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RESEARCH ARTICLE

Antibacterial activity and antioxidant capacity of dairy kefir beverages

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Kefir traditionally is based on pasteurised milk and the use of other substrates is unusual. Kefir beverages made with raw milk (RMK), pasteurised milk (PMK) or whey (WK), were obtained and fractionated in microbial cells and supernatants. Antioxidant and antibacterial activity of fractions against Cronobacter sakazakii *and* Escherichia coli *were analysed. The highest antioxidant activity was obtained for PMK with values between 8500 and 10 000 μm TE/mg. Microbial extracts from RMK and PMK showed a reduction of* C. sakazakii *of 7 log units; while microbial extracts and supernatants from RMK decreased* E. coli *in 3 and 5 log units, respectively. These results indicate that dairy kefir beverages can be an excellent source of defensive agents to fight against bacteria that cause gastrointestinal disorders.*

Keywords Kefir beverages, Kefir whey, Antioxidant, Emerging pathogens, *Cronobacter sakazakii*, *Escherichia coli*.

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INTRODUCTION

According to the Codex Alimentarius on Milk and Milk Products, kefir is a fermented milk product prepared from kefir grains consisting of *Lactobacillus kefiri*, and species of the genera *Leuconostoc*, *Lactococcus* and *Acetobacter*, growing in a solid specific relationship. Kefir granules are also constituted by both lactose-fermenting yeasts (*Kluyveromyces marxianus*) and nonlactose-fermenting yeasts (*Saccharomyces unisporus*, *Saccharomyces cerevisiae* and *Saccharomyces exiguus*) (Magalhães-Guedes and Magalhães 2016; FAO/WHO 2018). Native of the Balkan-Caucasus region, kefir is described as an acidic, viscous, and mildly alcoholic fermented milk, whose grains can vary in size from 0.3 to 3.5 cm of diameter (Tamang *et al.* 2020; Al-Mohammadi *et al.* 2021).

Kefir is produced by mixing grains with milk in empirically determined proportions. Afterwards, milk is fermented in a temperature range between 8 and 25°C, at a variable time from 10 to 40 h, although the most common incubation time is 24 h (Rosa *et al.* 2017). Kefir grains harbour a microbial diversity composed of homofermentative lactic acid bacteria, heterofermentative lactic acid bacteria, and lactose-assimilating and nonlactose-assimilating yeasts. These microorganisms influence the lactic and alcoholic fermentation of kefir, and their composition varies according to culture origin, substrate, and storage conditions (Prado *et al.* 2015).

Research studies have reported the positive health effects of kefir, mainly due to kefiran, its main polysaccharide. It is attributed with antitumour, anti-carcinogenic, anti-diabetic, immunomodulatory, anti-hypertensive and hypocholesterolaemic properties (Azizi *et al.* 2021). However, one of the most relevant characteristics attributed to kefir is its antimicrobial capacity, which has been reported against many microbial pathogens (Azizi *et al.* 2021; Gonzalez-Orozco *et al.* 2022). The inhibitory activity of kefir against pathogens is due to

metabolites produced by the microorganisms present in the grains. During fermentation, acidity increases while redox potential and nutrients decrease, affecting the growth and survival of pathogens (Rodríguez-Figueroa *et al*. 2017).

The World Health Organisation has recognised kefir as a functional food with potential therapeutic benefits due to its inhibitory effects on various pathogenic bacteria. Consequently, there is considerable interest in further research on kefir (Al-Mohammadi *et al*. 2021). Nevertheless, despite the acknowledgment of health benefits, the utilisation of dairy by-products like whey as a substrate for its production remains understudied.

Whey can be obtained from milk through acid precipitation of caseins, enzymatic coagulation or by combination of them, yielding acid, sweet, or mixed coagulation whey (Tânia and Malcata 2013). Whey obtained by coagulation in cheese manufacture, contains proteins that are considered as the ones with the highest quality in food industry (Sharma and Shah 2010), to which immunomodulatory, antioxidant and antimicrobial properties have been attributed (Tânia and Malcata 2013; Leon-Lopez *et al*. 2022). Whey proteins primarily include β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA) and lactoferrin (LF), which are also key contributors to the antioxidant activity of whey (Korhonen and Pihlanto 2006). The antioxidant activity of milk proteins is attributed to their hydrophobic and aromatic amino acids, which act as proton donators, reducing lipid peroxidation and scavenging free radicals (Kim *et al*. 2015; Leon-Lopez *et al*. 2022).

Some of the antimicrobial peptides derived from milk have been shown to exert activity against Gram-positive and Gram-negative bacteria, such as *Listeria*, *Salmonella*, *Escherichia*, *Staphylococcus* and *Helicobacter*; but also against fungi and yeasts (Korhonen and Pihlanto 2006). However, the capacity of kefir (Kim *et al*. 2016) or whey (Harouna *et al*. 2015) against some emerging pathogens, such as *Cronobacter sakazakii*, has not been deeply studied, being a very promising research line.

Cronobacter sakazakii belongs to the Enterobacteriaceae family and is a rod-shaped Gram-negative, motile, nonspore-forming and facultative anaerobic bacterium with resistance to osmotic stress, dryness and to a wide range of antibiotics (Shi *et al*. 2017; Bai *et al*. 2019). Considered as a newly emerging foodborne pathogen, it has been isolated from a wide range of food products, herbs, spices and starch (Shukla *et al*. 2016), as well as from water and soil (Harouna *et al*. 2020). It is capable of growing in a wide range of temperatures, from 6 to 47°C, and also has high resistance to low water activity (Abad *et al*. 2022a). Newborns and the elderly are the population groups most affected by *C. sakazakii* infection, with higher incidence and severity in preterm infants (Parra-Flores *et al*. 2018). Powdered infant formula milk is the major vehicle of transmission of this pathogen for neonates and infants, and its consumption is associated with the majority of *C. sakazakii* outbreaks (Shukla *et al*. 2016). When ingested by neonates, this bacterium causes disease conditions such as bacteraemia, necrotising enterocolitis, meningitis and sepsis; with a range of mortality from 40% to 80% (Ripolles *et al*. 2017; Gan *et al*. 2022), and survivors suffering from severe neurological disorders (Shi *et al*. 2017).

Escherichia coli belongs to the Enterobacteriaceae family and is a Gram-negative, rod-shaped, nonspore-forming, anaerobic, mesophilic bacterium. It is considered as the most important of foodborne pathogenic bacteria (Ekici and Dümen 2019). Based on virulence factors and phenotypic characteristics, pathotypes of diarrheagenic and extraintestinal pathogenic *E. coli* are differentiated from nonpathogenic *E. coli*. However, sometimes pathogenic *E. coli* cannot be distinguished from commensal *E. coli* based on specific virulence factors (Braz *et al*. 2020). Pathogenic *E. coli* strains, such as enterotoxigenic *E. coli*, are a major cause of the so-called traveller's diarrhoea and the childhood diarrhoea pathogen (Yang *et al*. 2017; Pokharel *et al*. 2023). Enterotoxigenic *E. coli* strains adhere to the intestinal lining and generate enterotoxins that initiate an ionic imbalance, leading to altered electrolyte homeostasis along with tissue fluid loss and the onset of diarrhoea (Pokharel *et al*. 2023). This type of enterotoxigenic *E. coli* infection normally causes watery diarrhoea, abdominal pain, nausea, vomiting and fever. Nevertheless, as a high dose of bacteria is necessary to produce infection, enterotoxigenic *E. coli* spreads through either food or water, rather than through human-to-human transmission (Yang *et al*. 2017). Moreover, *E. coli* strains have been found to be multi-drug resistant, indicating conceivable dangers to public health (Jang *et al*. 2017; Pokharel *et al*. 2023).

Therefore, the main objective of this study was to evaluate the antimicrobial capacity of dairy kefir products, obtained using three different types of substrates, against *C. sakazakii* and enterotoxigenic *E. coli*. In order to understand more deeply the function and potential of those products, the protein profile of each dairy beverage and their antioxidant activity have also been studied.

MATERIALS AND METHODS

Preparation of dairy kefir beverages

Three different dairy kefir beverages were produced using pasteurised cow milk, raw cow milk and whey from cow milk as substrates, obtaining pasteurised milk kefir (PMK), raw milk kefir (RMK) and whey kefir (WK). Pasteurised and raw cow milk were provided by the dairy industry Villacorona (El Burgo de Ebro, Zaragoza, Spain). The procedure to obtain whey from cow milk was performed as previously described (Buey *et al*. 2021). The kefir beverages were made using two types of starter cultures with the same lactic acid bacteria (LAB) and differing in a single

Table 1 Composition of the microorganisms of the two kefir starter cultures used in this study.

Strain	Kefir B	Kefir C
Lactococcus lactis subsp. *lactis*	+	+
Lactococcus lactis subsp. *cremoris*	+	+
Lactobacillus delbrueckii subsp. *bulgaricus*	+	+
Lactobacillus helveticus	+	+
Lacticaseibacillus rhamnosus	+	+
Lacticaseibacillus paracasei	+	+
Lactobacillus acidophilus	+	+
Streptococcus thermophilus	+	+
Bifidobacterium bifidum	+	+
Leuconostoc mesenteroides	+	–
Kluyveromyces marxianus	–	+

component: kefir B contained the bacterium *Leuconostoc mesenteroides*, whereas kefir C had the yeast *K. marxianus* (Abiasa, Pontevedra, Spain) (Table 1).

The different types of kefir were prepared in volumes of 600–800 mL and distributed in 250 mL glass containers. Fermentation lasted from 20 to 22 h at 25°C. All the kefir samples were subjected periodically to acidity and pH measurements. The sample taken from kefir beverages to measure pH and acidity was of 10 ml. The average pHs at the end of fermentation were of 4.35 for KPM, 4.36 for KRM and 4.25 for KW. The mean of acidity values was of 50–70 °Dornic for KPM, 70–90 °Dornic for KRM and 100 °Dornic for KW. The kefir beverages were labelled with the name of the beverage used (RMK, PMK, and WK) followed by the time in which measurements were performed (1, after fermentation; 2, after 7 days under refrigeration; and 3, after 14 days under refrigeration) and the starter culture used for fermentation (B or C).

Fractionation of dairy kefir beverages

A sample of each type of kefir drink was subjected to mechanical fractionation by using a refrigerated centrifuge GZ-1736R (Gyrozen, Kimpo, South Korea) for 15 min at 10 000 **g** to obtain two different fractions: the microbial fraction and the supernatant with the postbiotic components of the kefir beverages.

The microbial cell fractions were transferred to a vial of the Precellys CKMix Tissue Homogenising Lysing Kit (Bertin Instruments, Montigny-le-Bretonneux, France) and underwent a treatment by using the Precellys® 24 homogeniser (Bertin Instruments) for two cycles of 30 s at 6500 rpm and 10 s delay between cycles. Separation of the products obtained after lysis was completed with a centrifugation at 9500 **g** for 10 min at 4°C (Eppendorf, Hamburg, Germany) obtaining the microbial extracts.

Supernatants obtained were lyophilised and subsequently resuspended with the adequate volume of milli-Q water to obtain a final concentration of 40 mg/mL. All samples were sterilised using a 0.2 µm PES Whatman™ Filter Media (Cytiva, Marlborough, USA) and stored at −20°C until later use.

Lyophilised supernatants were brought to a 10 mg/mL solution and stirred magnetically for 15 min for their complete dissolution. A fraction of 4 mL was transferred to an Ultracel-10 K ultrafiltration tube (Milipore, Darmstadt, Germany), which was centrifuged at 4000 **g** for 5 min, obtaining two fractions: one with molecular weight greater than 10 kDa (>10 kDa) and another lower than 10 kDa (<10 kDa).

Determination of protein profiling and concentration

All kefir samples were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis to determine their protein profile. The SDS-PAGE was performed using precast 4–20% polyacrylamide gels on a Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories, Hercules, CA, USA) as previously described (Laemmli 1970). Following electrophoresis, gels were stained for 1 h with Coomassie Brilliant Blue R-250 and destained afterwards.

Protein concentration of supernatants and microbial extracts was determined by the bicinchoninic acid (BCA) assay (ThermoFisher Scientific, Waltham, MA, USA) following manufacturer instructions, using bovine serum albumin as standard and phosphate buffered saline (PBS) as diluent. The absorbance was measured at 560 nm. Each sample was assayed in triplicate.

Antioxidant assays

A colorimetric method based on DPPH (2,2- diphenyl-1-picrylhydrazyl) (Sigma Aldrich, San Louis, MO, USA) was used to assess the antioxidant capacity of the dairy kefir fractions. DPPH is a nitrogenous organic free radical, susceptible to react with antioxidant compounds, when those are capable of donate an electron. The reaction is carried out in a methanolic or ethanolic media and monitored spectrophotometrically at 520 nm, switching colour from violet to yellow when the reaction takes place (Huang *et al*. 2005).

Solutions of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) with various concentrations were made as standards and agitated for 30 min. Sample dilutions and standards, in a volume of 50 µL, were added to a 96-well microtitre plate by duplicate in two series, one of them with 150 µL of DPPH and the other one with 150 µL of ethanol: water (50:50). The plate was incubated in the dark for 60 min at 37°C and, afterwards, absorbance was measured at 520 nm. Firstly, control absorbance (A_{blank}) and sample absorbance (A_{sample}) were calculated as the difference between the

absorbance of the sample with DPPH and that without it. Subsequently, the percentage of DPPH reduction could be measured as the difference between the absorbance of control and sample, divided by the absorbance of control (Equation 1).

$$\mathrm{DPPH}\% = \left(\left(A_{\mathrm{blank}} - A_{\mathrm{sample}}\right)/A_{\mathrm{blank}}\right) \times 100 \qquad (1)$$

The DPPH reduction values for Trolox were used to obtain the standard curve, which was used to substitute in it the DPPH reduction values of the samples, thus expressing their antioxidant capacity as μM Trolox equivalent (TE). Considering the data obtained for the amount of protein by the BCA method, the antioxidant capacity could be expressed as μM TE/mg protein.

Culture of Gram-negative bacteria and antibacterial assays

The two Gram-negative bacteria strains used in this study were *C. sakazakii* CECT 858 (equivalent to strain ATCC 29544) supplied by the Spanish Type Culture Collection (CECT, Valencia, Spain) and a strain of enterotoxigenic *E. coli* isolated from a pig farm and donated by Pentabiol (Esquiroz, Spain). The strain of *C. sakazakii* is of clinical origin from a child throat and is recommended as reference strain by international standards ISO 22964:2017 (2017) and ISO 11133:2014/Amd 2:2020 (2020).

Both freeze-dried cultures of bacteria were processed following manufacturer instructions and maintained at −80°C in cryovials. The working culture was obtained by transferring a porous bead from the frozen stock into 10 mL of trypticase soy broth (TSB) (Merck, Darmstad, Germany) supplemented with 0.6% (w/v) yeast extract (YE) (Oxoid, Basingstoke, UK) in the case of *C. sakazakii*, and 10 mL of MacConkey broth (MCB) (ThermoFisher Scientific) in the case of *E. coli*. The cultures were incubated at 37°C for 24 h and, subsequently, were seeded by depletion on a trypticase soy agar (TSA) (Merck) supplemented with 0.6% (w/v) YE in the case of *C. sakazakii*; and MacConkey agar (MCA) (ThermoFisher Scientific) in the case of *E. coli*. Finally, the cultures were incubated at 37°C for 24 h. The antibacterial activity assays of dairy kefir beverages against both Gram-negative bacteria, were conducted using the cultures previously obtained at the stationary phase, following protocols of previous studies (Franco *et al*. 2013). In each case, a single colony of bacteria, formerly isolated, was incubated at 37°C in aerobiosis for 18–20 h in 10 mL TSB with 0.6% (w/v) YE or 10 mL MCB, for *C. sakazakii* and *E. coli*, respectively. Afterwards, serial dilutions of both bacterial suspensions were made with 1% (w/v) peptone water to reach a 4–5 log cfu/mL, which were later added to a 96-well microtitre plate (100 μL per well). Fractions derived from RMK, PMK and WK were mixed with the bacterial suspension at a 1:1 ratio (v/v). As a control of bacterial growth, 100 μL of peptone water was added to 100 μL of the bacterial suspension. All samples were analysed in duplicate in three independent experiments.

The plate was maintained at 37°C and the absorbance was measured at 620 nm in an ELISA reader (LabSystem Multiskan RC/MS/EX Microplate Reader, Pittsbutgh, PA, USA) at 0, 4, 8, 22 and 24 h of incubation, with shaking for 15 s before reading. Although the concentration of the bacterial cells was not analysed along the incubation period, the turbidity measurements were enough to monitor the bacterial growth. After 24 h of incubation, appropriate dilutions of the samples in the wells were made using 1% peptone water; and seeded on TSA or MCA plates, for *C. sakazakii* and *E. coli*, respectively. The colonies were counted after 24 h of incubation of the plates at 37 °C. The antibacterial activity was established as the disparity between the log number of colonies grown on plates inoculated with samples from control wells and the log number of colonies from samples from the rest of wells.

The antibacterial activity of fractions from microbial extracts obtained by ultrafiltration (<10 kDa and >10 kDa) from lyophilised samples were analysed against *C. sakazakii* with the same protocol as described above, to determine the fraction to which the highest antibacterial activity was associated.

Statistical analysis

Statistical analysis of the results was performed using the statistical software GraphPad Prism v8.0.2 (GraphPad Software, San Diego, CA, USA). The normality of data was verified with Shapiro–Wilk Test. For data that followed a normal distribution, an analysis of variance (ANOVA) was performed followed by a Bonferroni test for a multiple comparison test. For data that did not follow a normal distribution, a nonparametric Kruskal–Wallis test was done, followed by a Bonferroni test as a multiple comparison test. Differences with a P-value <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Protein profile of kefir fractions

The total protein content of the supernatants of dairy kefir beverages, determined by BCA, oscillated between 0.084 and 0.260 mg per mg of lyophilised product, with an average of 0.127 mg/mg of PMK, 0.131 mg/mg of WK and 0.224 mg/mg of RMK. In general terms, the RMK supernatants showed a higher protein content than the PMK supernatant, because since they did not undergo any type of heat treatment, proteins were not denatured and did not precipitate with the microorganisms in the centrifugation.

The protein profile of PMK and WK supernatants after fermentation and in the 2 weeks of storage under refrigeration, and of the microbial extracts (RMK, PMK and WK)

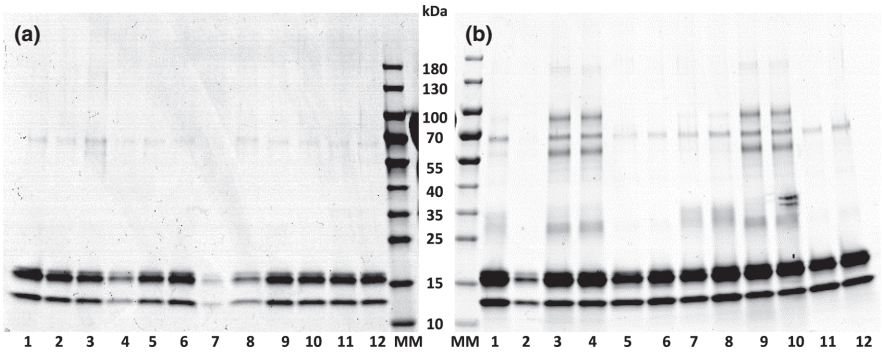

Figure 1 SDS-PAGE on 4–20% gradient polyacrylamide gel stained with Coomassie Blue of the two fractions obtained from the dairy kefir beverages over time. (a) SDS-PAGE of supernatants of the dairy kefir beverages: (MM) Molecular weight marker, (1) WK-1B, (2) WK-1C, (3) WK-2B, (4) WK-2C, (5) WK-3B, (6) WK-3C, (7) PMK-1B, (8) PMK-1C, (9) PMK-2B, (10) PMK-2C, (11) PMK-3B, (12) PMK-3C. (b) SDS-PAGE of the microbial extracts of the dairy kefir beverages: (MM) Molecular weight marker, (1) PMK-1B, (2) PMK-1C, (3) RMK-1B, (4) RMK-1C, (5) WK-1B, (6) WK-1C, (7) PMK-2B, (8) PMK-2C, (9) RMK-2B, (10) RMK-2C, (11) WK-2B, (12) WK-2C. Codes for samples correspond to: RMK, raw milk kefir; PMK, pasteurised milk kefir; WK, whey kefir; 1, after fermentation; 2, 7 days under refrigeration; 3, 14 days under refrigeration; B and C, starter cultures.

obtained from the different kefir beverages in the first 2 weeks after fermentation, was analysed by SDS-PAGE (Figure 1a,b). The electrophoretic protein profile of these fractions followed the characteristic pattern previously described in other studies (Chen *et al*. 2005; Yadav *et al*. 2015).

In Figure 1(a), showing the profile of the supernatants of the kefir beverages, two notable bands were observed. The band of 18 kDa corresponding to β-LG and the band of 14 kDa corresponding to α-LA, both are the major proteins in bovine whey.

In Figure 1(b), a greater number of protein bands were observed. In addition to the same bands shown in Figure 1 (a), the different types of caseins of around 35 kDa can be distinguished, as they have been separated together with the microbial cells in the centrifugation. Other types of proteins, located between 55 and 110 kDa, were observed in RMK. These proteins, such as LF (80 kDa) and BSA (66.5 kDa), have been probably denatured by the pasteurisation treatment in PMK and WK beverages, because they are susceptible to heat treatments (Brick *et al*. 2017). The lighter bands at the 150 kDa level, in the RMK samples, correspond to IgG, which are lost in the rest of the samples because the pasteurisation process completely denatures them (Wang *et al*. 2022b).

Several microbial extracts, two from kefir with starter culture B and two from kefir with starter culture C (Table 1), were also analysed after ultrafiltration (Figure 2). β-LG and α-LA, proteins that did not cross the membrane of 10 kDa pore, can be observed in the electrophoresis; whereas in the samples corresponding to the fraction <10 kDa, no bands were seen because the proteins and peptides that crossed the membrane were not properly stained or leaked out the gel due to their low molecular weight.

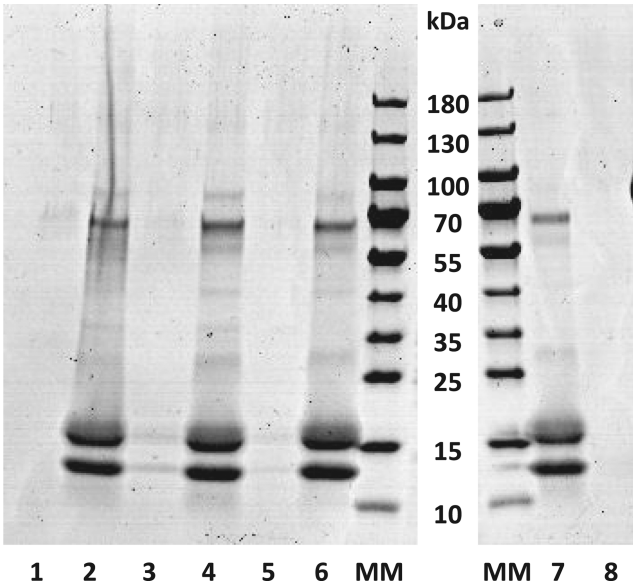

Figure 2 SDS-PAGE on 4–20% gradient polyacrylamide gel stained with Coomassie Blue of the fractions obtained by ultrafiltration of the supernatants of some kefir beverages. (MM) Molecular weight marker, (1) PMK-3C < 10 kDa, (2) PMK-3C > 10 kDa, (3) PMK-2B < 10 kDa, (4) PMK-2B > 10 kDa, (5) WK-3C < 10 kDa, (6) WK-3C > 10 kDa, (7) WK-3B > 10 kDa, (8) WK-3B < 10 kDa. Codes for samples correspond to: PMK, pasteurised milk kefir; WK, whey kefir; 2, 7 days under refrigeration; 3, 14 days under refrigeration; B and C, starter cultures.

Antioxidant activity

In this study, the ability of the dairy kefir beverages to scavenge DPPH free radicals was evaluated to analyse their antioxidant activity. DPPH radical has been extensively used to determine the antioxidant activity of compounds, since DPPH can accept hydrogen atoms or electrons to become a

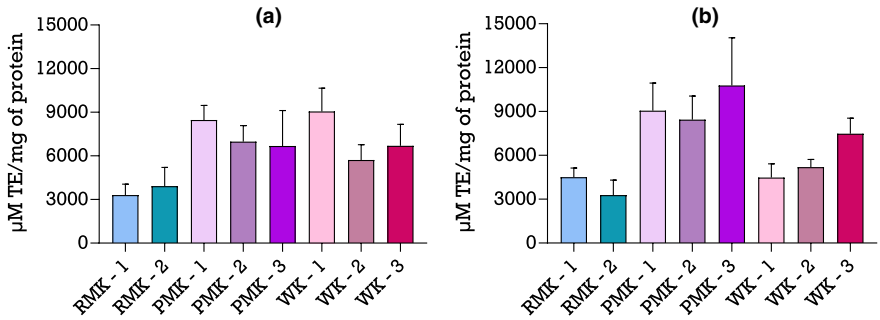

Figure 3 Antioxidant capacity of the supernatants of kefir beverages per mg of protein according to (a) the starter culture B or (b) the starter culture C. The samples correspond to raw milk kefir (RMK), pasteurised milk kefir (PMK) and whey kefir (WK), and their storage time: after fermentation (1), after 7 days (2) and after 14 days (3) under refrigeration. The values represent the mean ± standard error of the mean ($n = 4$). TE, Trolox equivalent.

stable molecule, producing a change of colour that can be easily measured spectrophotometrically (Xu *et al*. 2019).

The antioxidant capacity of kefir depends on numerous factors, such as the type of milk and its composition, the processing conditions, the type and amount of starter culture, or even the ability of antioxidant factors that are generated in fermentation to donate hydrogen atoms and electrons (Yilmaz-Ersan *et al*. 2018). Some studies have shown that *Leuconostoc mesenteroides* strains have an antioxidant activity due to its probiotic action, which becomes greater if they participate in fermentation (Hwang *et al*. 2019). On the other hand, the yeast *K. marxianus* has also been shown to have antioxidant capacity as an individual extract of its culture (Ceugniez *et al*. 2017) and as a component on kefir beverages together with other microorganisms (Cho *et al*. 2018), taking into account that the yeast strains used were different.

Results obtained from the antioxidant capacity of the supernatants of different types of kefir beverages and their fractions are represented in units of µM TE/mg protein in Figure 3, differentiating between the starter culture B (Figure 3a) and C (Figure 3b), the type of substrate (RM, PM or W) and time of storage time (1, 2 or 3). The mean values ranged from 3000 (RMK-1) to 9000 (WK-1) µM TE/mg protein for the starter culture B, while for starter culture C, they ranged from 3000 (RMK-2) to 11,000 µM TE/mg protein (PMK-3).

There is a clear fluctuation in the antioxidant activity with time. In kefir beverages with starter C (Figure 3b), the activity increases during storage in PMK (from 9000 to 11,000 µM TE/mg protein) and WK (from 4000 to 8000 µM TE/mg); whereas with starter B (Figure 3a), the activity decreases over time in the same substrates. The different antioxidant activity of the two types of WK could be linked to the presence of *Leuconostoc mesenteroides* in starter B, which is absent in starter C. It has been reported that this bacterium produces low molecular weight peptides (4–20 kDa) in milk, increasing the antioxidant activity of the hydrolysates at the end of the fermentation (Virtanen *et al*. 2007). The antioxidant activity of supernatants of PMK fermented with starter C, mean value about 10,000 µM TE/mg protein, presented a higher antioxidant activity than those of RMK (3800 µM TE/mg protein) and WK (5700 µM TE/mg protein). The presence of peptides from the hydrolysis of caseins, which are not so abundant in whey, could be the reason of the higher antioxidant activity of PMK supernatants, together with the effect of heat treatment on the susceptibility of milk proteins to proteolysis (Li *et al*. 2021).

In the same way, the data of antioxidant capacity were also represented according to µM TE/mg product, in order to consider all the intrinsic compounds of each kefir beverage (Figure 4), differentiating between the type of starter (Figure 4a,b).

The antioxidant activity of the supernatants from kefir elaborated with starter culture B (Table 1) was very similar, all ranging around 4 µM TE/mg of product, except for WK that reached values of 6 µM TE/mg of product. In kefir beverages with starter C, the activity of the RMK supernatants was lower than that of PMK when comparing mean values of samples 1 and 2 (∼4.1 vs. ∼4.8). These differences could be because raw milk has a higher protein content (Bonczar *et al*. 2016), so when expressing the results of µM TE in relation to protein, it shows a lower antioxidant activity than the other milks with a lower protein concentration. Regarding PMK, some of its peptides derived from major proteins, such as β-LG and α-LA, have shown to have a high antioxidant capacity (Corrochano *et al*. 2018). That is why in some cases, the WK supernatants have a similar or lower protein concentration than the PMK supernatants, especially with ferment B, which are observed to have a greater antioxidant capacity.

Antioxidant activity of the supernatants of some kefir beverages after fractionation by ultrafiltration was also evaluated. Random samples of both starter cultures (PMK-2B, PMK-3C, WK-3B and WK-3C) were selected for this

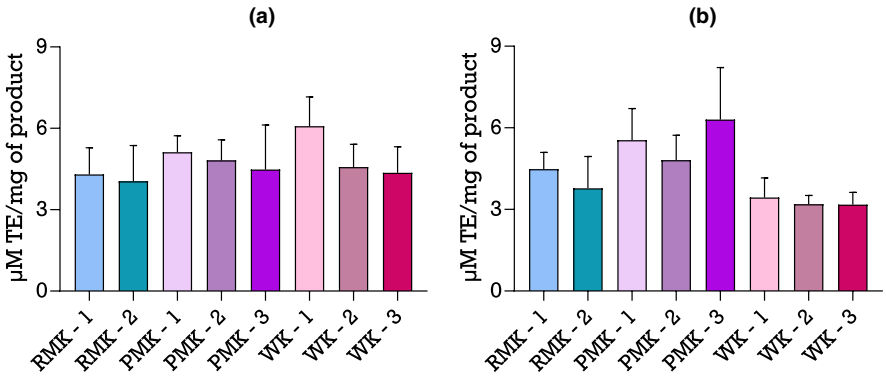

Figure 4 Antioxidant capacity of the supernatants of kefir beverages per mg of product according to (a) the starter culture B or (b) the starter culture C. The samples correspond to raw milk kefir (RMK), pasteurised milk kefir (PMK) and whey kefir (WK), and their storage time: after fermentation (1), after 7 days (2) and after 14 days (3) under refrigeration. The values represent the mean ± standard error of the mean ($n = 4$). TE, Trolox equivalent.

evaluation. Two fractions were obtained after ultrafiltration: a fraction >10 kDa and a fraction <10 kDa. In all supernatants from kefir beverages fermented with both starter cultures (Table 1), the antioxidant activity of the fractions >10 kDa was always almost half of those <10 kDa. The obtained values of activity for the fractions >10 kDa of PMK ranged from 1170 to 1280 µM TE/mg protein, while for the fractions <10 kDa, the range was between 2690 and 2750 µM TE/mg protein. In the case of WK supernatant fractions, the range of activity was from 800 to 1350 µM TE/mg protein in the fraction >10 kDa, whereas WK with both starters had an activity of 2200 µM TE/mg protein in the <10 kDa fractions. Therefore, the results of antioxidant activity obtained with fractions <10 kDa showed higher antioxidant activity per mg of protein than with fractions >10 kDa. This high activity may be due to the presence of peptides derived from α-casein, β-casein, κ-casein and α--lactalbumin (Wang *et al*. 2022a). It has been reported that the fractions that cross the 10 kDa membrane have a significantly higher antioxidant capacity, due to their scavenging ability and by an increase in the oxygen radical absorption capacity of bioactive peptides (Vieira *et al*. 2021).

Antibacterial activity of dairy kefir beverages against *C. sakazakii*

The antibacterial effect of the different dairy kefir beverages fermented with the two types of starter culture (Table 1) was analysed against *C. sakazakii* and *E. coli* in stationary phase of growth, at 24 h of incubation, including native bovine LF in the assay as a positive control of antibacterial activity. It has been reported previously that LF has antibacterial activity against *C. sakazakii* (Nakamura *et al*. 2022; Abad *et al*. 2022a). Regarding the duration of the experiment, RMK could only be maintained for 1 week, while PMK and WK could be stored under refrigeration for a total period of 2 weeks.

The supernatants of the different beverages did not show antibacterial activity against *C. sakazakii* from the outset and throughout the time of the experiment (results not shown). The microbial extracts obtained from RMK and PMK beverages exerted greater antibacterial activity than those with whey as substrate (Figure 5). The PMK exerted a decrease of bacterial growth of around 7 log units, while the RMK, depending on its starter culture, could also reach around 7 log units decrease with respect to the control group. Moreover, while with the samples of PMK and RMK, the antibacterial activity remained quite stable over time; with WK it decreased as time progressed, reaching levels similar to those of the control of bacterial growth by the end of the second week. Therefore, the effect observed after fermentation (especially with the sample of B starter) has been significantly reduced, probably due to the disappearance or inactivation over time of active proteins or other intrinsic components of the beverage.

This inhibitory capacity of kefir against *C. sakazakii* could be correlated with the results of other studies previously conducted, where its effect over the bacteria saw an important inhibitory capacity (Kim *et al*. 2015) due to some LAB, such as *Lacticaseibacillus rhamnosus* (Charchoghlyan *et al*. 2016), *Lactobacillus acidophilus* and *Lactococcus lactis* subsp. *lactis* (Gonzalez-Orozco *et al*. 2022), all present in the two types of kefir cultures used in our study. The drastically reduced antibacterial capacity of microbial extracts derived from WK, has also been reported in other studies, where LF antibacterial activity decreased when reconstituted in whey (Harouna *et al*. 2020; Abad *et al*. 2022b). Not only the low levels of LF, but also the absence of caseins (Hayes *et al*. 2006) or even IgG (Ripolles *et al*. 2017) in the whey may be a reason for the lack of antibacterial activity. No significant differences between the two starter cultures (Table 1) were seen in all kinds of kefir beverages. However, from these results, it is

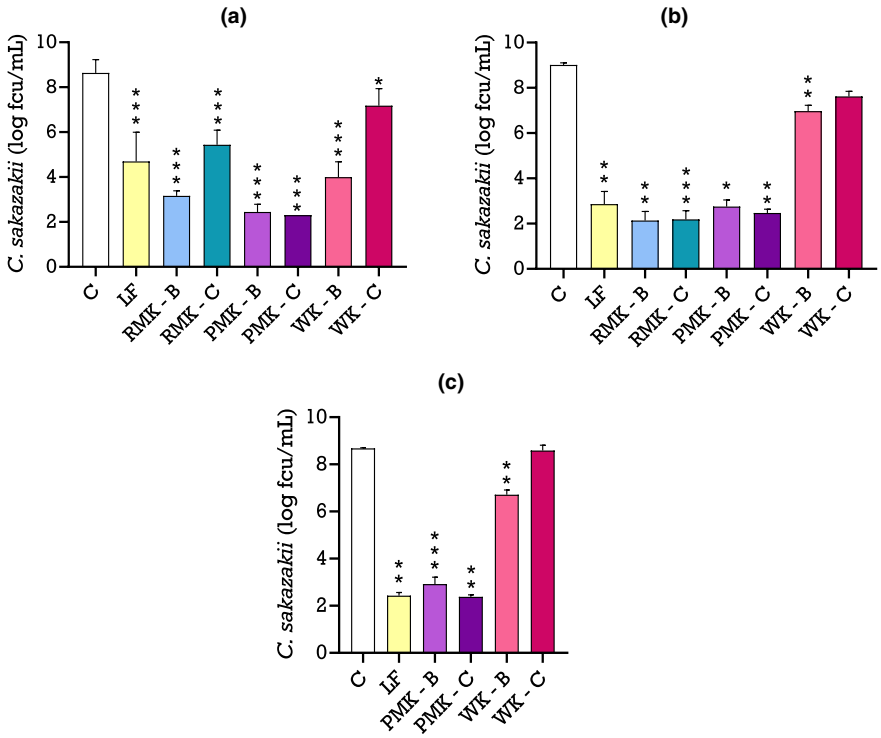

Figure 5 Antibacterial activity against *Cronobacter sakazakii* of the microbial extracts from different kefir beverages depending on their time of incubation: (a) after fermentation, (b) after 7 days and (c) after 14 days under refrigeration. C, control; LF, lactoferrin; RMK, raw milk kefir; PMK, pasteurised milk kefir; WK, whey kefir. The values represent the mean ± SE of the mean ($n = 4$). Significant differences compared to the control group are indicated by $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

important to highlight the significance of the antibacterial capacity of beverages in which fermentation with *K. marxianus* takes part, since there is a lack of research on the impact of yeasts on this emerging pathogen.

The microbial extracts from kefir beverages with both starter cultures with the highest antibacterial activity against *C. sakazakii* (from PMK-1 and RMK-2), were subjected to fractionation by ultrafiltration and the antibacterial activity of the fractions obtained was evaluated (Figure 6). There are no noteworthy differences between the two fractions derived from kefir beverages cultured with starter B and C, both having a significant antibacterial effect, with a decrease in bacterial growth of at least 4 log units in all fractions. Only in the case of starter B against *E. coli*, a lower antibacterial activity can be seen in the <10 kDa fraction compared to the >10 kDa fraction.

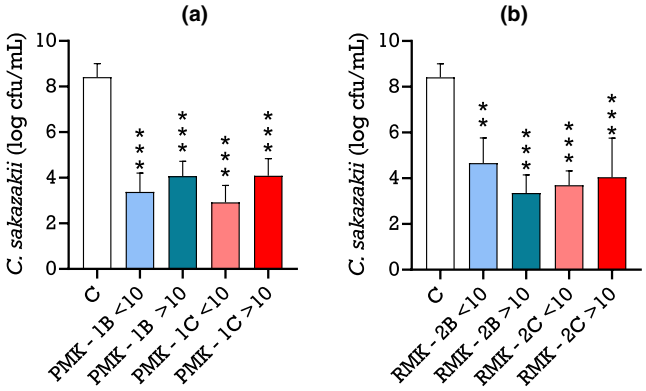

Figure 6 Antibacterial activity against *Cronobacter sakazakii* of the fractions >10 kDa and <10 kDa obtained by ultrafiltration from (a) microbial extracts of PMK after 7 days under refrigeration and (b) RMK after 14 days under refrigeration with starter cultures B and C. The values represent the mean ± SE of the mean ($n = 4$). Significant differences compared to control group are indicated by $**P < 0.01$; $***P < 0.001$.

Antibacterial activity of dairy kefir beverages against enterotoxigenic *E. coli*

Regarding the antibacterial assays against *E. coli* performed with the supernatants of kefir beverages, only RMK was the one with significant antibacterial activity (Figure 7). Slightly antibacterial activity was seen in the PMK with the starter culture C after 14 days of storage. The appearance of the significant antibacterial activity was not accompanied by any change in the acidity or pH of the beverage, because all the physicochemical characteristics of the beverage remained the same. The RMK beverages showed a reduction of around 5 log units with respect to the control, since

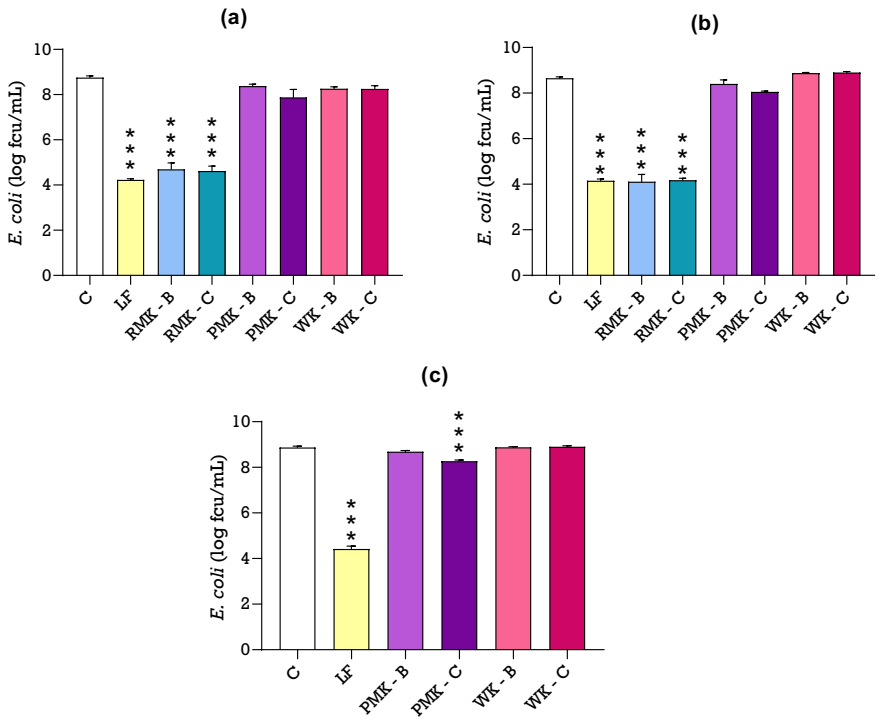

Figure 7 Antibacterial activity against *E. coli* of the supernatants of different kefir beverages depending on their time of incubation: (a) after fermentation, (b) after 7 days and (c) after 14 days under refrigeration. C, control; LF, lactoferrin; RMK, raw milk kefir; PMK, pasteurised milk kefir, WK, whey kefir. The values represent the mean ± SE of the mean ($n = 4$). Significant differences compared to the control group are indicated by ***$P < 0.001$.

the rest of the samples at most showed a reduction effect of 1 log unit.

In the case of the antibacterial activity of microbial extracts from kefir beverages against *E. coli*, those with greater activity were the ones with raw milk and whey as substrates (Figure 8). The PMK with starter culture B showed a slight significant antibacterial activity, with a decrease in bacterial growth of about 2 log units, but after only 1 week, this decrease disappeared and bacterial counts returned to levels similar to those of the control. This inhibitory capacity of kefir beverages could be correlated to other studies, where it was already proven against several digestive pathogenic strains of *E. coli* (Sulmiyati *et al*. 2019; Al-Mohammadi *et al*. 2021; Gonzalez-Orozco *et al*. 2022). On the other hand, the antimicrobial effect of WK samples, with only a decrease in bacterial growth of about 1.5–2 log units in our study, had already been demonstrated in previous *in vitro* studies with different cheese-whey derived samples (Pour 2014), where it was shown that this by-product, had an important inhibitory capacity. Nevertheless, in other studies, it was observed that kefir beverages made directly with pasteurised milk did show an antibacterial effect from the beginning or after 36–48 h of fermentation, maintaining this effect over time; contrasting with our results obtained with PMK beverage (Kim *et al*. 2016; Cortéz *et al*. 2023).

When comparing the kefir beverages fermented with the two starter cultures (Table 1), it was observed that those with culture B had a higher antibacterial activity in most samples, plus or minus 0.5 log units; correlating to other studies, where different strains of *L. mesenteroides* had shown to have similar activity against *E. coli* (Thangavel and Subramaniyam 2019).

Therefore, an antibacterial function can be observed in kefir, according to its intrinsic composition and the substrate on which it was prepared. This activity has been observed with respect to two of the most important foodborne Gram-negative pathogenic bacteria, and that, together with previous antibacterial studies (Kim *et al*. 2016; Marques *et al*. 2020; Al-Mohammadi *et al*. 2021), demonstrates the great antimicrobial capacity of this fermented beverage.

CONCLUSIONS

In conclusion, kefir beverages for consumption, depending on the substrate on which they are made, exhibit noteworthy antioxidant and antibacterial effects against two emerging pathogens of relevance, as being the cause of severe gastrointestinal infections nowadays. Importantly, their antibacterial activity endures over time, indicating the stability of the bioactive compounds.

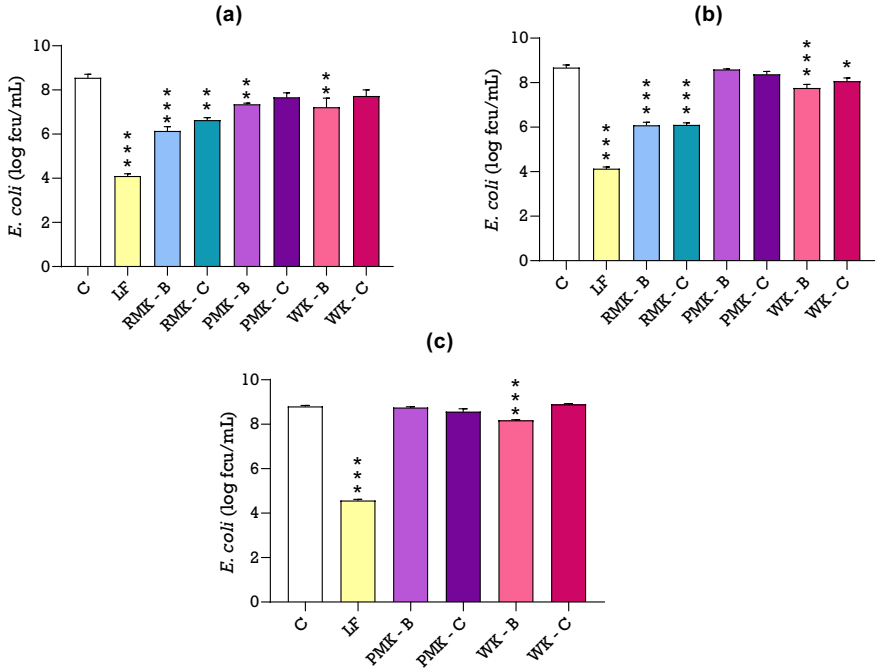

Figure 8 Antibacterial activity against *E. coli* of the microbial extracts of different kefir beverages depending on their time of incubation: (a) after fermentation, (b) after 7 days and (c) after 14 days under refrigeration. C, control; LF, lactoferrin; RMK, raw milk kefir; PMK, pasteurised milk kefir; WK, whey kefir. The values represent the mean ± SE of the mean ($n = 4$). Significant differences compared to the control group are indicated by $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Our results have shown that kefir beverages made on whole milk, either in raw or pasteurised form, have more bioactive properties than when elaborated on a milk by-product like whey. The loss of some components, such as proteins, due to processing, temperature changes or filtering may have a negative effect on the capacity of the substrate to generate bioactive compounds by the action of kefir cultures.

In either case, dairy products serve as rich sources of essential nutrients and defensive agents, benefiting not only the newborns, but also children and adults. However, further research should be conducted to improve our knowledge about the activity of dairy kefir beverages against other emerging pathogenic bacteria or virus affecting human population.

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AUTHOR CONTRIBUTIONS

Diego Aguirre-Ramírez: Conceptualization; methodology; data curation; investigation; formal analysis; writing – original draft. **Inés Abad:** Conceptualization; methodology; data curation. **Emma Pinilla:** Conceptualization; formal analysis; investigation; data curation; methodology; writing – original draft. **María D. Pérez:** Funding acquisition; project administration. **Laura Grasa:** Funding acquisition; project administration; writing – review and editing. **Lourdes Sánchez:** Conceptualization; project administration; writing – review and editing; funding acquisition; supervision.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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