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Kefir fermented fruit by-products: anti-*Alicyclobacillus* spp. activity, and antioxidant activity

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Abstract

This study aimed to investigate the antimicrobial activity of kefir fermented fruit by-products against strains of *Alicyclobacillus* spp., and determine their chemical characterization and antioxidant activity. According to the results, extracts fermented for a longer period (72 h) showed greater inhibition, and the extract of acerola by-product fermented for 72 h achieved the best results. For all strains, the Minimal Inhibitory Concentration (MIC) was 0.78%, except for *A. acidocaldarius* subsp. *rittmannii*, (1.56%). The same applies 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 detected by Scanning Electron Microscopy. Metabolite identification through liquid chromatography (UHPLC-Qtof-MS) showed that the fermented extracts had a greater number of compounds compared to the non-fermented ones, such as glucuronic, succinic and glutaric acids. In conclusion, the extracts of fruit by-products showed to have bioactive properties, such as antibacterial potential and antioxidant activity against the *Alicyclobacillus* strains tested in this study, not to mention the value added by the use of a food by-product.

Keywords: bio-compound; fermentation; antimicrobial compound.

Practical Application: Kefir fermented fruit by-products showed antimicrobial activity against *Alicyclobacillus*.

1 Introduction

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

There has been a great deal of research on the use of natural compounds to replace synthetic chemicals in the food industry, specifically regarding their possible application in food products. In their composition, there are biologically active compounds with antimicrobial effects, 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 that have already been reported in the literature. Fruit by-products can often have high levels of bioactive compounds compared to their 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).

According to the United Nations Environment Programme (2021), approximately 931 million tons (about 17%) of all the food available to consumers in 2019 were wasted by households, restaurants, and other retail food establishments. Therefore, it is important to find ways to reduce waste and, thus, help to preserve the environment. We need to adopt 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, wich is performed to extract economically important compounds using substrates, such as food by-products (Arun et al., 2020).

One of these natural compounds is kefir, which are grains constituted by polysaccharides in combination with a complex microbiota containing different lactic acids bacteria, acetic

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acids bacteria, and yeast. They are used to ferment fruit, honey, vegetables, tea, and juices (Moretti et al., 2022; Fiorda et al., 2016b; Gulitz et al., 2013; Marsh et al., 2014). The metabolites produced by the kefir fermentation, such as ethanol and organic acids, show antimicrobial activity against deteriorative and pathogenic microorganisms, such as Gram-positive and Gram-negative bacteria (Dias et al., 2016; Kim et al., 2016), as well as *Salmonella typhimurium, E. coli,* and *Staphylococcus aureus* (Romero-Luna et al., 2020). It has also proved to inhibit filamentous fungi, such as *Aspergillus flavus* (Gonda et al., 2019), and *A. ochraceus* (Velez & Peláez, 2015). Therefore, 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 may lead to death, not to mention economic, social, and environmental losses (Penha et al., 2017).

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 have studies involving the antimicrobial activity of kefir fermented fruit by-products against *Alicyclobacillus* spp. Thus, the goal of this research was to evaluate the antimicrobial activity of kefir fermented fruit (strawberry, grape, and acerola) by-products (peels, seeds, and pomace) against six strains of *Alicyclobacillus* spp.

2 Material and methods

2.1 Obtaining fruit by-products

To produce extracts fermented with kefir, we used by-products obtained from pulp processing (peels, seeds and pomace) of grape, acerola and strawberry. The by-products were donated by Redondo Polpa de Frutas Industry, Cambé, Paraná - Brazil. The materials were kept frozen at -20 °C until experimentation.

2.2 Kefir grains

Traditional water kefir grains were used as the inoculum in the fermentation process. They were donated by artisan producers from Maringá, in the state of Paraná, Brazil. The grains were kept refrigerated (4 °C) in glass flasks with water and 10% brown sugar, and were used for posterior 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 ratio 1:2 (by-product: water). The solutions were mixed by using a mixer, filtered with a previously sanitized plastic strainer, and 5% brown sugar was added. The extracts were fermented with kefir grains as an inoculum, and the fermentation temperature was 30 °C. The time varied (24, 48 and 72 h), and initial concentration of the standardized inoculum was 10% (Kim et al., 2016, with modifications).

After fermentation, the 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 procedure to produce a negative control.

2.4 Bacterial strains

Strains of the species *Alicyclobacillus* spp., obtained from the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI), at the Chemical, Biological and Agricultural Research Center (CPQBA) of the State University of Campinas (UNICAMP), were used as a reference in this study. They are *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, in Maringá, Paraná - Brazil.

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

MIC and MBC were determined by the microdilution technique in a 96-well microplate, according to the CLSI – M07 – A11 methodology (Clinical and Laboratory Standards Institute, 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, the samples were plated onto BAT agar and incubated again at 45 °C and 60 °C, for another 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 onto BAT agar and incubated at 45 °C and 60 °C for 24 h, where the smallest concentration capable of inactivating bacterial growth was considered the minimum bactericidal concentration. The experiments were carried out in triplicate and independently.

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 kefir fermented acerola by-product for 72 h (A72), kefir fermented strawberry by-product for 72 h (S72), and kefir fermented grape by-product for 72 h (G72), defined by the MIC antimicrobial activity, and

positive control (only the inoculum and culture medium). After incubation at 45 °C for 24 h, sample fixation was performed with glutaraldehyde at 2.5% in cacodylate buffer 0.1 M and adhesion to glass slides pretreated 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

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 and 2 mL of the standardized solution of 6.25×10^{-5} mol/L of DPPH were placed in dark flasks and kept for 30 min in the dark. Methyl alcohol was used to calibrate the spectrophotometer. Scanning was performed with a spectrophotometer at 517 nm, and a standard curve was constructed with the Trolox solution (acid (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic). The results were expressed as µM Trolox/mL of the extract.

ABTS method

Total antioxidant activity by ABTS followed the methods of Mizuta et al. (2020) and Rufino et al. (2007). Protected from light, a 30 µL aliquot of each extract dilution was transferred and mixed into test tubes with 3 mL of the $ABTS^{\bullet+}$ radical solution (5 mL of ABTS solution at 7 mmol/L and 88 µL of potassium persulfate at 140 mmol/L; reaction in the dark for 16 h). Reading was done at 734 nm, 6 min after mixing, and ethyl alcohol was used to calibrate the spectrophotometer. Quantification was done through the Trolox standard curve, and the result was expressed as µM Trolox/mL of the extract.

FRAP method

The Ferric Reducing Antioxidant Power Assay (FRAP) was performed according to Mizuta et al. (2020) and Rufino et al. (2006), with some modifications. Protected from light, a 90 µL aliquot of each extract was transferred. Then, we added 270 µL of distilled water and 2.7 mL of the FRAP reagent (25 mL of 0.3 M acetate buffer, 2.5 mL of 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) and 2.5 mL of 20 mM Iron chloride). After that, the solution was homogenized and incubated for 30 min. Reading was done at 595 nm, using the FRAP reagent to calibrate the spectrophotometer. Quantification was done through the ferrous sulfate standard curve, and the results were expressed as µM ferrous sulfate/mL of the extract.

2.8 Identification of the metabolites by UHPLC-Qtof-MS

Compound identification was done with the extracts of grape, acerola, and strawberry before and after fermentation for 72 h. The 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 two 30AD Pumps and a C_{18} Waters Symmetry® column (4.6 mm (inner diameter) x 75 mm (length) x 3.6 µm (particle size)) 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 the 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 desolvation gas flow 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 MS and MS/MS spectra 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 ($p < 0.05$), with 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 the kefir fermented fruit by-products 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, which proves that the fermentation process resulted in metabolites with antimicrobial capacity. The acerola extract fermented for 72 h stood out, for it presented MIC of 1.56% for the 0245^T strain, and 0.78% for the other strains. The same applies to acerola's A72 MBC, whose inhibition was superior to that of the other extracts, where 1.56% of the extract was enough to inativate 0297^T and 0299^T strains.

The bioactivity of kefir drinks has already been studied, and it has proved to contain substances with anti-inflammatory,

Table 1. Minimum inhibitory and bactericidal concentrations (MIC and MBC) by percentage of extracts without 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 and after fermentation for 24, 48, and 72 h, respectively; GWF, G24, G48 and G72: Grape extract without and after fermentation for 24, 48, and 72 h, respectively; AWF, A24, A48, and A72: Acerola extract without and after fermentation for 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^T).

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, although with kefir fermented in milk, and achieved a more efficient antibacterial activity from 36 to 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 this study 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, which it due to the substances synthesized during the process.

Mizuta et al. (2020) evaluated green tea and kombucha fermented tea for 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 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, 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 important to mention that 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).

Lactic acid, identified in most fermented samples, is the final product of fermentation and prevents the production of energy from certain bacteria, fungi and some other organisms (Black & Black, 2021), which can be a justification that affects the cellular components *of Alicyclobcillus* spp. and obtains more satisfactory results for fermented samples. Furthermore, Magalhães et al. (2010) describes that lactic acid is the result of homofermentative metabolism, and it is of great importance due to the inhibitory effect on pathogenic microorganisms.

3.2 Scanning Electron Microscopy (SEM)

Morphological changes in the vegetative cells of *A. acidoterrestris* (0244^T) exposed to the A72, S72, and G72 extracts were observed through SEM (Figure 1). Control cells of *A. acidoterrestris* not treated with the extracts (Figure 1A) visually presented a smooth cell surface with uniform and characteristic morphology. As for the cells treated with extracts fermented with kefir (Figure 1B-1D), there was a decrease in the number of cells related 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 1B), S72 (Figure 1C) and G72 (Figure 1D) extracts.

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).

Although the vegetative cells of *A. acidoterrestris* adapt to acidic environments due to their tolerance to lethal stresses, improvement of membrane integrity, decreased shrinkage, and roughness of their surface (Zhao et al., 2022), recent studies have shown that the association of organic acids with other chemical substances resulting from fermentation is responsible for cell

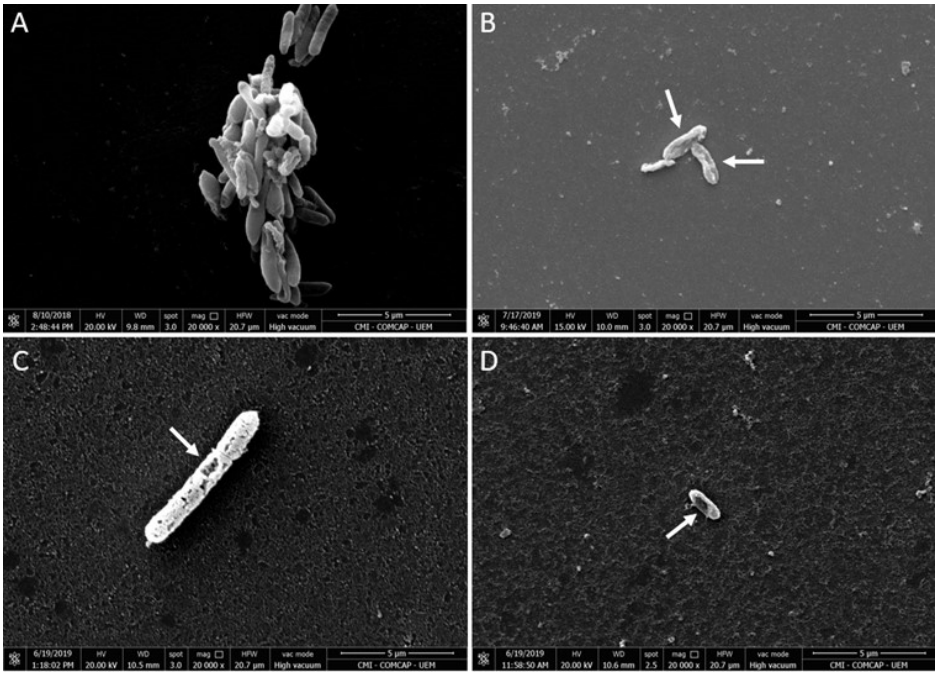

Figure 1. Scanning electron microscopy. A: control of *A. acidoterrestris* vegetative cells (Menezes et al., 2020); B: MIC of acerola extract fermented with kefir for 72 h against vegetative cells of *A. acidoterrestris*; C: MIC of strawberry extract fermented with kefir for 72 h against vegetative cells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

wall rupture, cell deformation and microbial growth reduction (Mizuta et al., 2020; Ju et al., 2021).

3.3 Antioxidant activity

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

The strawberry (SWF), grape (GWF) and acerola (AWF) extracts, as well as their respective extracts fermented with kefir in 24, 48 and 72 h (S24, S48, S72, G24, G48, G72, A24, A48, and A72), demonstrated significant difference compared to the different antioxidant tests (DPPH, ABTS and FRAP method), with the exception of SWF and A72 para FRAP method.

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

Sousa et al. (2011) evaluated the antioxidant capacity of acerola, guava, pineapple, soursop, bacuri, and cupuaçu by-products. The acerola by-product was the one with the greatest antioxidant capacity (aqueous extract) regarding the ABTS radical, which corroborates our finding.

Santos (2015), when fermenting different blueberry cultivars with *Saccharomyces cerevisiae,* also achieved a remarkable reduction of 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 found a reduction in the fermented product compared with the extract did not undergo fermented though the DPPH, ABTS and FRAP methods.

It is important to note that, 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). 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., 2016a).

3.4 Identification of metabolites by UHPLC-Qtof-MS

The UHPLC-Qtof-MS analysis for metabolite identification in fermented and non-fermented fruit extracts is shown in Figure 3. The metabolites were identified and confirmed by using an open-access mass spectrometry database, the *Human Metabolome Database* (HMDB). The was a variation in the compounds profile among the different samples. Such variation results from the different extracts of the fruit by-products, which are complemented by the action of the diversified microbiota of the kefir grains, capable of producing such compounds.

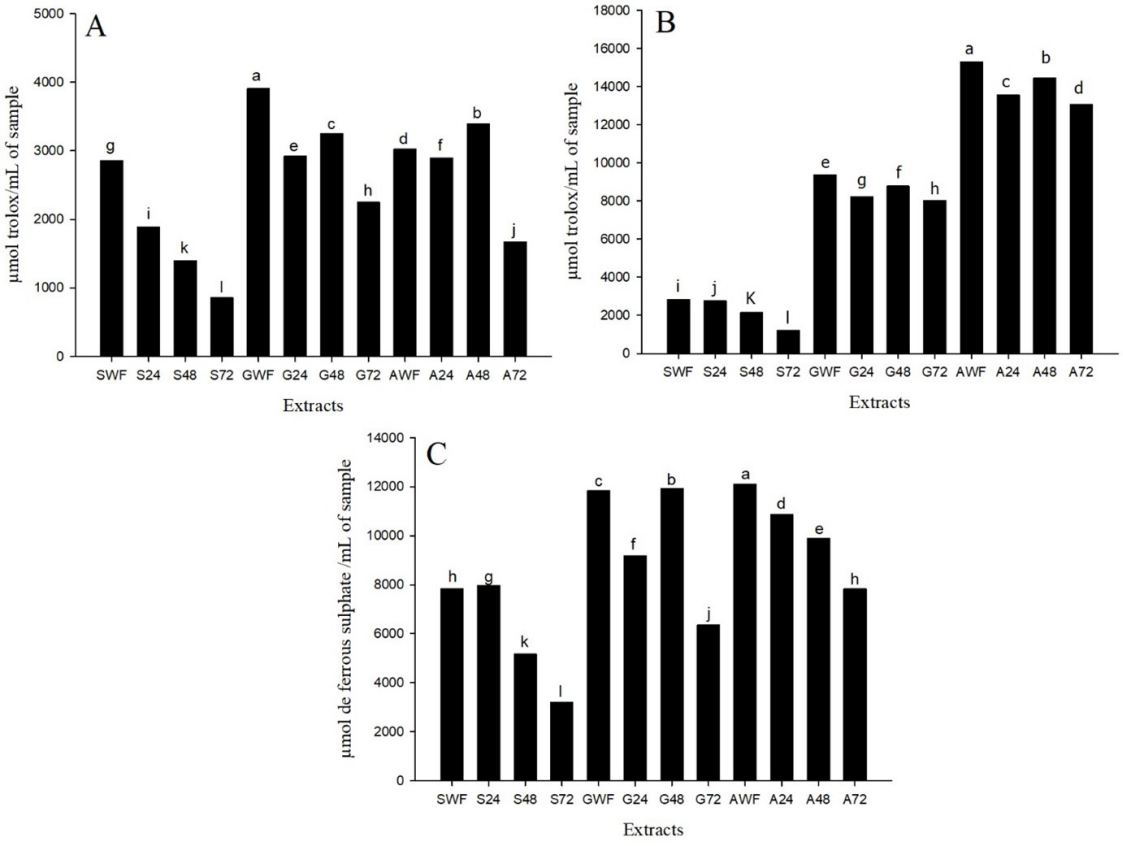

Figure 2. Antioxidant capacity by the ABTS, FRAP, and DPPH methods of extracts from by-products without fermentation and after fermentation. Values with different lower-case letters in the same column, referring to the same fruit by-product, are significantly different ($p < 0.05$) by the *Tukey* test. SWF, S24, S48, and S72: Strawberry extract without fermentation and exposed to fermentation for 24, 48, and 72 h, respectively; GWF, G24, G48, and G72: Grape extract without fermentation and exposed to fermentation for 24, 48, and 72 h, respectively; AWF, A24, A48, and A72: Acerola extract without fermentation and exposed to fermentation for 24, 48 and 72 h, respectively.

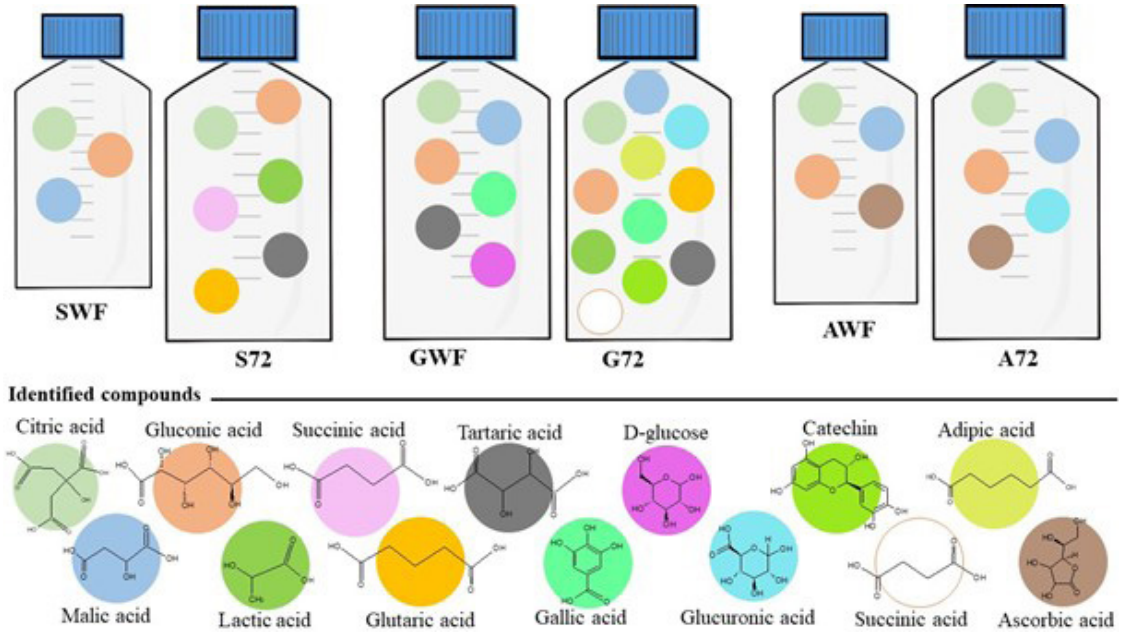

Figure 3. Identified compounds found in strawberry, grape, and acerola extracts without and after fermentation with kefir for 72 h, using UHPLC-MS analysis. SWF and S72: Strawberry without and after fermentation for 72 h, respectively; GWF and G72: Grape without and after fermentation for 72 h, respectively; AWF and A72: Acerola without and after fermentation for 72 h, respectively.

In total, thirteen compounds were identified, varying according to the extract analyzed. They are organic acids, such as citric acid ($C_6H_8O_7$, Theoretical weight (*m/z)* 191.0197), malic acid ($C_4H_6O_5$, *m/z* 133.0142), gluconic acid ($C_6H_{12}O_7$, *m/z* 195.0510), lactic acid ($C_3H_6O_3$, *m/z* 89.0244), succinic acid ($C_4H_6O_4$, *m/z* 117.0193), glutaric acid ($C_5H_8O_4$, *m/z* 131.0350), tartaric acid ($C_4H_6O_6$, *m/z* 149.0092), glucuronic acid ($C_6H_{10}O_7$, *m/z* 193.0355), adipic acid ($C_6H_{10}O_4$, *m/z* 145.0506), and ascorbic acid ($C_6H_8O_6$, *m/z* 175.0249); phenolic compounds, gallic acid ($C_7H_6O_5$, *m/z* 169.0142) and catechin ($C_{15}H_{14}O_6$, *m/z* 289.0718); and D-glucose ($C_6H_{12}O_6$, *m/z* 179.0561). 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), as Citric acid ($[M+H^-]^-$ 191.0197, Error (E): -1.0470 ppm), Gluconic acid ($[M+H^-]^-$ 195.0510, E: 0.0000 ppm), Malic acid ($[M+H^-]^-$ 133.0142, E: 0.0000 ppm), Gallic acid ($[M+H^-]^-$ 169.0137, E: -2.9583 ppm), Tartaric acid ($[M+H^-]^-$ 149.0092, E: 0.0000 ppm), Glutaric acid ($[M+H^-]^-$ 131.0344, E: -4.5789 ppm), Succinic acid ($[M+H^-]^-$ 117.0190, E: -2.5637 ppm), Glururonic acid ($[M+H^-]^-$ 193.0355, E: -0.5180 ppm), Lactic acid ($[M+H^-]^-$ 89.0242, E: -2.2466 ppm), Catechin ($[M+H^-]^-$ 289.0711, E: -2.4215 ppm) and Adipic acid ($[M+H^-]^-$ 145.0506, E: 0.0000 ppm).

For the other extracts, the compounds identified were: SWF: Citric acid ($[M+H^-]^-$ 191.0197, E: 0.000 ppm), Malic acid ($[M+H^-]^-$ 133.0142, E: 0.000 ppm) and Gluconic acid ($[M+H^-]^-$ 195.0511, E: 0.5127 ppm); S72: Citric acid ($[M+H^-]^-$ 191.0195, E: -1.0470 ppm), Gluconic acid ($[M+H^-]^-$ 195.0504, E: -3.0761 ppm), Lactic acid ($[M+H^-]^-$ 89.0239, E: -5.6164 ppm), Succinic acid ($[M+H^-]^-$ 117.0190, E: -2.5637 ppm), Glutaric acid ($[M+H^-]^-$ 131.0350, E: -6.1052 ppm) and Tartaric acid ($[M+H^-]^-$ 149.0085, E: -4.6977 ppm); GWF: Citric acid ($[M+H^-]^-$ 191.0197, E: -1.0470 ppm), Gluconic acid ($[M+H^-]^-$ 195.0510, E: 0.5127 ppm), Malic acid ($[M+H^-]^-$ 133.0143, E: 0.7518 ppm), Gallic acid ($[M+H^-]^-$ 169.0138, E: -2.3667 ppm), Tartaric acid ($[M+H^-]^-$ 149.0092, E: 0.0000 ppm) and D-glucose ($[M+H^-]^-$ 179.0561, E: 0.0000 ppm); AWF: Ascorbic acid ($[M+H^-]^-$ 175.0249, E: 0.0000 ppm), Citric acid ($[M+H^-]^-$ 191.0202, E: 2.6175 ppm), Malic acid ($[M+H^-]^-$ 133.0143, E: -0.7518 ppm) and Gluconic acid ($[M+H^-]^-$ 195.0510, E: 0.0000 ppm); and A72: Ascorbic acid ($[M+H^-]^-$ 175.0248, E: 0.0000 ppm), Citric acid $[M+H^-]^-$ 191.0198, E: 0.0000 ppm), Malic acid ($[M+H^-]^-$ 133.0139, E: 0.0000 ppm), Gluconic acid ($[M+H^-]^-$ 195.0510, E: 0.0000 ppm) and Glucuronic acid ($[M+H^-]^-$ 193.0354, E: 0.0000 ppm).

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, which 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 on 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. The term refers to a group of secondary plant metabolites that contain aromatic rings and can be replaced by hydroxyls. The phenolic structures, in turn, contribute to bioactive and antioxidant properties (Muhlack et al., 2018).

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

Bioactive compounds are found in fruit, and several metabolites are produced/synthesized during fermentation (Lopes et al., 2016). Furthermore, because of fermentation, some compounds can be converted into others, such as malic acid into succinic acid, due to the action of the fumarase enzyme (Corsetti et al., 2011). This is what may have happened to the strawberry extract, as malic acid was identified in the non-fermented strawberry extract, while only succinic acid was identified in the fermented S72 extract.

Citric and malic acids are commonly found in fermented fruit drinks, where they act as preservatives with antimicrobial properties. In addition, organic acids produced by yeast and bacterial species contribute to taste, unique aroma and texture, besides controlling 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.

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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