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Qualidade microbiológica e células somáticas de leite *in natura* produzido no Estado de Alagoas, Brasil

Microbiological quality and somatic cells of *in natura* milk produced in Alagoas State, Brazil

Calidad microbiológica y células somáticas de la leche *in natura* producida en el Estado de Alagoas, Brasil

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Bruno Fernando Oliveira Araújo
ORCID: https://orcid.org/0000-0001-9837-573X
Universidade Federal de Alagoas, Centro de Ciências Agrárias, Brasil
E-mail: bruno.oliveiraaraujo@hotmail.com
Sybelle Geórgia Mesquita da Silva
ORCID: https://orcid.org/0000-0002-9318-0332
Renorbio, Brasil
E-mail: belle_mesquita21@hotmail.com
João Manoel da Silva
ORCID: https://orcid.org/0000-0002-7654-5475
Renorbio, Brasil
E-mail: joao.manoel@iqb.ufal.br
Cícero Cerqueira Cavalcanti Neto
ORCID: https://orcid.org/0000-0002-4085-0849
Universidade Federal de Alagoas, Centro de Ciências Agrárias, Brasil
E-mail: cicerodurao@ig.com.br
Paula Cibelly Vilela da Silva
ORCID: https://orcid.org/0000-0003-2157-4698
Universidade Federal de Alagoas, Centro de Ciências Agrárias, Brasil
E-mail: paulacibelly17@gmail.com
Yamina Coentro Montaldo
ORCID: hhttps://orcid.org/0000-0003-1444-8642
Universidade Federal de Alagoas, Centro de Ciências Agrárias, Brasil
E-mail: ycmzte11@gmail.com

Jakes Halan de Queiroz Costa
ORCID: https://orcid.org/0000-0002-0018-9646
Universidade Federal de Alagoas, Centro de Ciências Agrárias, Brasil
E-mail: jakes.jakes@gmail.com
Elizabeth Simões do Amaral Alves
ORCID: https://orcid.org/0000-0001-5078-4104
Universidade Federal de Alagoas, Centro de Ciências Agrárias, Brasil
E-mail: jelizabetamaral@hotmail.com
Tania Marta Carvalho dos Santos
ORCID: https://orcid.org/0000-0002-1816-7840
Universidade Federal de Alagoas, Centro de Ciências Agrárias, Brasil
E-mail: jtmcs@ceca.ufal.br

Resumo

O leite cru é um alimento com grande consumo e valor econômico no Brasil. No entanto, é susceptível de contaminação por bactérias patogênicas. O objetivo deste estudo foi avaliar a qualidade do leite *in natura* com base em contagem de células somáticas (CCS), contagem bacteriana e sua composição físico-química. Foram feitas as seguintes análises microbiológicas: contagem de bactérias mesofílicas e psicrotróficas, coliformes a 30 ºC, coliformes a 45 ºC, *Staphylococcus* spp., *Listeria* spp. e CCS. As análises físico-químicas foram gordura, proteína, lactose, sólidos totais, uréia e caseína. Não houve evidência de *Salmonella* spp. e *Escherichia coli* em nenhuma amostra. De acordo com os padrões microbiológicos estabelecidos pela Instrução Normativa nº 76, apenas as contagens de coliformes a 30 ºC e 45 ºC estavam acima dos padrões. Houve uma diferença significativa ($p \leq 0,05$) entre as três fazendas estudadas quanto à maioria dos aspectos microbiológicos. Além disso, foi observada diferença ($p \leq 0,05$) para a maioria dos aspectos físico-químicos. Em geral, o leite produzido nas regiões do estado de Alagoas não consegue atender apenas a um critério constante da legislação vigente.

Palavras-chave: Sanidade alimentar; *Listeria;* qualidade microbiológica; *Salmonella; Staphylococcus.*

Abstract

Raw milk is a food with great consumption and economic value in Brazil. However, is susceptible of contamination by pathogenic bacteria. The aimed of this study was to evaluate the quality of *in natura* milk based on microbiological in three dairy farms, somatic cells counting (SCC), bacterial counting and his physical-chemical composition. Were made the following microbiological analysis: counting of mesophilic and psychrotrophic bacteria, coliforms at 30 ºC, coliforms at 45 ºC, *Staphylococcus* spp., *Listeria* spp., and SCC. The physical-chemical analysis was fat, protein, lactose, total solids, urea, and casein. There was no evidence of *Salmonella* spp. and *Escherichia coli* were identified in any samples. In accordance to the microbiological standards established by Normative Instruction 76 only coliforms 30 ºC and 45 ºC counts were above the standards. There was a significant difference ($p \leq 0.05$) between the three farms studied regarding most microbiological aspects. Also, was observed difference ($p \leq 0.05$) for most of physical-chemical aspects. Overall, the milk produced in the regions of Alagoas State fails to meet just a constant criterion in the current legislation.

Keywords: Food safety; *Listeria;* microbiological quality; *Salmonella; Staphylococcus.*

Resumen

La leche cruda es un alimento de gran consumo y valor económico en Brasil. Sin embargo, es susceptible a la contaminación por bacterias patógenas. El objetivo de este estudio fue evaluar la calidad de la leche *in natura* en base al recuento de células somáticas (CCS), el recuento de bacterias y su composición físico-química. Se realizaron los siguientes análisis microbiológicos: recuento de bacterias mesófilas y psicotróficas, coliformes a 30 ºC, coliformes a 45 ºC, *Staphylococcus* spp., *Listeria* spp. y CCS. Los análisis físico-químicos fueron de grasa, proteína, lactosa, sólidos totales, urea y caseína. No se identificaron pruebas de *Salmonella* spp. y *Escherichia* coli en ninguna muestra. De acuerdo con las normas microbiológicas establecidas por la Instrucción Normativa Nº 76, sólo los recuentos de coliformes a 30 ºC y 45 ºC estaban por encima de las normas. Hubo una diferencia significativa ($p \leq 0,05$) entre las tres granjas estudiadas en cuanto a la mayoría de los aspectos microbiológicos. Además, se observó una diferencia ($p \leq 0,05$) en la mayoría de los aspectos físico-químicos. En general, la leche producida en las regiones del estado de Alagoas no puede cumplir con un solo criterio de la legislación vigente.

Palabras clave: Salud alimentaria; *Listeria*; Calidad microbiológica; *Salmonella; Estafilococo.*

1. Introduction

Milk is a food considered one of the most complete, due to its nutritional value, consisting of proteins, carbohydrates, fats, minerals, vitamins and water, and, an important nutritional source, indispensable for human health. Because the milk is a product that is present in the diet of individuals of all ages, should be careful quality control in obtaining dairy products thus aiming to ensure their safety, because the high microbial load is frequent causes of health problems, and large economic losses, resulting in a low quality milk (Costa et al., 2020).

The microbial contamination rate of milk can be used as an indicator of its quality, and also the sanitary conditions of its production and, herd health. Because the potential of multiplication, bacteria found in the milk can cause chemical changes such as degradation of fat and protein and, may make the product unfit for consumption and/or industrialization. The quality and safety of raw milk are important in reducing the risk of foodborne illnesses associated with milk as raw milk is the starting point of the milk production chain for consumption (Oliveira et al., 2020).

For milk quality should take into account the chemical composition: total solids, fat, protein, lactose-mineral; Microbiological: total count of bacteria; organoleptic as smell, taste and appearance; and, the number of somatic cells. Such factors must meet the parameters required by legislation. Somatic cell count has been considered a worldwide standard of quality measure, because it is related to the composition, industrial output and food security.

The Brazilian Ministry of Agriculture, Livestock, and Supply (MAPA), regulated the criteria for quality for human consumption milk with Normative Instruction No. 76 (NI 76) of 26 November of 2018 which standards in production, identity and quality of milk types A, B, C, cooled pasteurized and raw, in addition to regulate the collect of raw milk and its refrigerated bulk transport (Brasil, 2018).

Thus, established minimum levels for fat, crude protein and, defatted dry extract for refrigerated raw milk. It was also envisaged the gradual decrease of the ceilings in Somatic Cell Count (SCC) and Standard Count Plate (SCP), allowing time needed to adapt the producers in these parameters. This was planned to end the division in milk type B and type C, terming it as refrigerated raw milk. As for the quality of milk, IN 76 requires maximum

SCC and SCP are 4.0 x 10^5 and the minimum values for fat and protein are, respectively, 3.0g (g/100g) (Brasil, 2018).

In view of the above the aim of this study was to evaluate three dairy producers to evaluating the quality of raw milk obtained and, their compliance with the current legislation.

2. Materials and Methods

Homogenization and Serial Dilution

Samples were collected from three dairy farms, one in Zona da Mata and two in Agreste of Alagoas. From the samples collected in the properties was made a composite sample for each. Was collected 50 ml of each animal. For the composite sample was withdrawn 10 ml of each collected sample (animal), totaling five samples of 50 ml from each farm. For the first serial dilution were transferred in 25 ml of composite sample for a bottle containing 225 ml of peptone water solution to 0.1% and then homogenized (10^{-1} dilution). From this first dilution were transferred to 1 ml three bottles containing 9 ml of Peptone Water 0.1% (dilution 10^{-2}). Following the same procedures were made dilution 10^{-2}, 10^{-3} and, 10^{-4}.

Mesophiles and psychrotrophic microorganisms

Was placed 1 ml dilutions: 10^{-2}, 10^{-3} and 10^{-4} in triplicate using the PCA culture medium in Petri dishes (Standard Agar Count). For mesophilic, the plates were incubated at 32 °C for 24 hours and to psicotrophycs were incubated at 11 ºC for 7 days. The plates that had counts between 30 and 300 Colony Forming Units (CFU) were selected for the colonies former unit count (CFU/mL) (Speck, 1976; Siqueira, 1995).

Determination of *Staphylococcus* spp.

To detecting the presence of *Staphylococcus* spp., stretch marks were used from dilution 10^{-1} in plates with selective culture medium agar Baird-Parker and incubated at 37 °C for 48 hours and observed the growth of colonies.

It was performed catalase and coagulase tests, using the broth enriched with Brain Heart Infusion (BHI). *Staphylococcus* aliquots were removed from the plates and selective medium were inoculated in 1 ml of broth (BHI) and were stored at 37 °C for 24 hours.

In catalase assay was used 0.7 ml of BHI broth, and added 0.7 ml of hydrogen peroxide in test tubes. The catalase unfolds the hydrogen peroxide into water and oxygen, it gives off bubbles forming. When there is blistering the test is considered positive.

Coagulase was used 0.3 ml of BHI broth and, added 0.5 ml of rabbit plasma tubes and take to water bath at 37 °C for 4 hours. The test is considered positive when there is clot formation. It is characteristic of *S. aureus* both when testing catalase and coagulase are positive.

Determination of total and fecal coliforms (30 and 45 ºC) and E*scherichia coli* confirmation

Was used the technique of the Most Probable Number (MPN), using 3 tubes series. In presumptive test were used three dilutions of each sample (10^{-1}, 10^{-2} and 10^{-3}). Aliquots of 1 ml were transferred to test tubes containing Lauryl Sulfate Tryptose culture medium (LST) and inverted Durham tube and, incubated at 37 °C for 24-48 hours. Positive tubes were considered those with turbidity and gas production into the Durham tube.

In the confirmatory test, 1 ml aliquots of the tubes with positive samples were transferred to tubes containing *Escherichia coli* culture medium (EC) and inverted Durham tube and incubated at 45 °C for 24 hours. Positive tubes were considered those with turbidity and gas production into the Durham tube. From the tubes containing EC, Aliquots of 1 ml tubes with positive samples were transferred to tubes containing Brilliant Green culture medium (BG) and Durham tubes inverted and incubated at 45 °C for 48 hours. Positive tubes were considered those with turbidity and gas production in the Durham tube.

***Salmonella* spp. assay**

For the study of *Salmonella* spp. was used selective culture medium Selenite Cystine broth and, inoculation of 1 ml of the 10^{-1} dilution of the samples in test tubes containing 9 ml of the above-mentioned enrichment broth and, incubated at 37 ºC for 24 hours. Samples were withdrawn from the enrichment medium and inoculated in Petri dishes containing selective culture medium Salmonella Shigella Agar (SS) and incubated at 37 °C for 24 hours. After this period, the SS colonies were transferred to tubes containing the culture medium agar Three Sugars Iron (TSI) and incubated for at 37 °C for 24 hours. Then biochemical hydrolysis tests urea and decarboxylation of lysine for confirmation of *Salmonella* spp. were performed.

To test the hydrolysis of urea were placed in test tubes 3 ml of urea broth, these tubes received TSI and, the inoculum were incubated at 37 ºC for 24-48 hours. The test is considered positive when the medium changes color, getting pink red. In lysine decarboxylation test were placed in test tubes 3 ml of broth lysine decarboxylase, these tubes received TSI and, the inoculum were incubated at 37 °C for 48 hours. The test is considered positive when there is blackening of the medium. It's characteristic of *Salmonella* spp., when the urease test is negative and is positive lysine.

Listeria spp. assay

For this evaluation was conducted enrichment method using 1 ml of the dilution 10^{-1} in MacConkey Agar Base broth and, incubated at 37 °C for 24 hours. After this period were plated in chromogenic medium (ALOA® Probac Brazil), and incubated at 37 °C for 72 hours, where growth of green colonies, characterizing the presence of *Listeria* spp..

Somatic Cell Count (SCC) and analysis of milk physical composition

The somatic cell count was performed by Somacount 300® machine, and the results are expressed in somatic cells per ml of milk. The chemical composition was determined by the equipment Bentley 2000®, which is used for infrared absorption. The results were transferred in percentages per ml (%/ml). The total solids (TS) was determined by the sum of the values of the components (fat, protein and lactose). The defatted dry extract (DDE) was calculated by the difference between ST and fats (Bentley Instruments, Inc., 2007).

Standard Count on Plates (SCP)

The standard count plate (SCP) was made through the Bacto Count IBC equipment (Bentley Instruments, Inc., 2007). Standardized bottles containing the preservative azidiol were used, which were duly identified and kept in refrigerated boxes for 48 hours. Samples were analyzed electronically by flow cytometry. The result is expressed in colony forming unit (CFU) (Bentley Instruments, Inc., 2007).

Statistical Analysis

The results obtained in this study were submitted to descriptive statistics (mean, standard deviation and coefficient of variation). Comparing the results, carried out between groups we used the comparison test between means of Student-Newman-Keuls (SNK) ($p \leq 0.05$).

3. Results and Discussion

In this study the average of aerobic mesophilic was $3.8x10^{-3}$ CFU/ml. This amount would not satisfy the requirements contained in the NI 76 (Brasil, 2018), how showed in Table 1.

Table 1 - Most Probable Number (MPN/ml) of coliforms at 30 ºC and 45 °C, psychophile and mesophilic microrganisms count (CFU/ml), somatic cell count (SC/ml) and Standard Count Plate of raw milk.

Mesophilic	Psychophile	SCP[1]	Coliforms (30 °C)	Coliforms (45 °C)	Somatic Cells/ml
(CFU/ml)[2]			MPN/ml[3]	MPN/ml	
3.8×10^3	6.7×10^2	4.3×10^3	10	9	495.733

Note:[1]Standard Count Plate. [2]Count forming Units. [3]Most Probable Number. Source: Author's elaboration (2020).

Higher values of microorganisms count were observed by Augusto et al. (2016) assessing the sanitary quality of raw milk on north of Paraná state, with <4 MPNml. The average obtained for psychrotrophic microorganisms was 6.7×10^{-2} CFU/ml. There is currently no specific legislation to for milk related to psychrotrophic microorganisms, so, there is a deficiency in research for this way, thus requiring more attention to this topic.

Is usually necessary that the milk has a count above 6×10^{-3} CFU/ml of psychrotrophic microorganisms to become noticeable aroma and taste changes of milk. Although it represents less than 10% of the initial microbiota in adequate conditions of hygiene, the population of psychrotrophic microorganisms can reach high levels with poor hygienic condition and/or a high number of somatic cells. Silva et al. (2017) found this group of microorganisms in the hand of milkers, being a factor that needs more attention. The hygiene of utensils used, and also the water used in sanitation, which might increase contamination by psychrotrophic microorganisms and, produce low quality products with shorter shelf life (Mörschbächer et al., 2017).

The origin of the contamination of milk products by psychrotrophic bacteria can occur through the poor quality water supply and hygiene and mastitis. Even in refrigerated conditions these microorganisms maintain their ability to multiply and tend to become predominant in the microbiota of raw milk.

In the present study, the average of coliforms at 30 °C was 10 MPN/ml. This value is above the recommended by the current legislation (4 MPN/ml) and, therefore pose a risk to consumer health because they are harmful bacteria to humans. However, to coliforms at 45 °C had an average of 9 MPN/mL, where the NI 62 could not exceed the value 2 MPN/mL.

The average count for SCP was $4.3x10^{-3}$ CFU/ml, meeting in accordance to compare with the current legislation (Brasil, 2018) ($6x10^{-5}$ CFU/ml). A high degree of contamination by microorganisms becomes detrimental to both the consumer and the industry, as they are responsible for undesirable changes in milk composition.

The average of SCC was 495,733 CS/ml of milk. Thus, being out the standard set by the NI 76 (Brasil, 2018). The practice of a correct handling milking (such as hygiene of milkers and, to clean the teat), regardless of herd size, implies a decrease in the risk of milk contamination. There is a need for producers to receive technical guidance and professional training to adhere to an adequate management system with the correct adoption of good practices in milking (Silva et al., 2017).

Tests conducted for *Staphylococcus* spp. indicated the presence of this bacteria in all analyzed samples. A result considered unsatisfactory, due to the high incidence. For samples that have been made the catalase and coagulase tests, most showed positive results. Although there is no legislation to contamination for coagulase positive *Staphylococcus* in milk, the presence of this microorganism is worrisome because concentrations of these microorganisms ranging from 5 and 6 x 10^{-6} CFU/mL are considered enough for the production of toxins harmful to human health.

The presence of *S. aureus* in milk is indicative of infection of the mammary gland and, indicates frailty in the control of mastitis, a major disease that occur in dairy cattle, besides the risk of enterotoxin production resistant to pasteurization where the milk is not held at refrigeration temperature below 7.2 ºC. Furthermore, it can influence the quality of by-products obtained from milk (Aragão et al., 2020).

The adoption of safety procedures has fundamental importance in preventing *S. aureus* contamination since its spread in the herd mainly occurs during milking, the hands of the milkers and milking equipment. So, these procedures are essential for the effective control of mastitis and, to reduce the number of microorganisms in milk (Barros et al., 2018).

The presence of *Listeria* spp. was found at 83.33% of the analyzed milk samples. While there is no standard in the legislation for the presence of *Listeria*, this microorganism is a risk to the health of consumers, thus deserving attention to their presence in food.

The average results, standard deviations and coefficients of variation of the analysis of raw milk physical composition are shown in Table 2.

Table 2 - Average, standard deviations (SD) and coefficients variation (CV) of protein, fat, lactose, solids, urea and casein.

	Fat (%)	Protein (%)	Lactose (%)	Solids (%)	Urea (%)	Casein (%)
Average	3.86	3.33	4.60	12.80	11.25	2.63
SD	1.17	0.40	0.22	1.40	3.94	0.33
CV	30.47	12.13	4.82	10.90	35.07	12.69

Source: Author's elaboration (2020).

The average of milk fat found in samples obtained satisfactory results, with averages of 3.81%. This fits within the legislation with minimum required (3%) (Brasil, 2018). Fat values can be influenced by age and race (Oliveira et al., 2020). The average obtained in the current study was 12.80% for total solids.

Comparing the results of microbiological analysis of milk from three farms studied, it was found that the average of mesophilic bacteria, SCP, coliforms at 30 °C, coliforms at 45 °C and SCC differ statistically ($p \leq 0.05$). Only psychrotrophic microorganisms obtained no significant statistically difference ($p \leq 0.05$) (Table 3). The farms 1 and 3 obtained the highest average for mesophilic microorganisms, coliforms at 30 °C, coliforms at 45 °C, differing statistically ($p \leq 0.05$).

Table 3 - Comparison means of microbiological parameters, Standard Count Plate (CPP) and Somatic Cells Count (CCS) of raw milk collected in three farms.

Farm	Mesophilic (CFU/ml)[1]	Psychrophyle	SCP[2]	Coliforms (30 °C) MPN/ml[3]	Coliforms (45 °C) MPN/ml	Somatic Cells (CS/ml)[4]
1	3.366b*	2.988 a	3.807 a	0.894 b	0.727 b	357.467 b
2	4.091 a	2.888 a	3.391 b	1.044 a	1.044 a	875.867 a
3	4.128 a	2.597 a	3.594 c	1.104 a	1.104 a	253.867 c

*Averages followed by the same letters in the same column are not statistically different from each other ($p \leq 0.05$). [1]Count Form Units. [2]Standard Count Plate. [3]Most Probable Number. [4]Somatic Cells. Source: Author's elaboration (2020).

Failures in hygiene procedures increase the contamination of milk by mesophilic bacteria. Milk residues present on the surfaces of equipment can become nutrients for the growth of bacteria thus contaminating the product in subsequent processing steps. The contact of the milk with dirty animals, inappropriate production environments, failures in the milk cooling rate and milk from animals with mastitis may also result in high microbial counts (Barros et al., 2018; Alves et al., 2020).

The SCP reflects the animal hygiene, environment, equipment, the milking and cooling procedures. Its importance for considering the potential to multiply the bacteria from milk may cause changes such as degradation of fats, proteins or carbohydrates, which may make the product unfit for consumption and industrial processing.

Among the three farms under study, there was a statistically significant difference in somatic cells count, and, the farm 2 showed higher average in relation to the farms 1 and 3. Several factors influence the somatic cells count in milk, however, the presence of intra-mammary infections is a fairly reliable indicator of the health of the mammary gland. In addition, other factors that can influence the SCC such as race, stage of lactation, milk production, number of lactations, age, milk production, stress caused by deficiencies in the management, nutritional problems, conditions climate and, diseases.

The farms differ in the milking parlor facilities, but also show differences in the equipment cleaning system and handling of animals. Such factors are decisive in the

microbiological differences found and may be related to failures in cleaning and sanitizing procedures.

Because of regional production characteristics it is difficult to determine the number of producers framed the rules of the NI number 76 (Brasil, 2018), because there is no control by the competent authorities and the local industry has not yet implemented the quality of payment system, already widespread in other Brazilian regions. Tests to check the quality of milk are made useful for greater awareness of the producers regarding the quality of milk.

There was a difference between the farms on the percentage of fat, protein, lactose, urea and casein. And these parameter settings showed differences statistically significant ($p \leq 0.05$). The farms 2 and 3 were those with the highest average for percentages of fat, protein and casein, with no statistically significant difference ($p \leq 0.05$) between them (Table 4).

Table 4 - Comparison mean of protein, fat, lactose, solids, urea and casein of raw milk in the three farms.

Farms	Fats (%)	Protein (%)	Lactose (%)	Solids (%)	Urea (%)	Casein (%)
1	3.213 b*	3.115 b	4.629 a	12.109 a	9.720 c	2.486 b
2	3.369 a	3.418 a	4.646 a	13.074 a	10.770 b	2.662 a
3	3.472 a	3.465 a	4.521 b	13.139 a	13.260 a	2.761 a

*Means followed by the same letters in the same column are not statistically different from each other ($p \leq 0.05$). Source: Author's elaboration (2020)

Lowest values for protein and fat can be attributed to lower supply of concentrate or the application of diets unbalanced by producers. The reduction in the protein and fat content in milk may also take place due to environmental factors such as temperature, humidity and type of available forage and, a supply of low-quality pastures (Alves, 2017).

The total solids did not differ significantly ($p \leq 0.05$) when compared to farms 1, 2 and 3. The variation in the total solids content is directly dependent variations of its components (fat, protein, lactose and ashes), thus the variation best explained by the differences of its components. As for the urea content in the farm one had higher mean levels over the farms 2 and 3 ($p \leq 0.05$). The urea content of the milk has high color-relation with the protein intake

diet, being essential to providing balanced diet, which may explain the difference between the properties, because they use different feeding management among them.

To obtain milk of best quality, should be an improvement in sanitary conditions; hygiene procedures in obtaining milk, such as: management of proper milking, cleaning of milking equipment and adequate refrigeration are important factors for reducing the bacterial count in milk.

4. Conclusions

According to the microbiological parameters evaluated for milk produced except for coliforms at 30 ºC and coliforms at 45 °C, the milk produced is within the standards required by current legislation in function of the other evaluated parameters. The farm 1 showed differences in their microbiological characteristics.

Milk quality was satisfactory According to the composition of milk (fat, protein, lactose, the total solids, urea and casein) having suitable values and in accordance with the NI 76.

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Porcentagem de contribuição de cada autor no manuscrito

Bruno Fernando Oliveira Araújo – 20%

Sybelle Geórgia Mesquita da Silva – 10 %

João Manoel da Silva – 10%

Cícero Cerqueira Cavalcanti Neto – 10%

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Yamina Coentro Montaldo – 10%

Jakes Halan de Queiroz Costa – 10%

Elizabeth Simões do Amaral Alves – 10%

Tania Marta Carvalho dos Santos – 10%