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Distribution of microorganisms on surface of Kefir biofilms associated with Açaí extract

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Resumo

Neste estudo, realizou-se uma análise morfológica e estatística da superfície de biofilmes de kefir associados ao extrato de açaí para determinar o biofilme que possui maior potencial terapêutico, o qual ainda não foi relatado quantitativamente na literatura. Seis amostras de diferentes concentrações dos biofilmes foram obtidas, as quais foram analisadas em um Microscópio de Força Atômica. Análises estatísticas foram realizadas com o objetivo de determinar o número e a cobertura superficial de microrganismos. A análise morfológica mostrou que as superfícies dos biofilmes são compostas por microrganismos como bactérias e leveduras, com o Açaí dando seu caráter antioxidante. A análise estatística mostrou que biofilmes com concentração de Açaí entre 10 e 40 mL apresentam maior número e cobertura de bactérias. Os resultados mostram que o biofilme com concentração de 20 mL de Açaí mostrou-se o mais adequado, visto que apresentou maior número e cobertura de bactérias

Palavras-Chave: Morfológica. Microrganismos. Antioxidante. Bactéria.

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In this study, a morphological and statistical analysis of the surface of Kefir biofilms associated with the Açaí extract was performed for to determine the biofilm which have greater therapeutic potential, which has not yet been reported quantitatively in the literature. Six samples of different concentrations of the biofilms were obtained, which were analyzed in an Atomic Force Microscope. Statistical analyzes were performed with the objective of determining the number and surface coverage of microorganisms. The morphological analysis showed that the surfaces of the biofilms are composed of microorganisms such as bacteria and yeasts with the Açaí giving its antioxidant character. Statistical analysis showed that biofilms with Açaí concentration between 10 and 40 mL present a greater number and coverage of bacteria. Results show that the biofilm with a concentration of 20 mL of Açaí proved to be most appropriate, considering that it presented a greater number and coverage of bacteria

Key-words: Morphological. Microorganisms. Antioxidant. Bacteria.

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1. Introduction

Kefir is a beverage derived from the fermentation of milk, in which the hydrolysis of lactose during fermentation occurs with the simultaneous action of bacteria and yeasts contained in the Kefir grains (HAMET et al., 2015). Its artisanal production is based on the tradition of the Caucasus peoples (LEITE et al., 2013). The Kefir grains consist of numerous small-grained units (JEONG et al., 2017) which are characterized by a hollow globular structure with a diameter between 2.0 and 9.0 mm (LU et al., 2014). (LAUREYS and VUYST, 2014) it have shown that the bactericidal composition of the beverage is generally composed of Lactobacillus. Kefir has been shown to be a very efficient antibactericide (JEONG et al., 2017; LU et al., 2014).

A Kefir biofilm is a film that contains a microbiota associated with Kefir grains (HAMET et al., 2015). It is the result of the fusion of an exopolysaccharide matrix (EPS) (JEONG et al., 2017) that has influence on bacterial aggregation, adhesion and survival (LEBEER et al., 2010). In this sense, finding out if there is a good distribution of bacteria in the biofilm provides evidence of the applicability potential of this as a curative in the healing process of wounds and burns, since the microbiota found in the biofilm is directly responsible for its therapeutic properties (LAUREYS and VUYST, 2014; MATOS et al., 2018).

In addition, the Açaí has three species that occur most abundantly: E. oleracea, E. precatoria and Euterpe edulis (SCHULZA et al., 2016; YAMAGUCHI et al., 2015), where Euterpe oleracea Mart was used in this research because it is the most used species in the study site. In Brazil, Açaí has been used, in view of its antioxidant potential (RANER et al., 2015), in the treatment of skin complications, digestive disorders and parasitic infections, and in recent years has been widely disseminated, for example, throughout the Internet (PORTINHO and ZIMMERMAN, 2012). In addition, Açaí has attracted attention because it is a nutraceutical food with rich content of anthocyanins (RANER et al., 2015; AMSELLEM-LAUFER, 2015), water-soluble pigments that designate the violet color of the fruit (PORTINHO and ZIMMERMAN, 2012; KANG et al., 2011; YAMAGUCHI et al., 2015).

Through specific programs it is possible to determine some important parameters for the analysis of elements that make up the surface of a certain image. ImageJ software was recently used for image processing by (DIAS, 2018; MORAES et al., 2013).

This work aims to use the superficial distribution of bacteria and yeasts on the surface of the biofilm as a parameter to determine the biofilm with greater therapeutic potential, since (MATOS et al., 2018) pointed out that these biofilms can be used as possible natural curatives, aiming at the antibacterial character of Kefir and the antioxidant, lipid regulator and tissue restorative character of Açaí.

2. Materials and Methods

Obtaining Biofilms

To obtain the biofilms of Kefir associated with the Açaí extract used in this work, the same procedures described in the methodology of (MATOS et al., 2018; OLIVEIRA, 2017) were strictly followed and the experimental procedure occurred in the period from March to July of 2016 in the laboratory of Research in Pharmaceuticals of the Federal University of Amapá, where six biofilms were obtained with the following configuration: 1 - biofilm of 40 g/L brown sugar solution with 40 g/L of Kefir and 10 mL dose of filtered Açaí; 2 - biofilm of 40 g/L brown sugar solution with 40 g/L of Kefir and 20 mL dose of filtered Açaí; 3 - biofilm of the 40 g/L brown sugar solution with 40 g/L of Kefir and 40 ml dose of filtered Açaí; 4 - biofilm of 40 g/L brown sugar solution with 40 g/L of Kefir and 60 mL dose of filtered Açaí; 5 - biofilm of 40 g/L brown sugar solution with 40 g/L of Kefir and 80 ml dose of filtered Açaí; 6 - biofilm of 40 g/L brown sugar solution with 40 g/L of Kefir and 100 mL dose of filtered Açaí.

In summary, the biofilms were produced by mixing in a beaker distilled water, brown sugar and non-alcoholic Acai extract, which was filtered on commercially obtained paper filters, which formed the substrates for the above-mentioned essays. This mixture was made manually. Subsequently, the grains of kefir were inoculated on the substrate in a UV chamber. All biofilms were formed at room temperature under a 51% relative humidity condition. The process of biofilm formation lasted 25 days. The biofilms are formed naturally and deposited manually in glass sheets of dimensions

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of 15 x 40 mm and thereafter are taken to the freezer for drying. The drying process lasted 10 days and then the biofilms were taken to the AFM.

Morphology Analysis

The images obtained for the analyzes in this work were made in the Materials Science laboratory of the Federal University of Amapá from July to August 2016. The images were obtained under ambient conditions (51% relative humidity) in an Atomic Force Microscope (AFM) of the company Nanosurf, model easyscan 2 controler in contact mode, with ContAL-G silicon cantilever, with resonance frequency of 13 kHz and elastic constant of 0.2 N/m, same conditions established by (MATOS et al., 2018). Twenty AFM deflection images made at random points of each sample were obtained from each biofilm in order to obtain a more statistically reliable value at the end of the analysis.

Statistical Analysis of the Biofilm Surface

From the obtained deflection images the calculated area of each bacterium or yeast was obtained using the ImageJ software and afterwards the counting of the number of particles present in the images was also made using the ImageJ software in order to obtain the average number of bacteria and yeasts of all images.

Twenty bacteria and twenty yeasts were randomly selected in biofilms of 10 to 100 mL. The studies were done considering the mean length dimensions of a lactobacillus, which is approximately 900 nm to 3 μm (BERGEY et al., 1989; BERGEY and HOLT, 1994), to find a statistical model that represents the best way to calculate the area of lactobacilli in biofilms. The same methodology was applied to the Yeasts, so that there is a comparison between the manually calculated area and the area measured by ImageJ. In this case, it has been defined that the length will be attributed to a greater distance from one end to another of a yeast or bacteria and the width the shortest distance, considering the forms of the yeasts and bacteria, which are roughly spherical and cylindrical respectively.

The determination of the most adequate equation to estimate the area of bacteria or yeast was studied from regression involving its width and length measurements, being the same of the linear type (Y = a + bx). The value Y estimated the area of the bacterial or the yeast limbus as a function of X, whose values can be the length (L), the width (W) or the product (L x W). The values of a and b represent the linear and angular coefficient of the obtained line. All the adjustments of the equations were made from the straight line, thus, all the equations used were linear.

The correlation coefficients μ, of adjusted determination and p were obtained with the variables X and Y, and the value of the bacterial or the yeast area measured was considered the dependent variable (Y) and length, width and product length x width as independent variables. The best fit model was tested and validated using the regression analysis between the estimated yeast area or bacterial area and the measured yeast area or bacterial area.

The mean particle number <N> and the mean particle density <N>/A, where A is the total area evaluated, were determined to analyze the degree of concentration of bacteria in the analyzed area. Subsequently, the percentage of coverage of the biofilm surface by bacteria or yeasts was determined, where the following equation was used:

$$Perc = \frac{100 \times measured\,area}{900} \qquad (1)$$

where Perc. represents the percentage of the area covered, measured area represents the area measured by the software Imagej and 900 represents the total area of the biofilm, considering that the images obtained were 30 x 30 micrometers.

The analysis of variance (ANOVA) was applied with the objective of comparing if different treatments have statistical behavior similar or not. In other words, it aimed to check whether there is difference in at least some treatment or whether they are the same, both for the area of bacteria and for yeast. In this case, the different concentrations represent the treatments.

The statistical model that describes the ANOVA (FAVERO and FAVERO, 2016) for the case on screen is:

$$y_{ij} = \mu_i + \varepsilon_{ij} \qquad (2)$$

where, is the observed variable (Area), is the mean effect of each treatment i = 10, 20, ..., 100, i.e. the concentrations, and and is the random error; j can assume the values 1, 2, ..., 20, that is, the repetitions in each treatment. In order for ANOVA to be performed, it met the assumptions of randomness, homogeneity of variances and normality of residues. Thus, the superficial distribution of bacteria and yeasts in biofilms was determined. For

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all statistical analyzes the application R (R CORE TEAM R, 2017) was used.

3. Results and Discussion

Morphological Analysis

Figure 1 show the biofilm image of Kefir with the Açaí extract, formed under the conditions described above. The AFM images obtained in this work are very similar to those obtained by (MATOS et al., 2018). It is possible to perceive from the figure 1 that the fermentation process is very accentuated, considering that there is a formation of a foam on the biofilm, but on the other hand the purple coloring indicates that after 25 days of formation and 10 days of storage there was no alter of the antioxidant potential provided by Açaí, in the same way as observed by (MATOS et al., 2018).

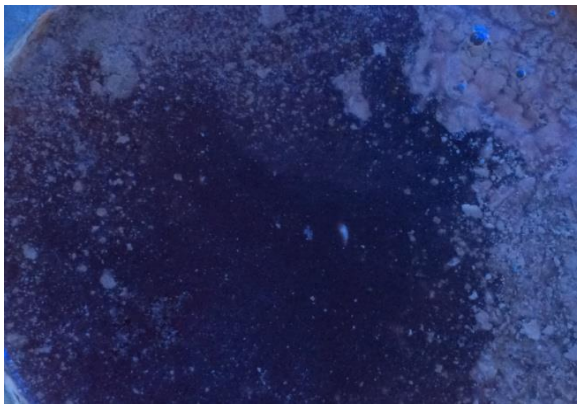

Figure 1. Kefir biofilm associated with Açaí extract.

Figures 2 (a) to (f) show the deflection images obtained in AFM (orange image), which were then binarized (gray image) and sequenced (black image) in the ImageJ software, for the calculation of the area of each bacterium or yeast. They are images with areas of 30x30 micrometers and show microstructural details of biofilms. It is possible to identify structures identical to those of the genus Lactobacillus, such as those described by (KATRINA, 2011) and other structures identical to yeasts such as those described by (GALLONE et al., 2016).

Yeasts, from a microbiological point of view, grew more in the presence of larger amounts of Açaí because the fermentation process increased, which causes a phenomenon known as yeast flocculation (the appearance of more yeasts), a fact that occurs mainly when it comes to pathogenic microorganisms as in the case seen by (NETO et al., 2012), which is a defense strategy that culminates with the colony increase of these microorganisms.

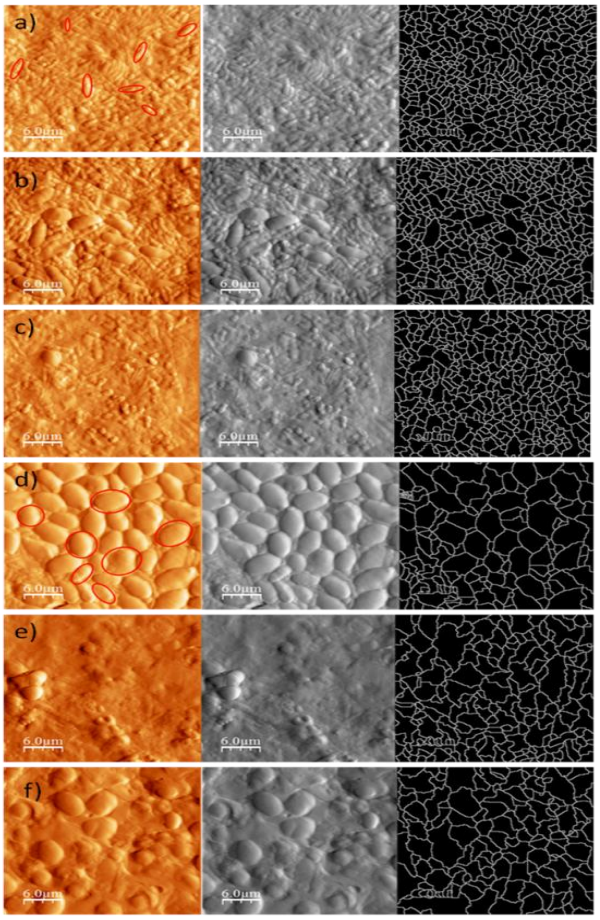

Figure 2. Deflection image, binarized image and segmented image, respectively, of a Kefir biofilm associated with the Açaí extract, obtained via AFM and ImageJ, for the concentration of (a) 10 mL, (b) 20 mL, (c) 40 mL, (d) 60 mL, (e) 80 mL and (f) 100 mL. The circulated areas on images show the various forms that bacteria and yeasts can present.

Topographic images of biofilms have also been discussed in (MATOS et al., 2018), where it was shown that the Roughness of the samples increased as the Açaí concentration increased. However, it has been described that there is a relative saturation in the transition from the concentration of 60 mL to 100 mL. In statistical terms it is very important to study this phenomenon, considering that it is an unknown distribution of microorganisms on the surface of the sample.

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Statistical Analysis

Table 1 shows the average values obtained for the length and width of Bacteria and Yeasts obtained with the ImageJ software, in which case the maximum and minimum values of the Calculated Area were used as the range of consideration in the calculation of the Measured Area. Considering the values defined in (BERGEY et al., 1989; BERGEY and HOLT, 1994) for the average length of a lactobacillus, which is 900 nm to 3 μm, the values found here are in relative agreement with the standard deviation found and approximate said values.

The highest values of standard deviation, representing the highest dispersion and the greatest distance from the extreme values of the mean, occurred for the Calculated Area of Yeasts, which is normal considering that the in vivo formation of yeasts depends on factors such as the proliferation of Bacteria that can change the way yeasts multiply. On the other hand, Table 2 shows the values found for the measured area of bacteria and yeasts.

Table 1. Mean values and standard deviation (in parentheses) of Length (L), Width (W) and Calculated Area (CA) of the biofilms, in which 20 lengths and 20 widths were taken for each microorganism, randomly chosen, in the samples.

Organism	Length-L(μm)	Width-W(μm)	Calc Area-AC (μm2)	Mín-Máx (μm2)
Bacteria	2.005 (± 0.59992)	0.76 (± 0.15111)	1.6983(±0.60714)	0.4988-2.7336
Yeasts	4.375 (±1.06149)	2.6 (± 0.50500)	12.0358(±5.32456)	7.46-24

Table 2. Mean values and standard deviation (in parentheses) of the Measured Area (MA) in the ImageJ software of biofilms for each analyzed sample, considering the 20 images obtained for each concentration.

Concentration (mL)	Bacteria	Yeasts
10	367.86795 (±161.52742)	140.4068 (±131.02885)
20	438.4296(±103.43194)	67.09625 (±32.643517)
40	438.56947 (±157.26698)	203.01565(±138.14308)
60	114.50959 (±52.094585)	540.06996 (±191.80217)
80	221.11515(±98.519969)	406.97465 (±94.432972)
100	149.08675(±39.3288772)	556.05115 (±942773996)

The most adequate regression equations were used to evaluate a possible improvement in the method of calculating the area of bacteria and yeasts by adjusting mathematical models, comparing the results of table 1 with those of table 2 which are shown in table 3. It was verified that no adjusted mathematical model was significant to estimate a better area of both Bacteria and Yeasts for the biofilms presented, taking into account the length and width of the microorganisms, since the equations always presented $p>0.001$. This particular result contrasts with that obtained by (MORAES et al., 2013), who managed to adjust mathematical models by simple linear regression to estimate the leaf area of different plant species, the difference is that in this case the leaves have an exponential growth pattern and here both yeasts and bacteria are microorganisms whose development is not a predictable event, as well as the formation of membranes in vivo (ISRAELACHVILI, 2011) as is the case of biofilm formation.

The values found for the mean number of bacteria and yeasts present in each sample are shown in table 4. It is observed that there are more bacteria than yeasts in the biofilms, for all the concentrations evaluated. This discrepancy is related to the particle size and the availability of energy that the microorganisms have. In the case of bacteria, which are much smaller microorganisms than yeasts, the reproduction is more pronounced, since they only depend on that amount of energy to develop. Yeasts, among other factors, need to adapt to the space left by bacteria and are developed more by the need to defend themselves against the abrupt multiplications of bacteria.

Table 5 shows the values found for the mean surface particle density for both bacteria and yeasts found in each sample. Superficial density as a measuring instrument for the degree of

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concentration of the number of particles on the surface, showed that the biofilm concentration of 20 g/L has a higher concentration of bacteria in detriment of yeasts. This particular result already improves the understanding of (MATOS et al., 2018), who qualitatively demonstrated that the 10 ml biofilm appeared to be the biofilm with the highest number of microorganisms on its surface. In particular, this fact helps in choosing the best concentration to be used in a possible treatment of infections, since Kefir has a well-known antibacterial capacity (PRADO et al., 2015; KIM et al., 2016; LEITE et al., 2015). On the other hand, all samples in the range of 10 to 40 g/L were shown to have a similar density.

The percentages of average surface coverage of bacteria and yeasts in the analyzed samples are shown in Table 5 and in graph of the figure 3. Outliers may be other microorganisms, or the same microorganisms that have grown above the stipulated mean. The graphical comparison shows that there is an inverse relationship of distribution of these microorganisms as the concentration increases. This aspect only intensifies the idea that the group comprising the concentrations between 10 and 40 mL of Açaí have a higher concentration of bacteria than of yeasts and therefore are the concentrations whose potential antibacterial are statistically higher.

Table 3. Equations adjusted for each concentration evaluated with the respective determination coefficient (R^2), correlation coefficient μ and p value.

Concentration	*Adjusted equation*	R^2	*μ*	*P*
10 mL/Bacteria	*1.3490+[0.0340*(C*L)]*	*0.0197*	*0.1844*	*0.5581*
10 mL/ Yeasts	*10.4799+[-.0556*(C*L)]*	*0.0293*	*0.2835*	*0.2240*
20 mL/ Bacteria	*1.4385+[-0.0373*(C*L)]*	*0.0274*	*0.1634*	*0.5025*
20 mL/ Yeasts	*9.3549+[-0.0227*(C*L)]*	*0.0395*	*0.1234*	*0.6099*
40 mL/ Bacteria	*1.6797+[-0.0217*(C*L)]*	*0.0496*	*0.0751*	*0.7509*
40 mL/ Yeasts	*8.8412+[-0.0094*(C*L)]*	*0.0538*	*0.0405*	*0.8594*
60 mL/ Bacteria	*1.2719+[0.0603*(C*L)]*	*0.0489*	*0.0791*	*0.739*
60 mL/ Yeasts	*12.1586+[0.0207*(C*L)]*	*0.0405*	*0.1195*	*0.6210*
80 mL/ Bacteria	*1.6721+[0.0711*(C*L)]*	*0.1215*	*0.4107*	*0.0091*
80 mL/ Yeasts	*10.0264+[0.0062*(C*L)]*	*0.0008*	*0.0547*	*0.9000*
100 mL/ Bacteria	*1.7973+[-0.0193*(C*L)]*	*0.0464*	*0.0932*	*0.6976*
100 mL/ Yeasts	*11.8642+[0.0586*(C*L)]*	*0.0464*	*0.3458*	*0.1321*

Table 4. Average surface number of particles, for bacteria and yeasts found on the surface of biofilms.
Average number of surface particles (<N>)

Average number of surface particles (<N>)		
Concentration	***Bacteria***	***Yeasts***
10 mL	*268.9*	*13.65*
20 mL	*324.75*	*7.5*
40 mL	*269.65*	*22.1*
60 mL	*90.15*	*43.7*
80 mL	*124.25*	*40.7*
100 mL	*84.75*	*50.35*

In fact, according to (DIA et al., 2018; KIM et al., 2016; ROSA et al., 2017) Lactobacillus acidophilus isolated from Kefir present inhibitory activity on several Gram-positive and Gram-negative bacteria. Among the different microorganisms isolated from Kefir, Lactococcus and acetic acid bacteria (AAB) are the ones with the highest inhibitory effect on coliforms (YENICE et al., 2014), and also have inhibitory activity on Staphylococcus aureus, Bacillus cereus, Clostridium tyrobutyricum and Listeria monocytogenes (SANTOS, 2008).

Table 5. Average surface particle density for bacteria and yeasts found on the surface of biofilms.

Surface Average Particle Density (<N>/A)		
Concentration	**Bacteria**	**Yeasts**
10 mL	0.298	0.015
20 mL	0.361	0.008
40 mL	0.300	0.024
60 mL	0.100	0.048
80 mL	0.138	0.045
100 mL	0.094	0.056

In order to know if at least some concentration influenced the distribution of the microorganisms on the surface, the analysis of variance (ANOVA)

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was applied to study the difference between averages for bacteria and yeasts. The results show that in the case of the coverage, for both bacteria and and yeasts and there was a significant difference in coverage between the concentrations analyzed. This shows that the concentration has influence on the distribution of microorganisms in the samples.

Table 6. Mean surface coverage of bacteria and yeasts in the analyzed samples, considering the concentrations of Açaí used.

Concentration	Bacteria (%)	Yeasts (%)	Outliers (%)
10 mL	40.87	15.60	43.53
20 mL	48.71	7.45	43.84
40 mL	48.72	22.55	28.73
60 mL	12.72	60.07	27.21
80 mL	24.56	45.21	30.23
100 mL	16.56	61.78	21.66

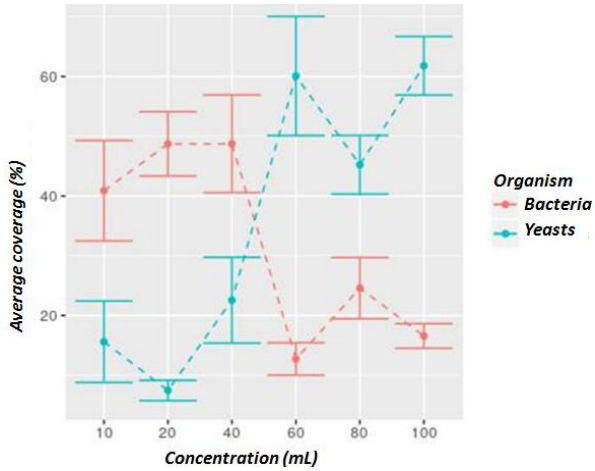

Figure 3. Average percentage of area occupied (center point connected by dotted lines), with 95% confidence interval (continuous lines, above and below points), for each concentration (ml) of Açaí for both bacteria and yeasts.

4. Conclusion

In this research was carried out a study of the morphology and superficial distribution of bacteria and yeasts in biofilms of Kefir associated to the Açaí extract. Morphologically, the particles found on the surfaces presented structures similar to those of bacteria of the genus Lactobacillus and of yeasts randomly distributed over the sample. The images obtained were similar to results found in the literature. The application of linear regression showed that it is not possible to adjust mathematical models for the calculation of the area of bacteria and yeasts. The results showed that biofilms with Açaí concentration between 10 and 40 mL present a greater number and coverage of bacteria, being the 20 mL concentration considered the most appropriate. ANOVA showed that the concentration influences the formation and distribution of microorganisms on the surface of biofilms. Therefore, regarding the statistical approach, biofilms whose Açaí concentrations are between 10 and 40 mL have a higher antibacterial potential. This research may help in the development of a kefir biofilm product with acai that serves as a substitute for the dressings used today. However, future research may help confirm this potential and the applicability of the biofilm in the pharmaceutical industry as curative, since the results presented here only show indications of a probable application.

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