UNIVERSIDADE ESTADUAL DE MARINGÁ
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CARACTERIZAÇÃO E BIOATIVIDADE DE SUBPRODUTOS ALIMENTARES FERMENTADOS COM KEFIR FRENTE À *Alicyclobacillus* spp.

JÉSSICA LIMA DE MENEZES

Maringá
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Tese apresentada à Universidade Estadual de Maringá, como parte das exigências do Programa de Pós-graduação em Ciência de Alimentos, para obtenção do grau de Doutor em Ciência de Alimentos.

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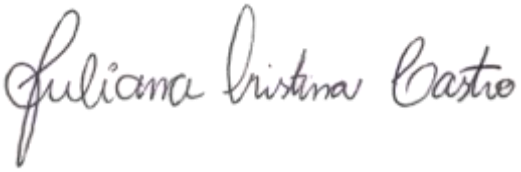

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Dedico

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APRESENTAÇÃO

Esta tese de doutorado está apresentada na forma de dois artigos científicos

1 **AUTORES:** JÉSSICA LIMA DE MENEZES, AMANDA GOUVEIA MIZUTA, TATIANE VIANA DUTRA, TAIANA VARELA FERREIRA, EDINÉIA BONIN, VICKY CRISTINE BRAGANTE THUMAZ, MÁRCIA MARIA DOS ANJOS, BENÍCIO ALVES DE ABREU FILHO.
TÍTULO: ANTIMICROBIAL ACTIVITY OF FERMENTED KEFIR WITH A BYPRODUCTION OF GRAPE FRUIT TO *Alicyclobacillus acidoterrestris*.
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GENERAL ABSTRACT

INTRODUCTION: Among the microorganisms that represent a great concern in the food industry, *Alicyclobacillus* spp. strains stand out. They are non-pathogenic spore-forming bacteria that are related to the deterioration of drinks and citric juices. Among the 25 species of *Alicyclobacillus* that exist today, *A. acidoterrestris* is the most studied and challenging for the food industry since it alters products' sensory characteristics. It is also the most isolated species in deteriorated and non-deteriorated sour products. Natural compounds as an alternative to replace synthetic chemicals in the food industry is something under frequent research regarding their possible application in food products. In their composition biologically active compounds with antimicrobial effects are present, especially in plants extracts, such as spices, herbs, fruits, and vegetables. Moreover, fruit by-products, such as pomace, peel, and seeds, have a series of bio-compounds already reported. Fruit by-products can often present high levels of bioactive compounds compared to their own pulp. Among these bio-compounds, the group of the phenolic and organic acids stands out with possible natural antimicrobial and antioxidant properties. Among the natural compounds we can mention kefir, which are grains constituted by polysaccharides in combination with a complex microbiota containing different lactic acid, acetic acid, bacteria and yeast. The metabolites produced by the fermentation of kefir, such as ethanol and organic acids, have antimicrobial activity against deteriorative and pathogenic microorganisms, such as Gram-positive and Gram-negative bacteria. In this manner, the use of fruit by-products rich in bioactive compounds as a substrate for kefir fermentation, is a strategy for obtaining products with higher levels of bioactive compounds and antimicrobial properties.

AIMS: The objective of this research was to evaluate the antimicrobial and antioxidant activity of different fruit by-products, such as grape, acerola and strawberry against different strains of *Alicyclobacillus* spp.

MATERIAL AND METHODS: Article 1: 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) for *A. acidoterrestris* was determined by the serial microdilution technique of extracts from 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). Article 2: 12 extracts were prepared using the subproducts of acerola, grape and strawberry without fermentation and after fermentation with Kefir for 24, 48 and 72 h. The extracts were centrifuged at 10,000 rpm for 5 min and the supernatant was submitted by membrane filtration (0.22 µm). The minimum inhibitory concentration (CIM) *A. acidoterrestris* 0244^{T}, *A. acidocaldarius* subsp. *rittmannii* 0245^{T}, *A. herbarius* 0246^{T}, *A. acidiphilus* 0247^{T}; *A. cycloheptanicus* 0297^{T}, *A. acidocaldarius* 0299^{T}, was determined by the serial microdilution technique of the extracts of 50 to 0.1% concentration in *Bacillus acidoterretris* (BAT) medium. The minimum bactericidal concentration (CBM) was also determined. For the MEV was used the *A. acidoterrestris* strain, and the inoculum was treated with the extracts of strawberry, grape and acerola fermented by 72 h. The antioxicant capacity of the extracts was measured through the methods of 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and Ferric Reducing Antioxidant Power (FRAP). And finally, the metabolites present in the extracts were identifier by UHPLC-Qtof-MS.

RESULTS AND DISCUSSION: Article 1: 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. Article 2: The results show that extracts fermented for a longer period (72 h) had greater inhibition, and the extract of acerola by-product fermented for 72 h had the best results. For all strains, the Minimal Inhibitory Concentration (MIC) was 0.78%, except for *A*. *acidocaldarius* subsp. *rittmannii*, which obtained 1.56%. The same applied to the Minimal Bactericidal Concentration (MBC), in which 1.56% of the extract was capable of inactivating *A. cycloheptanicus* and *A. acidocaldarius*. The bioactivity of kefir drinks has already been studied, and demonstrated to contain substances with anti-inflammatory, antioxidant and antimicrobial activities. Other authors analyzed the same fermentation times of kefir used in this study, however with kefir fermented in milk, and achieved a more efficient antibacterial activity from 36-48 h of fermentation against *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogene*s, *Enterococcus faecalis*, *Escherichia coli*, *Salmonella* Enteritidis, *Pseudomonas aeruginosa* and *Cronobacter sakazakii*. Other studies also observed the action of kefir against yeast and pathogenic bacteria with the inhibition of *Candida albicans*, *Salmonella* Typhi, *Shigella sonnei, E. coli* and *S. aureus* by kefir fermented for 144 h. Although the present work investigated extracts of fruit fermented with kefir against *Alicyclobacillus* spp., the aforementioned studies corroborate our findings, since they indicate that antimicrobial activity increases with fermentation time, and it is attributed to the substances synthesized during the process. In addition, damages to the structure of the microorganisms caused by the extracts were verified by Scanning Electron Microscopy. Metabolite identification through liquid chromatography (UHPLC-Qtof-MS) demonstrated that the fermented extracts presented a greater number of compounds compared to the non-fermented ones, such as glucuronic, succinic and glutaric acids.

CONCLUSIONS: The results show that the by-products of fruits, fermented or not with Kefir, presented bioactive properties, such as antimicrobial potential against tested *Alicylobacillus* strains, and antioxidant potential, which results in an aggregate value product. In addition, our findings show an increase in antimicrobial activity with longer fermentation periods, with potential to be explored as an antimicrobial agent in the food industry. However, more research is needed to evaluate the use of such extracts in citrus drinks that can deteriorate due to the presence of *Alicicylobacillus* spp..

Key words: bio-compound; fermentation; antimicrobial compound; preservation; deterioration, by-products.

RESUMO GERAL

INTRODUÇÃO: Entre os microrganismos que representam uma grande preocupação na indústria alimentícia, cepas de *Alicyclobacillus* spp. se destacam. São bactérias não patogênicas, formadoras de esporos, que estão relacionadas à deterioração de bebidas e sucos cítricas. Entre as 25 espécies de *Alicyclobacillus* que existem hoje, *A. acidoterestris* é a mais estudada e desafiadora para a indústria alimentícia, uma vez que altera as características sensoriais dos produtos. É também a espécie mais isolada em produtos deteriorados e não deteriorados. Os compostos naturais como uma alternativa para substituir os produtos químicos sintéticos na indústria alimentícia é algo sob pesquisa frequente sobre sua possível aplicação em produtos alimentícios. Em sua composição, os compostos biologicamente ativos com efeitos antimicrobianos estão presentes, especialmente em extratos de plantas, como especiarias, ervas, frutas e legumes. Além disso, subprodutos de frutas, como casca e sementes, têm uma série de biocompostos já relatados. Os subprodutos de frutas podem frequentemente apresentar altos níveis de compostos bioativos em comparação com a sua própria polpa. Entre esses, o grupo dos ácidos fenólicos e orgânicos se destaca com possíveis propriedades antimicrobianas e antioxidantes. Entre os compostos naturais, podemos mencionar Kefir, que são grãos constituídos por polissacarídeos em combinação com uma microbiota complexa contendo bactérias do ácido láctico, ácido acético e levedura. Os metabólitos produzidos pela fermentação de kefir, como etanol e ácidos orgânicos, têm atividade antimicrobiana contra microrganismos deteriorantes e patogênicos, tais como bactérias gram-positivas e gram-negativas. Dessa maneira, o uso de subprodutos de frutas ricos em compostos bioativos como substrato para a fermentação com Kefir, é uma estratégia para obter produtos com níveis mais altos de compostos bioativos e propriedades antimicrobianas.

OBJETIVOS: O objetivo dessa pesquisa foi avaliar a atividade antimicrobiana e antioxidante de diferentes subprodutos de frutas, tais como uva, acerola e morango frente a diferentes cepas de *Alicyclobacillus* spp.

MATERIAL E MÉTODOS: Artigo 1: 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 7 dias. 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) para *A. acidoterrestris* 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). Artigo 2: Foram preparados 12 extratos com utilização dos subprodutos de acerola, uva e morango sem fermentação e após fermentação com kefir por 24, 48 e 72 h. Os extratos foram centrifugados a 10.000 rpm por 5 min e o sobrenadante foi submetido a filtração por membrana (0,22 µm). A concentração inibitória mínima (CIM) das cepas *A. acidoterrestris* 0244^T, *A. acidocaldarius* subsp. *rittmannii* 0245^T, *A. herbarius* 0246^T, *A. acidiphilus* 0247^T; *A. cycloheptanicus* 0297^T, *A. acidocaldarius* 0299^T, 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. Para o MEV foi utilizada a cepa *A. acidoterrestris*, e o inóculo foi tratato com os extratos de morango, uva e acerola fermentados por 72 h. A capacidade antioxante dos extratos foi mensurada através dos métodos de 2,2-difenil-1-picrilhidrazil

(DPPH), 2,2-azinobis (3-etilbenzotiazolina-6-ácido sulfônico) (ABTS), e poder antioxidante de redução do ferro (FRAP). E por fim, os metabolitos presentes nos extratos foram identificador por UHPLC-Qtof-MS.

RESULTADOS E DISCUSSÃO: Artigo 1: 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. Artigo 2: Os resultados mostram que os extratos fermentados por um período mais longo (72 h) apresentaram maior inibição, e o extrato de subproduto de acerola fermentado por 72 h tinha os melhores resultados. Para todas as cepas, a concentração inibitória mínima (MIC) foi de 0,78%, exceto para *A. Acidocaldarius* subsp. *rittmannii*, que obteve 1,56%. O mesmo foi observado para MBC, onde, apenas 1,56% do extrato foi capaz de inativar *A. cycloheptanicus* e *A. acidocaldarius*. A bioatividade das bebidas kefir já foi estudada e demonstrou conter substâncias com atividades anti-inflamatórias, antioxidantes e antimicrobianos. Outros autores analisaram os mesmos tempos de fermentação de Kefir utilizados neste estudo, no entanto, com Kefir fermentado no leite, e alcançou uma atividade antibacteriana mais eficiente de 36-48 h de fermentação contra *Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes, Enterococcus faecalis, Escherichia coli*, *Salmonella* Enteritidis, *Pseudomonas aeruginosa* e *Cronobacter sakazakii*. Outros estudos também observaram a ação de Kefir contra as bactérias patogênicas e leveduras com a inibição de *Candida albicans, Salmonella* Typhi, *Shigella sonnei, E. coli* e *S. aureus* por Kefir fermentado por 144 h. Embora o atual trabalho investigasse extratos de frutas fermentado com Kefir contra *Alicyclobacillus* spp., estudos acima mencionados corroboram nossas descobertas, uma vez que indicam que a atividade antimicrobiana aumenta com o tempo de fermentação, e é atribuído às substâncias sintetizadas durante o processo. Em adição, danos na estrutura do micro-organismo causado pelos extratos fermentados foram observados pela técnica de Microscopia Eletrônica de Varredura. A identificação dos metabólitos presentes através de cromatografia líquida (UHPLC-Qtof-MS), demonstrou que os extratos fermentados apresentaram maior número de compostos comparado aos extratos não fermentados, dentre eles, ácido glucurônico, succínico e glutárico. E por fim, uma potencial capacidade antioxidante dos extratos foi observada pelos radicais DPPH, FRAP e ABTS.

CONCLUSÕES: Os resultados mostram que os subprodutos das frutas, fermentados ou não com kefir, apresentaram propriedades bioativas, como o potencial antimicrobiano contra as cepas *Alicyclobacillus* testadas, e o potencial antioxidante, o que resulta num produto de valor agregado. Além disso, nossos achados mostram um aumento na atividade antimicrobiana com períodos de fermentação mais longos, com potencial para serem explorados como um agente antimicrobiano na indústria alimentícia. No entanto, mais pesquisas são necessárias para avaliar o uso de tais extratos em bebidas cítricas que podem se deteriorar devido à presença de *Alicyclobacillus* spp..

Palavras chaves: biocompostos, fermentação, compostos antimicrobianos, preservação, deterioração, subprodutos.

Brazilian Journal of Development

ARTICLE 1

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

3.1 MINIMUM 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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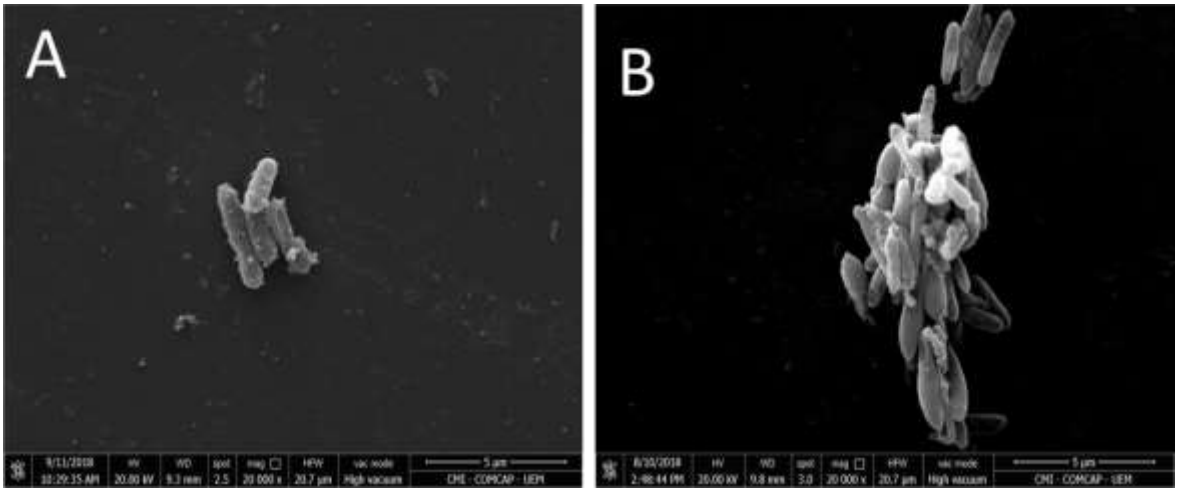
3.2 SCANNING ELECTRON MICROSCOPY (SEM)

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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ARTICLE 2

Relevância do trabalho: Temos a satisfação de encaminhar nosso trabalho para a apreciação e avaliação dessa conceituada revista para possível publicação. Nosso estudo investigou o potencial efeito de metabólitos, após fermentação de Kefir em vários subprodutos industriais de polpa de frutas como: morango, uva e acerola. Os metabólitos foram testados frente a *Alicyclobacillus* spp., bactéria deteriorante de suco de bebidas ácidas industrializadas como suco concentrado de laranja de exportação. Os resultados demonstraram atividade antibacteriana satisfatória, além de alta capacidade antioxidante. Assim, o que foi reportado em nosso artigo, sugere uma alternativa possível e sustentável para indústria de alimentos utilizar metabólitos ativos do fermentado de kefir com subprodutos de frutas.

Título em inglês: Fruit by-products fermented by kefir: anti-*Alicyclobacillus* spp. activity, and antioxidant activity

Título para cabeçalho: By-products fermented by kefir: anti-*Alicyclobacillus* spp.

Abstract

The present study aimed to investigate antimicrobial activity of fruit by-products fermented by kefir against strains of *Alicyclobacillus* spp., and to determine chemical characterization and antioxidant activity. The results show that extracts fermented for a longer period (72 h) had greater inhibition, and the extract of acerola by-product fermented for 72 h had the best results. For all strains, the Minimal Inhibitory Concentration (MIC) was 0.78%, except for *A. acidocaldarius* subsp. *rittmannii*, which obtained 1.56%. The same applied to the Minimal Bactericidal Concentration (MBC), in which 1.56% of the extract was capable of inactivating *A. cycloheptanicus* and *A. acidocaldarius*. In addition, damages to the structure of the microorganisms caused by the extracts were verified by Scanning Electron Microscopy. Metabolite identification through liquid chromatography (UHPLC-Qtof-MS) demonstrated that the fermented extracts presented a greater number of compounds compared to the non-fermented ones, such as glucuronic, succinic and glutaric acids. It is possible to conclude that the extracts of fruit by-products demonstrated bioactive properties, such as antibacterial potential and antioxidant activity against the *Alicyclobacillus* strains tested, not to mention the added value attributed to the use of a food by-product.

Practical Application: Fruit by-products fermented with kefir showed antimicrobial activity against *Alicyclobacillus*.

Keywords: Bio-compound; Fermentation; Antimicrobial compound; waste.

1. Introduction

Among the microorganisms that represent a great concern in the food industry, *Alicyclobacillus* spp. strains stand out. They are non-pathogenic spore-forming bacteria that are related to the deterioration of drinks and citric juices (Anjos et al., 2018). Among the 25 species of *Alicyclobacillus* that exist today (Sokołowska et al., 2020), *A. acidoterrestris* is the most studied and challenging for the food industry since it alters products' sensory characteristics. It is also the most isolated species in deteriorated and non-deteriorated sour products (Sant'Ana et al., 2014; Sokołowska et al., 2020).

Natural compounds as an alternative to replace synthetic chemicals in the food industry is something under frequent research regarding their possible application in food products. In their composition biologically active compounds with antimicrobial effects are present, especially in plants extracts, such as spices, herbs, fruits, and vegetables (Cai et al., 2019; Castro-Rosas et al., 2017; Gyawali et al., 2015; Miao et al., 2016; Mostafa et al., 2018; Pascoli et al., 2018).

Moreover, fruit by-products, such as pomace, peel, and seeds, have a series of bio-compounds already reported. Fruit by-products can often present high levels of bioactive compounds compared to their own pulp. Among these bio-compounds, the group of the phenolic and organic acids stands out with possible natural antimicrobial and antioxidant properties (Arbos et al., 2013; Manna et al., 2015; Plaza et al., 2016; Rezende et al., 2017; Rochelle et al., 2016; Sousa et al., 2011).

Food by-products are a source of a wide variety of bioactive molecules such as vitamins, minerals, enzymes, pigments, functional ingredients, micronutrients, nutraceutical products, active pharmaceutical compounds, phytochemicals, biomaterials, and others. Approximately 1.3 billion tons of by-products are discarded annually all over the world (Arun et al., 2020; FAO, 2014). Therefore, it is important to think of ways to reduce waste and help to preserve

the environment. We need to resort to methods and technologies that allow converting by-products into value-added products. One of these methods is solid state fermentation, or submerged and liquid fermentation, performed to extract economically important compounds using substrates such as food by-products (Arun et al., 2020).

Among the natural compounds we can mention kefir, which are grains constituted by polysaccharides in combination with a complex microbiota containing different lactic acid, acetic acid, and yeast bacteria. They are used to ferment fruit, honey, vegetables, tea, and juices (Fiorda et al., 2016a; Gulitz et al., 2013; Marsh et al., 2014). The metabolites produced by the fermentation of kefir, such as ethanol and organic acids, have antimicrobial activity against deteriorative and pathogenic microorganisms, such as Gram-positive and Gram-negative bacteria (Dias et al., 2016; Kim et al., 2016). In this manner, the use of fruit by-products rich in bioactive compounds as a substrate for kefir fermentation, is a strategy for obtaining products with higher levels of bioactive compounds and antimicrobial properties.

Food contamination is a growing concern for consumers, regulatory organs, the food industry, and for public health in general, as it can cause diseases that can lead to death, besides economic, social, and environmental losses (Penha et al., 2017).

To avoid food deterioration and contamination, the food industry uses synthetic additives and physical methods like pasteurization that is widely used to inactivate deteriorative and pathogenic microorganisms. Yet, the spores of some species of the genera *Alicyclobacillus* can survive it and, consequently, deteriorate the product (Anjos et al., 2018; Anjos et al., 2016). As for synthetic chemicals, when used inadequately, can expose consumers to health risks. Besides, microorganisms can still resist some preservatives used (Machado et al., 2011; Mostafa et al., 2018). Due to these factors, the use of natural preservatives in food has been studied as a promising alternative to ensure safety and maintenance of nutritional and sensorial

attributes of food products (Cai et al., 2019; Machado et al., 2011; Mostafa et al., 2018; Pascoli et al., 2018).

The use of natural antimicrobial compounds in food can be an option to inactivate microorganisms and guarantee the final quality of a commercialized product. It is important to mention that the literature still does not present studies involving antimicrobial activity of fruit by-products fermented by kefir against *Alicyclobacillus* spp. Thus, the goal of this research was evaluate the antimicrobial activity of fruit (strawberry, grape and acerola) by-products (peels, seeds and pomace) fermented with kefir against six strains of *Alicyclobacillus* spp..

2. Material and Methods

2.1. Obtaining fruit by-products

In order to produce extracts fermented with kefir, we used the by-products from pulp processing (peels, seeds and pomace) of grape, acerola and strawberry. The by-products were obtained from Redondo Polpa de Frutas Industry, located in Cambé, Paraná - Brazil. The materials were kept frozen at -20 ºC until experimentation.

2.2. Kefir grains

Traditional water kefir grains were used as an inoculum in the fermentation process and were donated by artisanal producers from the city of Maringá, Paraná - Brazil. The grains were kept refrigerated (4 ºC) in glass flasks with water and 10% brown sugar and were used for the following fermentation.

2.3. Preparation of the fermented extracts

Strawberry, grape and acerola extracts were prepared from the wet and frozen fruit by-products in a 1: 2 ratio by-product: water. The solutions were mixed using a mixer then filtered with a previously sanitized plastic strainer, and 5% brown sugar was added. The extracts were fermented using kefir grains as an inoculum, fermentation temperature was 30 ºC, and time

varied between 24, 48 and 72 hours with an initial concentration of standardized inoculum at 10% (Kim et al., 2016, with modifications).

After fermentation, by-products were centrifuged at 10.000 rpm for 5 min and the supernatant underwent cold sterilization using a 0.22 µm filtering membrane (Millipore, São Paulo, Brazil). The extract of each by-product without fermentation or sugar was also centrifuged and filtered in the same manner to produce a negative control.

2.4. Bacterial strains

Strains of the species *Alicyclobacillus* spp. were used from the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI), located at the Chemical, Biological and Agricultural Research Center (CPQBA/UNICAMP), as a reference to this study: *A. acidoterrestris* 0244^{T}; *A. acidocaldarius* subsp. *rittmannii* 0245^{T}, *A. herbarius* 0246^{T}; *A. acidiphilus* 0247^{T}; *A. cycloheptanicus* 0297^{T}; *A. acidocaldarius* 0299^{T}. All strains remained stored at -20 °C in the Laboratory of Water, Environment and Food Microbiology of the State University of Maringá.

2.5. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The inhibitory and bactericidal minimum concentrations were determined with the microdilution technique in a 96-well microplate, according to the methodology CLSI – M07 – A11 (2018), with BAT (*Bacillus acidoterrestris*) broth as the culture medium. The microorganism was activated in the BAT broth for 48 h before the experiment and incubated at 45 °C (for the 0244^{T}, 0246^{T}, 0247^{T} and 0297^{T} strains) and 60 °C (for the 0245^{T} and 0299^{T} strains).

After 24 h, plating on BAT agar was performed and there was a new incubation at 45 °C and 60 °C, for 24 h. A standard saline suspension was prepared for the experiment in accordance with the McFarland 0.5 scale, equivalent to 10^{8} CFU/mL. The serial dilution of the fermented

extracts was performed with concentrations from 50 to 0.1%, with a final volume of 100 µL in each well.

The microorganism suspension was inoculated at the concentration of 10^4 CFU/mL in each well, after dilution of the standardized inoculum. The microplates were incubated at 45 °C and 60 °C for 24 h. The MIC was defined as the smallest extract concentration capable of inhibiting visual bacterial growth. After that period, 20 µL of each well were plated in BAT agar and incubated at 45 °C and 60 °C for 24 h, in which the smallest concentration capable of inactivating bacterial growth was considered the minimum bactericidal concentration. The experiments were carried out in triplicate and within three independent experiments.

2.6. Scanning electron microscopy (SEM)

The strain used was *A. acidoterrestris,* since it is the most isolated species in deteriorated sour products. The inoculum of *A. acidoterrestris* was treated with the extracts of acerola by-product fermented with kefir for 72 h (A72), strawberry by-product fermented with kefir for 72 h (S72) and grape by-product fermented with kefir for 72 h (G72), defined by the MIC antimicrobial activity, and positive control (only inoculum and culture medium). After incubation of the samples at 45 °C for 24 h, fixation was performed with glutaraldehyde at 2.5% in cacodylate buffer 0.1M and adhesion to glass slides pre-treated with poly-L-lysine. That was followed by dehydration with an increasing ethanolic series (30% - 100%), critical point with CO_2, gold plating and analysis in a scanning electron microscope Quanta-250 (Endo et al., 2010).

2.7. Antioxidant capacity

2.7.1. DPPH method

Antioxidant capacity was measured by sequestration of DPPH radicals (2,2-diphenyl-1-picrylhydrazyl), as in Dutra et al. (2019), Ma et al. (2011) and Mizuta et al. (2020), with modifications. 25 µL of each extract dilution and 2 mL of the standardized solution of 6.25 ×

174 10^{-5} mol/L of DPPH, were placed in dark flasks and kept for 30 min in the dark. Methyl alcohol
175 was used to calibrate the spectrophotometer. Scanning was performed with a spectrophotometer
176 at 517 nm, and a standard curve was constructed with the Trolox solution (acid (±)-6-hydroxy-
177 2,5,7,8-tetramethylchroman-2-carboxylic). The results were expressed as μM Trolox/mL of
178 extract.

179 **2.7.2. ABTS method**

180 Total antioxidant activity by ABTS followed the methods of Mizuta et al. (2020) and Rufino
181 et al. (2007b). Protected from light a 30 μL aliquot of each extract dilution was transferred and
182 mixed into test tubes with 3.0 mL of the ABTS radical solution (5 mL of ABTS solution at 7
183 mmol/L and 88 μL of potassium persulfate at 140 mmol/L; reaction in the dark for 16 h).
184 Reading was done at 734 nm 6 minutes after mixing and ethyl alcohol was used as blank.
185 Quantification was done through the Trolox standard curve, and the result was expressed as μM
186 Trolox/mL of extract.

187 **2.7.3. FRAP method**

188 The Ferric Reducing Antioxidant Power Assay (FRAP) was performed according to
189 Mizuta et al. (2020) and Rufino et al. (2006), with some modifications. Protected from light, a
190 90 μL aliquot of each extract was transferred, and added 270 μL of distilled water and 2.7 mL
191 of the FRAP reagent (25 mL of 0.3 M acetate buffer, 2.5 mL of 10 mM TPTZ and 2.5 mL of
192 20 mM Iron chloride), the solution was homogenized and incubated for 30 min. The reading
193 was done at 595 nm, using the FRAP reagent to calibrate the spectrophotometer. Quantification
194 was done through the ferrous sulfate standard curve, and the results were expressed as μM
195 ferrous sulfate /mL of extract.

196 **2.8. Identification of the metabolites by UHPLC-Qtof-MS**

197 Compound identification was carried out with the extracts of grape, acerola, and strawberry
198 before and after fermentation for 72 h. Aliquots were analyzed by UHPLC-HRMS using an

ultra-high performance liquid chromatography system Nexera X2 coupled to a mass spectrometer (Q-tofImpact II, Bruker, Germany). The chromatographic system was equipped with 2 30AD Pumps and a C_{18} Waters Symmetry® column (4.6 x 75 mm x 3.6 μm) kept at 40 °C with a linear gradient solution, using water (0.1% formic acid) (A) and acetonitrile (0.1% of formic acid) (B) as solvents, both of LC-MS purity grade. Chromatographic separation was done in 20 min. The gradient used was: 1 min, 95% of solvent A and 5% of solvent B; 10 min, 50% of solvent A and 50% of solvent B; 12 min, 5% of solvent A and 95% of solvent B; 13 min, 5% of solvent A and 95% of solvent B; 17 min, 95% of solvent A and 5% of solvent B; and 20 min, 95% of solvent A and 5% of solvent B. Flow was maintained at 0.20 mL/min during the whole chromatographic separation period. The mass spectrometer with an ionization source by *electrospray* (ESI) was operated in negative mode of ionization, with capillary voltage adjusted to 4.50 kV and 3.0 kV, respectively. The source temperature was kept at 200 °C, and the flow of desolvation gas at 8 L/min. The three most intense ions of each chromatographic peak were selected for fragmentation. The spectra were obtained at the range of *m/z* 50-800, and the acquisition rate was 5 Hz (MS and MS/MS). The fragmentation spectra were obtained by collision-induced dissociation at the range of 15-40 eV for negative mode (Castro et al., 2018; Mizuta et al., 2020, with modifications).

The ion chromatogram and the spectra MS and MS/MS were visualized by using the software *Data Analysis* 4.3, in comparison and analyzed in accordance with the free access mass spectrometry database - *The Human Metabolome Database* (HMDB) (Fahy et al., 2009).

2.9. Data treatment

The analyses were carried out in triplicate. The antioxidant data were treated through the variance analysis (ANOVA) and the *Tukey* test at the level of 5% significance ($p<0.05$) making use of the software SISVAR 5.3.

3. Results and Discussion

3.1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The antimicrobial properties of the extracts of fruit by-products fermented with kefir were tested against six species of *Alicyclobacillus* spp., as previously described. The results are shown in Table 1.

MIC and MBC results show that the extracts of the fruit by-products fermented for a longer period (72 h) had significant results against all strains tested. Proving that the fermentation process resulted in metabolites with antimicrobial capacity. The acerola extract fermented for 72 h stood out, presenting a MIC of 1.56% for the 0245^{T} strain, and 0.78% for the other strains, the same applies to acerola's A72 MBC with superior inhibition to the other strains. Only 1.56% of the extract was capable of inactivating 0297^{T} and 0299^{T} strains.

The bioactivity of kefir drinks has already been studied, and demonstrated to contain substances with anti-inflammatory, antioxidant and antimicrobial activities (Fiorda et al., 2017; Rodrigues et al., 2016). Kim et al. (2016) analyzed the same fermentation times of kefir used in this study, however with kefir fermented in milk, and achieved a more efficient antibacterial activity from 36-48 h of fermentation against *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogene*s, *Enterococcus faecalis*, *Escherichia coli*, *Salmonella enteritidis*, *Pseudomonas aeruginosa* and *Cronobacter sakazakii*. Silvia et al. (2009) also observed the action of kefir against yeast and pathogenic bacteria with the inhibition of *Candida albicans*, *Salmonella typhi*, *Shigella sonnei*, *E. coli* and *S. aureus* by kefir fermented for 144 h. Although the present work investigated extracts of fruit fermented with kefir against *Alicyclobacillus* spp., the aforementioned studies corroborate our findings, since they indicate that antimicrobial activity increases with fermentation time, and it is attributed to the substances synthesized during the process.

Mizuta et al. (2020) evaluated green tea and tea fermented by kombucha during 7 (K07) and 14 (K14) days against five species of *Alicyclobacillus* (*A. acidoterrestris* 0244^T, *A. herbarius* 0246^T, *A. acidiphilus* 0247^T, *A. cycloheptanicus* 0297^T and *A. hesperidum* 0298^T). The results showed that K07 and K14 were the most satisfactory, since they presented a MIC of 1.563 and 0.195%, respectively, for all strains. As for the MBC results, they varied. For K07 it was >50% for 0244^T and 0297^T, and 50% for the other strains. As for K14, the MBC was 25% for 0244^T and 0297^T strains, 12.5% for 0247^T and 0298^T, and 6.25% for 0246^T. In comparison, our results for A72 were more effective for the same strains tested.

It is relevant to mention that the kefir supernatant contains various metabolites and inhibitory compounds, such as organic acids, hydrogen peroxides, ethyl alcohol, diacetyl, peptides and possibly bacteriocins, which can contribute to the antimicrobial effects (Kim et al., 2016).

3.2.Scanning electron microscopy (SEM)

Morphological changes in the vegetative cells of *A. acidoterrestris* (0244^T) exposed to the extracts A72, S72 and G72 were observed through SEM (Figure 1). Control cells of *A. acidoterrestris* not treated with the extracts (Figure 1.A) visually presented a smooth cell surface with uniform and characteristic morphology. As for the cells treated with extracts fermented with kefir (Figure 1. B, C e D), there was a decrease in the number of cells relative to the control, with structural and morphological changes, in addition to disruption of the cell wall and, consequently, damage to the integrity of the bacterial cell and loss of genetic material, due to the antimicrobial effect of A72 (Figure 1.B), S72 (Figure 1.C) and G72 (Figure 1.D) extracts.

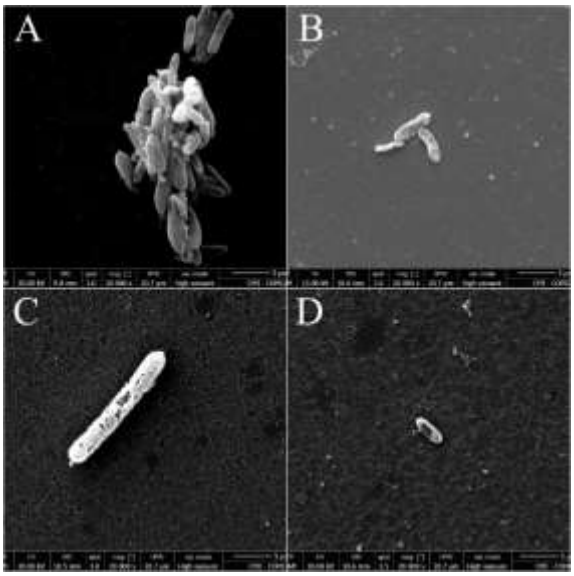

Figure 1. Scanning electron microscopy. A - control of *A. acidoterrestris* vegetative cells (Menezes, et al., 2020); B - MIC of acerola extract fermented with kefir for 72 hours against vegetative cells of *A. acidoterrestris*; C - MIC of strawberry extract fermented with kefir for 72 hours against vegetative cells of *A. acidoterrestris*; D - MIC of grape extract fermented with kefir for 72 hours against vegetative cells of *A. acidoterrestris*. Bar: 5 µm; Magnification: 20,000 X.

The action mechanisms of the natural compounds in relation to antimicrobial activity have not been elucidated yet. However, some studies mention factors such as the disruption of the cell membrane, which leads to extravasation of cellular content; organic acids can interfere with permeability of the membrane and inhibit NADH oxidation; the natural compounds attack the bilayer of phospholipids, interruption of enzymes systems and damage of genetic material, among others (Gyawali et al., 2015; Machado et al., 2011).

Table 1. Minimum Inhibitory and Bactericidal Concentrations (MIC and MBC) in percentage of extracts without fermentation and after fermentation with kefir (24, 48 and 72 h) against strains of *Alicyclobacillus* spp.

(%)	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Extract/strain	**0244^T**		**0245^T**		**0246^T**		**0247^T**		**0297^T**		**0299^T**	
SWF	25,00	>50,00	50,00	>50,00	25,00	>50,00	25,00	>50,00	25,00	>50,00	12,50	50,00
S24	6,25	25,00	12,50	50,00	6,25	25,00	6,25	>50,00	6,25	50,00	6,25	12,50
S48	1,56	25,00	3,13	6,25	1,56	6,25	3,13	>50,00	1,56	6,25	3,13	12,50
S72	0,78	25,00	1,56	3,13	0,78	3,13	1,56	50,00	0,78	3,13	1,56	6,25
GWF	25,00	50,00	25,00	25,00	25,00	50,00	25,00	50,00	25,00	>50,00	12,50	25,00
G24	12,50	50,00	12,50	12,50	6,25	25,00	12,50	50,00	6,25	12,50	6,25	25,00
G48	6,25	50,00	6,25	12,50	3,13	12,50	6,25	50,00	1,56	6,25	6,25	12,50
G72	3,13	50,00	1,56	12,50	3,13	12,50	3,13	50,00	0,78	1,56	3,13	6,25
AWF	3,13	>50,00	25,00	50,00	1,56	1,56	3,13	50,00	6,25	12,50	3,13	6,25
A24	3,13	50,00	6,25	12,50	1,56	3,13	3,13	50,00	3,13	6,25	3,13	6,25
A48	1,56	25,00	3,13	3,13	0,78	3,13	1,56	50,00	1,56	3,13	1,56	1,56
A72	0,78	12,50	1,56	3,13	0,78	3,13	0,78	25,00	0,78	1,56	0,78	1,56

SWF, S24, S48 and S72: Strawberry extract without fermentation and fermentation for 24, 48 and 72 h, respectively; GWF, G24, G48 and G72: Grape extract without fermentation and with fermentation of 24, 48 and 72 h, respectively; AWF, A24, A48 and A72: Acerola extract without fermentation and with fermentation of 24, 48 and 72 h, respectively. Strains of *A. acidoterrestris* (0244^T), *A. acidocaldarius* subsp. *rittmannii* (0245^T), *A. herbarius* (0246^T), *A. acidiphilus* 0247^T, *A. cycloheptanicus* (0297^T) and *A. acidocaldarius* (0299

3.3.Antioxidant activity

Antioxidant substances are the ones that slow or prevent food oxidation (Sucupira et al., 2012), and decrease oxidative damages caused to the human body by free radicals (Arshad et al., 2019). Natural antioxidants include several compounds, such as tocopherol, vitamin C, phenolic compounds, among others, which are present in plants and fruits (Arshad et al., 2019; Sucupira et al., 2012).

Table 2 presents the antioxidant activity values of the extracts of strawberry, grape and acerola with and without fermentation with kefir for 24, 48 and 72 h.

Regarding the strawberry extract, there was no significant difference between the samples of strawberry without fermentation and S24. The highest values were obtained for the strawberry extract. As for S48 and S72, they were different from the others, for both ABTS and FRAP. Regarding DPPH, there was a significant difference between all the fermentation times, except for S24 compared with S48, and S48 compared with S72.

For the grape extracts, the ABTS analysis resulted in no significant difference among the samples. The FRAP analysis showed significant difference among all samples, except for grape without fermentation and G48. Regarding DPPH, there was no significant difference between grape without fermentation and G48, G24 and G48, G24 and G72.

As for the acerola extracts, there was no significant difference among the samples regarding the analysis through the ABTS method. As for the FRAP analysis, there was a significant difference among all samples. DPPH pointed to a significant difference among all samples, except for acerola without fermentation and A24.

The results obtained in this study varied depending on the fruit by-product used and different fermentation times. In general, the extracts of the fruit by-products without fermentation presented a stronger antioxidant capacity compared to the three methods applied.

317 **Table 2.** Antioxidant capacity by ABTS, FRAP and DPPH of extracts from by-products without
318 fermentation and after fermentation.

	ABTS (µmol trolox/mL of sample)	FRAP (µmol de ferrous sulphate /mL of sample)	DPPH (µmol trolox/mL of sample)
SWF	2840,22[a]	7837,48[a]	2855,00[a]
S24	2745,04[a]	7955,28[a]	1882,50[b]
S48	2163,19[b]	5170,37[b]	1394,17[bc]
S72	1200,96[c]	3202,50[c]	855,83[c]
GWF	9376,28[a]	11831,48[a]	3910,00[a]
G24	8227,67[a]	9181,94[b]	2915,00[bc]
G48	8774,56[a]	11926,11[a]	3245,00[ab]
G72	8020,85[a]	6345,56[c]	2250,00[c]
AWF	15290,52[a]	12112,23[a]	3027,00[b]
A24	13564,96[a]	10876,12[b]	2894,43[b]
A48	14444,41[a]	9896,56[c]	3390,44[a]
A72	13057,19[a]	7832,90[d]	1665,89[c]

319 * Values with different lower-case letters in the same column, referring to the same fruit by-product, are significantly different
320 ($p < 0.05$) by the *Tukey*.
321 SWF, S24, S48 and S72: Strawberry extract without fermentation and fermentation for 24, 48 and 72 h,
322 respectively; GWF, G24, G48 and G72: Grape extract without fermentation and with fermentation of 24, 48 and
323 72 h, respectively; AWF, A24, A48 and A72: Acerola extract without fermentation and with fermentation of 24,
324 48 and 72 h, respectively.
325
326 Sousa et al. (2011) evaluated the antioxidant capacity of acerola, guava, pineapple, soursop,
327 bacuri and cupuaçu by-products. The acerola by-product was the one that presented the greatest

antioxidant capacity (aqueous extract) regarding the ABTS radical, thus, corroborating this study.

The fermentation process can synthesize or degrade compounds that present biological activity (Behera et al., 2018; Brito et al., 2012). As for the antioxidant capacity of the fermented extracts, there was a reduction compared with the fruit extracts that did not undergo fermentation. Santos (2015), when fermenting different cultivars of blueberry with *Saccharomyces cerevisiae* also obtained a remarkable reduction of the antioxidant capacity in comparison with the non-fermented fruit. Ferrandin (2014), when evaluating the antioxidant capacity of apple pomace extract and its alcoholic fermented product also verified that there was a reduction of the fermented product compared with not fermented extract by DPPH, ABTS and FRAP.

Products with antioxidant properties can reduce the risks of diseases through positive actions in the biological functions of the human body. Besides, the presence of antioxidants preserves the lifespan of drinks and avoids the development of unwanted tastes (Fiorda et al., 2016b).

3.4. Identification of metabolites by UHPLC-Qtof-MS

The UHPLC-Qtof-MS analysis for metabolite identification present in fermented and non-fermented fruit extracts is shown in Table 3. The metabolites were identified and confirmed by using an open access mass spectrometry database, called *Human Metabolome Database* (HMDB) (Fahy et al., 2009).

A variation of the compounds profile among the different samples was verified. Such variation results from the different extracts of the fruit by-products, and they are complemented by the action of the diversified microbiota of the kefir grains, capable of producing such compounds.

In total, thirteen compounds were identified, varying according to the extract analyzed. They are organic acids, such as citric acid, malic acid, gluconic acid, lactic acid, succinic acid, glutaric acid, tartaric acid, glucuronic acid, adipic acid, ascorbic acid; phenolic compounds, gallic acid and catechin; and D-glucose. Citric and gluconic acids were identified in all extracts. It was also verified that the fermented extracts obtained a greater number of compounds identified when compared with the non-fermented ones. Eleven compounds were identified in the extract of grape by-product fermented for 72 h (G72).

Microbiota in kefir grains produces organic acids, peptides, bacteriocins and fatty acids with antifungal, antibacterial and antioxidant activities (Ismaiel et al., 2011; Gerez et al., 2013; Erdogan et al., 2019). Organic acids, that result from the carbohydrate catabolism, contribute to a decrease in pH, making the environment hostile to most of the undesirable microorganisms (Dias et al., 2016). Garrote et al. (2000) in their studies with kefir against *E. coli* attributed the bacteriostatic effect to the organic acids metabolized during kefir fermentation.

Some compounds identified in this study are phenolic compounds, such as gallic acid and catechin. Such term refers to a group of secondary plants metabolites which contain aromatic rings, and can be replaced by hydroxyls, and the phenolic structures contribute to bioactive and antioxidant properties (Muhlack et al., 2018).

Silva et al. (2019), in their studies, found tartaric, malic and citric acid in grape juice samples, thus, corroborating our findings, which, besides these compounds, included gluconic and gallic acid in the grape extract without fermentation. This variation in the bioactive composition depends mainly on the grape used. The concentration of these compounds can also vary depending on the species, climate conditions, maturation stage, among other factors.

Bioactive compounds are found in fruit, and several metabolites are produced / synthesized during fermentation (Lopes et al., 2016). Moreover, with fermentation, some compounds can be converted into others, such as malic acid, converted to succinic acid due to the action of the

fumarase enzyme (Corsetti et al., 2016), which may have occurred with the strawberry extract, since in the non-fermented strawberry extract malic acid was identified, and in the fermented S72 extract only succinic acid was identified.

Citric and malic acids are commonly found in fermented fruit drinks, where they act as preservatives with antimicrobial properties. Besides, organic acids produced by yeast and bacterial species contribute to taste, unique aroma and texture, and they control the growth of undesirable microorganisms in food (Viana et al., 2017).

Therefore, the use of fruit by-products rich in bioactive compounds as a substrate for fermentation with kefir is a promising alternative to obtain products with a higher level of compounds with antimicrobial effect.

Table 3. Detection and identification of compounds present in strawberry, grape and acerola extracts without fermenting and after fermentation with kefir for 72 h, using UHPLC-MS analysis.

Compounds	**Molecular formula**	**Theoretical weight (*m/z*)**	**$[M+H^-]^-$**	**Error (ppm)**
		SWF		
Citric acid	$C_6H_8O_7$	191,0197	191,0197	0,0000
Malic acid	$C_4H_6O_5$	133,0142	133,0142	0,0000
Gluconic acid	$C_6H_{12}O_7$	195,0510	195,0511	0,5127
		S72		
Citric acid	$C_6H_8O_7$	191,0197	191,0195	-1,0470
Gluconic acid	$C_6H_{12}O_7$	195,0510	195,0504	-3,0761
Lactic acid	$C_3H_6O_3$	89,0244	89,0239	-5,6164
Succinic acid	$C_4H_6O_4$	117,0193	117,0190	-2,5637

Glutaric acid	$C_5H_8O_4$	131,0350	131,0342	-6,1052
Tartaric acid	$C_4H_6O_6$	149,0092	149,0085	-4,6977
GWF				
Citric acid	$C_6H_8O_7$	191,0199	191,0197	-1,0470
Malic acid	$C_4H_6O_5$	133,0142	133,0143	0,7518
Gluconic acid	$C_6H_{12}O_7$	195,0510	195,0511	0,5127
Gallic acid	$C_7H_6O_5$	169,0142	169,0138	-2,3667
Tartaric acid	$C_4H_6O_6$	149,0092	149,0092	0,0000
D-glucose	$C_6H_{12}O_6$	179,0561	179,0561	0,0000
G72				
Citric acid	$C_6H_8O_7$	191,0199	191,0197	-1,0470
Malic acid	$C_4H_6O_5$	133,0142	133,0142	0,0000
Gluconic acid	$C_6H_{12}O_7$	195,0510	195,0510	0,0000
Glucuronic acid	$C_6H_{10}O_7$	193,0355	193,0354	-0,5180
Lactic acid	$C_3H_6O_3$	89,0244	89,0242	-2,2466
Catechin	$C_{15}H_{14}O_6$	289,0718	289,0711	-2,4215
Gallic acid	$C_7H_6O_5$	169,0142	169,0137	-2,9583
Succinic acid	$C_4H_6O_4$	117,0193	117,0190	-2,5637
Glutaric acid	$C_5H_8O_4$	131,0350	131,0344	-4,5789

Adipic acid	$C_6H_{10}O_4$	145,0506	145,0506	0,0000
Tartaric acid	$C_4H_6O_6$	149,0092	149,0092	0,0000
AWF				
Ascorbic acid	$C_6H_8O_6$	175,0249	175,0249	0,0000
Citric acid	$C_6H_8O_7$	191,0197	191,0202	2,6175
Malic acid	$C_4H_6O_5$	133,0144	133,0143	-0,7518
Gluconic acid	$C_6H_{12}O_7$	195,0510	195,0510	0,0000
A72				
Ascorbic acid	$C_6H_8O_6$	175,0248	175,0248	0,0000
Citric acid	$C_6H_8O_7$	191,0198	191,0198	0,0000
Malic acid	$C_4H_6O_5$	133,0139	1330139	0,0000
Gluconic acid	$C_6H_{12}O_7$	195,0510	195,0510	0,0000
Glucuronic acid	$C_6H_{10}O_7$	193,0354	193,0354	0,0000

SWF, S24, S48 and S72: Strawberry without fermentation and fermentation for 24, 48 and 72 h, respectively; GWF, G24, G48 and G72: Grape without fermentation and fermentation of 24, 48 and 72 h, respectively; AWF, A24, A48 and A72: Acerola without fermentation and with fermentation of 24, 48 and 72 h, respectively.

4. Conclusion

The non-fermented extracts of fruit by-products and the ones after fermentation with kefir presented bioactive properties, such as antimicrobial potential against the *Alicyclobacillus* strains tested, and antioxidant potential, which results in a value-added product. Moreover, our findings show an increase in antimicrobial activity with longer fermentation periods, and identified the number of metabolites through UHPLC-Qtof-MS.

Among the extracts, A72 had the best MIC and MBC results, with potential to be explored as an antimicrobial agent in the food industry. Yet, further research is necessary to evaluate the use of such extracts in citrus drinks that could deteriorate due to the presence of *Alicyclobacillus*.

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