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Study of kefir biofilm associated with hydroethanolic extract of *Euterpe oleracea* Mart. (açai)

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Kefir is a microbial complex capable of producing biofilms. This study aims to obtain biofilms associated with hydroethanolic extract of Euterpe oleracea Mart. (BEHEEo) as fixing method of recovery anthocyanins in kefir biofilm. Thus, BEHEEo formation kinetics was made at different concentrations of aqueous extract of E. oleracea, of kefir grains (KG) and of brown sugar (BS). Thereafter, macro and microelements of BEHEEo were determined; the rheological and structural characteristics were analyzed using atomic force microscopy (AFM), and anthocyanins dosage in BEHEEo was obtained. The best concentration of BS to form BEHEEo was 40 g/l with 100 ml of EHEEo (12.62±0.16 g), as well as a release of the BEHEEo anthocyanins occurring after 5 min (4.3±0.6 mg/100 g) with maximum peak at 60 min (20.8±0.3). The concentration of calcium iron and magnesium was 0.089, 0.1740 and 0.1808 mg/kg, respectively, indicating the concentration of Zn2+ which was 0.533 mg/kg. The AFM analysis revealed differences in the peaks of roughness depending on the concentration of kefir grains, with presence of Lactobacillus and yeast. The concentration of anthocyanins in BEHEEo two years after the incorporation was 26 mg/100 g. Therefore, it is suggested that the BEHEEo incorporating EHEEo anthocyanins has potential for therapeutic applications in several pathologies necessary for antioxidative processes.

Key words: Biofilms, kefir, açaí, *Euterpe oleracea* Mart., anthocyanins, atomic force microscopy (AFM).

INTRODUCTION

Kefir is a beverage produced by fermenting substrates such as milk or sugar water by kefir grains (Rodrigues et al., 2005; Laureys and De Vuyst, 2014). The growth and the function of bacteria inside a population are crucial for their survival (Davey and O'toole, 2000). In kefir, there is a predominance of *Lactobacillus* which has high catabolic

capacity and is complemented by yeast with high biosynthetic capacity.

When growing kefir in water for more than twenty days, there is the formation of biofilms with therapeutic properties against pathogenic organisms. These biofilms are surfaces with a high rate of structural organization associated with bacterial colonies held together by a polymer secreted by their cells, which is called extracellular polymeric substance (EPS) (Watnick and Kolter, 2000, Donlan, 2002; Zhang et al., 2015). Among many strategies used for the study of biofilms, the one that is more highlighted is the use of the Atomic Force Microscopy (AFM), which allows measurement of the interaction forces between cells or cell-cell interactions (Dufrêne, 2015).

One of the substances that have been intensified in recent years are the anthocyanins, due to their protective and healing properties. Anthocyanins have an important role in physiological functions related to human health (Lee et al., 2013). The antioxidant effect of anthocyanins have been known and proven fact. The action mechanism of the anthocyanin is inhibiting the free radicals that promote oxidation. The antioxidant agents capable of binding to the free radicals are found in the medium.

The açaí (*Euterpe oleracea* Mart.) plant species is found in wetlands along rivers and streams, in the lowlands and wetlands, along the full extent of the Amazon (Malcher, 2011). The high anthocyanin content with its consequent antioxidant power has awakened growing interest in the study of the fruits of this plant. This fact is related to the antimutagenic effect (Galotta and Boaventura, 2005). The fruits of palm tree have high value as antioxidants, and are therefore considered a great source of antioxidants (Santos et al., 2008). This study aims to obtain kefir biofilms associated with aqueous extract of *E. oleracea* (EHEEo) as means of fixation and future use of anthocyanins.

MATERIALS AND METHODS

Study area

Kefir grains (2 kg) were obtained in the Laboratory of Drugs Research at the Federal University of Amapá, Amapá, Brazil. Five kilogram of the ripe fruit of açaí (*E. oleracea*) was collected in August, 2015 in a fragment of Amazon forest (16° 30′ to 16° 44′ S and 56° 20′ to 56°30′ W) in Mazagão city, State of Amapá, Brazil.

Sample collection and preparation

A total of 500g of the ripe fruit of açaí (*E. oleracea*) was weighed and placed in maceration at ambient temperature (23°C) in ethanol 98% acidified with 2% of formic acid for 3 days. After this procedure, the extraction solution was filtered and the mass discarded. The EHEEo was concentrated on rotaevaporator (Hikal, India), and the determination of anthocyanins was performed based on the AOAC method (2005-02).

To obtain the biofilm associated with hydroethanolic extract of *E. oleracea,* bottles with 4 L capacity were used and then placed in a laminar flow cabinet under ultraviolet UV radiation for two hours. A brown sugar (BS) solution (40 g/l) with KG (0.25, 0.5, 1, 2, 5, 10, 20, 40, 60 and 80 g/l) were added to 100 ml of EHEEo and conditioned at 25°C for 20 days. After this time, it was removed and weighed. The volume and pH of the remaining solution were measured and or the best concentration of KG was considered for the formation BEHEEo in terms of biomass consistency, in other words, the one that could be separated without loss of integrity.

KG (40 g/l) were inoculated at different concentrations of BS solution (0.25, 0.5, 1, 2, 5, 10, 20, 40, 60 and 80 g/l) in which was poured EHEEo (100 ml) and the final volume was 500 ml. The experiment was performed in triplicate. After 20 days pH, volume and weight of BEHEEo were measured; treatment being considered the best is the one that presented the highest BEHEEo biomass consistently.

The amounts of 10, 25, 50 and 100 g of EHEEo were inoculated completing the volume of 500 ml with BS solution (40 g/l) with kefir grains 20 g/l. After 20 days, the grains and BEHEEo formed were weighed separately. The pH, temperature, volume were measured and the dosage of anthocyanins in the BEHEEo and in the culture medium was performed based on the AOAC method (2005-02). The best concentration was the one that presented the highest concentration of anthocyanins.

The quantification of anthocyanins was carried out using the AOAC method (2005-02). KG was inoculated (20 g/l) in 400 ml of BS solution (40 g/l) in which was poured in 100 ml of EHEEo. After 20 days, the BEHEEo were removed and weighed on an analytical balance and further dehydrated in oven at 110°C. After that, 1 g BEHEEo was placed in 100 ml of distilled water and left to stand for 24 h protected from light at room temperature (25°C). 10 ml of this dilution was then removed to assay anthocyanin. 10 ml of the culture medium for dosage and 1 g of the EHEEo was also removed. Both samples were filtered through paper Whatmam No. 3.

The samples of EHEEo (1 g) of the culture medium with kefir and of the EHEEo (10 ml) were diluted in buffer (KCl 0.03 M, pH 1), and in sodium acetate buffer (0.4 M, pH 4.5), respectively and the volume was completed to 50 mL. Then, the readings were performed on Shimadzu UVMini 1240 (Shimadzu Corporation, Kyoto, Japan) at a wavelength of 520 and 700 nm for anthocyanin, respectively. For calculating the anthocyanin concentration, the following formula was employed:

$$TAC = A/e \times l \times MW \times DF \times M/W \times 100\%$$

Where, TAC = total anthocyanin content in %; $A = (A_{520\ nm} - A_{700\ nm})_{pH1.0} - (A_{520\ nm} - A_{700\ nm})_{pH4.5}$; MW (molecular weight) = 449.2 mol^{-1}; DF = dilution factor; W = sample weight in mg; l = optical path in cm; ε = 26.900 M extinction coefficient in L $mol^{-1}cm^{-1}$ to cyd-3-glu
The anthocyanin of the açaí fruits was expressed as cyanidin-3-glucose (Inácio et al., 2013) and this method is based on the fact that the monomeric anthocyanins undergo reversible structural change in function of the pH.

In this assay the equipment Dissolution Model 299-6 was used (New Ethics, Ltda. Sao Paulo, Brazil) with rotation speed of 75 rpm. In each vessel of the equipment was placed 1 g of BEHEEo and

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100 mL of distilled water pH 7.0, which was used as a dissolutor means. The temperature was maintained at 37±1°C. The samples were collected at 5, 10, 30 and 60 min respectively. After the end of the process, the samples were filtered through Whatman paper n°1, and then performed the determination of anthocyanins using the AOAC method (2005-02).

A total of 100 g of the BEHEEo samples were used for the determination of macro and microelements using the calcination method described by Salazar et al. (2011). The analysis was made in Laboratório of Absorção Atômica e Bioprospecção – UNIFAP by atomic absorption spectrophotometry model 6300 Shimadzu AAS.

BEHEEo structural analysis by atomic force microscopy (AFM)

The surface morphology of BEHEE was analyzed by atomic force microscopy (AFM Easyscan2/STM, Nanosurf; USA). The relative humidity during the measurements was maintained at 51%. The scan was of 30 mm × 30 mm, and the images were obtained in contact mode. The cantilever has rectangular shape with spring constant of 0.77 nN/m and constant strength of 0.2 N/m, resonance frequency of 13 KHz. For the analysis of BEHEEo, they were dehydrated and electrodeposited on slides with gold films after 03/10 scanning cycles and analyzed by cyclic voltammetry on its device.

Twenty images of different regions were examined and analyzed, and the roughness values (RMS) were calculated. The parameter analyzed was the average roughness (Rm) and its effective value (RMS), defined by the equations described by Khulbe et al. (2008);

$$R_m = \int Z(x,y)dxdy$$

Where R_m is the average roughness and Z (x, y) is the vertical profile of the area

$$RMS = \sqrt{\iint |Z(x,y)|^2 dx\, dy}$$

RMS expresses the average of the roughness square root.

Analysis of kefir biofilms through scanning electron microscopy (SEM)

The analysis of the microstructure and surface morphology of the biofilms were investigated by scanning electron microscopy (TM3030Plus, Hitachi, Japan). The accelerating voltage of 15 kV was used. The magnification used was 2.5 K.

Stability of the anthocyanin in the biofilms

It was sought to verify if the anthocyanins present in the biofilms remained stable over time. Therefore the anthocyanins incorporated in biofilms were quantified. These biofilms were produced 12 and 24 months ago using the same analytical methodology previously described. So, to evaluate if the anthocyanins present in biofilm were stable throughout the time, the anthocyanin content at 12 and 24 months after the biofilms' preparation was evaluated. The same analytical methodology was used as mentioned before. Three different biofilms (BHE1, BHE2 and BHE3) made in the same conditions as described before, were used. The biofilms were held at 25ºC, in amber flask with liner of PVC and tight closed for 24 months. The analysis was made in triplicate.

Statistical analysis

For statistical analysis of the results the Statgraphics Centurion XVI software (STATPOINT Technologies, 2012) was used. ANOVA was used followed by Tukey's test, and the average weights of BEHEEo were analyzed using the Student t test, and results with $p < 0.05$ were considered significant.

RESULTS

Obtainment of BEHEEo

It was observed the formation of BEHEEo biomass was dependent on the increase of the concentration of the KG (Figure 1). It was observed that the change in pH occurred, which was 4.42 at the beginning, and then declined in accordance with the concentration of KG (Figure 1). ANOVA showed the difference among the treatments, and according Tukey test, above 10 g/l of the KG the biomas of biofilm (13.10±0.30 g) represented no significant increases. At 40 g/l of the KG (14.52±0.45 g) BEHEEo was obtained biofilm with good integrity, however at lower concentrations it was broken up easily.

An important parameter for obtaining BEHEEo is the consistency. The consistency here is simply defined as the formation of cohesive BEHEEo in their structures. BEHEEo without consistency break up easily and disperse in the medium. The consistency was obtained only when using concentrations equal to or greater than 40 g/l of the KG (14.52 ± 0.45 g of the biomas).

Determination of the concentration of substrate to form biofilms with EHEEo

The variation of the BS concentration in EHEEo resulted in different BEHEEo cohesiveness. Without BS and only EHEEo, the BEHEEo formed showed no cohesiveness. With the BS and the BEHEEo from 20 g/l of the BS (12.44 ± 0.332 g the biomas) BEHEEo was presented with cohesiveness and the anthocyanins were incorporated to them (Figure 1). ANOVA showed a significant difference between treatments with different concentrations of BS up to 10 g/L. Through the Tukey test it was shown that only above 10 g/L (12.68 ± 0.3272 g) there were no significant differences in the treatments. However, to obtain BEHEEo cohesiveness is necessary concentration of 40 g/l (12.62 ± 0.1626 g) (Figure 1). The formation of BEHEEo was stabilized around 10 g/l of BS and the pH fell from 4.61 to 2.72 over 24 days (Figure 1).

Determination of the concentration of EHEEo to the biofilm formation

When only the EHEEo used without BS, fragmented biofilms with no cohesiveness and with the medium pH around 6.5 was obtained. With the addition of the BS to

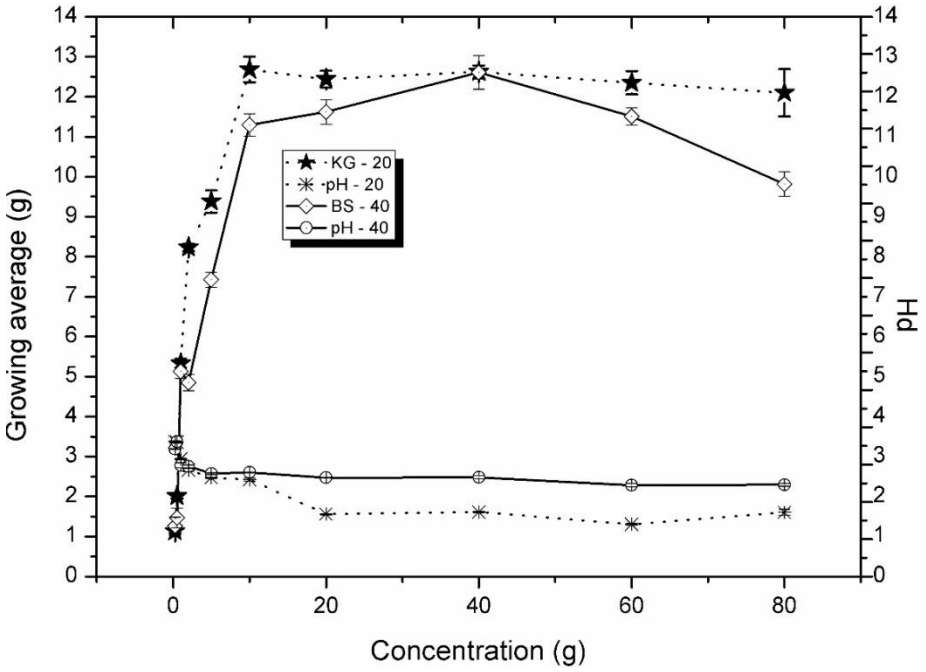

Figure 1. Growing average of the biomas of biofilms: ★ Formation of the biofilms associated with hydroethanolic extract of *E. oleracea* Mart. (BEHEEo) in different concentrations of kefir grains (KG) in brown sugar solution (BS) (40 g/l). ◇ Formation of the biofilms associated with hydroethanolic extract of *E. oleracea* Mart (BEHEEo) at different concentrations of brown sugar (BS) with kefir grains (KG) (20 g/l). * Altering of pH at variation of concentrations of kefir grains (KG) in brown sugar solution (BS) (40 g/l). ○ Altering of pH at variation of at different concentrations of brown sugar (BS) with kefir grains (KG) (20 g/l).

the culture medium in EHEEo, biofilms showed cohesiveness.

Before starting the experiment, the pH of the EHEEo was 4.6 and with the EHEEo concentration increasing, the biofilms showed higher concentration of anthocyanins.

Biomass which presented undifferentiated growth and ANOVA showed no differences between treatments ($p>0.05$). There were, however, clear increase in incorporation of anthocyanin in BEHEEo, with an increasing of EHEEo concentration in the medium. This increase was detected in both BEHEEo as in the medium.

The analysis of BEHEEo through AFM confirmed the predominance of *Lactobacillus* in lower concentrations of EHEEo (Figure 2a and c) and in higher concentrations yeast were predominant (Figure 2e). These results are in agreement with Stadie et al. (2013) which showed Lactobacillus preparing the condition for yeasts.

Profile of liberation and dissolution of anthocyanins from BEHEEo

The dissolution profile in aqueous medium showed that the release of anthocyanins from BEHEEo occurred after 5 min reaching the release peak at 60 min (Table 1).

Extraction and dosage of anthocyanin of the BEHEEo and medium of culture

Table 2 shows anthocyanin concentration in biofilm associated with hydroethanolic extract of *E. oleracea* (BEHEEo) formed with kefir grains (20 g/l) in brown sugar solution (BS) (40 g/l) with different concentrations of aqueous extract of *E. oleracea*. (EHEEo). The pH climbed from 2.23±0.02 to 3.89±002 (Table 2) in proportion to the increasing of the concentration of EHEEo. The culture medium of two months still showed anthocyanins (19±0.5 mg/100 g).

Content of macro and microelements of BEHEEo

The presence of microelements in the biofilm was evaluated. This is important because, first the calcium concentration help to maintain the consistence of the biofilm network, and secondly because these elements could contribute to the antioxidant activity of the anthocyanin (Grumbein et al., 2014). The identified elements were: Zinc 0.53 mg/kg, iron 0.089 mg/kg, magnesium 0.183 mg/kg and calcium 0.140 mg/kg. The results correlated with the results of previous researches. According to one of the studies, it has been reported that

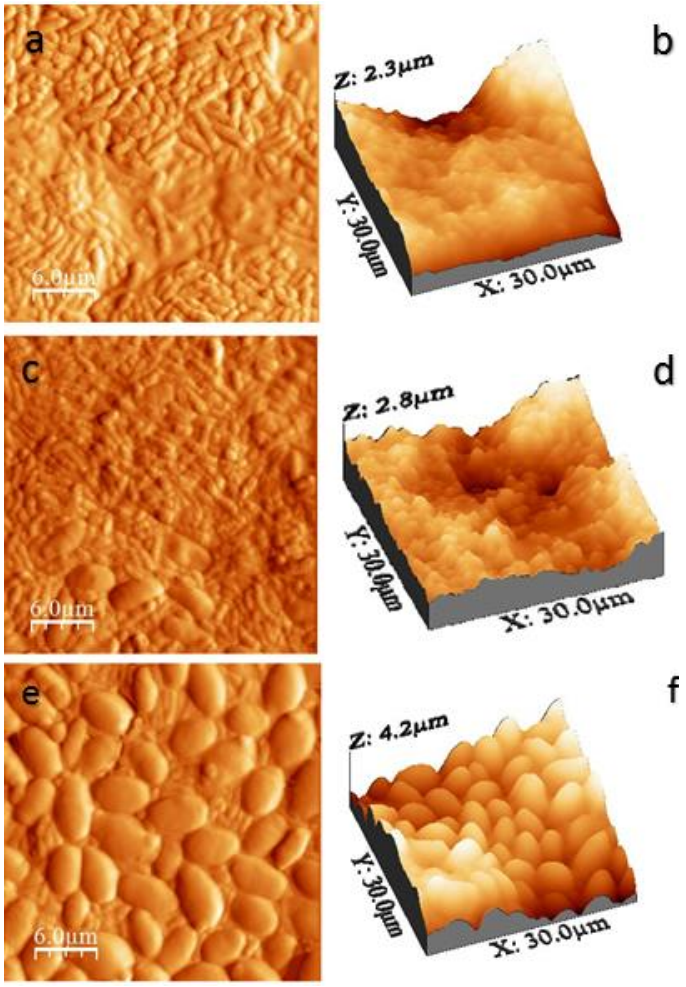

Figure 2. Graphic image by atomic force microscopy (AFM) with BEHEEo (a) (10 g/l), (c) BEHEEo (20 g/l) noteworthy is the predominant presence of *Lactobacillus* and (e) BEHEEo (60 g/l) where occur the predominant presence of yeast. Topographic image by Atomic Force Microscopy (AFM) with (b) BEHEEo (10 g/l), (d) BEHEEo (20 g/l) and (f) BEHEEo (60 g/l).

the occurrence of Zn, Fe and Ca have been observed in the biofilm matrix formed by *Lactobacillus* strains (Grumbein et al., 2014). The presence of these polyvalent elements can contribute to the rigidness of the biofilms once dried (Table 3).

BEHEEo structural analysis by atomic force microscopy

In the analysis of BEHEEo by AFM, 3D images with different concentrations of EHEEo was obtained (Figure 2b, d, e and f), which revealed different configurations of roughness (RMS), dependent of the concentration (Figures 2b, d, e and f). There is an increased in roughness in proportion to the increasing concentration of EHEEo in the culture medium. With lower concentrations (10 g/L), the predominant presence of bacillus was revealed (Figures 2a and c). They are presumed to be Lactobacillus because they constitute up to 70% of the flora present in kefir (Stadie et al., 2013).

Analysis of kefir biofilms through scanning electron microscopy (SEM)

Figure 3 show Scanning electron microscopy (SEM) microphotograph of the biofilm surface obtained when 20 g/L of the kefir grain and 40 g/L of brown sugar, loaded with anthocyanin was used.

Determination of temperature on the formation of BEHEEo

In relation to the BEHEEo formation, temperature is one of the most important parameters. With the culture medium cultivated at room temperature (25 ± 2°C), BEHEEo with a significant biomass (11.46±0.50 g) with relative cohesiveness was obtained whereas at a temperature of 15°C the formation of structures without consistency, breaking in the middle was observed. The optimum temperature was 30°C and there was no formation to 38°C.

Stability of the anthocyanin in the biofilms

In this work the content of anthocyanins of a vegetal extract was recovered and fixed in a complex matrix of a biofilm, obtained by using kefir grains (20 g/L) and brown sugar (40 g/L). In these conditions, the content of anthocyanins remained stable for two years (Table 4).

DISCUSSION

The initial adhesion of the cells is crucial in the biofilm formation period, and the characteristic of the contact surface has great influence at this stage (Pereira et al., 2015). Therefore, the biofilm formation depends on the variation of the substances concentration and on the flow conditions at the surface and on the culture medium turbulence (Perni et al., 2006).

The three most important factors to form the BEHEEo were: Time, temperature and substrate concentration (Figure 1). Biofilms may be formed in just 24 h (Alimova et al., 2006; Heydorn et al., 2000; Stoodley et al., 2002). The increase of the cells in the biofilm is part of its own maturation process, occurring up to 10 days after initial adhesion. This increase is as a result of cell division and co-adherence of other cells present in the system in their planktonic state. The BEHEEo formation occurred only after 20 days, although the formation of biofilm structures

Table 1. Profile of liberation and dissolution of anthocyanins from biofilms associated with hydroethanolic extract of *Euterpe oleracea* Mart. (BEHEEo).

Time (minutes)	Anthocyanins (mg/100 g)	Percentage
5	4.3±0.6	0.0040
15	3.8±0.8	0.0038
30	14.5±0.5	0.0145
60	20.8±0.3	0.0208

The numbers represent the average ± standard deviation of n = 3.

Table 2. Anthocyanin concentration in biofilm associated with hydroethanolic extract of *E. oleracea* Mart. (BEHEEo) formed with kefir grains (20 g/l) in brown sugar solution (BS) (40 g/L) with different concentrations of aqueous extract of *E. oleracea* Mart. (EHEEo).

Conc. of EHEEo (g/L)	Antocyanins (mg/100 g)	pH
10	0.00±0.00	2.23±0.02
25	4.93±0.40	2.32±0.01
50	13.10±0.26	3.13±0.01
100	18.06±0.21	3.89±0.02

The numbers represent the average ± SD of n = 3.

Table 3. Concentration of macro and microelements of aqueous extract of *E. oleracea* Mart. (EHEEo) and biofilm associated with hydroethanolic extract of *E. oleracea* Mart (BEHEEo) in brown sugar solution (BS).

Macro and microelements	BS (mg/kg)	EHEEo (mg/kg)	BEHEEo (mg/kg)
Mg^{2+}	0.1856	0.1823	0.1808
Zn^{2+}	0.5740	0.2438	0.5330
Fe^{2+}	0.2496	0.2446	0.089
Cu^{2+}	0.0269	0.0034	0
Ca^{2+}	0.1836	0.1572	0.1384

can already be observed from the fifth day. These structures are aggregated by a binding substance secreted in the medium that form the biofilm.

The BEHEEo were formed at ambient temperature (around 25°C). At 15°C, the biofilms were not formed, only fragments which were dispersed in the medium were observed. Asadishad et al. (2014) reported that low temperatures (below 10°C) paralyze the production of biofilms by *Bacillus subtilis*. Biofilms formed by *Listeria* sp. are more resistant to temperature of 15°C than at 37°C (Shimamura et al., 2015). BEHEEo are not formed at 37°C. The optimum temperature for the formation was 30°C (27.53±0.92 g) while at room temperature the biomass formed was 11.46±0.50 g.

When using low substrate concentrations of BS only biofilms structures were formed. These structures did not merge, remaining dispersed in the culture medium. Biofilms were formed only when concentrations above 20 g/l were used, but were not yet cohesive and fragmented with the mildest agitation of the medium. At concentrations above 40 g/l, the biofilms produced showed cohesiveness. Interestingly at this concentration, an increase in biofilm roughness was identified by atomic force microscopy (AFM).

When free nutrient concentration is increases proportionally, there is an increase on the number of microorganisms of the biofilm. This is a very important factor for the adsorption of molecules present in the biofilm, which precede the bacteria adsorption (Rubio et al., 2006). When the substrate was insufficient or when it sought to use only the EHEEo as substrate and no BS, the biofilms that were formed showed lack of cohesiveness and so only biofilms fragments were formed, which were dispersed in the medium.

The substrate concentration increasing (BS) was accompanied by pH decay much more significant than the change in concentration of KG grains, which suggests the occurrence of the increase on the microorganism activity over the substrate, resulting in final products that make the medium acidified by the presence of lactic and

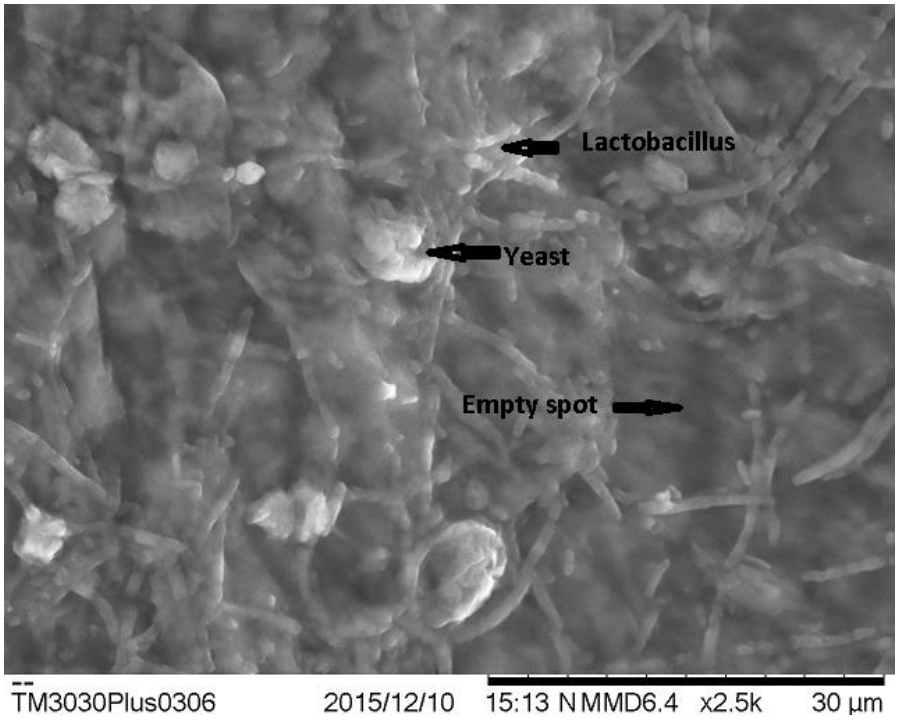

Figure 3. SEM microphotograph of the biofilm surface obtained when 20 g/L of the kefir grain and 40 g/L of brown sugar loaded with anthocyanin was used.

Table 4. Anthocyanin content evaluated along the time of 24 month.

Time (month)	BHE1	BHE2	BHE3
0	70.30 ± 2.52	71.36 ± 2.25	64.30 ± 1.25
12	71.50 ± 1.70	69.26 ± 1.70	62.50 ± 0.70
24	70.33 ± 1.57	71.33 ± 2.57	65.33 ± 0.57

ANOVA test F = 233.25; p = 0.0000 BHE, biofilm loaded with anthocyanin, n = 3. BHE1 batch one, batch 2 and so on.

acetic acid. The culture medium with kefir after 18 months remained with no unchanged pH (2.81) and free from contaminants. According to Abe et al. (2013) low pH constitutes a protection against contamination. Although pH below or above neutral directly affects the biofilm development by interfering with the motor proton force, which is the force used by the bacteria to generate its electrochemical gradient (Pereira, 2001) low pH did not interfere in the formation of the BEHEEo.

The microorganisms present in kefir are incorporated in the biofilm (Figures 2a, c and e). Thus, they ensure a contaminant-free environment. The pH drops until a protection level was reached against contaminants. Probiotics such as kefir are organisms that live in consortium repelling pathogenic organisms (Santos et al., 2003).

The anthocyanins present in the EHEEo were incorporated into BEHEEo which remained stable 24 months after formation. These anthocyanins suffered BEHEEo release in aqueous medium at pH 7.0 without losing its characteristics. This fact is important because the acai (*Euterpe oleracea* Mart.) anthocyanins inevitably suffer degradation from the harvest until reach the consumer. Rogez et al. (2012) concluded that the degradation kinetics of the anthocyanin results in a half-life of 50 h, occurring, concomitantly, with the infection by microorganisms such as yeast, mesophilic bacterias and fecal coliforms. The anthocyanins degradation can occur during the extraction process or during the storage of food and drugs (Rogez et al., 2012). A major challenge has been to find a matrix where anthocyanins can remain stabilized. These anthocyanins may then be used for incorporation into foods and pharmaceuticals. In this work the content of anthocyanins of a vegetal extract was recovered and fixed in a complex matrix of a biofilm, obtained by using kefir grains (20 g/L) and brown sugar (40 g/L). In these conditions, the content of anthocyanins remained stable for two years (Table 4). The ANOVA test

showed statistical significant differences among the mean of the biofilm mass, for the three batches used. The Tukey HSD test showed that was no statically significant differences between the time 0 and 24 month, for the three batch. In this work a simple technique is used to extract and stabilize the anthocyanins. In this sense this result suggest that this biofilm can be used for recovery and stabilizing anthocyanin for further utilization both in food, as a possible probiotic by the content of yeast and lactobacteria, and for pharmaceuticals as a proved antioxidant.

The release of the anthocyanin from the BEHEEo occurred from 5 min reaching a peak after 60 min. This time-dependent kinetics of release is an important factor for application of BEHEEo, since the gradual increase in anthocyanin release suggests that there may also be an efficiency increase over time.

In the culture medium of kefir, the anthocyanins were stable for at least two months. After this period, they started to be degraded along with the culture medium. Bobbio et al. (2000) described two types of EHEEo in anthocyanins, cyanidin-3-arabinoside and cyanidin-3-arabinoside-arabinosyl, and considered them as the two major anthocyanins of the *E. oleracea* fruits peel, with tenor of 50.00 ± 5 nmg/100 g in the peel of the fruit. They concluded that the fruit as a whole must have 263 mg/100 g of peel, which is only 19% of the fruit and the *E. oleracea* anthocyanins are concentrated only in the peel.

In this study, 110 mg/100 g of anthocyanin was found in the EHEEo. In the BEHEEo, 36 mg/100 g was found. It is noteworthy that the dosage was performed in BEHEEo with six months of acquisition. Açaí is a source of carbohydrates, therefore, the possibility that the formation of biofilms could occurred with the use exclusive of açai as the source of carbon was sopposed. In fact the formation of a biofilm occurred, but also an unpleasant smell of these biofilms fragmented easily. The culture medium without BS containing just the EHEEo as substrate presented biofilms with low cohesiveness, fragmented and with pH of 6.7, suggesting actions of different kinds of microorganisms

The release and dissolution profiles of BEHEEo synthesized after 12 months released 34.5±0.70 mg/100 g of cyanidin-3-glucose, while BEHEEo synthesized after 24 months released 33.87±0.57 mg/100 g of cyanidin-3-glucose. In relation to the presence of macro and microelements in EHEEo and BEHEEo, 0.553 mg/kg of zinc was found, and Rogez (2000) found 7 mg/100 g in extract and Menezes et al. (2008) found 2.82 mg/100 g in lyophilized açai extract.

The biofilms may use the absorption capacity of some metals in its ionic form, such as zinc, copper, iron and aluminum contained in its matrix to avoid erosion forces (Grumbein et al., 2014). These ionic metals are toxic for planktonic forms of the bacteria, components of the biofilm, but its toxicity is suppressed in the biofilm matrix (Grumbein et al., 2014).

The analysis by AFM can be used to evaluate the morphological progression of biofilms (Chatterjee et al., 2014; Pereira et al., 2015) as well as providing an understanding of the biophysical mechanisms of the impact of the organizational structure of the poly-saccharide capsule in biofilm formation (Wang et al., 2015).

The AFM makes mechanical nano measures providing data that show how the capsular organization influences in the adhesion of cells and consequently in the formation of biofilms. In this study, through AFM, the presence of *Lactobacillus* at high quantity in the biofilms was recorded (Figures 2a and c) especially at lower concentrations of EHEEo (10 g/L). At higher concentrations (60 g/l) the predominant presence of yeast was noted (Figure 2e). The *Lactobacillus* acidify the medium and thus provide optimum conditions for the metabolism of yeast (Stadie et al., 2013) that appeared in BEHEEo with increasing concentration of EHEEo to 60 g/L. In kefir, the yeasts provide the essential amino acids and vitamins, as folic acid to the lactobacilos and the *Lactobacillus* reduce the pH creating ideal conditions for the yeast, in a mutualistic relationship enlightened by Stadie et al. (2013).

It is known that the substrate influences biofilm formation, and also the extent of microbial colonization increases with increasing surface roughness (Characklis, 1984). An increase in the concentration of the substrate causes a biofilm growth with the consequent increase in roughness (Viana, 2009) and with the analysis by AFM; it is possible to detect these roughness changes on the surface of the biofilm (López-Jiménez et al., 2015). The variation of the concentration of EHEEo in the culture medium was accompanied by changes in the roughness of the BEHEEo (Figures 2b, d and f). The increase in the roughness is a very important factor for indicating increased retention capacity of the cells and incorporation of substances like anthocyanins in this study. The AFM analysis confirmed the increased roughness of the biofilm depend on the concentration of the amount of kefir grains. The presence of *Lactobacillus* and yeast in the biofilms loaded with anthocyanins was observed.

Conclusion

The BEHEEo formed showed capacity to retain and preserve the anthocyanins. The analysis by AFM confirmed the increased roughness of BEHEEo dependent on the concentration of EHEEo, and showed *Lactobacillus* and yeast in large quantities in these biofilms. Also, the BEHEEo retained anthocyanins of EHEEo for relatively long periods, and, the anthocyanins of BEHEEo were released without losing its characteristics *in vitro* conditions (pH 7.0). In the present study, it was established that biofilms incorporated anthocyanins at a satisfactory level and that fermentation with kefir grains did not reduce the amounts of

anthocyanins. In biofilms incorporation of the anthocyanins took 20 days of cultivation. It is suggested that the biofilms with incorporated anthocyanins have potential for therapeutic applications in several pathologies wherein the antioxidative processes is necessary. This work suggests a solution to the problem of anthocyanins instability. They remain stable in biofilms for at least 24 months. Biofilms, therefore, constitute vehicles for the transposition of bioactive compounds, notably anthocyanins.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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