *Streptococcus*, followed by the genera *Enterococcus* and *Lactcoccus*. The species *S. infantarius* subsp. *infantarius* was the most prevalent (36.4%). The genus *Streptococcus* showed the greatest variety of species identified in all cheeses. In this work four species have been identified: *Streptococcus infantarius* subsp. *infantarius, Streptococcus lutetiensis, Streptococcus macedonicus* and *Streptococcus waiu*. The *Streptococcus infantarius* subsp. *infantarius* is highly prevalent in artisanal fermented products produced in Africa (Jans, Follador, Lacroix, Meile, & Stevens, 2012). Bacteria from the species *S. lutetiensis* and *S. infantarius* were identified in the Italian cheese Vastedda della Valle Del Belìce (Gaglio et al., 2014). According to Winn et al. (2008), *S. lutetiensis* is a reclassification of *S. infantariuis* subsp. *coli*, which belongs to the *S. bovis/Streptococcus equinus* bacterial complex (Pacini, Cariolato, Andrighetto, & Lombardi, 2006; Schlegel et al., 2000). Members of the *Streptococcus* genus were also found in other studies carried out with Coalho cheese in Pernambuco (Guedes Neto, 2008) and in Ceará (Albuquerque, 2010), in Brazil. Orsahin also isolated *S. lutetiensis* from Armola cheese produced in Turkey (Orsahin, 2012). In the samples from the micro-region of Cajazeiras (R2), no species of the genus *Streptococcus* was found.

The bacteria from the *Lactobacillus* genus isolated from Coalho cheese samples belonged to the species *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus plantarum* subsp. *plantarum* and *Lactobacillus rhamnosus*. Bacteria from this genus had already been identified by Carvalho (2007), in Coalho cheese in the State of Ceará, in Brazil, and by Veljovic et al. (2007) in Zlatar cheese, during the maturation process. Despite its importance for dairy products, in this study, the genus *Lactobacillus* was isolated only in the micro-regions of Cajazeiras (R2), Sousa (R3) and Patos (R4), with a variable prevalence of 32.8%, 10.0% and 1.4%, respectively. The presence of other LABs, which are not so abundant in dairy products subject to pasteurization, might limit the proliferation of bacteria from the genus *Lactobacillus*; in particular, the highest counts for bacteria of this genus occurred in the Cajazeiras micro-region (R2), where the mesophilic cocci presented the lowest number of colonies.

The *Leuconostoc* genus, also included in lactic microbiota, is considered important in the production of dairy products due to its contribution to the development of aroma characteristics (Hassan & Frank, 2001). In this research, *Leuconostoc mesenteroides* subsp. *mesenteroides* was isolated from Coalho cheese in the micro-regions of Catole do Rocha (R1) and Patos (R4).

Weissella paramesenteroides was identified and isolated from Coalho cheese produced in the micro-region of Patos (R4). Borelli (2006) also found this species in artisanal Minas cheese produced in the Serra da Canastra region. In studies of the microbial diversity of artisanal cheeses from Spain (Mas et al., 2002) and Greece (Gerasi, Litopoulou-Tzanetaki, & Tzanetakis, 2003), *W. paramesenteroides* was also found. Ferreira and co-workers isolated *W. paramesenteroides* from Marajó cheese in the State of Pará, Brazil (Ferreira, Seixas, Eller, Nero, & Carvalho, 2015).

The distribution of LAB genera was not homogeneous in all the micro-regions of Sertão: the LAB of the *Streptococcus* genus prevailed in the micro-region of Catolé do Rocha (R1), which is a state in the north, the LABs of the *Enterococcus* genus were dominant in the micro-regions of Cajazeiras (R2), Sousa (R3) and Patos (R4), which correspond to the central region of the meso-region Sertão, and the LABs from the *Lactococcus* genus were more abundant in the micro-regions of Itaporanga (R5), Piancó (R6) and Serra do Teixeira (R7), located in the southern part of the Sertão region. Other authors have observed the influence of the geographical origin in the distribution of LAB species from dairy products such as the regional differences in the distribution of *Streptococcus infantarius* subsp. *infantarius* in fermented

milk in Africa (Jans et al., 2013). However, Casalta and Montel (2008) noted that the presence of *Lactococcus* genus in raw milk is due to contamination of the fodder during milk collection. Since the cheese-processing methods are similar in all the micro-regions of Sertão, this observation may reflect the influence of edaphoclimatic conditions and animal genetics in the microbiology of the milk and by consequence the cheese. The absence of standardization of the Coalho cheese produced in Paraíba is probably a crucial factor for these heterogeneous distributions of LAB genera that contribute to the variability of the organoleptic properties of this traditional product.

The identification of the species that make up the lactic acid microbiota is of relevance in the characterization of Coalho cheese produced in the State of Paraíba. The results obtained in this study contribute to the knowledge of the diversity of the LAB microbiota present in Coalho cheeses from the Sertão region, and may be used to develop a *starter* culture to be used in the production of Coalho cheese from pasteurized milk, ensuring safety conditions for the consumer, maintaining the organoleptic characteristics of the artisanal cheeses and even improving the homogeneity of the sensorial characteristics of the cheeses produced in the different micro-regions.

Owing to cultural and socioeconomic importance of the production of artisanal Coalho cheese in the State of Paraíba, it is highly relevant to seek alternatives that reduce the risk of exposing the population to pathogenic microorganisms present in the raw milk, but still preserve the traditional characteristics of this product, which justify its market acceptance.

5. Conclusions

The artisanal Coalho cheese produced in Sertão of the State of Paraíba presents a diversified microbiota of LAB, represented by the genera *Enterococcus, Lactococcus, Streptococcus, Lactobacillus, Leuconostoc* and *Weissella*. The sequencing of the 16S rRNA gene was a very efficient tool for the identification and differentiation of these microorganisms. The most prevalent species were *Enterococcus faecium, Lactococcus lactis* subsp. *lactis, Lactococcus garvieae* and *Streptococcus infantarius* susbp. *infantarius*. The distribution of the different LAB species was not the same in all the micro-regions of the Sertão region of the State of Paraíba.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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