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Microbiological evaluation of type B Coalho cheese with the addition of aqueous extract and hydrolat obtained from *Croton campestris*

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Abstract

Coalho cheese is a typical product of the Northeast region of Brazil, presenting significant importance in the economy of the nine states that make up this geographical area. This dairy concentrate of animal origin has cow's milk as its raw material and, when it is artisanal produced, can transmit pathogenic microorganisms to the consumer. The aim of the present work is to propose the evaluation of the use of aqueous extract of Croton campestris leaves, against of microorganisms common to the milking environment and Coalho cheese production environment, such as the total and thermotolerant coliforms, Salmonella sp., Staphylococcus aureus, mold and yeast. All samples were not contaminated by Salmonella sp. However, it was possible to observe that the extract, in the different applied volumes, was able to

promote the reduction of microbial loads for total and thermotolerant coliforms, Staphylococcus aureus and mold and yeasts. It is important to note that only for Staphylococcus aureus, the leaves aqueous extract of Velame managed to reduce the microbial load to the values below the limits established by RDC no. 12/2001 of the National Health Surveillance Agency (ANVISA).

Keywords: rennet cheese, natural product, microbiology, plant, sertão

INTRODUCTION

The origin of cheese dates to centuries before Christ. Some authors, such as Beux (2011), point out that the first cheeses were produced around 8000 BC, using cow, goat, sheep, buffalo milks as raw material. Various cheese types are produced worldwide, such, as Camembert and Roquefort of French origin, the English Cheddar and Cottage and the Canastra, Minas and Coalho cheeses, produced in Brazil.

Brazilian cheeses gain flavor and specific characteristics of their regions, totaling more than seventy types. Among them, the Coalho cheese stands out having its origin in the northeast region. Initially produced in the State of Pernambuco, reached the other states of the northeast region and becoming one of the main socioeconomic factors in the region as it is one of the main activities of the productive chain of the dairy sector, inserted in family farming. (PACHECO, 2011). Today, Coalho cheese is already produced in some northern states and sold in some cities in the southeast region of Brazil.

The production method is simple and does not require sophisticated equipment, in addition to having a low investment cost in its manufacture. Coalho cheese is produced by fermentation and coagulation of cow's milk from addition of specific enzymes or bacteria as well as isolated or combined organic acids, resulting in two by-products: fresh or smear-ripened dough curd and a fraction of serum. This product is divided into two categories: A and B; the first is subjected to the pasteurization process and the second does not go through the thermal process, being, therefore, a artisanal and natural

product (GOMES, 2012; PAQUEREAU, et al., 2016). This milk concentrate has a white color, semi-hard and elastic consistency, rectangular shaped, compact and soft texture, medium to high humidity, its mass can be raw, semi-cooked or cooked, with fat content between 35.0% and 60.0%. It has a slightly acidic and salty or spicy taste; thin, crack-free crust. It can be consumed raw, roasted, fried, pure or as a garnish (DUARTE et al., 2005; SILVA et al., 2012; PAQUEREAU, et al., 2016).

The microbiological parameters of Coalho type B cheese vary during milking and during manufacturing process. This is due to the lack of hygienic-sanitary care, ineffective conservation methods and the presence of microorganisms common to the milking and production environments, such as total and thermotolerant coliforms, *Salmonella* sp., *Staphylococcus aureus*, mold and yeast, these being the main contaminants of this product (DANTAS, 2012; SILVA et al., 2018).

The bacteria from the coliform groups and *Staphylococcus aureus* stand out as the microorganisms most easily present in Coalho cheeses, as they can be found naturally in the cow's udder and can already contaminate the milk during the milking process. The group of coliforms belonging to the *Enterobacteriaceae* family classified as total and thermotolerant Coliforms, may be present in high quantity per gram of cheese produced depending on this value, may or may not cause damage to health. The Brazilian National Health Surveillance Agency (ANVISA) through Resolution of the Collegiate Board (RDC) No. 12 of January 2, 2001 [RDC nº 12/2001], does not establish to total coliforms limits for Coalho cheese, however, for thermotolerant coliforms, the limit is 5×10^2 (MPN/g) (Most Probable Number per gram) (BRASIL, 2001).

The *Staphylococcus aureus* bacterium, of the *Micrococca*e family, has a spherical structure, from the group of gram-positive cocci. When present in larger quantities and if in favorable conditions of temperature, water and gas activity, oxygen and pH (Hydrogenionic potential), they will possibly produce staphylococcal enterotoxins in food that, after ingestion, will cause intoxication (SANTANA et al. 2008; ETGES 2011). The RDC nº 12/2001, establishes a growth tolerance for coagulase-positive staphylococci (*Staphylococcus aureus*)

of a maximum of $5x10^2$ CFU/g (Colony Forming Units per gram), with moisture content greater than 55%, including Coalho cheese with corresponding moisture.

Salmonella sp. (Salmonellose) is a bacterium that, like coliforms, also belongs to the *Enterobacteriaceae* family, being responsible for causing serious infections and even death by ingestion contaminated food and water (FEITOSA et al., 2003). According to the RDC no. 12/2001, there are no tolerable limits for the presence of *Salmonella* sp. in food, being classified as inappropriate for consumption the food that presents this bacterium (BRASIL, 2001).

The group of molds and yeasts constitutes a significant evaluation standard as an indicator of the quality of dairy products, especially Coalho cheese, since their presence is causally linked to deterioration and changes in the acceptability profile, for altering the flavor. It is important to point out that there is still no normative standard that emphasizes a limit profile for their presence in the product (KAMLEH et al., 2012).

In general, the presence of *Salmonella* sp., or the presence of other microorganisms in quantities above the permitted limits, makes foods unfit for consumption (BRASIL, 2001). However, several studies have shown that these limits are not respected during the manufactured of artisanal Coalho cheese, mainly due to the lack of adoption of good hygienic practices during the milking and manufacturing process (Borges et al., 2003; Santana et al., 2008; Sousa et al., 2014; Bezerra et al., 2017; Dias et al., 2015).

Given this, it is evident the need for new alternatives, such as use products of plant origin, to reduce the microbiological contamination of Coalho cheese artisanal, since it does not undergo a pasteurization process to eliminate microorganisms. One suggestion would be to use products origin natural (as such plants), aims to minimize changes in visual and sensory characteristics, in addition to changes in white mass and rectangular shape. It is not new in Brazil, to add of natural products, such as “cumaru” and “pequi” (fruits originating in northern Brazil) or other condiments, to Coalho cheese. However, this occurs with the purpose of expanding supply to the market with new flavors and winning new consumers and the

possibility of improving producers' income, and not for sanitary purposes.

The aim of the present work is to propose the evaluation of the use of aqueous extract of Croton campestris leaves, against of microorganisms common to the milking environment and Coalho cheese production environment, such as the total and thermotolerant coliforms, *Salmonella* sp., *Staphylococcus aureus*, mold and yeast.

METHODOLOGY

Plant material: Leaves of "Velame do Campo" (Croton campestris) were collected from the base of the tree top of six specimens at The municipality of Venturosa (latitude 08º34'29 "south and at longitude 36º52'27" west), located in the Mesoregion of Agreste, Microregion of Vale do Ipanema, Pernambuco, Brazil. Only whole leaves, without signs of insects, herbivory, fungi, or sunburns, were used in this study. Metabolite changes due to climate, temperature, and seasonality were not part of this study. A specimen voucher was sent to the Herbarium IPA (Agronomic Instituto of Pernambuco - IPA).

Obtaining the aqueous extract: The collected leaves were washed with distilled water and dried with paper. To obtain the aqueous extract, 20 grams of Croton campestris leaves were used, which were placed in the appropriate filter paper cartridge for the Soxhlet apparatus (Merck, Darmstadt, Germany). Then, 250 mL of distilled water were added to the round-bottom flask. The extraction lasted as long necessary, for the water to pass through the Soxhlet 3 times. At the end of the process, 250 mL of aqueous extract of leaves were obtained, at a concentration of 80 □g/mL.

Addition of aqueous extract l to fresh Coalho cheese type B
Before the process of adding the aqueous extract is described, it is necessary to understand the production process of Coalho cheese. The process goes through seven stages, described below: (i) straining the milk to remove macro-impurities; (ii) adding rennet to promote coagulation (duration 40 to 60 minutes); (iii) separation of the whey of

the curds; (iv) put up, to its edge, the curd in rectangular shapes, for the pressing processe, so that the maximum possible elimination of the serum occurs (duration of 5 to 10 minutes); (vi) removal of the shapes and process of salting with table salt, for a period of 1 to 5 h. (vii) After this period, the cheese is left at room temperature, for a period ranging from 2 to 5 hour and then stored in a freezer or cold chamber at a temperature ranging from 10ºC to 18 ° C, for 24 h.

The chosen moment for the addition of the aqueous extract was one day after its manufacture (after the step vii), time was the release of the highest percentage of the residual whey from the production occurs. In total, 1.2 kg of cheese was separated into 4 samples of 300 g, each put up in trays with dimensions of 200 mm x 72 mm x 125 mm. Each tray was identified according to the volume of aqueous extract, at a concentration of 80 □g/mL, added to the cheese, as follows: Tray A, addition of 5 mL; Tray B, addition of 10 mL; Tray C, addition of 15 mL, and; Tray D, without to adding extract (control sample). All samples were stored in a refrigerator at 12ºC and analyzed at 1, 5, 10 and 15 days after the addition of the aqueous extract.

Microbiological Analysis: The analyzes carried out in the present study allowed the identification of the concentrations of mold and yeast, Coliforms and *Staphylococcus* aureus and presence or absence of *Salmonella* sp.. The methodological process was applied in accordance with Normative Instruction No. 62, of August 26, 2003, as described below:

Coliform Analysis: The inoculation occurred from the addition of 1mL of the initial 10^{-1} dilution in sodium lauryl sulfate (Sigma-Aldrich, St. Louis, MO) broth solution in simple concentration. Then, 1 ml of the 10^{-1} dilution was inoculated into three tubes to obtain the 10^{-2} dilution and from this 1 ml was inoculated in a series of 3 tubes to obtain the 10^{-3} dilution. All dilutions were prepared in triplicate and in test tubes containing inverted Durham tubes.

Then the tubes were incubated at 35°C for 48 hours, after which time it was checked whether there was gas production in the Durham tubes. Once positive, the number of tubes in each dilution series was

recorded and the confirmatory test was carried out for Total and Thermotolerant Coliforms, according to the following criteria:

i. Total Coliforms: Each positive tube was peaked into a tube containing 2% lactose bile bright green broth (Merck, Darmstadt, Germany). Then, the tubes were incubated at 36 ± 1°C for 48 hours. After this period, a reading was performed to confirm or not the presence of total coliforms due to gas formation. Thus, the MPN (Most Probable Number) method was used for the probabilistic counting of the result.

ii. Thermotolerant Coliforms: With the aid of a loop, positive tubes from the confirmatory test were peaked to tubes containing EC broth (Merck, Darmstadt, Germany). Subsequently, the tubes were incubated at 45 ± 0.2°C for 48 hours in a water bath with agitation. After this period, the reading was carried out, giving a positive result to those who presented gas formation in the Durhan tube, thus noting the result obtained for each tube in order to calculate the MPN.

Mold and Yeast Count: The counting was performed in a Petri dish, containing three dilutions (10^{-1}, 10^{-2} and 10^{-3}) for the four groups analyzed, all being applied in triplicate. In this, the medium of culture Agar potato dextrose 2% acidified with tartaric acid 10% (Sigma-Aldrich, St. Louis, MO) was used to reduce the pH until close to 3,5. The plates were incubated at a temperature of 25± 1ºC for a period of seven days. After this time interval, the colonies present on the plates were counted and the result expressed in CFU/g (Colony Forming Units per gram).

Staphylococcus aureus: The four analyzes for the four groups were done in Petri dish, containing three dilutions (10^{-1}, 10^{-2} and 10^{-3}) with inoculation of 0.1 mL of the sample for each dilution in their respective plates, in triplicate. These was prepared in Baird-Parker agar culture medium (Sigma-Aldrich, St. Louis, MO). The inverted plates were incubated in C.O.D at 36 ± 1ºC for a period of 48 hours. In the next stage, the colonies present on the plates were counted and the result expressed in CFU.

Salmonella sp: The evaluation, performed in triplicate, followed the following procedure: in the pre-enrichment stage, 25 ± 0.2 g of each sample of Coalho cheese were used, with the addition of each of the volumes of aqueous extract studied (the control, 5 ml, 10 ml and 15 ml), which were added in 4 tubes containing 225 ml of 1% buffered peptide saline solution (Acumedia, MI, USA). These mixtures were homogenized for 1 minute in the Stomacher (Model MA440, Piracicaba, Brazil) and, then, the tubes containing the mixtures were incubated at 36ºC for 18 hours. In the selective enrichment stage, three aliquots were removed from each of the 4 pre-enriched samples for simultaneous inoculation in the selective liquid meday as shown below;

- 0.1 mL was added to a tube containing 10 mL of Rappaport Vassiliadis broth (Acumedia, MI, USA);
- 1 ml added in a tube containing 10 ml of cystine selenite broth (Acumedia, MI, USA), and;
- 1 mL added to a tube containing 10 mL of tetrathionate broth (Acumedia, MI, USA), totaling 12 tubes.

All these tubes were incubated in a water bath, with shaking, for 24 hours at 41ºC. Subsequently, isolation was performed, where, from the selective enrichment broths, the previously dried plate surfaces, two for each solid medium, were sown and streaked in order to obtain isolated colonies. These plates were incubated, inverted, at 36 ºC for 24 hours. After this period, the characteristic colonies were subjected to biochemical tests to assess the presence or absence of Salmonella sp in 25 g of food.

Statistical analyses: The Lilliefors test was employed to evaluate the normality of the data. The Mann - Whitney test was employed for comparison of two independent samples. Values for $p \leq 0.05$ were considered significant.

RESULTS

Table I presents the values of the Most Probable Numbers per gram (MNP/g) of total and thermotolerant coliforms found in the samples of

Coalho cheese analyzed in the present work, over the 15 days of analysis.

Table I: Total and thermotolerant coliforms values for Coalho cheese samples.

	Total Coliform (MPN/g)**			
	Observation day***			
Volume (mL)	1º	5º	10º	15º
00*	4.3×10^4	4.3×10^4	1.2×10^5	1.2×10^5
05	4.3×10^4	1.5×10^4	1.5×10^4	1.5×10^4
10	4.3×10^4	1.5×10^4	1.5×10^4	9.2×10^3
15	4.3×10^4	9.2×10^3	9.2×10^3	9.2×10^3
	Thermotolerant Coliforms (MPN/g)**			
	Observation day***			
Volume (mL)	1º	5º	10º	15º
00*	2.3×10^4	2.3×10^4	4.3×10^4	4.3×10^4
05	2.3×10^4	2.3×10^4	4.3×10^4	4.3×10^4
10	2.3×10^4	9.2×10^3	9.2×10^3	9.2×10^3
15	2.3×10^4	9.2×10^3	6.1×10^3	6.1×10^3

* Control sample; ** (MNP/g) = Most Probable Number per gram (BRASIL, 2001); ***After the add the aqueous extract.

When evaluating the relationship between the different volumes of the aqueous extract of leaves from *Croton campestris* A. St. Hill added to Coalho cheese, a reduction was verified from the sixth day of analysis, for total coliforms, especially when comparing the control group to the group containing 15mL of the extract. It was also observed that on the fifteenth day of storage, there was a variation from 1.2×10^5 to $9,2 \times 10^3$ (MPN/g).

It can be evidenced that this natural product applied in the cheese promoted a lower microbiological load for total coliform when compared with cheeses analyzed by Silva et al. (2018) who evaluated 36 samples obtained from four open markets in the city of Petrolina - PE and found a value above the order of 105 for all samples. However, comparing our values with those found by Santana et al. (2008), Dias et al. (2015) and by Bezerra et al. (2017), it is possible to observed a higher level of contamination for the samples analyzed here, although, as previously informed, the MPN/g limits for total coliforms in Coalho cheese are not yet recommended by RDC no. 12/2001. It is observed, from the counting data for thermotolerant coliforms, that the contamination values are one to two orders of magnitude higher than

those of Santana et al. (2008), Dias et al., (2015) and by Bezerra et al. (2017), but smaller than that observed by Silva et al., (2018).

The statistical analysis indicated that, for total and thermotolerant coliforms, there is no influence of the exposure time in the reduction of the microbiological count (p-value = 0.2482). However, there is an indication of the influence of the applied aqueous extract volumes (p-value = 0.0152) on the decrease in the total coliform count. For the thermotolerant coliforms, the volume applied only allowed the microbial load to decrease, from the volume of 10 mL (p-value = 0.0217), however, there was no statistically significant difference (p-value = 0, 3865) between 10 mL and 15 mL volumes. So, the values obtained in that study for samples of Coalho cheese with the addition of the different volumes of the aqueous extract leaves of Croton campestris, show that the extract promoted reductions in the total and thermotolerant coliform counts, when compared with the control sample, despite these data being beyond the limit established by the RDC No. 12, for thermotolerant coliform.

Table II presents the results for the analysis of mold and yeast, over the 15 days of analysis. From the analysis of the data presented, it is possible to observe concentrations in the order of 10^4 (CFU/g) in the control sample, throughout the observation period. The order of magnitude of mold and yeast concentrations only starts to decrease after the sixth day of observation, for the concentration of 10 mL of aqueous extract.

Peixoto et al., (2012) evaluated 4 dairy products in the state of Paraíba (Northeast region of Brazil) and showed that all of them had a high mold and yeast count and that most samples had a value of $2,4x10^4$ CFU/g. Souza et al., (2011) stand out in their research that the content of mold and yeast reached $5,2x10^9$ CFU/g configuring according to the same an elevated contamination index of Coalho cheese.

Silva, Furtado and Garcia (2016) when evaluating the growth of molds and yeasts in Coalho cheese produced with pasteurized milk sold in a public market in Manaus - AM, found a count ranging from $2.88x10^5$ to $1.33x10^7$ CFU/g showing that the pasteurization process it is not enough to eradicate or reduce these microorganisms. However, in the present study, the data found are lower than those evidenced in the

study by Silva, Furtado and Garcia (2016) for the treatments carried out and without the milk having gone through the pasteurization process.

Table II: Mold and yeast values for Coalho cheese samples: control and with add extract aqueous of Croton campestris.

	Mold and yeast (CFU/g)**			
	Observation day***			
Volume (mL)	1º	5º	10º	15º
00*	>1.0 x 10^4	>1.0 x 10^4	>1.0 x 10^4	>1.0 x 10^4
05	6.0 x 10^3	3.2 x 10^3	1.6 x 10^3	9.0 x 10^2
10	3.3 x 10^3	2.4 x 10^3	1.6 x 10^3	5.0 x 10^2
15	3.1 x 10^3	1.0 x 10^3	8.0 x 10^2	3.0 x 10^2

*Control sample; **(CFU/g) = Colony Forming Units per gram; ***After the add the aqueous extract.

The statistical analysis indicates that there was no influence of the exposure time in the reduction of the mold and yeast count (p-value = 0.3123). However, the influence of applied volumes was observed (p-value = 0.0105); the greater the volume, the less the load of mold and yeast. The volumes of 5 ml, 10 ml and 15 ml did not differ from each other (p-value = 0.2482).

Thus, the aqueous extract promoted decrease in the colony forming units per gram of mold and yeast in Coalho cheese. Although there is not yet a limit pre-established by Brazilian legislation, these microorganisms are configured as an indicator of contamination. DUGAT-BONY *et al*., (2016) stand out that these microorganisms, depending on the quantity, can produce a diversified number of enzymes that, in turn, will promote the faster deterioration of the cheese.

Table III presents the values for the count of Staphylococcus aureus in the samples of Coalho cheese. The values determined here to the control sample, are one order of magnitude lower than those reported by SANTANA et al (2008), that evaluated the occurrence of Staphylococcus aureus in Coalho cheese sold in Aracaju city, Sergipe state (Northeast Region of Brazil). Their results showed that 46.7% of the analyzed samples were positive for coagulase-positive staphylococci, with mean values of 6,30 x 10^5 (CFU/g).

Table III: Values of *Staphylococcus aureus* for Coalho cheese samples: control and with add extract aqueous of Croton campestris A. St. Hill leaves.

	Staphylococcus aureus (CFU/g)**			
	Observation day***			
Volume (mL)	1º	5º	10º	15º
00*	1.0×10^4	1.3×10^4	1.6×10^4	1.0×10^5
05	1.0×10^4	1.0×10^3	7.1×10^2	6.7×10^2
10	1.0×10^4	4.8×10^2	3.1×10^2	1.9×10^2
15	1.0×10^4	3.5×10^2	2.0×10^2	1.6×10^2

*Control sample; **(CFU/g) = Colony Forming Units per gram; ***After the add the aqueous extract.

Sousa *et al*. (2014), evaluated the microbiological profile of Coalho cheeses marketed in six states (Pernambuco, Piauí, Ceará, Rio Grande do Norte, Sergipe and Paraíba) in northeastern Brazil. The authors found that 96.15% of samples analyzed were outside the recommended limits for coagulase-positive staphylococci. Bezerra *et al*. (2017), in a study that aimed to identify the presence of this bacterium in samples of Coalho cheese produced in the city of Souza, Paraíba state (Northeast region of Brazil), showed that 65% of the analyzed samples were outside the recommended limits for coagulase-positive staphylococci, with values above 10^3 (CFU/g). Pereira et al. (2017), investigated the presence of this bacteria in samples of artisanal Coalho cheese, produced in São Rafael, Rio Grande do Norte (Northeast Brazil) and concluded that all samples presented high *Staphylococcus aureus* counts. Oliveira *et al*. (2019), investigated the occurrence of coagulase-positive staphylococci in Coalho cheeses (artisanal and industrial) produced in seventeen municipalities in the State of Ceará. It was observed that 64.9% of artisanal cheese samples showed growth for coagulase-positive staphylococci outside the current microbiological standards, while it was observed that 31% of industrialized samples were outside these standards.

According to Carmo and Bergdoll (1990), foods with counts in the range between 10^4 to 10^8 (CFU/g) will produce enterotoxins. Forsythe (2000), Carmo et al., (2002) and Picoli et al., (2006) stand out that if these enterotoxins develop a count from 10^5 (CFU/g) they can cause intoxication in consumers, thus pointing out that the extract are

efficient to provide greater security to the cheese, mainly in the volumes of 10 and 15mL.

It is possible to observe (Table III) that the aqueous extract reduced the bacterial load already from the sixth day of evaluation, for the volume of 10 mL, to values lower than the limits established by RDC no. 12/2001. On the fifteenth day, there was a marked decrease from 10^5 (CFU/g) (control sample) to 1.2 x 10^2 (CFU/g) (sample added with 15 mL of aqueous extract). The statistical analysis indicated that this decrease did not occur due to the influence of the observation time (p value = 0.2482), but due to the volume of aqueous extract added (p value = 0.0152).

Microbiological analyzes indicated the absence of Salmonella sp. for all samples of Coalho cheese evaluated. This observation is in line with the work of Pereira et al. (2017), who reported the absence of Salmonella sp. in all samples of Coalho cheeses artisanal produced in the city of São Rafael, Rio Grande do Norte (Northeast Brazil), and in accordance with the result found by Sousa et al. (2014), who reported that only in one of the 104 samples of Coalho cheese collected in several states in the Northeast of Brazil, the presence of Salmonella was detected. However, some studies have reported the presence of this bacterium, such as Santana et al. (2008), who reported the presence of Salmonella sp. in 73.33% of the Coalho cheese samples collected at the Central Market in the city of Aracaju, SE, from August to November 2007. Oliveira et al. (2010) reported that 9.52% of the Coalho cheese samples collected in the municipality of Cabo de Santo Agostinho, Pernambuco, Brazil, showed the presence of Salmonella sp. Bezerra et al. (2017), reported that in 40% of the cheese samples analyzed, was detected the presence of Salmonella sp.

CONCLUSION

Since the control samples were not contaminated by Salmonella sp., It was not possible to evaluate the influence of the aqueous extract of Velame leaves on the contamination of Coalho cheese for these microorganisms. However, it was possible to observe that the extract, in the different applied volumes, was able to promote the reduction of

microbial loads for total and thermotolerant coliforms, *Staphylococcus aureus* and mold and yeasts. It is important to note that only for *Staphylococcus aureus*, the leaves aqueous extract of Velame managed to reduce the microbial load to the values below the limits established by RDC no. 12/2001 of the National Health Surveillance Agency (ANVISA).

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