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Atividade antimicrobiana de kefir fermentado com subproduto de uva contra *Alicyclobacillus acidoterrestris*

Antimicrobial activity of fermented kefir with a byproduction of grape fruit to *Alicyclobacillus acidoterrestris*

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ABSTRACT

Alicyclobacillus spp. they are spore-forming bacteria that deteriorate acidic fruit-based drinks, causing economic losses, with *A. acidoterrestris* being the most studied, responsible for causing sensory changes, especially in orange juice. The use of natural antimicrobial agents in foods can be an option with great advantages for the industry and the consumer. Therefore, the objective of this work was to investigate the antimicrobial activity of extracts fermented by kefir, from a grape by-product, against *A. acidoterrestris*. Four extracts were prepared with kefir grains, being 1 - grape extract and kefir grains; 2 - grape extract, brown sugar and kefir grains; 3 - grape extract, ultrasound and kefir grains; and 4 - grape extract, ultrasound, brown sugar and kefir grains. The four extracts were fermented at 28 ºC for 7 days. The extracts were centrifuged at 10,000 rpm for 10 min and the supernatant was subjected to membrane filtration (0.22 μm). The minimum inhibitory concentration (MIC) was determined by the serial microdilution technique of extracts of 50 to 0.1% concentration in *Bacillus acidoterrestris* (BAT) medium. The minimum bactericidal concentration (CBM) was also determined. Structural changes in cells after treatment were evaluated by scanning electron microscopy (SEM). The MIC value capable of inhibiting the visible growth of *A. acidoterrestris* for all extracts was 1.6%, while the CBM was 50% for extracts 1 and 3, while for extracts 2 and 4 the CBM was 25%. The results show that extracts 2 and 4 obtained better CBM value, possibly because kefir produced more secondary metabolites with the addition of brown sugar, in addition, the use of ultrasound did not interfere. The results of the inhibitory and/or bactericidal concentration indicate that the extracts have activity against *A. acidotrrestris*.

Keywords: deterioration, preservation, scanning electron microscopy.

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RESUMO

Alicyclobacillus spp. são bactérias formadoras de esporos que deterioram bebidas ácidas à base de frutas, causando prejuízos econômicos, sendo *A. acidoterrestris* a mais estudada, responsável por causar alterações sensoriais, principalmente em suco de laranja. O uso de agentes antimicrobianos naturais em alimentos pode ser uma opção com grandes vantagens para aindústria e o consumidor. Portanto, o objetivo deste trabalho foi investigar a atividade antimicrobiana de extratos fermentados por kefir, a partir de um subproduto da uva, contra *A. acidoterrestris*. Foram preparados 4 extratos com grãos de kefir, sendo 1 – extrato de uva e grãos de kefir; 2 – extrato de uva, açúcar mascavo e grãos de kefir; 3 – extrato de uva, ultrassom e grãos de kefir; e 4 – extrato de uva, ultrassom, açúcar mascavo e grãos de kefir. Os 4 extratos foram fermentados a 28 ºC por 7dias. Os extratos foram centrifugados a 10.000 rpm por 10 min e o sobrenadante foi submetido a filtração por membrana (0,22 μm). A concentração inibitória mínima (CIM) foi determinada pela técnica de microdiluição em série dos extratos de 50 a 0,1% de concentração em meio *Bacillus acidoterrestris* (BAT). A concentração bactericida mínima (CBM) também foi determinada. As alterações estruturais das células após o tratamento foram avaliadas por microscopia eletrônica de varredura (MEV). O valor da CIM capaz de inibir o crescimento visível de *A. acidoterrestris* para todos os extratos foi de 1,6%, enquanto o CBM foi de 50% para os extratos 1 e 3, enquanto para os extratos 2 e 4 o CBM foi de 25%. Os resultados mostram que os extratos 2 e 4 obtiveram melhor valor de CBM, possivelmente porque o kefir produziu mais metabólitos secundários com a adição de açúcar mascavo, além disso, o uso do ultrassom não interferiu. Os resultados da concentração inibitória e/ou bactericida indicam que os extratos possuem atividade contra *A. acidotrrestris.*

Palavras-chave: deterioração, preservação, microscopia eletrônica de varredura.

1 INTRODUCTION

Alicyclobacillus acidoterrestris is a thermophilic, acidophilic and spore-forming microorganism. Spores are found mainly in the soil and are therefore easily transferred to industrial food production. As these bacteria survive high temperatures, they can withstand commercial pasteurization applied to fruit juices and subsequently germinate and produce guaiacol, an odorous compound (Hu et al., 2020). Thus, this microorganism is a major threat to the acidic beverage industry, because in addition to 2-methoxyphenol (guaiacol), they also produce 2,6-dibromophenol, 2,6-dichlorophenol, responsible for causing strange flavors, without changing the pH (Pascoli et al., 2018).

Food safety is a global and important factor for the food industry and public health, so the concern with the use of synthetic preservatives, the discovery of microorganisms resistant to antimicrobial agents and the increase in consumption for natural foods are challenges for the industry of food (Miao et al., 2016). In this sense, natural antibacterial products have been

studied to control the spoilage caused by the *A. acidoterrestris*, such essential oil of oregano, *Piperaceae* extracts, citral essential oils, rosemary extracts, grape seed extract (Dutra et al., 2019; Pascoli et al., 2018; Huertas et al., 2014; Piskernik et al., 2016; Molva & Baysal, 2015). Use of natural antibacterial agents has been used as a sustainable alternative.

Kefir is a microbial symbiose that produces jelly-like grains as it grows, that contain both lactic acid bacteria (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Acetobacter* and *Streptococcus* spp.) and yeasts (*Kluyveromyces*, *Torula*, *Candida* and *Saccharomyces* spp.) (Rodrigues et al., 2005). Water kefir is a slightly sour, alcoholic and fruity fermented drink, fermentation is started with the microbial population of kefir grains. The potential health benefits contribute significantly to the interest and consumption in this product (Coma et al., 2019). It is known as a probiotic product with several health-promoting properties, such as reduced fat deposition, immunological, anti-tumor, hypocholesterolemic, antioxidant and antibacterial effects. In addition to the microorganisms themselves, various metabolites are released by the microorganisms during fermentation, they may have bioactive properties (Savastano et al., 2020).

Antimicrobial peptide F1, antimicrobial peptide from kefir, have shown strong antimicrobial activity against *Escherichia coli* (Miao et al., 2016). Probiotic kefir offers many benefits, including antimicrobial effects, but there are no studies that show its antimicrobial activity against the deteriorating bacteria *A. acidoterrestris*.

Grapes are sources of phenolic compounds, besides the usable part of the fruits, by-products generated during the fruit processing are also rich in bioactive phenolics (Zambrano et al., 2019). In addition, methods such as ultrasound have been used to extract residual biocomposites from vegetables (Lima et al., 2018). Thus, this study objective to investigate the antimicrobial activity of kefir fermented with grape waste prepared in different concentrations of sugar against *A. acidoterrestris* and in addition, to investigate the influence of ultrasound on these fermentates.

2 MATERIAL AND METHODS

2.1. MATERIAL

For the elaboration of extracts fermented by kefir, grape waste was used, which were composed of parts of the skin, seeds and bagasse. These were provided by Indústria Redondo Polpa de Frutas, located in the city of Cambé - PR.

Brown sugar was also used, sourced from local stores, in the city of Maringá - PR. And the distilled water obtained in the laboratory.

The grains of water kefir used as an inoculum in the fermentation process were obtained through donation, from artisanal cultivation in the city of Maringá - PR.

The reference strain *A. acidoterrestris* 0244^T was used from the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI), located at the Chemical, Biological and Agricultural Research Center (CPQBA / UNICAMP). The strain is stored at -20 °C in the Water, Environment and Food Microbiology Laboratory of the State University of Maringá.

2.2 METHODS

2.2.1 Preparation of extracts

Four extracts were prepared using grape by-product in water (1: 2).

- Extract 1: GBP + 10% kefir grains (KG);
- Extract 2: GBP + 2% brown sugar (CHO) + 10% KG;
- Extract 3: GBP (15'ultrasound) + 10% KG;
- Extract 4: GBP (15'ultrasound) + 2% CHO + 10% KG;

The ultrasonic bath used was Unique, 25kHz. The four extracts were fermented at 28 °C for 7 days. The extracts were centrifuged at 10,000 rpm for 10 min and the supernatant was subjected to membrane filtration (0.22 μm), to subsequently assess its antimicrobial activity against *A. acidoterrestris.*

2.2.2 Minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) and bactericidal concentration (MBC) for each extract was determined on 96-well microdilution plates (TPP®, Switzerland), following the CLSI Methodology M7-A11 (2018). Serial dilutions of the extract were carried out with an initial fermentation concentration of 50 to 0.1% performed and with *Bacillus acidoterrestris* medium - BAT broth (Deinhard et al., 1987). Then, 5 μL of vegetative cell suspension was added after standardization with the McFarland scale at 10^8 CFU/mL, followed by 1:10 dilution. The culture volume in each well was 100 μL and the initial level of the inoculum was 10^4 CFU/mL. The 96-well plate was incubated at 45 °C for 24 h. After that, the turbidity of the well was observed visually. The minimum inhibitory concentration was the

lowest concentration resulting in growth inhibition as defined by visual observation. The tests were performed individually for each extract.

The bactericidal concentration was determined by subculture of 20 μL of each negative well on the surface of an BAT agar plate that has been incubated at 45 °C for 24 h. The tests were performed in triplicate.

2.2.3 Scanning Electron Microscopy (SEM)

The inoculum of *A. acidoterrestris* 0244^T was treated with extract 2 at concentrations defined by MIC and control antimicrobial activity (only with *A. acidoterrestris* cells without addition of the extract) The cells were subjected to scanning electron microscopy, according to the protocol proposed by Endo et al. (2010).

The samples were washed in saline and fixed in 2.5% gluteraldehyde (Sigma-Aldrich, St. Louis, MO) and 0.1 M sodium cacodylate buffer (SEM, Hatfield, PA). They were then washed in 0.1 M sodium cacodylate buffer and coverslipped with poly-L-lysine, followed by dehydration with ethanol, critical point drying with CO_2, gold plating and observation under a Scanning Electron Microscope (Quanta 250, FEI Company).

3 RESULTS AND DISCUSSION

3.1MINIMUM INHIBITORY CONCENTRATION AND MINIMUM BACTERICIDAL CONCENTRATION

Plant extracts have a diversity of compounds essential for plant survival, and studies have shown that their secondary metabolites, that is, their bioactive compounds, have antimicrobial activity against bacteria, fungi and yeasts (Jardim et al., 2019). Thus, the use of grape extract has great potential for use as an antimicrobial agent. In addition, by going through the fermentation process, with the use of kefir, this antimicrobial potential can be increased due to the formation of metabolites produced by the microorganisms present in the kefir.

Studies has been verify the water kefir effective antimicrobial activity against some species of pathogenic microorganisms, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella tiphymurium*, *Escherichia coli*, *Listeria monocytogenes*, and *Candida albicans* (Golowczyc et al., 2007, Rodrigues et al., 2005). In addition, in this study the

antibacterial activity of water kefir against the deteriorating bacillus *A. acidoterrestris* is shown in Table 1.

TABLE 1. Results obtained for MIC and MBC of kefir fermented extracts against *A. acidoterrestris.*

Extracts	**MIC (%)**	**MBC (%)**
1	1,6	50
2	1,6	25
3	1,6	50
4	1,6	25

*1: Extract BPG + 10% KG; 2: Extract BPG + 2% CHO + 10% KG; 3: Extract BPG (15'ultrasound) + 10% KG; 4: Extract BPG (15'ultrasound) + 2% CHO + 10% KG

There was no difference between the minimum inhibitory and bactericidal concentrations of the extracts submitted or not to ultrasonic bath (Unique, 25kHz), demonstrating the indifference in their use.

The added 2% brown sugar extracts showed superior results, due to the fact that sugar serves as a substrate for fermentation, possibly leading to the production of more metabolites with antimicrobial capacity.

Fiorda et al. 2016 isolated lactic acid bacteria from kefir grains and found their antimicrobial action, by producing antimicrobial substances by such bacteria.

Beverages based on grape kefir, that is, fermented water kefir with added fruit, are already consumed in southern Italy as acidic, refreshing, lightly carbonated and low alcoholic drinks (Gaware et al., 2011); standing out in this work also as a natural antimicrobial potential.

In comparison to other natural compounds, the kefir extracts analyzed in this study obtained values of minimum inhibitory concentrations against *A. acidoterrestris* higher than the essential oil of oregano (Dutra et al., 2019) and bromelain (Anjos et al., 2016). Although the values of the bactericidal concentrations did not show the same result, the kefir extracts showed effective antimicrobial activity against the deteriorating microorganism.

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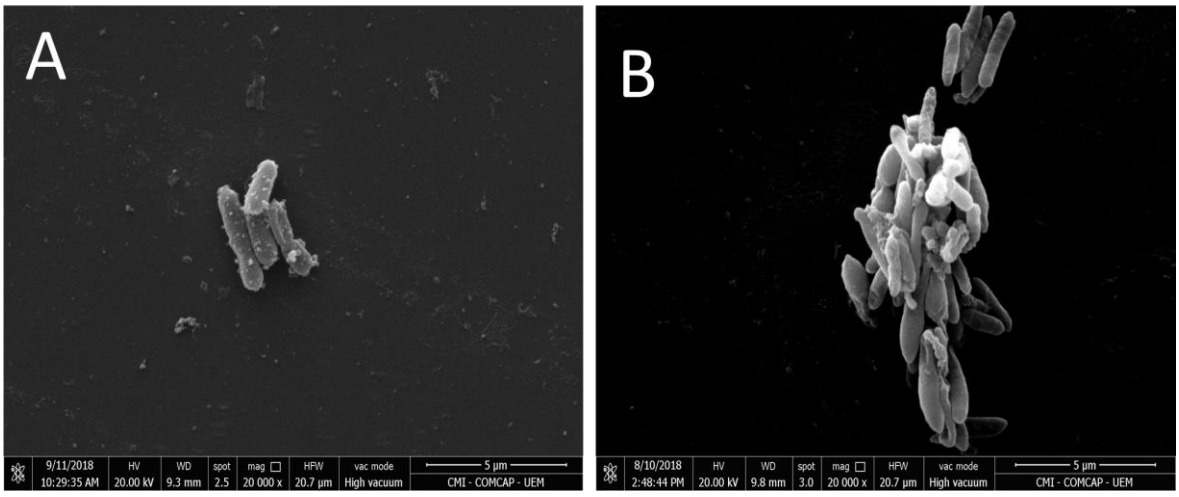
3.2 SCANNING ELECTRON MICROSCOPY (SEM)

The effect of extract 2 on *A. acidoterrestris* cells can be observed (Figure 1). The morphological alterations, were observed by scanning electron microscopy (SEM). Untreated control cells of *A. acidoterrestris* (B), were visually showed higher numerical quantities, a smooth cell surface with uniform and characteristic morphology. whereas the cells treated with of kefir (A), were observed reduced cells in the presence of kefir and wrinkled and wilted appearance, thus confirming an antimicrobial effect of kefir.

FIGURE 1 - Scanning electron microscopy images. (A) MIC of extract 2 with vegetative cells of *A. acidoterrestris*. (B) Untreated control of vegetative cells of *A. acidoterrestris*



4 CONCLUSION

The results showed that the extracts that were added with brown sugar obtained a better MBC value, possibly because the kefir produced more secondary metabolites with the addition of this product. The use of ultrasound was not effective for the elimination of the evaluated microorganism. The results of the inhibitory and / or bactericidal concentration indicate that extracts fermented with kefir from the grape by-product have great potential for antibacterial activity against A. acidoterrestris, thus further studies are needed for its application as a natural antimicrobial in foods.

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