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Title	Berry kefirs as functional non-dairy beverages: linking microbial activity, phenolic profiles, and immunomodulatory properties
Authors	Salas-Millán, J.Á.;Iguaz, A.;Aguayo, E.;Melgar, S.;Aznar, A.
Publication date	2026-05-04
Original Citation	Salas-Millán, J Á, Iguaz, A, Aguayo, E, Melgar, S & Aznar, A 2026, 'Berry kefirs as functional non-dairy beverages: linking microbial activity, phenolic profiles, and immunomodulatory properties', J. Agric. Food. Res., vol. 28, 102963. < https://www.scopus.com/pages/publications/105037735149?origin=resultslist >
Type of publication	Article (peer-reviewed)
Link to publisher's version	https://www.scopus.com/pages/publications/105037735149, https://www.scopus.com/pages/publications/105037735149?origin=resultslist
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Download date	2026-10-31 00:24:50
Item downloaded from	https://hdl.handle.net/10468/18753

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Journal of Agriculture and Food Research 28 (2026) 102963

Contents lists available at ScienceDirect

Journal of Agriculture and Food Research

journal homepage: www.sciencedirect.com/journal/journal-of-agriculture-and-food-research

Berry kefirs as functional non-dairy beverages: Linking microbial activity, phenolic profiles, and immunomodulatory properties

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ARTICLE INFO

Keywords:
Fruit-based beverage
Polyphenols
LC-MS
Strawberry
Blueberry
Blackberry
Epithelial cells
Inflammation

ABSTRACT

The present study investigated the physicochemical, sensory, and biological properties of non-dairy kefirs produced by fermenting strawberry, blueberry, and blackberry juices with kefir grains. Fermentation was performed at 21 ± 1 °C for 48 h, followed by refrigerated storage at 4 ± 1 °C for 7 days. All fruit kefirs exhibited a decrease in total soluble solids and pH, and an increase in lactic and acetic acids, confirming the active metabolism of the kefir consortia. Viable mesophilic and lactic acid bacteria counts increased from 2.4–4.2 to 7.0–9.0 log CFU/mL after fermentation and remained stable during refrigeration, whereas microbial viability declined in the water kefir control. Polyphenolic profiling by HPLC-QTOF revealed extensive transformation of anthocyanins, phenolic acids, and flavonols; the blackberry kefir retained the highest content of cyanidin derivatives and showed greater phenolic stability. Total phenolic compounds and antioxidant activity values decreased after fermentation (up to 40%) but remained stable during refrigeration in the blackberry and blueberry kefirs, whilst being negligible in the water kefir control. Sensory evaluation indicated high consumer acceptance (flavour ≈4/5), particularly for the blueberry kefir, with flavour emerging as the main driver of acceptability, and polyphenol composition (notably total anthocyanins and TPC) shaping sensory quality. *In vitro* assays using HCT116-Dual reporter cells demonstrated the anti-inflammatory potential of preferentially strawberry juice, and after fermentation and storage under basal and inflammatory conditions. Blackberry kefir partly inhibited TNF-α-induced IL-8 secretion, consistent with its anthocyanin profile. These findings demonstrate that fruit-based kefirs are promising non-dairy fermented beverages in which the fruit matrix enhances microbial performance and phytochemical stability, representing a new generation of naturally fermented beverages with health-promoting potential.

1. Introduction

In recent years, there has been increasing interest focused on the use of fruits and vegetables as substrates for fermented beverages [1]. Several fruits, including apples, grapes, melons, plums, peaches and strawberries, have been used as substrates for novel beverage formulations. While alcoholic fermentation by yeast remains the most common technique, alternative methods have also been investigated to create new fruit-based beverages [2]. In addition to alcoholic beverages, numerous non-alcoholic fruit-based fermented beverages have been developed. Lactic fermentation, carried out by bacteria such as *Lactiplantibacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus acidophilus* and *Lacticaseibacillus casei*, is widely used for fermenting fruits, while *Acetobacter* spp. serves as a typical starter for acetic acid fermentation [3].

Kefir is among the most popular functional beverages that are emerging, with a projected market value of USD 1.6 billion by 2028 [4]. Kefir is produced using complex microbial consortia consisting of yeasts, lactic acid bacteria, and acetic acid bacteria [5], and is traditionally obtained from milk. However, alternative substrates, such as coffee, cocoa, cereals, herbal infusions, legumes, tea, vegetables and fruits, have

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https://doi.org/10.1016/j.jafr.2026.102963
Received 30 January 2026; Received in revised form 30 March 2026; Accepted 26 April 2026
Available online 4 May 2026
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also been successfully tested [6], highlighting kefir's versatility for the development of non-dairy fermented beverages. Therefore, the use of fruits as a fermentation substrate for kefir is a promising way to create novel, aromatic and healthy beverages. Several studies have explored different fruit substrates for kefir fermentation, including pumpkin, orange, pomegranate, mandarin, persimmon, passion fruit, and mixed fruits and vegetables matrices [7–13]. Nevertheless, most such studies remain descriptive and rarely integrate fermentation-driven phytochemical changes with sensory perception and validated biological effects within the same experimental framework. Since these bioactivities are largely driven by the polyphenolic composition of the fruits, it is essential to elucidate how fermentation modifies these compounds and their associated health-promoting potential. Polyphenols, including phenolic acids, flavonoids and non-flavonoids (such as stilbenes and lignans), exhibit anti-inflammatory, antioxidant, anticarcinogenic and antidiabetic properties [14].

Anthocyanins are the key pigments responsible for the red-to-blue coloration of many fruits and vegetables. The most predominant anthocyanidins are cyanidin, delphinidin, malvidin, peonidin, petunidin and pelargonidin, and their profile is fruit-dependent [15]. In this line, berries are among the richest dietary sources of these pigments, and numerous studies have reported their beneficial effects on cardiovascular health, weight management, and the modulation of inflammatory responses [16,17]. However, the specific anthocyanin profiles vary considerably across species**:** strawberries are dominated by pelargonidin derivatives, blueberries by malvidin, and blackberries by delphinidin and cyanidin [18–20]. These structural differences influence anthocyanin stability and biological function, thereby determining their overall bioactivity and potential health effects. During fermentation, microbial metabolism interacts with polyphenols through enzymatic hydrolysis, oxidation, and deglycosylation, producing simpler metabolites such as aglycones or phenolic acids. These transformations can alter both the concentration and bioavailability of phenolic compounds, potentially modulating their biological activity [14]. Additionally, the use of industry-grade berries, which are usually considered a by-product of fresh-market sorting, aligns with current strategies for adding value to lower-value parts of the harvest and developing sustainable food products. Therefore, this study aimed to develop and comprehensively characterise strawberry, blueberry and blackberry-based kefirs, linking the physicochemical and phytochemical evolution during fermentation and storage with the sensory quality and anti-inflammatory responses in epithelial cells.

2. Material and methods

2.1. Materials

Strawberries (*Fragaria* × *ananassa*), blueberries (*Vaccinium corymbosum*) and blackberries (*Rubus spp.*) were sourced from the Masiá Ciscar Company in the southwest of Spain (Lepe, Huelva). The supplied fruit was classified as industry-grade (not meeting fresh-market standards in terms of size, shape or appearance). In essence, it was considered a by-product of the fresh-market sorting process. Kefir grains were obtained from Burumart Commerce S.L. (Arrasate, Gipuzkoa, Spain).

2.2. Production of fruit-based kefirs and water kefir (control)

Fruit-based kefirs were produced by fermenting strawberry, blueberry, and blackberry juices with kefir grains. The berries were first washed by immersion in an aqueous sodium hypochlorite solution containing approximately 80 mg/L of free chlorine, prepared from commercial bleach (3.75% active Cl_2) at a ratio of 2 mL/L of water for 10 min. The fruits were rinsed in tap water to remove residual chlorine and then liquefied (Silvercrest Mod. SSJ 150 A1, Lidl, China) to separate the juice from the pulp. In the case of strawberries, peduncles were removed prior to processing (Fig. 1).

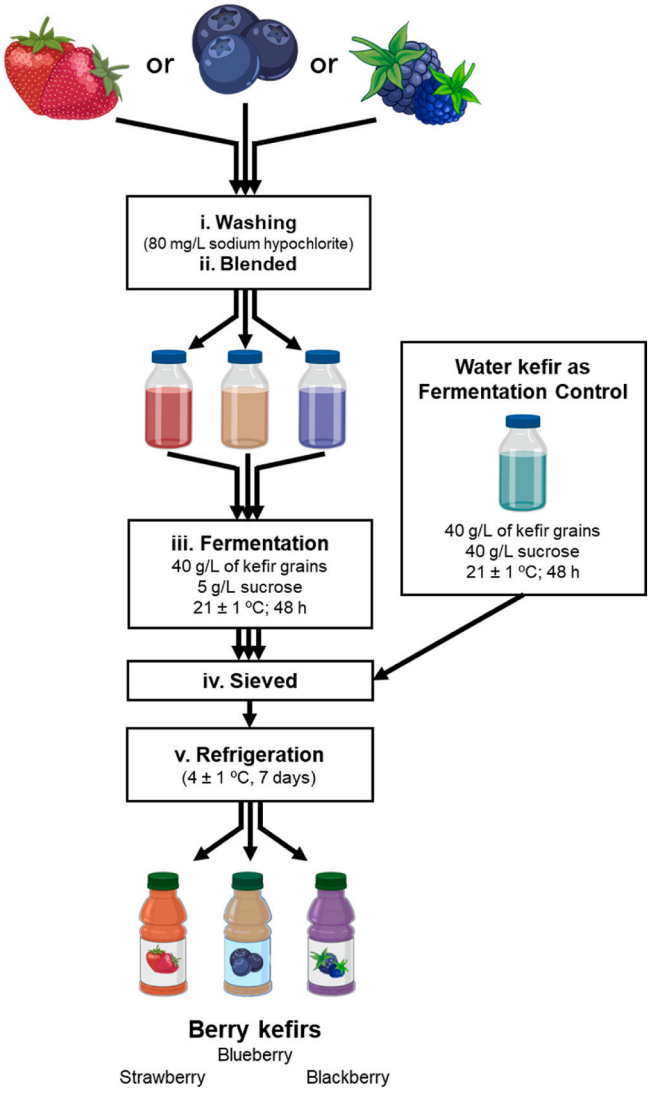


Fig. 1. Processing of berry kefirs from strawberry, blueberry and blackberry fruits, and water kefir as control.

The juice (J) obtained from each berry was sieved to remove lumps and tempered to 20 °C prior to processing. Berry juices were inoculated with 40 g/L of kefir grains and 5 g/L sucrose, followed by fermentation at 21 ± 1 °C for 48 h (K). After fermentation, the berry kefirs were sieved to remove the kefir grains and bottled. Finally, the fermented berry kefirs were refrigerated (RK) at 4 ± 1 °C for 7 days. A water-based kefir prepared without berry juice was used as the control. For that purpose, decalcified water was inoculated with 40 g/L of kefir grains and 40 g/L of sucrose. A small amount of sucrose was added to facilitate the initiation of fermentation in each matrix. Therefore, each beverage formulation was considered as an independent fermentation matrix from the beginning of the process.

2.3. Physicochemical analysis

Physicochemical parameters such as pH, total soluble solids (TSS as °Brix) using a digital refractometer (Atago Company Ltd. Tokyo), and colour, were determined in accordance with Salas-Millán et al. [2]. The total polyphenol content (TPC) and the antioxidant capacity, expressed as the ferric reduction antioxidant potential (FRAP), were measured using a multiscan plate spectrophotometer (Tecan infinite M200, Männedorf, Switzerland) after dilution of the berry kefirs in Milli-Q water. The TPC was expressed as mg of gallic acid equivalent per 100

mL of berry kefir (mg GAE/100 mL) and the FRAP as mmol of Fe^{2+} per 100 mL of berry kefir.

2.4. Determination of individual sugars and organic acids

Individual sugars were analysed using an ultra-high-performance liquid chromatography (UHPLC) instrument (Shimadzu, Kyoto, Japan) equipped with a DGU-20 A degasser, LC-170 30AD quaternary pump, SIL-30AC autosampler, CTO-10AS column oven, and a refractive index detector (RID). Chromatographic separation was performed at 65 °C using a mobile phase consisting of 20% (v/v) acetonitrile in Milli-Q water, at a flow rate of 0.6 mL/min for 30 min, with a Luna® Omega SUGAR column (150 × 2.1 mm, 3 μm particle size; Phenomenex, Macclesfield, UK). For sample preparation, 5 mL of fermented berry kefir was centrifuged at 14,000×*g* for 15 min at 4 °C, and the supernatant was further purified by solid phase extraction using C18-SPE columns [21]. The purified extracts were filtered through 0.20 μm polyamide syringe filters and diluted to reach a dilution of 20% (v/v) acetonitrile prior to analysis.

The analysis of individual organic acids was performed using an Agilent 1200 liquid chromatography (Santa Clara, CA, USA) equipped with a G1311B quaternary pump, G1329B standard autosampler and G1316A column oven, coupled to a 6420 triple-quadrupole mass spectrometer (QqQ) with an electrospray ionisation (ESI) source. Chromatographic separation was performed as per Sandín-España et al. [22]. The capillary voltage, gas temperature, gas flow and nebuliser were set at 4000 V, 350 °C, 10 L/min and 35 psi, respectively. In the analysis of individual sugars and organic acids, authentic standards (fructose, glucose, sucrose, citric, malic, and succinic acids) were used for their identification and quantification (Sigma-Aldrich, St Louis, MO, USA). Results were expressed as g of each sugar or organic acid per 100 mL of berry kefir.

2.5. Viable microbiological counts

Microbiological analyses were carried out following standard methods. Viable counts of mesophilic bacteria, lactic acid bacteria (LAB), yeasts and moulds and enterobacteria were performed on different agars including Plate Count Agar (PCA), de Man, Rogose and Sharpe agar (MRS), Rose Bengal (RB) and chloramphenicol agar, and Violet Red Bile Glucose Agar (VRBGA), respectively. The plates were incubated at 30 °C for 48 h (PCA and MRS), 25 °C for 72 h (RBC), and 37 °C for 24 h (VRBGA). Serial 1:10 dilutions of the berry kefir samples were prepared in sterile peptone water and inoculated (1 mL) onto the corresponding media. Results were expressed as log CFU/mL.

2.6. Characterisation and quantification of individual polyphenols in berry kefirs

To characterise the fermented beverages, two complementary analytical instruments were employed. Liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer (LC-QTOF-MS) was used for phytochemical identification, whereas liquid chromatography coupled to a triple quadrupole mass spectrometer (LC-QQQ-MS) was used for their quantification.

The qualitative characterisation of anthocyanins and flavonoids was carried out on an Agilent 1290 Infinity II HPLC system (Santa Clara, CA, USA) equipped with a G7104A quaternary pump, G7167B autosampler, and G7116B column oven coupled to a 6546-hybrid Q-TOF mass spectrometer with a Dual Agilent Jet Stream ionisation source. Samples were filtered through a 0.22 μm nylon filter and injected onto a reverse-phase column (ZORBAX Eclipse Plus C18; 3.0 × 100 mm; 1.8 μm). The mobile phase consisted of two solvents: (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The analysis was performed in negative ion mode for phenolic acids and flavonols, and in positive ion mode for anthocyanins. The drying gas flow (N_2) was set at 13 L/min, the nebuliser pressure at 40 psi, the gas drying temperature was 350 °C, and the sheath gas flow and temperature were 12 L/min and 350 °C, respectively. The instrument was set with an *m/z* range of 50–1700, a capillary voltage of 4000 V and a fragmentor of 200 V. Data acquisition and processing were performed in MassHunter Workstation software (Agilent Technologies). Tentative identification of anthocyanin compounds and phenolic acids was based on mass spectrometry (MS) and tandem mass spectrometry (MS^2) fragmentation patterns, which were compared with available bibliographic data and reference spectra from the (MassBank Europe [23] and Human Metabolome [24] databases.

Quantification of the anthocyanins and phenolic acids was carried out with an Agilent 1200 HPLC (Santa Clara, CA, USA) equipped with a G1311B quaternary pump, G1329B autosampler, G1316A column oven, and coupled to a 6420 QQQ mass spectrometer (QqQ) with an electrospray ionisation source (ESI). Separation was achieved on a reverse-phase Luna Omega C18 column (2.1 × 100 mm; 3 μm; Phenomenex, Macclesfield, UK) using a mobile phase consisting of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The capillary voltage, gas temperature, gas flow and nebuliser were set at 4000 V, 350 °C, 11 L/min, and 40 psi, respectively. Anthocyanins were quantified using authentic standards (Table S1) when available. Compounds lacking available standards were semi-quantified and expressed as equivalents of representative anthocyanins, due to the limited availability of commercial standards for complex anthocyanin derivatives.

2.7. Sensory evaluation

Sensory evaluation was carried out in a standardised tasting room kept at 22 °C. Glasses labelled with blind codes, each containing 15 mL of berry kefir samples, were presented to the panellists. Samples were served in a randomised order. Water and breadsticks were provided for palate cleansing to minimise potential carryover effects between sample. The semi-trained sensory panel was composed of 19 members of the laboratory staff (ten women and nine men), aged between 30 and 55 years old. The panellists evaluated the degree of satisfaction of different attributes such as aroma, colour, flavour and global acceptability, using a 5-point hedonic scale, 1 = extremely poor, 3 = acceptable, and 5 = excellent, for each attribute. Overall acceptability was assessed based on the panellists' general liking of the berry kefir, considering all the sensory attributes mentioned. A score of 3 was considered as the minimum commercial limit. Samples were served in random order to minimise positional and carry-over effects.

This sensory evaluation did not require ethical approval, as it involved no invasive procedures or health-related interventions. Prior to the evaluation, participants were provided with detailed information about the study, including its purpose, the identity of the researchers, data protection measures, privacy and data retention policies, the voluntary nature of participation, the right to withdraw at any time, and contact details for further inquiries. All participants provided written informed consent, confirming that they had read and understood the information and that any questions had been addressed.

2.8. In vitro cell culture of HCT116-Dual reporter cells assessing NF-κB pathway activation, anti-inflammatory potential and viability

The human intestinal epithelial reporter cell line, HCT116-Dual™, was purchased from InvivoGen (Toulouse, France). Cells were grown in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, Missouri, USA), supplemented with 10 % fetal bovine serum, and 1 % penicillin/streptomycin (Sigma-Aldrich), 10 μg/mL of blasticidin, 100 μg/mL Zeocin®, and 100 μg/mL of normocin™ (Invivogen) at 37 °C, in a humidified atmosphere of 5 % CO_2 and 95 % air, as previously reported [25]. Activation of the NF-κB pathway was quantified by using the Quanti-Blue™ assay (InvivoGen, Toulouse, France) following the manufacturer's instructions. Briefly, cell culture supernatant from the different treatments was mixed with Quanti-Blue reagent and incubated at 37 °C

for 1 h followed by absorbance reading at 630 nm using Synergy 2 multimode microplate reader (Biotek, Winooski, VT, USA). Cell culture supernatants were collected and stored at −80 °C until analysis. To assess cell viability, the cells were stained with 1 μM SYTOX™ Green stain (ThermoFisher, Massachusetts, USA) for 20 min at 37 °C, followed by fluorescence reading at 485 nm excitation and 530 nm emission using a Synergy 2 multimode microplate reader. Cells treated with 1% Triton X-100 lysis buffer for 45 min were used as a positive control of reduced cell viability.

To study the anti-inflammatory potential of the kefirs, cells were pre-treated for 24 h with 10 % of berry juice or kefir, followed by stimulation with human recombinant (hr)-IL-1β or hr-TNF-α at 10 and 20 ng/mL, respectively, for 24 h. Untreated cells served as negative controls. Cells between passages 6 and 14 were used in this study. The experiments were performed in technical triplicates, the results of which were averaged using samples from three independent fermentation experiments.

2.9. Enzyme-linked immuno sorbent assay (ELISA)

Interleukin-8 (IL-8) levels were measured by ELISA in cell culture supernatants collected from HCT116-Dual™ cells after the different treatments using the Human IL-8/CXCL8 DuoSet ELISA kit (DY208, R&D Systems, Minneapolis, MN, USA), in accordance with the manufacturer's instructions. Sample dilutions were adjusted to ensure that absorbance values fell within the linear range of the IL-8 standard curve. Absorbance was measured at 450 nm with wavelength correction at 570 nm. The standard curve ranged from 15.625 to 2000 pg/mL, diluted in a 2-fold serial dilution in Reagent Diluent. The lower limit of detection (LOD) of the assay was calculated as the mean blank signal plus 3 × SD of blanks and was found to be 8.5 pg/mL. Unstimulated cells treated with cytokines were diluted 1:1, while cytokine-stimulated cells were diluted 1:40 in Reagent Diluent. The diluent consisted of 1% bovine serum albumin (BSA) in PBS, prepared according to the manufacturer's instructions.

2.10. Statistical analysis

Statistical differences were assessed using a Student's t-test with Holm-Sidák correction for multiple comparisons when comparing fermented samples to their corresponding fruit juices. For comparisons among different berry juices, kefirs, and control kefirs, one-way ANOVA followed by Tukey's post hoc multiple comparisons test was applied. Statistical significance was set at $p < 0.05$. Results are presented as mean ± SEM from three independent fermentations ($n = 3$). A hierarchical heatmap was produced for each treatment using MetaboAnalyst 6.0 (www.metaboanalyst.ca). Ward's clustering method was applied, and Euclidean distance was used to measure similarities between normalised data, which were processed using $\log_{10}$ transformation and min–max scaling. Correlation analysis was performed using Pearson's correlation coefficient (r), and p-values were adjusted for multiple testing using the Benjamini–Hochberg procedure, with adjusted p-values <0.05 considered statistically significant. Debiased Sparse Partial Correlation (DSPC) networks were visualised in MetaboAnalyst 6.0, with nodes filtered using a degree cutoff of 1, a betweenness cutoff of 1, and an adjusted p-value <0.05.

3. Results and discussion

3.1. Physicochemical analysis and profiles of sugars and organic acids in berry kefirs

The physicochemical parameters of the berry kefirs throughout the processing stages are presented in Table S2. The fruit juices used in this study exhibited pH values below 4, which is consistent with previous reports [26–28]. With these low initial pH values, no significant pH variations were observed during fermentation, except in the blueberry kefir, where the pH decreased from 3.9 to 3.4 after fermentation and subsequent refrigeration. In contrast, the typical reduction in pH during fermentation was only observed in the control water kefir, which dropped from 5.2 to 3.6 after fermentation. As expected, the TSS decreased notably in all the berry kefirs following fermentation, with values dropping from 7.0 to 2.6 °Brix in strawberry, from 10.7 to 6.9 °Brix in blackberry, and from 14.6 to 11.8 °Brix in blueberry. After refrigeration, a further decrease was observed in all cases. In contrast, no significant changes were detected in the control water kefir during processing. This drop in TSS during fermentation reflects the metabolic activity of kefir microorganisms, which consume fermentable sugars (mainly glucose and fructose) as substrates to produce organic acids, ethanol, and carbon dioxide [29].

Table S2 also shows colour changes in all the samples. As expected, no significant variations were observed in the control kefir water after fermentation and refrigeration. However, the strawberry kefir exhibited a significant increase in L* after fermentation (from 24.4 to 26.1) and a further rise after refrigeration (to 33.7), suggesting that the beverage became visually brighter. The h° also increased slightly from 0.3° to 0.6°, indicating a minor shift towards a more orange-red tone. Moreover, C* values increased progressively during processing, reaching 17.9 after refrigeration, which denotes a marked increase in colour saturation. The higher C* value observed in strawberry kefir may be attributed to a reduction in pH and an increase in organic acids. Anthocyanins are pH-sensitive phytochemicals that undergo structural changes in response to pH, resulting in increased colour saturation at lower pH values [30]. On the other hand, these significant changes were not observed in the blackberry kefir, while the blueberry kefir showed a significant decrease in C* (from 16.7 in the juice to 9.5 after fermentation and 8.8 after refrigeration), indicating a reduction in colour saturation. This decrease may be associated with anthocyanin degradation or structural transformations occurring during fermentation and cold storage [3]. It is well known that, even though berries are rich in anthocyanins, they are a diverse family and that different anthocyanins, such as delphinidin, cyanidin and pelargonidin, react differently to pH and produce different colours and brightnesses [31]; this might confer variable colour trends throughout the fermentation process. The differences in the composition of individual anthocyanins are detailed in section 3.4.

Changes in concentrations of individual sugars and organic acids during the fermentation and subsequent refrigeration of the strawberry, blackberry, and blueberry juices are shown in Fig. 2. Individual sugars levels declined during fermentation, in agreement with the previously observed reduction in TSS (Table S2). This reflects their use as carbon sources by the yeast-bacteria consortium of the kefir grain inoculum [21]. In parallel, the significant increase in lactic and acetic acids across all matrices confirms the common metabolic pattern of bacteria and yeasts within the kefir consortium [32]. Interestingly, water kefir produced higher levels of acetic acid than the berry-based kefirs, suggesting that acetic acid bacteria might have become dominant in the absence of readily available nutrients, oxidising ethanol derived from yeast metabolism [33]. This hypothesis was not further explored, as the present study focused on berry kefirs and their phytochemical evolution during processing. Other organic acids naturally present in the juices exhibited different trends during fermentation (Fig. 2).

Malic acid decreased significantly in the blackberry and blueberry kefirs, whereas succinic acid increased significantly during fermentation in all the berry matrices. The formation of succinic acid, which increased significantly after fermentation in all the berry kefirs, suggests active yeast metabolism through the tricarboxylic acid (TCA) cycle under microaerophilic conditions. This compound often acts as a metabolic intermediate linking carbohydrate catabolism and amino acid degradation, and its accumulation has been reported in other mixed fermentations involving yeasts [34]. Conversely, the drop in malic acid in the blackberry and blueberry kefirs can be attributed to the malolactic

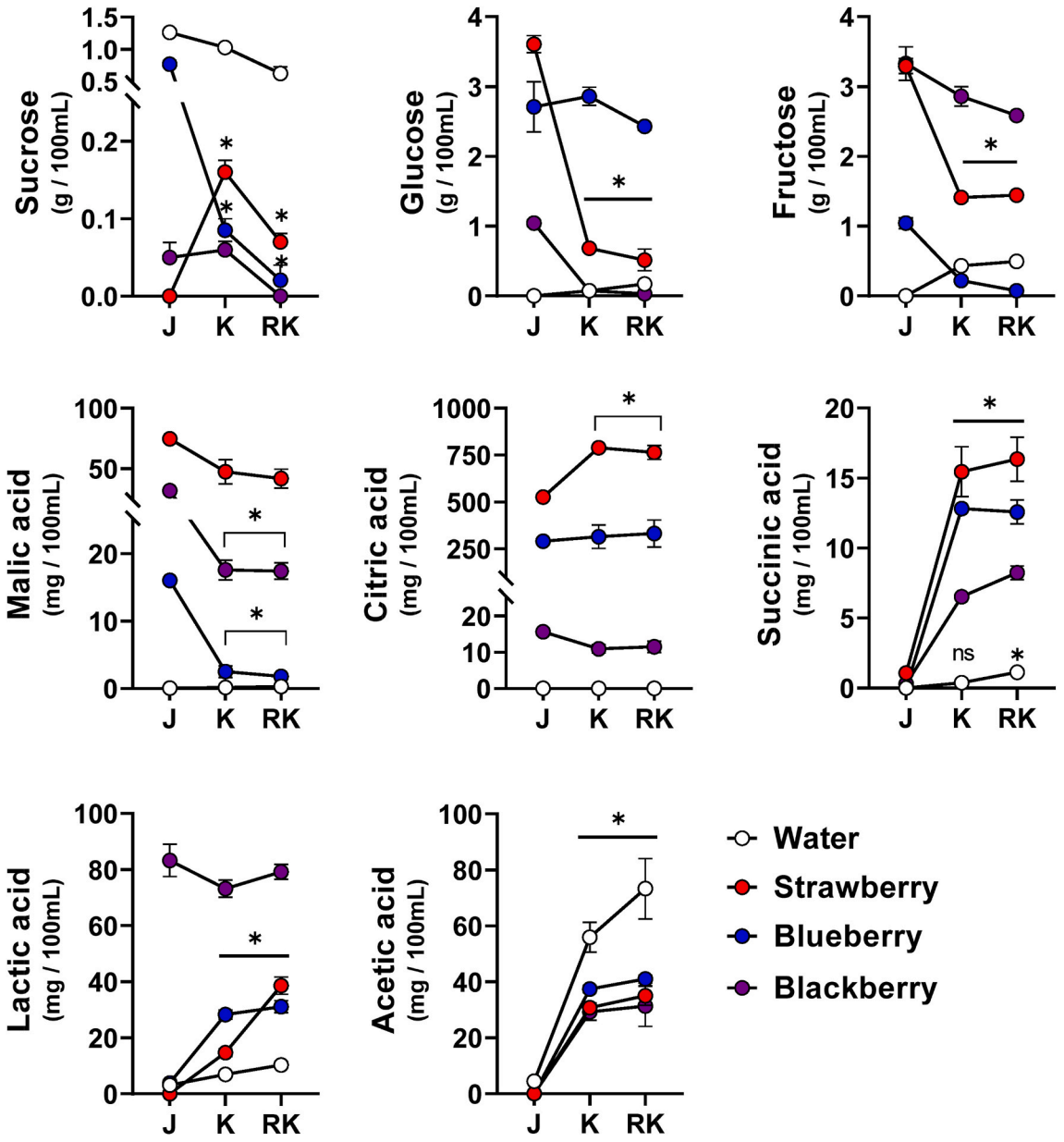


Fig. 2. Individual sugars (sucrose, glucose, and fructose; g/100 mL) and organic acids (malic, citric, succinic, lactic, and acetic acids; mg/100 mL) in berry kefirs prepared from strawberry ■, blueberry ■, and blackberry ■, and in water kefir as control □, before fermentation (J, juice); after fermentation with kefir grains (K) at 21 ± 1 °C for 48 h; and after refrigeration storage (RK) at 4 ± 1 °C for 7 days. Data are expressed as mean ± SEM (n = 3). Asterisks (*) indicate statistically significant differences (Student's t-test, $p < 0.05$) between fermented samples (K and RK) and their corresponding unfermented beverage (J) within the same fruit matrix. For the water kefir control, fermented samples were compared with the initial unfermented water control.

conversion carried out by certain LAB strains, which decarboxylate malic acid into lactic acid, contributing to acidity stabilisation and flavour smoothness. This conversion is commonly observed in fruit-based fermentations with low pH and moderate sugar content [35]. In the case of citric acid, its stability or slight increase in the strawberry kefir suggests that the dominant LAB does not efficiently metabolise it under these conditions, whereas in the blueberry and blackberry kefirs, the constant levels found indicate limited utilisation of citrate as an energy source. Differences among the berry matrices could therefore be associated with both the initial organic acid composition as well as the selective growth of microbial populations adapted to each substrate and different metabolic rates [36].

3.2. Microbiological viable counts

The fermentation process carried out in this study used an inoculation strategy involving a mixed consortium of yeast and bacteria from kefir grains. As a result, the counts of mesophiles microorganisms and LAB increased from 2.4–4.2 log CFU/mL at the initial stage to 7.0-9.0 log CFU/mL after 48 h of fermentation (Fig. 3). During refrigerated storage, the blueberry kefir increased its viable mesophile and LAB counts, whereas a decrease was observed in the control water kefir. This reduction may be attributed to nutrient depletion in the water matrix compared with the fruit-based substrates, which provide readily available sugars, amino acids, anthocyanins, and other bioactive compounds that can support microbial survival and adaptation [37]. Several LAB species present in fermented foods are mesophilic, showing optimal growth at 25–30 °C [38]; this is consistent with the trends observed in

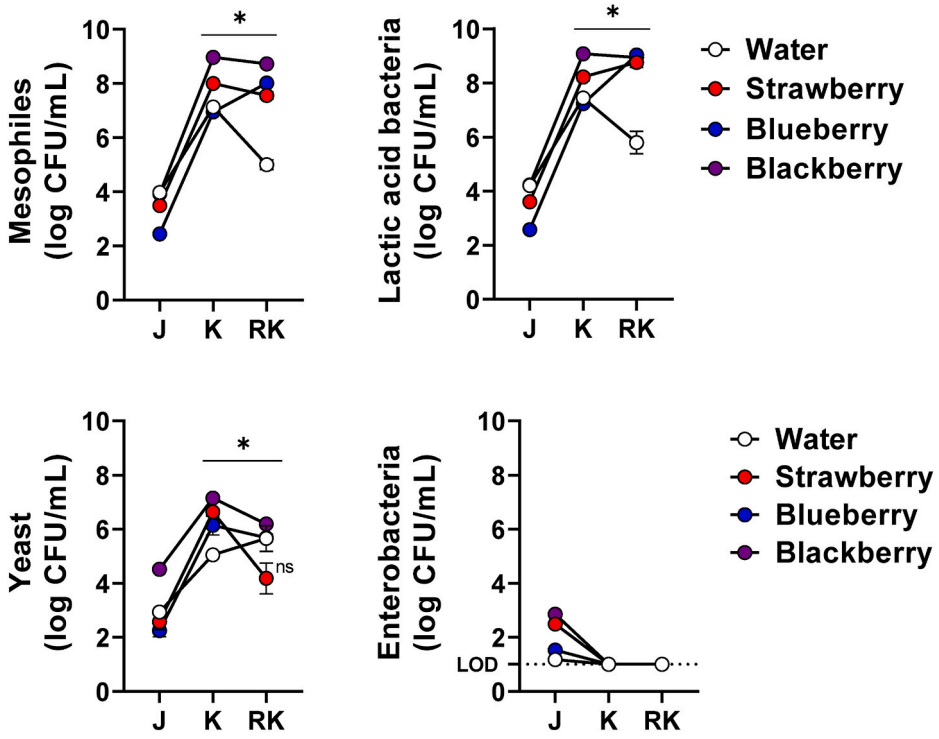


Fig. 3. Microbiological viable counts of mesophiles, lactic acid bacteria, yeasts, and enterobacteria in berry kefirs prepared from strawberry ■, blueberry ■, and blackberry ■, and in water kefir as control □ before fermentation (J, juice); after fermentation with kefir grains (K) at 21 ± 1 °C for 48 h; and after refrigeration storage (RK) at 4 ± 1 °C for 7 days. Data are expressed as means ± SEM of the results (n = 3). Asterisks (*) indicate statistically significant differences (Student's t-test, $p < 0.05$) between fermented samples (K and RK) and their corresponding unfermented beverage (J) within the same fruit matrix. For the water kefir control, fermented samples were compared with the initial unfermented water control. LOD: limit of detection (<1 log CFU mL^{-1}).

both the mesophile and the LAB populations in the berry kefirs. Yeasts also showed an increase in viable counts after fermentation, confirming their active role within the symbiotic consortium. Enterobacteria were initially detected at low levels (<3 log CFU/mL) in both the water and the berry juices, but their counts were far below the detection limit (<1log CFU/mL) after fermentation. This reduction is likely due to the sharp drop in pH, which inhibits the growth of acid-sensitive bacteria [21].

3.3. Total phenolic compounds (TPC) and antioxidant activity

Initially, the TPC content of the berry juices ranged from 97 to 190 mg GAE/100 mL (Fig. 4A), with the blackberry juice showing the highest concentration, followed by the strawberry and blueberry juices. However, the FRAP assay (Fig. 4B) revealed a different trend in antioxidant potential, where the blackberry juice exhibited the highest activity, followed by the blueberry and strawberry juices. As expected, water kefir contained negligible amounts of phenolic compounds and displayed almost no antioxidant activity throughout the process. After fermentation, TPC values decreased by approximately 20% in the strawberry kefir and 40% in the blueberry kefir, whereas the blackberry kefir only fell slightly (~8%). A similar trend was observed in the FRAP results, indicating that fermentation reduced the antioxidant capacity in the strawberry and blueberry kefirs but had little effect in the blackberry one. During refrigerated storage, the TPC values decreased by around a further 10% and 42% in the strawberry and blueberry kefirs, respectively, whilst the blackberry kefir remained stable, with no significant changes. In contrast to the TPC, the antioxidant potential of the strawberry kefir exhibited a 2-fold reduction after refrigeration, whereas the blueberry kefir maintained a stable FRAP response. These differences between the TPC and FRAP assays might be related to the distinct reactivity of polyphenols and other matrix components that may interfere with the reducing assays [39]. Similar discrepancies in the comparison of TPC and antioxidant responses with the concentration of individual flavonoids and phenolic acids have been reported previously [21]. A limitation of this study was that only a single assay (FRAP) was used to evaluate the antioxidant potential of the fermented beverages. However, the detailed analysis of individual phytochemical transformations presented in the following section provides further insight into these results. Overall, the observed decrease after fermentation suggests partial degradation or transformation of the phenolic and antioxidant compounds by kefir microorganisms during processing [40].

3.4. Characterisation and quantification of individual polyphenols in berry kefirs

This section further explores the findings on the TPC by analysing changes in individual anthocyanins, thereby providing insight into the mechanisms behind the results obtained. Fig. 4C–F shows the quantification of the major phytochemicals identified in berry kefir fruits. The heatmaps present normalised data obtained using log_{10} and max–min scaling, while the corresponding absolute concentrations of anthocyanins, phenolic acids, and flavonols are presented in Table S3. Overall, the data highlight significant differences in the relative distribution of polyphenol families in the fruits before and after fermentation and refrigeration (Fig. 4D–F).

In the strawberry juice, the proportion of anthocyanins-to-phenolic acids (A:PA) was extremely high (Fig. 4C): anthocyanins represented nearly 99.9% of the total quantified polyphenols, while phenolic acids accounted for about 0.1%. This ratio remained practically constant throughout fermentation and refrigerated storage. However, no flavonol derivatives were detected in any of the strawberry samples. Consistent with the TPC values, the total anthocyanins concentration decreased significantly after fermentation and refrigeration, whilst the total phenolic acids showed a slight reduction during processing.

In the blueberry juice, the phenolic profile was dominated by

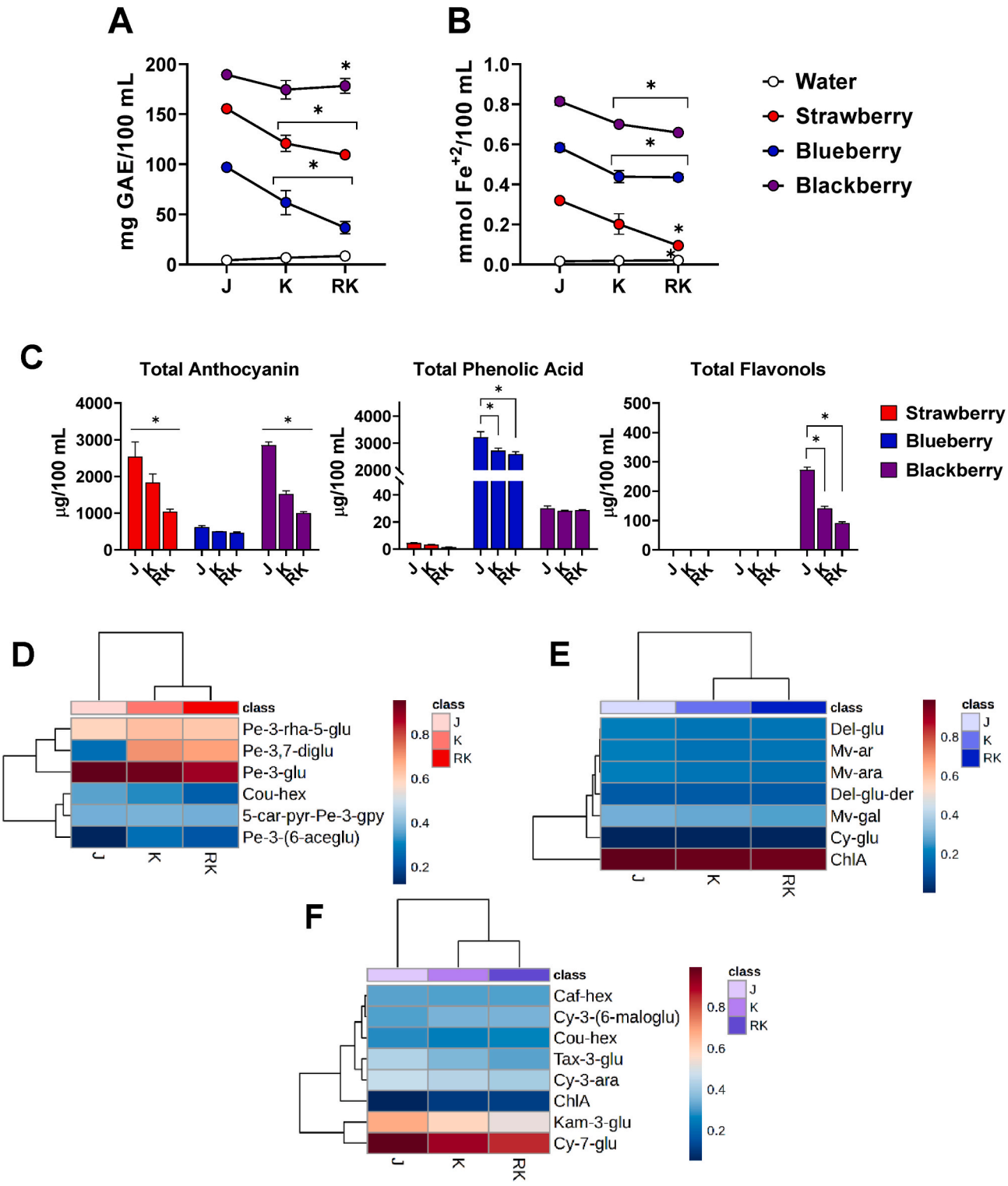


Fig. 4. Quantification of (A) total phenolic content (mg GAE/100 mL), (B) ferric equivalent antioxidant potential (mmol Fe^{2+}/100 mL), and sum of the total characterised polyphenolic class (C), including anthocyanin, phenolic acids, and flavonols in berry kefirs prepared from strawberry ■, blueberry ■, and blackberry ■, before fermentation (juice, J); after fermentation with kefir grains (K) at 21 ± 1 °C for 48 h; and after refrigeration storage (RK) at 4 ± 1 °C for 7 days. The graph shows the means ± SEM of the results (n= 3). Asterisks (*) indicate statistically significant differences (Student's t-test, $p < 0.05$) between fermented samples (K and RK) and their corresponding unfermented beverage (J) within the same fruit matrix. For the water kefir control, fermented samples were compared with the initial unfermented water control. Heatmaps showing changes and distribution of individual anthocyanins and related compounds in strawberry (D), blueberry (E), and blackberry (F) kefirs. The colour intensity corresponds to relative abundance. Abbreviations: Pelargonidin (Pe), cyanidin (Cy), delphinidin (Del), malvidin (Mv), kaempferol (Kam), taxifolin (Tax), chlorogenic acid (ChlA), p-coumaroyl (Cou), caffeoyl (Caf); glucoside (glu), rhamnoside (rha), galactoside (gal), arabinoside (ara), hexoside (hex), acetyl (ace), malonyl (mal), diglu (diglucoside), gpy (glucopyranoside), and 5-car-pyr (pyruvyl or carboxypyranoyl group). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

phenolic acids, with an initial anthocyanin-to-phenolic acid (A:PA) ratio of 16:84 in the juice. During fermentation, this proportion remained almost unchanged, resulting in a ratio of 15:85. The initial phenolic acid content decreased significantly after the refrigeration process, whilst the total anthocyanin content decreased, albeit not significantly. No flavonol derivatives were detected in the blueberry samples.

In the blackberry juice, the proportion of anthocyanins-to-phenolic acids-to-flavonols (A:PA:F) changed slightly from 90:1:9 in the juice to 89:3:8 after fermentation and refrigerated storage. This variation was mainly driven by a significant reduction in anthocyanins after the fermentation and refrigerated processing. In contrast, phenolic acids remained relatively stable, while flavonols dropped significantly during processing. These results are consistent with the TPC and FRAP measurements which identified the blackberry kefir as the product with the highest values in both assays. In contrast, the discrepancies observed between the TPC and FRAP values for the strawberry and blueberry kefirs likely arise from the differences in the chemical reactivity and antioxidant mechanisms of anthocyanins and phenolic acids, since each compound class responds differently to the reducing agents employed in those assays [41,42]. The following section provides a detailed quantification and discussion of all individual polyphenol derivatives identified in each fruit-based kefir (Table S4).

3.4.1. Strawberry kefir

The predominant anthocyanins identified in the strawberry kefir were pelargonidin derivatives (Fig. 4D). Among these, pelargonidin-3-glucoside (Pe-3-glu) was the main compound found in all the samples. The content of this compound decreased after fermentation and further declined after refrigerated storage. This trend concurs with previous studies of fermented strawberry products, where Pe-3-glu was reported as the main pigment and the most labile during fermentation processing [18].

In contrast, pelargonidin-3,7-diglucoside (Pe-3,7-diglu) increased significantly during fermentation, and remained stable after refrigeration storage. This apparent increase could be attributed to the enzymatic release of bound forms from the fruit matrix or partial transformation of other pelargonidin glycosides under acidic conditions [21]. Minor anthocyanins, such as pelargonidin-3-O-rhamnoside-5-O-glucoside (Pe-3-rha-5-glu), Pe-3-(6-acetylglucoside), and 5-carboxylpyranopelargonidin-3-O-glucopyranoside (5-car-pyr-Pe-3-gpy), were detected at lower concentrations but showed moderate stability during the fermentation and storage; this would suggest that not all anthocyanin degradation follows the same pattern as Pe-3-glu. Regarding phenolic acids, coumaroyl-hexoside (Cou-hex) decreased progressively during processing. This trend may be linked to the esterase activity of kefir microorganisms capable of hydrolysing phenolic ester bonds [43]. Overall, fermentation substantially altered the anthocyanin and phenolic acid composition of the strawberry kefir, characterised by the pronounced loss of Pe-3-glu, the partial preservation of minor derivatives, and selective transformations that modified the phytochemical profile of the beverage. Such alterations align with the reductions in TPC, and antioxidant capacity observed in Fig. 4A and B, respectively.

3.4.2. Blueberry kefir

The anthocyanin profile of the blueberry kefir was more diverse than that of the strawberry and blackberry kefirs, and consisted of mainly malvidin and delphinidin derivatives, with minor contributions from cyanidin (Fig. 4E–Table S4). These anthocyanins have been previously reported as the predominant pigments in blueberry [19]. Malvidin-3-galactoside (Mv-gal) was the main anthocyanin. However, it decreased after fermentation and further still after refrigerated storage. A similar downward trend was observed for other malvidin derivatives, including Mv-arabinoside (Mv-ara 1 and 2), which fell by approximately 25–35% compared to the initial juice.

These findings concur with previous studies indicating the higher chemical stability of Mv-based anthocyanins compared to other classes. In particular, Mv-gal and Mv-ara have been reported to exhibit enhanced resistance to alkaline conditions and thermal degradation due to the presence of additional methoxy groups in the B-ring, which increase molecular stability [44]. Nevertheless, our results demonstrate that, despite this inherent stability, malvidin glycosides still undergo partial degradation under fermentative and storage conditions. Our results also show that, while malvidin glycosides are more stable than pelargonidin or cyanidin derivatives, they are still susceptible to degradation under fermentative conditions. Delphinidin-3-glucoside (Del-glu 1) showed a similar pattern, decreasing after fermentation and stabilising during the refrigeration stage. In contrast, cyanidin-3-glucoside (Cy-glu) remained relatively constant throughout the process.

Among the non-anthocyanin compounds, chlorogenic acid (ChlA) was the most abundant phenolic compound in the blueberry juice, but decreased after refrigeration storage. This gradual decrease indicates that phenolic acids are also subject to moderate degradation during fermentation and storage. In summary, the blueberry kefir retained a relatively complex and diverse anthocyanin profile dominated by malvidin derivatives, which, although partially degraded, remained more stable than pelargonidin or cyanidin compounds under the same fermentation and refrigeration conditions.

3.4.3. Blackberry kefir

The anthocyanin composition of the blackberry kefir was dominated by cyanidin derivatives, particularly cyanidin-7-glucoside (Cy-7-glu), which was the major pigment in all samples (Fig. 4F–Table S4). However, its concentration decreased markedly during fermentation, and further still after refrigerated storage. Such a pattern indicates that Cy-7-glu is highly susceptible to degradation during fermentation and the subsequent refrigeration period; this is consistent with previous studies on fermented blackberry beverages that reported the limited stability of cyanidin-based anthocyanins under acidic and fermentative conditions [20].

Other minor anthocyanins were also identified. Cyanidin-3-arabinoside (Cy-3-ara) decreased progressively in the juice after fermentation and in refrigerated storage, reflecting a moderate but constant loss over time. In contrast, cyanidin-3-(6-malonylglucoside) increased slightly after fermentation but remained stable thereafter. This aligns with previous findings that acylated anthocyanins, such as malonylglucosides, are comparatively more stable and therefore more resistant to degradation [45,46].

Other non-anthocyanin polyphenols were detected in the blackberry kefir beverage, including taxifolin-3-glucoside (Tax-3-glu) and kaempferol-3-glucoside (Kam-3-glu), both of which had previously been reported in blackberry extracts [47]. Following the glycosylated anthocyanin trend, their concentrations decreased after fermentation, following the same trend observed for glycosylated anthocyanins. In contrast, the levels of phenolic acids, coumaroyl-hexoside (Cou-hex), caffeoyl-hexoside (Caf-hex), and chlorogenic acid (ChlA), remained relatively stable throughout fermentation and storage, indicating their greater resilience to microbial and/or pH-driven degradation.

Overall, the blackberry kefir exhibited a significant reduction in Cy-7-glu and other cyanidin derivatives as well as in non-anthocyanin polyphenols. Conversely, the acylated anthocyanins and phenolic acids remained relatively stable, suggesting the selective preservation of certain polyphenols during fermentation and refrigeration.

3.5. Sensory evaluation

A panel of 19 semi-trained assessors from the laboratory evaluated the sensory attributes of the fruit-based kefirs (Fig. 5). No significant differences were found among them in terms of aroma and colour, with average scores ranging from 3 to 4 on the five-point hedonic scale. This indicates moderate to high acceptance of these attributes across all the fruit kefirs. In contrast, flavour perception and global acceptability presented significant differences. The blueberry kefir received a higher

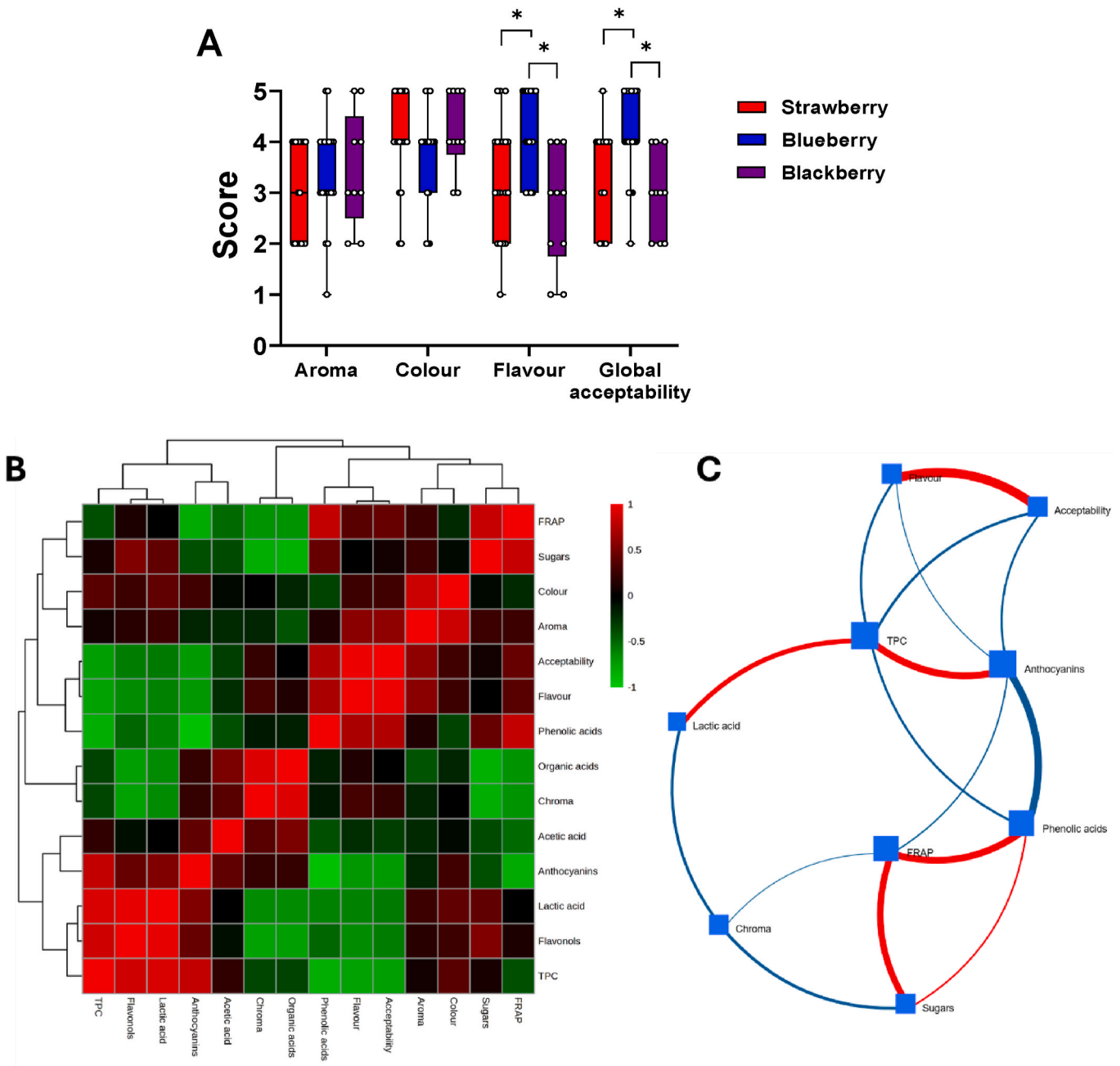


Fig. 5. Sensory analysis (A) in berry kefirs from strawberry, blueberry, and blackberry, and in water kefir (control fermentation) after refrigeration storage (RK) at 4 ± 1 °C for 7 days. Sensory scores were evaluated on a 5-point hedonic scale (1 = extremely poor, 3 = acceptable, 5 = excellent) and expressed as mean values (n = 19). Multiple *t*-test corrected by Holm-Sidak method showed significant effects for the main sensory parameters. Pearson correlation (*r*) matrix (B) of the main physicochemical, phytochemical, and sensory parameters achieved from refrigerated fruit kefirs (strawberry, blueberry, and blackberry). Debiased Sparse Partial Correlation diagram (C) showing the main parameters as nodes and their interconnection within the kefir system. Degree cutoff: 2; Betweenness cutoff: 1; Adjust *p*-value <0.05. Red and blue edges indicate positive and negative partial correlations, respectively, with the edge thickness being proportional to the strength of the association. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

flavour score than both the strawberry and the blackberry kefirs, whilst no significant difference was observed between the latter two. Global acceptability followed a similar trend, with the blueberry kefir achieving the highest overall score (median >4), which was significantly higher than the strawberry and blackberry kefirs, with averages of around 3. These findings indicate that all the berry kefirs were well accepted in terms of aroma and colour, although the blueberry kefir was perceived as having a more pleasant flavour and higher overall acceptability.

To explore potential relationships between chemical composition and sensory perception, correlation and network analyses were performed (Fig. 5). Fig. 5B shows the Pearson *r* correlation matrix including the main physicochemical (total sugars, total organic acids, acetic acid, lactic acid, and chroma), phytochemical (total anthocyanins, phenolic acids, flavonoids, FRAP, and TPC), and sensory parameters (flavour, colour, and aroma) of the refrigerated berry kefirs (Tables S5 and S6). Flavour and global acceptability were strongly and positively correlated ($r > 0.99$; $p < 0.05$; Tables S3 and S4), which would suggest that flavour was the sensory parameter that most strongly influenced overall liking.

On the other hand, phytochemical parameters such as TPC, total

anthocyanin and flavonoids exhibited significant negative correlations with both flavour and global acceptability ($r = -0.60$ to -0.75; Fig. S1). This inverse association may be attributed to the astringency and harsher astringency sub-qualities [48] conferred by anthocyanins and polyphenols, which are known to affect flavour perception. As expected, TPC correlated positively with total anthocyanins and flavonoids. Despite total organic acids, acetic acid, and lactic acid also negatively correlating with flavour and global acceptability, such associations did not reach statistical significance ($p > 0.05$, Table S6).

The Debiased Sparse Partial Correlation (DSPC) diagram is presented in Fig. 5C, with nodes representing the main variables and edges indicate statistically significant associations. After applying the statistical thresholds, only the most relevant nodes were retained. In the resulting network (Table S7), TPC was the most central node, exhibiting the highest degree (5) and betweenness (6.67), followed by total anthocyanins (degree = 5 and betweenness = 5.83), FRAP (degree = 4 and betweenness = 3.67) and phenolic acids (degree = 4 and betweenness = 3.33). This confirmed the structural relevance of polyphenols in defining the kefir matrix. From a sensory perspective, flavour and acceptability (degree = 3) are endpoints in the chemical network rather than intermediate variables (betweenness = 0.0). Additionally, they are positively affected by each other but negatively affected by the TPC and anthocyanins content, being the main negative drivers of the sensorial parameters. In summary, sensory evaluation, correlation, and network analysis consistently showed that flavour was the key driver of consumer acceptability in the berry kefirs, and that polyphenol composition (especially total anthocyanins and TPC) played a central role in shaping both the sensory and structural characteristics of the beverages.

3.6. Anti-inflammatory potential

Optimisation studies were conducted to determine the optimal sample concentration of the different formulations to be cultured with HCT116-Dual cells and using cell viability as a screening tool. A Sytox-Green™ probe was used, following a 48h sample exposure at 5%, 10%, and 20% dilutions (Fig. S2); this revealed that the 20% solution caused a marked and significant reduction in cell viability. Nevertheless, the 5% and 10% solutions did not significantly affect cell viability at similar levels as the cells treated with culture medium. Based on these findings, a concentration of 10% juice, berry kefirs, and refrigerated berry kefirs was selected for subsequent analyses i.e., NF-κB activation and IL-8 secretion at basal state upon exposure to the different formulations or after stimulations with the pro-inflammatory cytokines IL-1β or TNF-α (Fig. 6).

A contrasting pattern was observed in epithelial cells cultured with the strawberry juice, its fermented kefir, or its refrigerated kefir sample: it showed a significantly lower NF-κB activation while inducing IL-8 secretion from the cells (Fig. 6A). The blueberry juice showed no effect on the cells under basal conditions, whilst the blueberry kefir or refrigerated kefir significantly reduced NF-κB activation (Fig. 6C and E). The blackberry juice significantly induced NF-κB activation and both the blackberry juice and its kefir sample induced significant IL-8 secretion from the epithelial cells at basal level (Fig. 6B and D). When examining the response of these formulations under an inflammatory state created

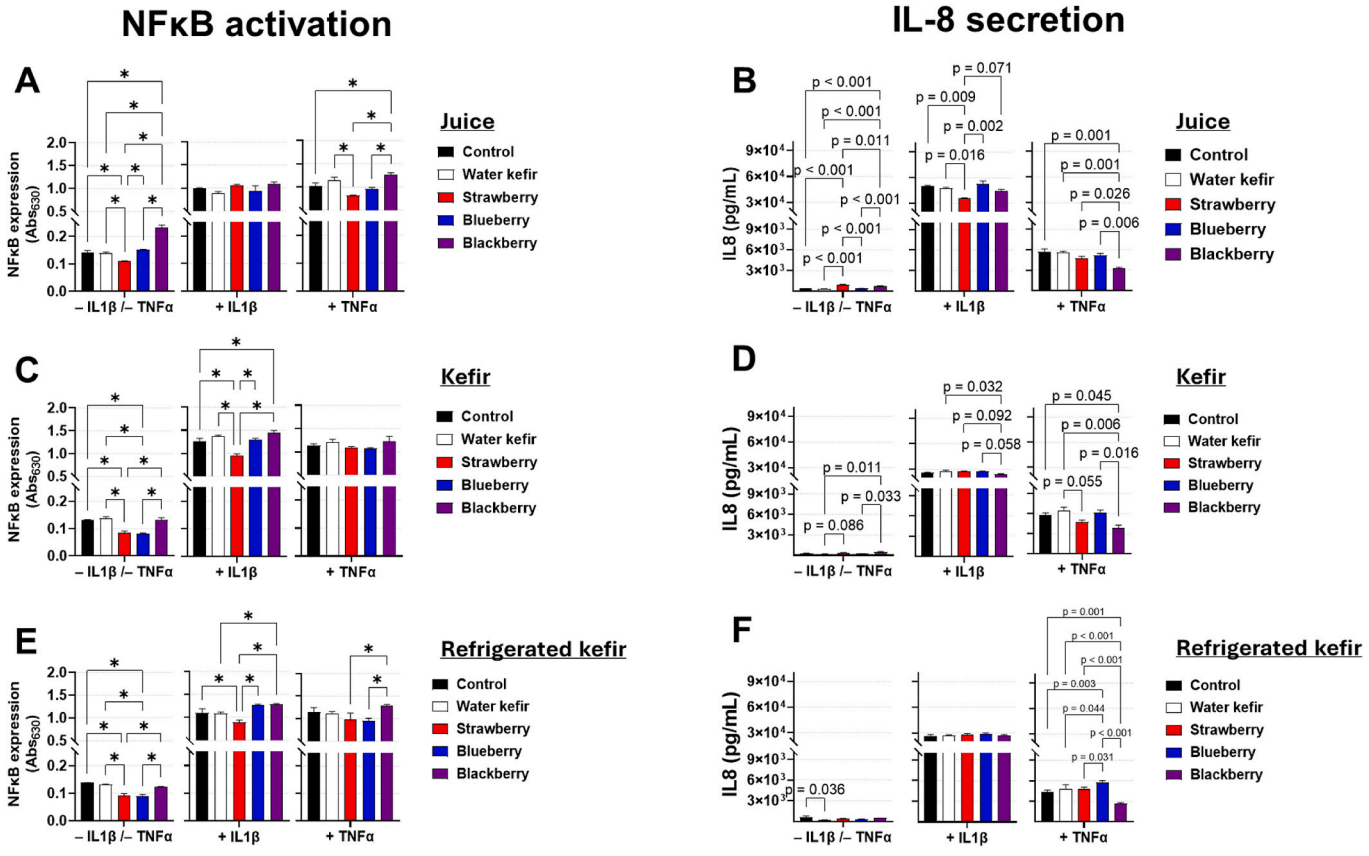


Fig. 6. Anti-inflammatory potential of berry juices and their kefirs (fermented and refrigerated) in HCT116-Dual reporter cells. NF-κB pathway activation (A, C, and E) and IL-8 secretion (B, E, and F) assessed by ELISA were evaluated following 24 h pre-treatment of epithelial cells with berry juices (A, B), berry kefirs (C, D), and refrigerated berry kefirs (E,F), followed by an additional 24h incubation under basal conditions (no cytokines, −IL1β/−TNFα) or in the presence of the pro-inflammatory cytokines (+IL1β or + TNFα). Water kefir was included as a control for the fermentation process. Cells treated with cytokines alone (control + IL-1β or control + TNFα) served as a positive control for NF-κB activation and IL8 secretion. The graph shows the means ± SEM of the results (n= 3). Asterisks (*) indicate statistically significant differences among samples within each processing stage (juice, kefir, refrigerated kefir), determined by one-way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$).

by IL-1β or TNF-α stimulations, the strawberry juice significantly reduced TNF-α induced NF-κB activation and IL-1β-induced IL-8 secretion, while the strawberry kefir significantly reduced TNF-α-induced IL-8 secretion and the refrigerated strawberry kefir showed a trend in reducing IL-1β-induced NF-κB activation (Fig. 6E). Neither the blueberry juice nor its kefir presented an anti-inflammatory potential on cytokine-treated cells, whilst the refrigerated blueberry kefir significantly increased TNF-α induced IL-8 secretion (Fig. 6F). The blueberry juice had no impact on cytokine-induced NF-κB activation or IL-8 secretion, whilst the blackberry kefir and its refrigerated kefir significantly reduced TNF-α induced IL-8 secretion (Fig. 6D and F). These data indicate that the strawberry juice or its kefir fermented form showed functional properties related to inflammation modulation in cell-based assays, while the blueberry and blackberry showed low to moderate immunomodulatory potential on these assays.

In contrast, blackberry juice and kefir exhibited an opposing pattern, with increased NF-κB reporter activation alongside decreased IL-8 secretion. While the reporter assay reflects upstream transcriptional activation, IL-8 secretion represents a downstream outcome dependent on additional regulatory mechanisms, including promoter-specific control, mRNA stability, translation and cytokine release (Priester et al., 2022). These findings suggest that blackberry-derived phytochemicals modulate multiple checkpoints in inflammatory signalling. Anthocyanin- and polyphenol-rich extracts have been shown to reduce IL-8 levels by targeting NF-κB and intersecting kinase pathways such as MAPK and AP-1, even when upstream inflammatory signalling remains active [49,50]. This apparent divergence between the reporter activity and IL-8 release highlights the multilayered regulation of the NF-κB pathway and indicates that fermentation-derived metabolites may differentially influence transcriptional and post-transcriptional processes. This dual-level modulation provides mechanistic insight into the anti-inflammatory potential of blackberry-based kefir and juice.

Under the tested conditions, the fermentation process reduced the anti-inflammatory bioactivity of the berry kefirs. However, the blackberry kefir exhibited a distinct response to TNF-α stimulation, partially preserving its bioactive potential. This loss of bioactivity is most likely associated with the reduction in polyphenolic compounds, particularly anthocyanins, phenolic acids and flavonols, as observed after fermentation and refrigeration (Fig. 4). Furthermore, the higher proportion of phenolic acids relative to flavonoids in the blueberry juice suggests that flavonoids are the main contributors to NF-κB regulation of these matrices; in line with a previous report on Caco-2 cells by Romier et al. [51].

Similarly, Cremonini et al. [52] demonstrated that the inhibition of NF-κB activation by anthocyanins depends on their chemical structure and concentration. Cyanidin exhibited the strongest inhibitory effect, followed by delphinidin, while malvidin, petunidin, and peonidin had no significant effect at the concentrations tested. These findings are consistent with the current results, which show that blackberry kefir, rich in cyanidin derivatives, effectively reduces TNF-α-induced IL-8 secretion, indicating indirect inhibition of the NF-κB pathway. The anti-inflammatory potential of the fresh berry juices, in particular the strawberry juice, differs from a previous study on TNF-α-treated HT-29 cells [53], in which lower juice concentrations (0.5% and 2%) and shorter culture time (1 h) were used, consequently resulting in a diminished effect when compared to the current study conditions.

Several studies have demonstrated that anthocyanins exert anti-inflammatory potential against TNF-α-induced NF-κB activation by reducing the phosphorylation of IKK and transcription factor p65 [52,54,55]. However, *in vitro* studies investigating IL-1β-induced inflammation have mainly focused on aglycone flavonoids in Caco-2 cells [51], overlooking glycosylated anthocyanin structures, which are more representative of those naturally present in berry matrices.

4. Conclusions

Strawberry, blueberry, and blackberry juices were fermented with kefir grains, confirming their suitability as substrates to produce non-dairy fruit kefirs with nutritional and functional properties. Fermentation followed by refrigerated storage notably modified their physicochemical, phytochemical, sensory, and biological characteristics compared with the water kefir control. During fermentation, all berry kefirs showed a decrease in TSS, accompanied by an increase in lactic and acetic acids, confirming the active metabolism of the kefir consortium. Microbial growth was evidenced by an increase in mesophilic and LAB counts after fermentation, and remained stable or increased slightly during refrigeration, but fell in the water kefir control. Polyphenolic profiling revealed substantial transformation of anthocyanins, phenolic acids, and flavonols during fermentation, with losses ranging from 35 to 60%, particularly in strawberry and blackberry kefirs. The blueberry kefir achieved the highest scores for flavour and overall acceptability (~4/5) in the sensory evaluation, while all the berry kefirs maintained good colour and aroma attributes, despite compositional changes. Correlation and network analyses highlighted TPC and anthocyanins as central parameters connecting chemical composition and sensory quality.

Epithelial cell studies demonstrated that fermentation and the refrigeration stage affect the anti-inflammatory potential of berry kefirs. However, the blackberry kefir partially preserved its inhibitory effect against TNF-α-induced responses, which is consistent with its cyanidin-rich profile and higher residual antioxidant activity. These results highlight that the functional bioactivity and microbiological performance of fermented berry kefirs depend strongly on the phenolic composition of the initial fruit matrix and the chemical transformations that occur during fermentation and refrigerated storage. While fermentation enhances the nutritional, sensory and microbiological attributes of berry kefirs, it may also reduce their anti-inflammatory potential due to the degradation of key polyphenolic compounds.

In conclusion, fruit-based kefirs represent promising non-dairy functional beverages, in which fermentation dynamics and fruit matrix composition determine both their chemical stability and their bioactivity. Moreover, the use of industry-grade fruit, typically considered a by-product of fresh-market sorting, supports product diversification and added-value generation while promoting more sustainable and efficient resource use. Optimising fermentation conditions to minimise polyphenol degradation, particularly of anthocyanins, while preserving sensory quality, could further enhance the health-promoting potential and consumer appeal of these naturally fermented kefir beverages.

CRediT authorship contribution statement

José Ángel Salas-Millán: Writing – original draft, Visualization, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Asunción Iguaz:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Encarna Aguayo:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Silvia Melgar:** Writing – review & editing, Methodology. **Arantxa Aznar:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors gratefully acknowledge the company Masía Ciscar S.A. (Huelva, Spain) for providing the financial support and supplying the fresh fruit materials used in this study. This work is also part of the AGROALNEXT programme and was supported by the Ministry of Science, Innovation and Universities (MICIU) with funding from the European Union Next Generation EU (PRTR-C17.I1) and by Fundación Séneca – Science and Technology Agency of the Region of Murcia (CARM) and by a centre grant from Science Foundation Ireland (SFI) to APC Microbiome Ireland (SFI/12/RC/2273_P2). We thank Concetta Libero and Carmela Livigni for collaborating on this study during their Bachelor's degree Erasmus exchange programme.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jafr.2026.102963.

Data availability

https://doi.org/10.5281/zenodo.18270470.

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