UFSM

CIÊNCIA e NATURA

ISSN 2179-460X
Acesso aberto

Ci. e Nat., Santa Maria, v. 44, e47, 2022 • https://doi.org/10.5902/2179460X40913
Submitted: 02/11/2019 • Approved: 09/06/2022 • Published: 27/09/2022

Environment

Study of biofilms with kefir associated with soy germs (*Glycine max* (L.) Merril)

Estudo de biofilmes de kefir associado com gérmen de soja (*Glycine max* (L.) Merril)

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ABSTRACT

The aim of this study was to investigate biofilm formation with kefir grains in the presence of soy extract. Kefir grains and soy germs at different concentrations were grown in the culture medium comprising brown sugar solution (40 gl^{-1}) for 20 days. Biofilms that formed in this period were then removed and the pH of the culture medium were measured. Isoflavones of the medium of culture were extracted and quantified by high-performance liquid chromatography (HPLC). The superficial properties of the selected biofilms were analyzed by atomic force microscopy (AFM) and scanning electron microscopy (SEM). The culture medium after 20 days was found to have 19.59±3.57 μgl^{-1} of glycitein and 23.86±2.21 µgl-1 of genistein. The best concentration of kefir grains in order to extract isoflavone was 40 gl-1, with yield levels at 11.67 μgl^{-1} of glycitein and 17.78 μgl^{-1} of genistein. The analysis by AFM and SEM confirmed the increased roughness of the biofilm, dependent of the concentration of the amount of kefir grains. It is suggested that the biofilms incorporated the isoflavones and has potential for therapeutic applications in several pathologies wherein it is necessary the antioxidative processes.

Keywords: Biofilms; Kefir; Soy germs; Isoflavones; AFM

RESUMO

O principal objetivo deste estudo foi investigar a formação de biofilme com grãos de kefir na presença de extrato de soja. Diferentes concentrações de grãos de kefir e de gérmen de soja foram cultivados em meio de cultura constituido de solução de acúcar mascavo (40 gl^{-1}) durante 20 dias. Os biofilmes formados neste período foram então removidos tendo sido determinado o pH do meio de cultura. As

isoflavonas do meio de cultura foram extraidas e quantificadas por Cromotografia Líquida de Alta Perfomance (CLAP). As propriedades superficiais dos biofilmes selecionados foram analisadas por microscopia de força atômica (AFM) e microscopia eletrônica de varredura (MEV). O meio de cultura após 20 dias apresentou 19,59±3,57 μgl^{-1} de gliciteína e 23,86±2,21 μgl^{-1} de genisteína. A melhor concentração de grãos de kefir para extração de isoflavona foi de 40 gl^{-1}, com níveis de rendimento de 11,67 μgl^{-1} de gliciteína e 17,78 μgl^{-1} de genisteína. A análise por AFM e MEV confirmou o aumento da rugosidade do biofilme, dependente da concentração da quantidade de grãos de kefir. Sugere-se que os biofilmes incorporam as isoflavonas e tem potencial para aplicações terapêuticas em diversas patologias em que se faz necessário os processos antioxidantes.

Palavras-chave: Biofilmes; Kefir; Germes de soja; Isoflavonas; AFM

1 INTRODUCTION

In recent years the effects of isoflavones have been continuously studied on account of their antioxidant properties. Isoflavones comprise daidzein, genistein and glycitein, the b-glycosides and their malonyl glucosides conjugates, and acetyl-glycosides.

In addition to their potent antioxidant benefits, isoflavones have a hormonal effect (Imai 2015) on the human body as phytoestrogens assets besides having recognized hypocholesterolemia capacities (Rossi *et al*. 2004).

Among all the beans studied so far, soybean has the highest isoflavone content related to genetic and environmental factors (Carrão-Panizzi *et al*. 2009).

Kefir is a probiotic substance consisting of a complex, symbiotic biomass of lactic acid bacteria and acetic acid. The resultant fermented product performs the synthesis and better absorption of protein, vitamins and minerals, benefiting the host (Farnworth 2005).

Kefir has the capacity to participate in biofilm formation processes (Oliveira *et al*. 2017). Biofilms are surfaces with a high degree of structural organization; they are associated with bacterial colonies that are held together by an extracellular polymeric substance secreted by the bacteria (PES) (Zhang *et al*. 2015).

This study investigated the formation of biofilms with kefir in the presence of soy germ extract. The objective was to determine the optimal concentration of kefir grains and soy germ concentration for the production of biofilms and perform

the extraction. The study also quantified isoflavones that were incorporated in the biofilms and isoflavones present in the culture medium with kefir.

2 MATERIALS AND METHODS

To obtain the biofilms with soy germ, we used vials with a capacity of 3.6 L sterilized with ethanol 70% and placed in a laminar flow cabinet under ultraviolet UV radiation for two hours.

Different soybean germ concentrations were added (0, 1, 2, 5, 10, 20 gl^{-1}) (40 gl^{-1}) with 20 gl^{-1} of kefir grains (KG) in a brown sugar solution (AM) as medium.

The flasks with the medium were then placed at an ambient temperature of 25±2°C for 20 days. After this period the formed biofilms were removed and weighed. The volume and pH of the remaining solution were measured.

The dosing was performed for both isoflavones in the culture medium and also in biofilms. Soybean germ concentration that showed biomass with better consistency, i.e. one that can be separated from the medium without loss of integrity with the highest concentration of isoflavones, was considered the optimal.

Standards of glycitein and genistein were utilized (Sigma-Aldrich) at a concentration of 0.0125 mg/ml. For preparation of the standard solution, isoflavones were extracted from soy germ of 20 gl^{-1} concentration, using 70% ethanol for two hours.

2.1 Determination of the concentration of kefir grain needed for formation of biofilms with soy germ

Brown sugar solutions of concentration 40 gl^{-1} were prepared and sterilized at 121°C for 20 minutes in an autoclave (Phoenix, Brazil). Next, 20 gl^{-1} soybean germs were added with kefir grains of concentrations of 5, 10, 20, 40, 60 and 80 gl^{-1} in the respective vials in a laminar flow chamber.

The flasks were stored at 25°C for 20 days. After this period, the formed biofilms were separated and weighed. The determination of isoflavones was performed in the culture medium as well as in the biofilms. The volume and pH of the solutions were also measured.

2.2 Determination of the concentration of isoflavones in the medium of culture and in the biofilms

The extraction of isoflavones was performed according to the methodology proposed by Carrão-Panizzi *et al*. (2002) Samples of 100 mL were taken from each vial of the culture medium with sugar solution, and kefir and soy germ were filtered on Whatman paper containing micropores of diameter 8 µ.

Then 1.5mL was filtered through a membrane with pores of 0.45 micrometres, the "Millipore" mark. For injection into the liquid chromatograph, 20 µL of the filtered extract were used. Separation and quantification of isoflavones were performed according to the alternative methodology recommended by Berhow (2002). The analyses were performed on high performance liquid chromatograph-HPLC (Shimadzu Corporation) equipped with autoinjector and diode array detector to scan 190-500 nm.

Chromatographic conditions: Chromatograms obtained at 254 nm with a furnace temperature maintained at 30°C, reverse phase column, Shim-pack VP-ODS (150 x 4.6 mm; 5 um), 10 µL injection volume, using as phase (A) 0.1% water acidified with acetic acid, and as phase (B) methanol (60:40) with a flow rate of 1 ml/min.

Quantification of glycitein and genistein was performed by comparison of the areas of the peaks obtained from the prepared standard solutions containing 10 µg/ml glycitein and 15 µg/ml genistein, diluted in methanol and then filtered through a membrane filter with pores of 0.45 micrometre (Millepore®) (Caesar *et al*. 2007).

The isoflavone content was expressed as mg/100 g samples on dry basis. Separated and quantified isoflavones comprised aglycone (genistein and glycitein) (Coward *et al*. 1993). The isoflavone values were presented in aglycone equivalents (Góes-Favoni *et al*. 2010).

2.3 Structural analysis of biofilms of kefir with soy germ by atomic force microscopy (AFM)

The morphology of the biofilm surfaces was analyzed by AFM (Easyscan2 AFM/STM, Nanosurf Co.). Relative humidity was maintained at 51%. The scan sample size was 30 μm×30 μm, and the images were obtained on contact mode. The cantilever was of rectangular shape with a spring constant of 0.77nN/m, a force constant of 0.2 N/m, and a resonance frequency of 13 kHz. The biofilms were dehydrated and electrodeposited on slides with gold films to take these measurements, and 10-3 scan cycles were analyzed by cyclic voltammetry on the device. Images from 20 different regions were examined and analyzed with the AFM software to calculate the roughness values (RMS).

The parameters used in this analysis included average roughness (Rm) and its effective value (RMS), which were calculated using the following equations (Khulbe *et al*. 2008):

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

Where Rm is the average roughness and Z (x, y) is a function of the height of the area.

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

Here RMS is expressed as the square root of average roughness.

2.4 Structural analysis of the biofilms of kefir with soy germ through scanning electron microscopy (SEM)

Scanning Electron Microscopy (SEM) was used to analyze the structure and composition of microbial biofilms of kefir with soy germ. The study used the Scanning Electron Microscope TM3030Plus of the Research Laboratory in Drugs of UNIFAP, Macapa, Amapá, Brazil. The kefir biofilm samples (dry) were directly introduced into the Deben accessory of the scanning electron microscope (TM3030Plus - Tabletop microscope - Hitachi) and the images were captured using TM3030Plus software.

2.5 Statistical Analysis

The Stat Graphics Centurium v. XVII software (StatEasy Co. MA, USA) was used for statistical analysis. ANOVA was performed to evaluate the statistically significant differences between the weights of the biofilms. This was followed by Tukey's test, and the average weight of the biofilms was analyzed using the Student's t-test. Results with $p < 0.05$ were considered as significant.

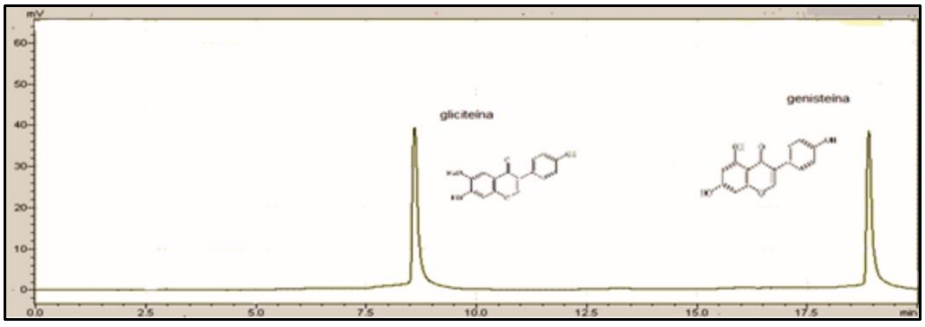
3 RESULTS

3.1 Determination of the concentration of soy germ needed for formation of biofilms with kefir

The standard sample of glycitein presented an equivalent dosage concentration of 12 µg/ml, while the sample of genistein showed concentration of 15 µg/ml (Figure 1).

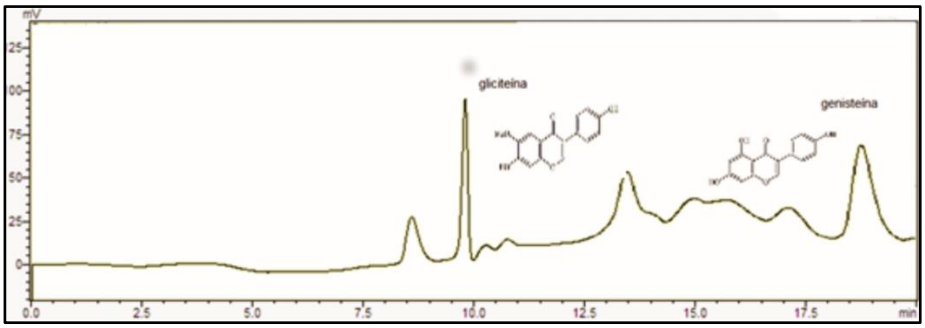
The soybean germ extract showed a concentration of 21.48±1.45 µg/ml for genistein, while for glycitein it was 24.77±2.56 µg/ml (Figure 2).

Figure 1 – Chromatographic profiles by HPLC standard for aglycones (Sigma- Aldrich) by high-performance liquid chromatography (HPLC) for genistein and glycitein, at retention times of 9.2 minutes and 18.7 minutes respectively



Fonte: Author's (2022)

Figure 2 – Chromatographic profiles by HPLC standard for the compounds obtained by combining soybean germ extract with glycitein and genistein at retention times of 9.60 minutes and 18.50 minutes respectively. The isoflavones were extracted from 20 g/L soy germ by treating it with 70% ethanol for two hours



Fonte: Author's (2022)

Higher concentrations of soybean germ in the culture medium coincided with increase of the isoflavone content, when both contents were quantified simultaneously.

Similarly the growth of the biomass of the biofilm was higher when soybean germ content increased in the medium (Table 1). However, the biofilm's biomass did not show any correlation with increased isoflavone incorporation into the medium.

The concentration of 10 gl^{-1} soy germ showed the greatest incorporation of isoflavones (39.63±0.64 gl^{-1} of glycitein and 42.24±0.4 gl^{-1} of genistein) (Table 1). The pH remained constant and low between 2.22 and 2.91 by 5 gl^{-1}soy germ (Table 1).

Furthermore, the analysis showed statistically significant differences with regard to the incorporation of isoflavones using different concentrations of soy germ (ANOVA, F = 207.39, p <0.0001).

It was seen that increasing concentrations of soy germ resulted in increased isoflavone incorporation in the culture medium, with a peak of 10 gl^{-1} (39.63±0.64 mgl^{-1} using glycitein and 42, 24±0,4 gl^{-1} of genistein).

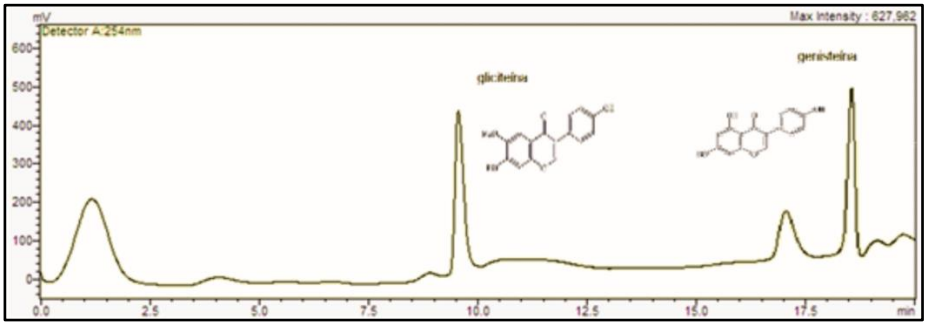
There was no statistical difference between isoflavones presented at concentration of 20 μgl^{-1} (19.80±0.36 μgl^{-1} of glycitein and 23.59±0.23 μgl^{-1} of genistein) and concentration 20 gl^{-1} (Table 1).

Table 1 – Biofilm formation with KG (20g/L) and BS (40 g/L) at different concentrations of soy germ (SG)

Conc. de SG (g)	**Grow (g) Média**	**Isoflavones**		**pH**	**Volume**
		glicitein	**genistein**		
0	6,75±0,14	0	0	2,28±0,02	480
1	10,06±0,26	7.65±0,20	9.57±0,10	2,29±0,01	485
2,0	17,30±1,02	11,54±0,10	10,41±0,06	2,40±0,02	480
5,0	7,63±0,46	17.50±0,07	13.75±0,09	2,91±0,02	475
10,0	16,52±0,95	39,63±0,64	42.24±0,40	2,33±0,01	465
20.0	46,37±0,4	19.80±0,36	23.59±0,23	2,43±0,02	465

KG = kefir grains; BS= sugar brown
Fonte: Author's (2022)

Figure 3 – Chromatographic profile by HPLC of the culture medium with kefir (20 g/L), brown sugar (40 g/L) and soybean germ extract (10 g/L) obtained through high-performance liquid chromatography (HPLC), in the process of conversion to glycitein and genistein for retention times of 9.8 minutes and 19.8 minutes respectively



Fonte: Author's (2022)

3.2 Determination of Concentration of Kefir Grains Needed for Formation of Biofilms with Soy Germ

There were statistical differences in the results obtained when using different concentrations of kefir grains in culture medium with brown sugar and soy germ (ANOVA, F = 40.01 and p <0.0001). The optimal concentration for biofilm formation was 40 gl^{-1} (44.49±2.09 g) (Table 2).

With respect to the incorporation of isoflavones, the concentration of 60 gl^{-1} was the highest (32.65±3.98 mgl^{-1} of glycitein and 39.89±3.25 mgl^{-1} of genistein) (Table 2). Time required for biofilm formation and incorporation of isoflavones in the biofilm of kefir associated with soy germ was twenty days (Table 3).

Table 2 – Biofilm formation with soy germ SG (40 g/L) and brown sugar BS (40 g/l) at different concentrations of kefir grains (KG)

Conc. of KG (g)	Grow(g) Mean	Isoflavones		pH	Volume
		Gliciteín	genisteín		
10	23.87±0.15	8.76±0.67	9.44±1.43	4.03±0.01	485
20	19.48±0.35	10.35±0.54	11.78±2.55	3.76±0.02	480
40	44.49±2.09	17.48±2.41	14.09±1.45	3.80±0.15	475
60	32.85±0.95	32.65±3.98	39.89±3.25	3.80±0.01	465
80	32.55±0.70	19.59±3.57	22.78±8.21	3.77±0.0	465
100	34.39±0.45	22.34±1.54	23.45±2.23	3.49±0.02	471

SG = gérmen de soja; BS= sugar Brown
Fonte: Author's (2022)

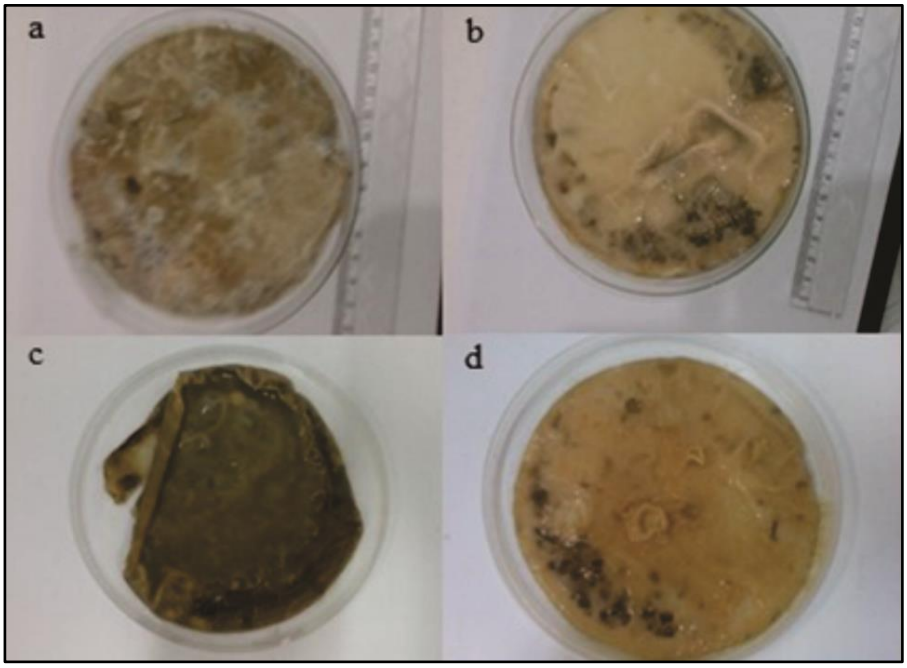
Table 3 – Determination of the time required for biofilm formation and incorporation of isoflavones in the biofilm of kefir associated with soy germ

Time (Days)	Biomass (g)	pH	Vol (mL)	Isoflavones (µg/g)	
				glicitein	genistein
5	2.95±0.24	3.66	204±3.51	5.47±0.52	7.4±0.49
15	5.81±0.23	3.57	185±1.67	17.51±0.47	18.56±0.63
20	10.54±1.10	3.49	188±1.27	19.59±3.57	23.86±2.21

The numbers represent the mean ± standard deviation
Fonte: Author's (2022)

Figure 4 shows the biofilms obtained with kefir grains (20 gl^{-1}) with brown sugar (40 gl^{-1}) with different concentrations of soy germ (a) 20 gl^{-1}, b) 40 gl^{-1}, c) 80 $gl^{-1}$and d) 100 gl^{-1}).

Figure 4 – Photo of keir biofilms (20g/L) with brown sugar (40 g/L) and soy germ a) 20 g/L, b) 40 g/L, c) 80 g/L and d) 100 g/L

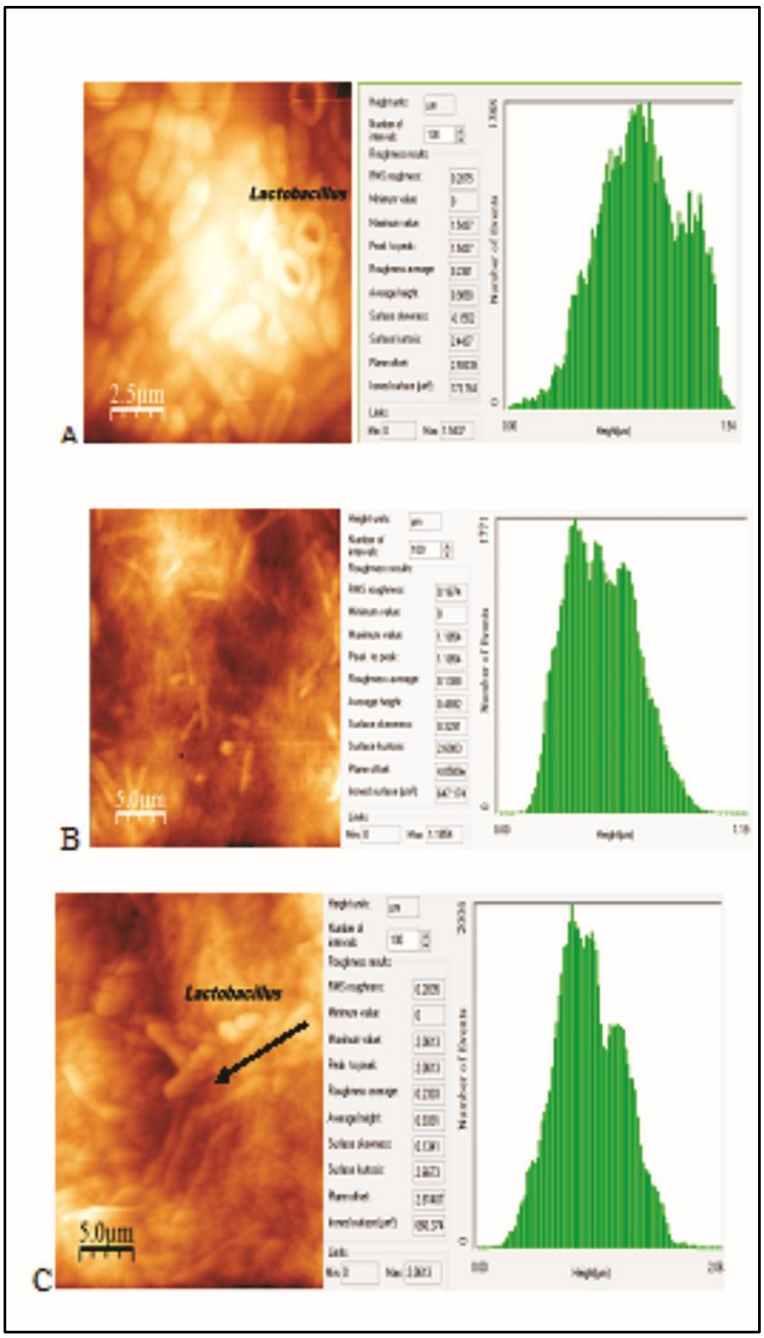


Fonte: Author's (2022)

3.3 Structural analysis of biofilms of kefir with soy germ by atomic force microscope and scanning electron microscopy (SEM)

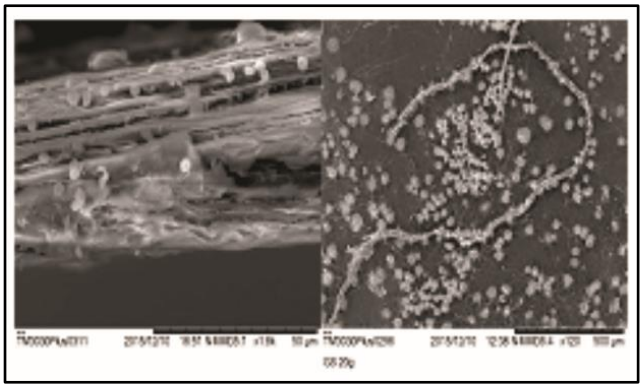
Analysis of the biofilms by AFM images were obtained with different concentrations of soy germ (Figure 5). The profile image obtained by scanning electron microscopy (SEM) shows the formation structure of biofilms with overlapping layers (Figure 6a). Still through scanning electron micrograph (SEM) of kefir biofilm (40 gl^{-1}) with brown sugar (40 gl^{-1}) and soy germ (20 gl^{-1}), is displayed hyphae with molecules linked to them (Figure 6b).

Figure 5 – Graphic image obtained by atomic force microscopy (AFM) showing: A), the roughness (0.287 mM) of kefir biofilm formed by 2 g/L soy germ. Note the presence of *Lactobacillus.* B) Biofilm with soy germ (10g/L) with roughness (0. 283 µm.). C) Biofilm with soy germ (80g/L) with RMS roughness: 0.167 µM. Increasing of soy germ concentration did not alter the roughness significantly



Fonte: Author's (2022)

Figure 6 – Scanning electron micrograph (SEM) of kefir biofilm (40 g/L) with brown sugar (40 g/L) and soy germ (20 g/L), A) The displayed profile is observed to form overlying biofilm layers. B) displaying hyphae with molecules linked to them



Fonte: Author's (2022)

4 DISCUSSION

The fermentation process of kefir resulted in no decrease in the amount of isoflavones compared to the extraction carried out with soybean germ extract by the usual procedure (with 70% ethanol). While the soybean germ extract yielded concentrations of 21.48±1.45 µg/ml for glycitein and 24.77±2.56 µg/ml for genistein (Figure 2), extraction carried out by fermentation with kefir associated with the soybean germ extract at a concentration of 10 gl^{-1} resulted in peak concentrations of 39.63±0.64 μgl^{-1} of glycitein and 42.24±0.40 gl^{-1} of genistein. Therefore, although Rossi *et al*. (2004) had reported that the fermentation caused reduction in the amount of isoflavonas, the fermentation process with kefir did not cause any significant degradation.

With the use of kefir, the fermentation process was effective in extracting the isoflavones without causing qualitative degradation. This finding is in agreement with the data obtained by Puri *et al*. (Puri *et al*. 2015) who found that fermentation of soymilk with various microorganisms using monoculture can yield more efficient

nutritional value for the fermented product due to the positive correlation between the level of enzymes and the content of isoflavones.

The fermentation process may alter the bioavailability of isoflavones and can also convert the isoflavone glycoside into aglycone (Yoo *et al*. 2015, Xu *et al*. 2014). These compounds have effectiveness concerns for human use because normally the body cannot absorb isoflavones in glycosylated forms (Oh *et al*. 2016).

The use of kefir brought a protective effect in the culture medium by preventing denaturation of the soy proteins and thereby delaying the onset of putrefaction. When the fermentation was stopped by some factor (temperature below 20°C. for example) protein denaturation of the soybean germ extract occurred with a consequent increase in the pH (ranging from 6.5 to 7.5), color change, and odor of the medium. It was also found that in these conditions when the fermentation was stopped, the isoflavones were degraded.

Analysis of biofilms obtained with different soybean germ concentrations by AFM images revealed that the roughness settings (RMS) were concentration-dependent (Figures 5). Scanning electron micrograph (SEM) of kefir biofilm (40 gl^{-1}) with brown sugar (40 gl^{-1}) and soy germ (20 gl^{-1}), displaying hyphae with molecules linked to them.

Stress conditions, change of pH and dysbiosis hamper the intestinal absorption of drugs and compounds, the concomitant intake of probiotics can be an effective strategy to achieve homeostasis in healthy microflora and thereby to obtain efficient drug absorption (Prudhviraj *et al*. 2015). Also, the biological effects of phenolic compounds depend on their mechanism of action in the body, which in turn is affected by the bioconversion of the colon microflora (Breynaert *et al*. 2015).

Kefir biofilms incorporated with isoflavone enable the effective absorption of isoflavones, thereby presenting the conditions to maintain intestinal homeostasis.

In the present study we established that kefir biofilms incorporated isoflavones at a satisfactory level and that fermentation with kefir grains did not reduce the amounts of isoflavones. With respect to the culture time for most of the isoflavone, the best concentration of kefir grains for production of biomass was 40 gl^{-1}. However, for the incorporation of aglycones the optimal concentration was 60 gl^{-1}. In biofilms incorporation of the aglycones took 20 days of cultivation. There was no change of roughness with increasing soy germ concentration.

5 CONCLUSION

The aim of this study was to investigate biofilm formation with kefir grains in the presence of soy extract. Kefir grains and soy germs at different concentrations were grown in the culture medium comprising brown sugar solution for 20 days. Biofilms that formed in this period with isoflavones of the medium of culture were extracted and quantified. The best concentration of kefir grains in order to extract isoflavone was 40 gl^{-1}, with yield levels at 11.67 μgl^{-1} of glycitein and 17.78 μgl^{-1} of genistein. The analysis by AFM and SEM confirmed the increased roughness of the biofilm, dependent of the concentration of the amount of kefir grains. It is suggested that the biofilms incorporated the isoflavones and has potential for therapeutic applications in several pathologies wherein it is necessary the antioxidative processes.

ACKNOWLEDGEMENTS

This work was supported by the CNPq. Authors would like to thank CAPES (nº 3292/2013 AUXPE), and CNPq Proc. 402332/2013-0 (Biotec) for the financial support.

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How to quote this article

OLIVEIRA, A.F.; FLORENTINO, A. C.; MACIEL, A. F.; VALENTE, A. S.; BEZERRA, R. M.; ZAMORA, R. R. M.; CARVALHO, J. C.T. Study of biofilms with kefir associated with soy germs (*Glycine max* (L.) Merril). **Ciência e Natura**, Santa Maria, v. 44, e47, 2022. DOI: https://doi.org/10.5902/2179460X40913.