Vol. 07, e2732019JBFS, 2020
https://dx.doi.org/10.18067/jbfs.v7i2.273
ISSN 2359-2710 Online Edition

Study of the superficial distribution of microorganisms in kefir biofilms prepared with Cupuaçu juice

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Received: 2019.06.23; **Revised:** 2020.01.16; **Accepted:** 2020.01.25; **Published:** 2020.01.04

Highlights: "Kefir Biofilms" and is important because currently kefir has been highly studied for its antibacterial, anticancerogenic and antitumoral potential. On another hand, the cupuaçu has antioxidant potential. In addition, an exhausting work of the researchers try to perfect methods of obtaining materials, with excellent superficial characteristics, that are biodegradable.

Section: This paper was submitted in Biotechnology, a section of the *J. Bioen Food Sci.*

Competing interests: There is not conflict of interest in the research conducted.

Funding: The author received no specific funding for this work.

Citation as (APA): Ferraro, M.A.N., Pinto, E.P., & Matos, R.S. (2020). Study of the superficial distribution of microorganisms in kefir biofilms prepared with Cupuaçu juice, *Journal of Bioenergy and Food Science*, *7*(2) e2732019JBFS. doi: 10.18067/jbfs.v7i2.273

Edited by Dr. Victor Hugo Gomes Sales - Federal Institute of Amapá, Macapá-AP, Brazil.

Review processes: 2732019R01 (Brazil) | 2732019R03 (Brazil)

ABSTRACT - Recently, much research in the field of biomaterials has focused on finding better material to serve as a dressing or temporary skin replacement, often without success. Kefir and Cupuaçu (*Theobroma grandiflorum* Schum) have therapeutic potential for use in this field. In this study, a morphological and statistical analysis of the superficial distribution of bacteria and yeasts in kefir biofilms prepared with Cupuaçu juice, obtained from the inoculation of kefir grains in brown sugar solution was performed. Six samples of different concentrations of the biofilms were obtained, which were analyzed in an Atomic Force Microscope to obtain topography images. Statistical analyses were performed on the surface parameters in order to determine the number and surface coverage of microorganisms in biofilms. The morphological analysis showed that the surfaces of the biofilms are composed of microorganisms similar to bacteria and yeasts. Statistical analysis showed

that biofilms with a concentration of Cupuaçu between 10 and 40 $g.L^{-1}$ present a greater number and coverage of bacteria. All results show that the Cupuaçu 10 $g.L^{-1}$ biofilm presented the most appropriate, considering that it presented a higher number and coverage of bacteria, which is the antibacterial agent. In this perspective, the results indicate that there is an expectation of biofilms to result in a future application in the pharmaceutical industry.

Keywords: Biofilms; Cupuaçu; Morphology; Statistics.

INTRODUCTION

A few years ago, microbiology was much more studied by the harmful potential of many microorganisms. Currently, furthermore, many studies find to use several of these microorganisms for benefits to human health, as is the case of probiotics and prebiotics (Morais and Jacob, 2006), such as kefir grains microorganisms.

In this sense, Kefir, which originates in the Caucasus mountains, is a fermented milk species (Gul et al., 2015) has been well studied by the therapeutic properties of the microorganisms contained in its composition. It is slightly acid and produced from so-called kefir grains when mixed with milk (Zanirati et al., 2015). Kefir is a natural probiotic, that is, it is a food that contains live bacteria (Salminena et al., 1998). The microorganisms that are grouped in a polysaccharide matrix called Kefiran (Otsoa et al., 2006).

The Kefir grains are small rigid granules yellowish that have their irregular shape (Seydim et al., 2000). Containing bacteria and yeasts, kefir grains also have proteins and polysaccharides (Garrote et al., 2001). It has a mixture of several bacteria, such as various species of lactobacilli, lactococci, leuconostocs, and acetobacteria and yeasts (lactose fermentation and lactose-free fermentation) (Otles et al., 2003).

Several studies have demonstrated that Kefir has several therapeutic activities, such as Antitumoral (Shiomi et al., 1982), Antimicrobial (Rodrigues et al., 2005; Santos et al., 2003; Ismaiel et al., 2011), Antifungal (Cevikbas, 1994) and Anticarcinogenic (Ahmed et al., 2013), which has promoted a series of researches on the benefits of kefir to human health.

Biofilms are developed by bacteria on several different surfaces, such as natural aquatic environments and soil, living tissues, medical devices, or potable or industrial water piping systems (Vu et al., 2009). They are due to the organization of bacteria on a surface where they begin to develop in a layer that stays constant maintenance until the environment is no longer favorable (Stoodley et al., 2002).

Kefir biofilms can be formed by the inoculation of Kefir grains in distilled water and brown sugar solution (Matos et al., 2018), where it has been discussed qualitatively that the associations of kefir and Cupuaçu are more abundant that the yeasts, but it was not verified if the fact has a reliable statistical answer since it can have beneficial utility as a bioremediation mechanism (Oliveira et al., 2016).

In addition, Cupuaçu (*Theobroma grandiflorum* Schum) is a fruit native to the Brazilian Amazonia (Alves et al., 2007), and stands out for its high economic potential that comes the exquisite sensorial appeal of the pulp (Carvalho et al. 2006). It is widely used in the candy and confectionery industry (Salgado et al., 2011). This fruit is also used for juices and ice creams (Venturieri, 1994).

Cupuaçu is mainly composed of ethyl butanoate, ethyl hexanoate, and linalool, also containing vitamin C, neutral sugar chains such as arabinans, galactans or arabinogalactans, and rare sugars such as apiose, acetic acid, 2-O-methyl fucose and Dha (Vriesmann et al., 2009), having nutrients such as iron, calcium, phosphorus and high natural acidity (Matos, 2007). Kefir biofilms associated with the Cupuaçu extract showed that it has excellent surface morphological characteristics and can be used as a bioremediatory (Matos et al., 2018).

On another hand, in the field of biomaterials, the search for materials synthesized with natural sources to be used as a natural dressing or temporary skin substitute has not yet yielded satisfactory results. These products often run into a lack of biocompatibility and toxicity. In this sense, what we hope is that the therapeutic properties of kefir biofilms associated with Cupuaçu antioxidants may represent a viable solution to this problem. For this, it is necessary to understand the dynamics of the surface of these materials to evaluate if these biomaterials offer minimum conditions for their applicability.

Thus, this paper aims to evaluate how Cupuaçu concentration affects the distribution of microorganisms on the surface of kefir biofilms. Statistical analysis was performed using atomic force microscopy image processing to achieve this objective. This fact may provide a better understanding of the organization of these microorganisms on the surface as well as determine the best theoretical point of production of biofilms, since (Matos et al., 2018) showed that these biofilms have a random distribution of microorganisms along the surface of kefir biofilms, which may affect a possible antimicrobial action of lactobacilli present on the surface of these films.

MATERIAL AND METHODS

Obtaining biofilms

To obtain the biofilms of this work, the same procedures described in the methodology of Matos et al. (2018) were rigorously followed and the experimental procedure occurred in the period from March to July of 2018. The kefir grains (Fig. 1a) were obtained in the laboratory of research in Drugs of the Federal University of Amapá.

The biofilm production essay related to the experimental procedure was performed with the following configuration: a) a film without kefir with the concentration of 40 $g.L^{-1}$ of brown sugar as control of the experiment; b) six biofilms of the distilled water solution with 40 $g.L^{-1}$ of brown sugar for the following variations in kefir concentration: 10 $g.L^{-1}$, 20 $g.L^{-1}$, 40 $g.L^{-1}$, 60 $g.L^{-1}$, 80 $g.L^{-1}$, and 100 $g.L^{-1}$; c) six biofilms of the distilled water solution with 40 g.L-1 of brown sugar and 40 $g.L^{-1}$ of Kefir inoculated for the following variations in the concentrations of commercial Cupuaçu pulp: 10 $g.L^{-1}$, 20 $g.L^{-1}$, 40 $g.L^{-1}$, 60 $g.L^{-1}$, 80 $g.L^{-1}$ and 100 $g.L^{-1}$.

The procedure consisted of mixing the sugar, distilled water, and Cupuaçu pulp to form the substrate, which was sterilized in a UV chamber to eliminate any contamination in bottles and substrate. Subsequently, the Kefir grains were inoculated and kept under the ambient condition for 25 days (Fig. 1b). Thereafter, the biofilms were deposited in 15x40 mm rectangular glass plates. The biofilms (Fig. 2) were dried under the ambient condition to be analyzed in the AFM. Figure 1 shows the Kefir grains that were inoculated in a solution of distilled water and brown sugar to form a biofilm. After twenty-five days the biofilms are formed.

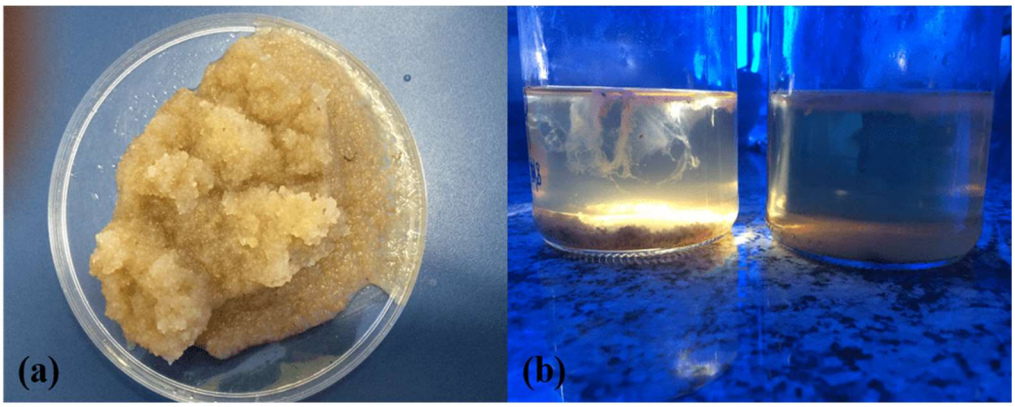

Figure 1. Kefir grains used (a) and biofilms information (b).

Figure 2. Kefir Biofilm. The obtained biofilms have approximately 10 cm of diameter

Morphology analysis

All analyses of the topography images were performed in the Materials Science laboratory of the Federal University of Amapá from July to August 2018. Under ambient conditions (51% relative humidity), the images were obtained in an Atomic Force Microscope (AFM) of the company Nanosurf, model Easyscan 2 controller in contact mode, with contal-G silicon cantilever, with the resonance frequency of 13 kHz and elastic constant of 0.2 N / m, same conditions established by Matos et al. (2018). For all biofilms, 20 images of 30x30 micrometers of topography and deflection in the AFM were obtained.

Statistical analysis of the surface of biofilms

Topographic images were processed in Image Processing and Analysis in Java software (ImageJ, Wayne Rasband, National Institutes of Health, USA) as described by Abràmoff et al. (2004), where the measured area of each bacterium or yeast was calculated together with a count of the number of particles that each biofilm had on its surface. After, the average number of particles was also calculated.

Thus, 20 bacteria and 20 yeasts were randomly selected for the statistical analyzes concerning the estimated area of the bacteria, since it is necessary to verify whether a statistical model can find more reliable values for the calculation of the area of the microorganisms than with the software.

In this aspect, the calculation of the calculated area and the estimated area was based on the mean length dimensions of a *Lactobacillus*, which is approximately 900 nm to 3 μm (Holt et al., 1994; Holt et al., 1993), so that if one could arrive at a statistical model that best represents the way of calculating the area of bacteria in biofilms.

In the same way, it was done with yeasts (yeasts grow disorderly because they depend on numerous factors, and there is not standard of length and breadth of these microorganisms) to conclude the comparison between the area measured by ImageJ and the area estimated by the measurements of the lengths and widths of microorganisms. Considering that the form of the bacteria (Lactobacilli) and yeasts found by Matos et al. (2018) are approximately cylindrical and spherical, respectively, it was defined that the length will be attributed to the greater distance from one end to another of yeast or bacteria and the width the shortest distance.

A model that could be describing the most adequate equation to estimate the area of bacteria or yeast was determined by linear regression ($Y = a + bx$) involving its width and length measurements. The value Y estimated the area of the bacterial or yeast limbus as a function of X, whose values can be the length (C), the width (L) or the product (C x L). 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 line, thus, all the equations used were linear.

The analyzed parameters, such as the correlation coefficients μ, of the adjusted values R^2 and *p* were obtained with the variables X and Y, where the estimated value of the bacterial or yeast area was considered as the dependent variable (Y) and the length, the width and the product length x width as independent variables.

Subsequently, the mean particle number <N> and the mean particle density <N> / A, where A is the total area evaluated, were found to study the degree of concentration of bacteria in the analyzed area.

Soon after this, the percentages of coverage of the biofilm surface by bacteria or yeasts were determined, using the equation:

$$Perc = (100\ x\ covered\ area)/900 \qquad \text{Eq. (1)}$$

Where: Perc. represents the percentage of the covered area, the measured area represents an area measured by the ImageJ and 900 software represents an evaluated area, considering that the images it had 30 x 30 micrometers.

Analysis of variance (ANOVA) was performed to compare the different treatments (coverage of the bacteria and yeasts area). For all the statistical analyzes the application R was used (Core Team, 2017).

RESULTS AND DISCUSSION

Morphological Analysis

The morphological analyzes showed that the biofilms obtained are very similar to those obtained by Matos *et al.* (2018). As in this work reported in the literature on films without kefir, it was not possible to visualize microorganisms with a consistent amount as in films containing kefir. Some microorganisms can be visualized due to contamination. Considering that the shape of the bacteria presents in kefir grains, as many researches point out, being cylindrical and that yeasts have approximately spherical shapes, the images leave much evidence that at low concentrations of kefir and Cupuaçu biofilms contain more bacteria than yeasts, which is not seen in the case at high concentrations, as can be seen in Fig. 3. It is important to note that they visually spread on the surface of approximately homogeneously.

Figures 3a, 3c, 3e, 3g, 3i, and 3l show the deflection images obtained in AFM for biofilms containing only kefir. They are images with areas of 30x30 micrometers and show structural topographical details of biofilms. These images, especially at low concentrations, show that there are structures identical to microorganisms of the genus Lactobacillus, the same described by Ray (2011).

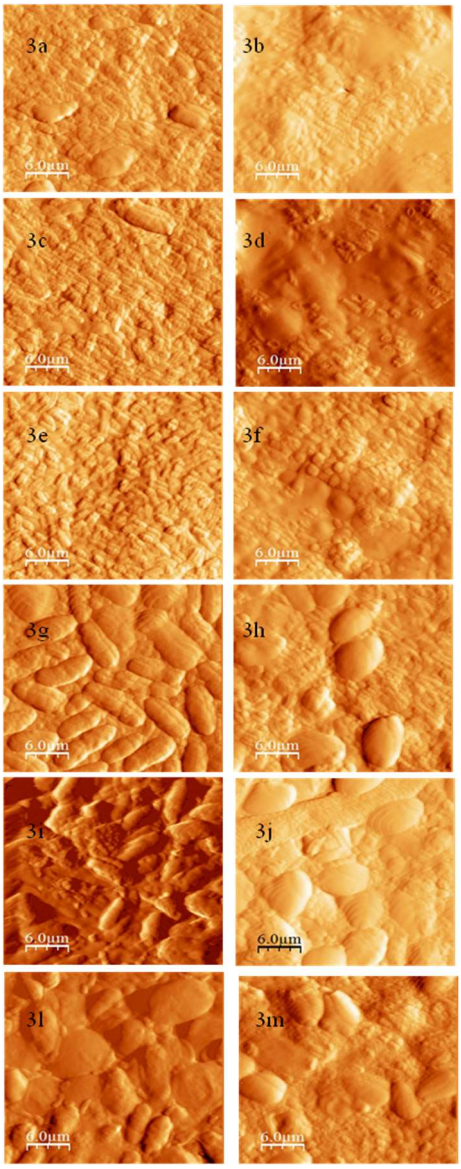

Figure 3. Topographic deflection images of Kefir and Kefir biofilms associated with Cupuaçu extract, with (3a) - 10 g.L^{-1} Kefir, (3b) -10 g.L^{-1} Cupuaçu, (3c) - 20 g.L^{-1} Kefir, (3d) - 20 g.L^{-1} Cupuaçu, (3e) - 40 g.L^{-1} Kefir, (3g) - 60 g.L^{-1} Kefir, (3g) - 60 g.L^{-1} Kefir, (3h) - 60 g.L^{-1} Cupuaçu, (3i) - 80 g.L^{-1} Kefir, (3j) - 80 g.L^{-1} Cupuaçu, (3l) - g.L^{-1} Kefir, (3m) -100 g.L^{-1} Cupuaçu, respectively.

Lactobacilli appear to develop better when there is a lower concentration of kefir, which can be explained by the fact that because there are few microorganisms and a reasonable amount of sugar, a greater predominance of these individuals is not absurd. Besides, other structures are identical to yeasts in the same way as those identified by Gallone et al. (2016).

In the other images are the topography of biofilms of kefir associated with the Cupuaçu extract, with the same proportions as the previous ones. The same consequence occurred with the case of lactobacilli for biofilms containing only kefir is observed for the other biofilms.

Statistical Analysis

The mean length of a lactobacillus, which can vary from 900 nm to 3 μm (Holt *et al*., 1994), was considered to obtain the lengths and widths of the bacteria, which can be seen in Table 1.

Table 1. Mean values and standard deviation of Length (L), Width (W), Calculated Area (CA), and Minimum-Maximum of the Calculated Area of the biofilms, in which 20 lengths and_20 widths were taken for each microorganism, randomly chosen, in the samples.

Type	C (μm)	L (μm)	CA-CxL (μm^2)	Min-Max (μm^2)
#1	7.072 ± 1.185	2.917 ± 0,621	20.989 ± 5,934	5.178-30.12
#2	2.174 ± 0.402	0.905 ± 0,153	2.017 ± 0.672	0.858-3.695
#3	7.099 ± 1.102	3.044 ± 0,548	21.669 ± 5,146	9.395-35.46
#4	2.183 ± 0.408	0.884 ± 0,116	1.954 ± 0.573	1.276-3.498

#1 Refers to yeast in kefir biofilms, #2 Refers to bacteria in kefir biofilms, #3 Refers to yeast in kefir biofilms prepared with Cupuaçu juice, and #4 Refers to bacteria in kefir biofilms prepared with Cupuaçu juice

These results show that the means are very nearby to the two types of biofilms analyzed. A t-Test was applied to compare these means and it was proved that for both bacteria ($p = 0.303$) and yeasts ($p = 0.318$) there was not a significant difference between the means of the calculated areas for a confidence interval of 0.05. This implies that microorganisms have a similar pattern of development even though they are produced in different solutions.

Tables 2 and 3 show the adjusted regression equations that were determined to try to verify if there is a statistical model that finds a more reliable area value than the Calculated Area or the Measured Area, which is here called the Estimated Area.

Table 2. Equations adjusted for each concentration of kefir biofilms evaluated with the respective determination coefficient (**R²**), correlation coefficient **μ** and ***p***-value.

Concentration	Adjusted equation	R^2	μ	p
	Kefir Biofilms With Cupuaçu			
10 g.L^{-1} (Bacteria)	1.8718+[-0.0008*(C*L)]	-0.0555	0.0050	0.9812
10 g.L^{-1}(Yeasts)	10.0733+[-0.0610*(C*L)]	-0.0257	0.1605	0.5056
20 g.L^{-1} (Bacteria)	1.8326+[0.0203*(C*L)]	-0.0484	0.0821	0.7301
20 g.L^{-1} (Yeasts)	9.5273+[0.0034*(C*L)]	-0.0555	0.0094	0.9675
40 g.L^{-1} (Bacteria)	1.7659+[0.0442*(C*L)]	-0.0193	0.1854	0.5607
40 g.L^{-1} (Yeasts)	6.8844+[0.0565*(C*L)]	-0.0466	0.0919	0.7013
60 g.L^{-1} (Bacteria)	2.0793+[0.0316*(C*L)]	-0.0151	0.1958	0.5873
60 g.L^{-1} (Yeasts)	11.7885+[0.0026*(C*L)]	-0.0553	0.0165	0.9433
80 g.L^{-1} (Bacteria)	2.2619+[-0.0242*(C*L)]	-0.0400	0.1212	0.6160
80 g.L^{-1} (Yeasts)	11.5958+[-0.0283*(C*L)]	0.0340	0.1844	0.5582
100 g.L^{-1} (Bacteria)	2.1929+[0.0526*(C*L)]	0.0309	0.2862	0.219
100 g.L^{-1} (Yeasts)	13.4443+[-0.0410*(C*L)]	0.0583	0.3285	0.1544

Table 3. Equations adjusted for each concentration of kefir biofilms with Cupuaçu evaluated with the respective determination coefficient (**R²**), correlation coefficient **μ** and ***p***-value.

Concentration	Adjusted equation Kefir Biofilms Without Cupuaçu	R^2	μ	p
10 g.L^{-1} (Bacteria)	2.1786+[-0.0142*(C*L)]	-0,0511	0.0650	0.7814
10 g.L^{-1}(Yeasts)	8.6409+[0.0132*(C*L)]	-0.0142	0.1980	0.5929
20 g.L^{-1} (Bacteria)	2.3734+[-0.0984*(C*L)]	0.0504	0.3168	0.1707
20 g.L^{-1} (Yeasts)	11.1911+[-0.0151*(C*L)]	-0.0305	0.1542	0.5227
40 g.L^{-1} (Bacteria)	2.1876+[0.0304*(C*L)]	-0.0415	0.1152	0.6335
40 g.L^{-1} (Yeasts)	7.9958+[0.0307*(C*L)]	0.0257	0.2774	0.2348
60 g.L^{-1} (Bacteria)	2.0761+[0.0522*(C*L)]	-0.0052	0.2183	0.6425
60 g.L^{-1} (Yeasts)	10.1717+[-0.0216*(C*L)]	0.0266	0.2790	0.2319
80 g.L^{-1} (Bacteria)	2.2509+[-0.0075*(C*L)]	-0.0548	0.0270	0.9062
80 g.L^{-1} (Yeasts)	11.1176+[-0.0246*(C*L)]	0.0126	0.2541	0.2794
100 g.L^{-1} (Bacteria)	2.2566+[-0.1191*(C*L)]	0.0675	0.3415	0.1374
100 g.L^{-1} (Yeasts)	9.7020+[0.0118*(C*L)]	-0.0476	0.0868	0.7164

These results show that it is not possible to determine a statistical model that improves the measurement of the area so that the only two possible options for this case are the Measured Area and the Calculated Area. A t-Test for two paired samples was applied to verify the difference between the means and showed that there is a large significant difference with p always much less than 0.001 in all samples. This explains the lack of correlation between the samples presented and that may be related to the accuracy of the method.

In particular, Moraes *et al*. (2013), adjusted mathematical models by simple linear regression and estimated the leaf area of different plant species, but the leaves of vegetables, in that case, follow a pattern of growth, since yeasts and bacteria, because they are living microorganisms do not. It is important to mention that the formation of membranes in vivo does not follow a specified standard (Israechivilli, 1992), which is the most likely event to occur with the formation of biofilms.

Table 4 shows the mean number and density of particles found on the surfaces of biofilms. The data show that there are more bacteria than yeasts in biofilms, for all concentrations evaluated. This difference can be associated with the development environment of the microorganisms and the biological conditions of each. Table 5 shows similar behavior for Cupuaçu containing biofilms.

Table 4. Average number and density of particles, for bacteria and yeasts found on the surface of kefir biofilms.

Mean number <N> and Particle density <N>.A^{-1} without Cupuaçu				
Conc.	Bacteria <N>	Yeasts <N>	Bacteria <N>. A^{-1}	Yeasts <N>.A^{-1}
10 g.L^{-1}	143.5	43.85	0.159	0.049
20 g.L^{-1}	88.95	44.7	0.099	0.050
40 g.L^{-1}	129.25	48.05	0.144	0.053
60 g.L^{-1}	158.55	44.35	0.176	0.049
80 g.L^{-1}	113.35	43.95	0.126	0.049
100 g.L^{-1}	150.4	36.35	0.167	0.040

Table 5. Average number and density of particles, for bacteria and yeasts found on the surface of kefir biofilms with Cupuaçu.

Mean number <N> and Particle density <N>.A^{-1} with Cupuaçu				
Conc.	Bacteria <N>	Yeasts <N>	Bacteria <N>. A^{-1}	Yeasts <N>.A^{-1}
10 g.L^{-1}	438.65	5.35	0.487	0.006
20 g.L^{-1}	431.4	7.15	0.479	0.008
40 g.L^{-1}	452.7	2.6	0.503	0.003
60 g.L^{-1}	137.65	29.15	0.153	0.032
80 g.L^{-1}	150.55	30.4	0.167	0.034
100 g.L^{-1}	69.85	35.75	0.078	0.040

On the other hand, the difference between the number of bacteria found in biofilms containing only kefir and the one containing Cupuaçu is evident, which is evidence that Cupuaçu potentiates the growth of more bacteria type microorganisms on the surface of biofilms. This can be due to the acidic character of Cupuaçu (Nascimento et al., 2019) which can accentuate the fermentative process and to improve the proliferation of microorganisms (Hernández et al., 2019). This can also be explained by yeast flocculation, as occurs when there are pathogenic microorganisms (Neto et al., 2012). Besides, yeasts have a higher tolerance to lower pH than bacteria (Melo, 2006), which may also explain the greater presence of yeasts for concentrations of 60 to 100 g.L^{-1} of Cupuaçu juice. Now, the increase in kefir concentration seems to increase the competition of bacteria for energy, since the concentration of sugar does not change, hindering the growth of bacteria and favors the growth of yeasts.

Another interesting fact to note is that the 40 g.L^{-1} Cupuaçu concentration has a higher density of particles (surface particle concentration) on its surface, which corroborates the fact discussed by (Matos *et al.* 2018) that in the range of 10-40 g.L^{-1} biofilms have a higher concentration of bacteria.

The mean surface coverage percentages of bacteria and yeasts in both cases are shown in Tables 6 and 7, respectively, where the outliers may be other microorganisms or microorganisms that have grown above the stipulated mean.

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

Average Percentage of Occupied Area			
Conc.	Bacteria (%)	Yeasts (%)	Outliers (%)
10 g.L^{-1}	30.67	45.03	24.30
20 g.L^{-1}	19.49	51.72	28.79
40 g.L^{-1}	28.33	49.91	21.76
60 g.L^{-1}	32.27	45.67	22.06
80 g.L^{-1}	24.53	49.26	26.21
100 g.L^{-1}	30.31	41.00	28.69

Table 7. Mean surface coverage of bacteria and yeasts in the analyzed samples, considering the concentrations of Cupuaçu used.

Average Percentage of Occupied Area			
Conc.	Bacteria (%)	Yeasts (%)	Outliers (%)
10 g.L^{-1}	57.41	5.65	36.94
20 g.L^{-1}	51.35	7.76	40.89
40 g.L^{-1}	54.92	2.66	42.42
60 g.L^{-1}	24.49	38.47	37.04
80 g.L^{-1}	27.17	36.89	35.94
100 g.L^{-1}	14.37	49.91	35.72

These data show that the biofilm with the highest concentration of bacteria occurred at the concentration of 10 $g.L^{-1}$ of Cupuaçu, again reinforcing the qualitative analysis of Matos et al. (2018), where it is possible to identify that of 10-40 $g.L^{-1}$ of Cupuaçu bacteria coverage is in the range of 50%, which is not seen from 60 to 100. Biofilms containing only kefir show that the percentage of surface coating did not change drastically with the concentration. However, when Cupuaçu is added, this coverage increases, which indicates that Cupuaçu further improves the therapeutic capacity of the kefir biofilm.

The study of the averages, by Anova, showed that for the case of the biofilms of kefir with Cupuaçu, for both bacteria (*F = 41.2 and p-value < 0.001*) and for yeasts (*F = 14.34 and p-value < 0.001*) there was a significant difference between the analyzed concentrations. This shows that concentration influences the distribution of microorganisms in the samples. For biofilms without Cupuaçu for both bacteria (*F = 4.222 and p-value > 0.001*) and yeasts (*F = 4.15 and p-value > 0.001*), there was no significant difference between the analyzed concentrations, showing that in this case, the concentration of kefir does not influence the distribution of microorganisms in the biofilm formation.

CONCLUSION

In this research was carried out a study of the morphology and the superficial distribution of bacteria and yeasts in Kefir biofilms prepared with Cupuaçu juice. Morphologically, the particles found on the surfaces of the two types of analyzed biofilms presented structures similar to those of bacteria of the genus Lactobacillus and yeasts randomly distributed over the sample. The images obtained were similar to the results found in the literature. The application of linear regression showed that it was not possible to adjust mathematical models for the calculation of the area of bacteria and yeasts in the studied biofilms. The results showed that biofilms with Cupuaçu concentrations between 10 and 40 $g.L^{-1}$ present a greater number and coverage of bacteria, with a concentration of 10 $g.L^{-1}$ considered more optimal. In fact, the higher the concentration of bacteria and the lower the concentration of yeasts, the greater the therapeutic activity of the biofilm must be, since bacteria are responsible for such activities, as mentioned in the literature cited here.

The Anova showed that the concentration influences the formation and distribution of microorganisms on the surface of the kefir biofilms associated with Cupuaçu extract, which is not the case of biofilms with only kefir. Therefore, concerning the statistical approach, biofilms whose Cupuaçu concentrations are between 10 and 40 $g.L^{-1}$ have greater therapeutic potential, since in this concentration range biofilms exhibited a higher density of lactobacillus bacteria. However, future research may help to confirm this potential and the applicability of biofilm in the pharmaceutical industry as bio-curative, since the results presented here only show evidence of a probable application.

ACKNOWLEDGMENTS

To the Federal University of Amapá-UNIFAP, for the granting of scholarship through the Program of Scholarships of Scientific Initiation-PROBIC for the student to carry out the present study and to the Department of Research of the Federal University of Amapá, for the management of the scholarship.

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