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Original Article

Investigation of the Viability of Probiotic Microorganisms in a Synbiotic Kefir Beverage Enriched with Pistachio (*Pistacia vera*) and Hazelnut (*Corylus avellana* L.) Oils

Hesam Zangeneh[a]; Aliasghar Bagheri Kashtali[b]; Abbas Ali Dehpour Joybari[a] ;Marjan nouri[c]

[a] *Department of Biology, QaS.C., Islamic Azad University, Qaemshahr,Iran*
[b] *Department of Biology, Ro.C., Islamic Azad University, Roudehen, Iran*
[c] *Department of Food Science and Technology, Ro.C., Islamic Azad University, Roudehen, Iran*

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Corressponding Authors:
[1]Aliasghar Bagheri Kashtali
aliasghar@iau.ac.ir
[2]Abbas Ali Dehpour Joybari
dehpour@gmail.com

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ABSTRACT

Kefir is a fermented beverage rich in probiotic microorganisms whose viability during storage is limited by environmental stresses, so prebiotic compounds are often needed to improve their survival. This study evaluated the effect of adding pistachio and hazelnut oils, as prebiotic and bioactive compounds, on the viability of the probiotic microorganisms of kefir and on its physicochemical, antioxidant and sensory properties. The oils were extracted by Soxhlet apparatus and characterised, and synbiotic kefir was then prepared with them at single (1.5% and 3%) and combined levels and compared with an oil-free control over 20 days of refrigerated storage. Both oils had low acidity and low peroxide values. Hazelnut oil gave the lower half-maximal inhibitory concentration (IC_{50}) values in the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays, whereas pistachio oil showed the stronger antimicrobial activity, with the lowest minimum inhibitory concentration (MIC) against Listeria monocytogenes. Throughout storage, oil addition significantly maintained a higher pH and a lower titratable acidity than the control ($P < 0.05$). The combined P2H2 treatment (1.5% pistachio + 1.5% hazelnut oil) retained the highest total phenolic content and antioxidant activity, preserved the highest microbial counts on day 20, and gave the highest cell-surface hydrophobicity, while the milder P1H1 treatment achieved the best overall sensory acceptance. In conclusion, pistachio and hazelnut oils acted as effective prebiotic agents that improved probiotic survival and enhanced the functional value of kefir, offering a practical option for developing more stable probiotic products.

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1. Introduction

Increasing awareness of the factors that influence health has raised the demand for healthy food among all sections of society, a trend confirmed by the growing market for functional foods (Turek & Wszłek, 2021). Functional foods are foods that, beyond their nutritional value, contain substances that act specifically on body functions related to controlling or reducing the risk of certain diseases. Probiotic fermented dairy products occupy a special place among them. Kefir is a traditional acidic–alcoholic fermented beverage obtained by fermenting milk with kefir grains, which are a symbiotic consortium of bacteria (lactic acid bacteria and acetic acid bacteria) and yeasts embedded in a polysaccharide matrix (kefiran). Consumption of kefir provides numerous health benefits — antimicrobial, anti-cancer, cholesterol-lowering and immune-modulating effects — through its probiotic microorganisms and bioactive metabolites (Yilmaz et al., 2022; Moghimani et al., 2023). The primary condition for the efficacy of such products is that the probiotics remain viable during storage and survive the harsh conditions of the gastrointestinal tract. Prebiotics are non-digestible compounds that selectively stimulate the growth and activity of beneficial gut bacteria, and a product that combines probiotics and prebiotics is termed a synbiotic (Markowiak & Śliżewska, 2017).

Nuts and vegetable oils have high potential as prebiotic and functional ingredients because of their content of unsaturated fatty acids, phenolic compounds and antioxidants. Pistachio oil (Pistacia vera) is rich in oleic acid, phytosterols and tocopherols, which are associated with good oxidative stability (Ryan et al., 2006;

Miraliakbari & Shahidi, 2008). Hazelnut oil (Corylus avellana L.) is likewise an excellent source of monounsaturated fatty acids and phenolic compounds, and has been reported to have a high total phenol and flavonoid content (Alasalvar & Bolling, 2015; Balik, 2021). Several studies have enriched kefir with plant materials — hazelnut milk, wheat germ, lyophilised wild garlic powder, ginger extract, hazelnut press cake, and walnut oil (Turek & Wszłek, 2021; Tsisaryk et al., 2022) — but a review of the Iranian and international literature indicates that pistachio and hazelnut oils have not previously been used together in kefir production. The aim of this study was therefore to evaluate the effect of adding pistachio and hazelnut oils on the viability of the probiotic microorganisms of kefir (Lactobacillus, Lactococcus & Bifidobacterium) and on the physicochemical, antioxidant and sensory properties of the resulting synbiotic kefir during 20 days of refrigerated storage.

2. Materials and Methods

2.1. Extraction and Evaluation of Pistachio and Hazelnut Oils

2.1.1 Oil extraction

Pistachio and hazelnut kernels were ground and sieved to a particle size of 2–5 mm. Oil was extracted with a Soxhlet apparatus at 50 °C for 5 h using petroleum ether as the solvent. At the end of the process the solvent was evaporated under vacuum at room temperature and the recovered oil was kept in a dark bottle at −18 °C until use (Özcan et al., 2019).

2.1.2 Physicochemical tests

The acidity and peroxide value of the pistachio and hazelnut oils were determined according to the standard methods of the Association of Official Analytical Chemists (AOAC) (Horwitz, 2010).

2.1.3 Total flavonoid content

Total flavonoid content was measured by the aluminium chloride colorimetric method. One millilitre of oil (0.1 mg/mL) was combined with 1.25 mL distilled water and 75 µL of 5% sodium nitrite and held at 24 °C in the dark. Then 150 µL of 10% aluminium trichloride was added and the mixture was kept for 5 min at 24 °C in the dark, after which 1 mL of 1 M sodium hydroxide was added. The absorbance was recorded at 510 nm (Hossain & Rahman, 2011).

2.1.4 Total phenolic content

The Folin–Ciocalteu reagent was used with gallic acid as the standard. Gallic acid standards of 0, 50, 100, 250, 500 and 1000 mg/L were prepared for the calibration curve. For the assay, 20 µL of oil (10 g/L) was added to 2 mL distilled water and 100 µL Folin–Ciocalteu reagent; after 3 min, 300 µL sodium bicarbonate solution was added and the mixture was stirred on a magnetic stirrer for 2 h. Absorbance was recorded at 765 nm with a spectrophotometer (Sigma 3-30k) and the result was expressed as gallic acid equivalent (Hossain & Rahman, 2011).

2.1.5 DPPH radical scavenging

A 3.9 mL aliquot of 2,2-diphenyl-1-picrylhydrazyl (DPPH) stock solution (0.004 g DPPH in 100 mL methanol) was transferred to a test tube, 0.1 mL of each oil was added, and the tube was held in the dark for 30 min before the absorbance was read at 517 nm. The results were expressed as the half-maximal inhibitory concentration (IC_{50}), that is, the amount of antioxidant required to reduce the DPPH concentration to 50% of its initial value (Falah et al., 2021).

2.1.6 ABTS radical scavenging

A solution containing 7 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2.4 mM potassium persulfate (1:1) was held in the dark at 24 °C for 14 h and then diluted with ethanol to an absorbance of 0.7 ± 0.02 at 734 nm. Two millilitres of the resulting solution was mixed with the oil at concentrations of 10, 50, 100, 250 and 500 µg/mL, held at room temperature for 30 min, and the absorbance was read at 734 nm. Results were reported as IC_{50} (Labiad et al., 2017).

2.1.7 Antimicrobial tests

Salmonella typhimurium, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Listeria monocytogenes and Shigella dysenteriae, obtained from the Iranian Biological Resource Center, were used as the pathogenic test strains; overnight cultures were prepared in Mueller–Hinton broth at 37 °C. For the disk diffusion method (DDM), oil solutions at 20 mg/mL were sterilised through a 0.45 µm syringe filter and blank disks were soaked in them for 15 min. Plates were inoculated with a microbial suspension equivalent to the 0.5 McFarland standard (1.5×10^8 CFU/mL), the disks were placed on the surface, and the plates were incubated at 37 °C for 24 h; the antimicrobial effect was reported as the inhibition zone. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by the serial dilution method, using oil dilutions from 3.75 to 600 µg/mL in 96-well microtiter plates inoculated with 100 µL of an 18 h culture at the 1 McFarland standard (3×10^8 CFU/mL). After 24 h at 37 °C, the lowest oil concentration showing no visible growth was recorded as the MIC; for the MBC, 5 µL from each well was transferred to oil-free medium and incubated at 37 °C for 24 h, and the lowest concentration preventing growth was recorded (El-Atki et al., 2020).

2.1.8 Scanning electron microscopy (SEM)

E. coli was cultured in broth at the MIC concentration and incubated at 37 °C, centrifuged at 5000 × g for 5 min,

washed with 0.1 M phosphate buffer (pH 7), filtered, fixed in 2.5% glutaraldehyde for 2 h at 4 °C, washed repeatedly with distilled water, dehydrated through a graded ethanol series (30, 50, 70, 80, 90 and 100%) for 10 min each, dried, gold-coated and examined with a scanning electron microscope (Zeiss (LEO) 1450 VP, Germany) (Ghazanfari et al., 2020).

2.2. Preparation of the Synbiotic Kefir Samples

Pasteurised cow milk (2.5% fat) was cooled to 22 °C, inoculated with 3% (w/v) kefir grains and mixed for 15 min. Pistachio and hazelnut oils were then added, separately and in combination, at defined levels (1.5% and 3%). After oil addition the milk was homogenised three times in an Armfield Ltd. (Ringwood, UK) homogeniser at 7 MPa. The treatments were packaged in glass bottles, incubated at 25 °C until the target acidity was reached, and then cooled to 4 °C. The treatments are coded in Table 1 and were evaluated on days 1, 10 and 20 of refrigerated storage.

Table 1. Kefir treatments used in the production of fermented kefir milk with pistachio and hazelnut oil.

Treatment code	Oil composition	Treatment description
C	–	Kefir without oil (control)
P1	Pistachio oil	Addition of 1.5% pistachio oil
P2	Pistachio oil	Addition of 3% pistachio oil
H1	Hazelnut oil	Addition of 1.5% hazelnut oil
H2	Hazelnut oil	Addition of 3% hazelnut oil
P1H1	Pistachio + hazelnut	Combined addition of 0.75% pistachio oil and 0.75% hazelnut oil
P2H2	Pistachio + hazelnut	Combined addition of 1.5% pistachio oil and 1.5% hazelnut oil

2.3. Evaluation of the Kefir Samples

2.3.1. Physicochemical properties

pH was measured potentiometrically with a pH meter (Seven Compact pH/Ion S220, Schwerzenbach, Switzerland). Titratable acidity was expressed as g lactic acid per 100 mL (Dornic degree) and determined according to AOAC (2000) (Cavia et al., 2002).

2.3.2 Total phenolic content and antioxidant activity

Total phenolic content and DPPH radical scavenging activity of the kefir samples were determined by the same methods described above for the oil samples.

2.3.3 Probiotic viability

Under sterile conditions, samples were taken from each treatment on days 1, 10 and 20 of storage. One millilitre of kefir was added to 100 mL de Man, Rogosa and Sharpe (MRS) medium; after incubation, serial dilutions were prepared in buffered peptone water. The dilutions were used to count Lactobacillus spp. (MRS, 37 °C, 72 h), Lactococcus spp. (M17, 20 °C, 120 h) and Bifidobacterium spp. (MRS-NNLP, containing nalidixic acid, neomycin sulphate, lithium chloride and paromomycin sulphate, 37 °C, 72 h). Viable yeasts were counted on a medium containing 0.5% yeast extract, 1% glucose, 1.5% agar and 0.5% polypeptone after incubation at 30 °C for 48 h. Counts were reported as log colony-forming units per millilitre (log CFU/mL) (Turek & Wszłek, 2021).

2.3.4 Cell-surface hydrophobicity

Because probiotic microorganisms must adhere to the intestinal wall in order to function properly, hydrophobicity was assessed with the n-hexadecane method. Lactobacillus, Lactococcus and Bifidobacterium isolates were cultured under the conditions given above until the optical density (OD_0) reached 0.6–0.7. Three millilitres of the microbial suspension was added to 1 mL n-hexadecane and incubated for 15 min. The tubes were vortexed for 3 min, held at room temperature for 30 min, and the OD was measured (Barzegar et al., 2021).

2.3.5 FTIR spectroscopy

The functional groups of the kefir samples were examined with a Fourier-transform infrared (FTIR) spectrophotometer (Perkin Elmer, USA) over the wavenumber range 400–4000 cm^{-1}.

2.3.6 Sensory evaluation

Sensory evaluation of the kefir beverage was carried out by 60 untrained panellists. The treatments were presented randomly in transparent glass cups to male and female participants, who scored them on a scale from dislike extremely (1) to like extremely (5) for appearance, flavour, consistency, odour and overall acceptance (Özcan et al., 2019).

2.4. Statistical Analysis

The whole experiment was carried out in a completely randomised design with three independent replicates (n = 3), and all measurements were made in triplicate. Results are expressed as mean ± standard deviation. Data were analysed by one-way analysis of variance (ANOVA) in SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA); the effects of treatment and of storage time were tested separately, and means were compared with Duncan's multiple range test at the 95% confidence level. Differences were considered significant at $P < 0.05$ and are indicated in the tables and figures by different letters (a–g between treatments within a column and A–C between storage days within a row).

3. Results

3.1. Properties of the Extracted Oils

3.1.1 Acidity and peroxide value

For pistachio oil, an acidity of 0.29% (as oleic acid) and a peroxide value of 0.18 meq O_2/kg oil were obtained. For hazelnut oil the acidity was 0.7% and the peroxide value 1.51 meq O_2/kg oil.

3.1.2. Total phenolic and flavonoid content

In the pistachio oil sample, a total phenolic content of 539.1 mg gallic acid equivalent per 100 g dry matter and a total flavonoid content of 13.23 per 100 g were found. For hazelnut oil, the total phenolic content was 110.15 gallic acid equivalent per 100 g and the reported flavonoid content was 8.91 quercetin equivalent per 100 g.

3.1.3 Antioxidant activity

The antioxidant activity, expressed as the percentage of DPPH and ABTS radical inhibition, is presented in Figure 1. Both pistachio and hazelnut oils increased the DPPH radical inhibition in a dose-dependent manner as the concentration rose from 100 to 500 µg/mL. Hazelnut oil showed a higher inhibition percentage than pistachio oil at every concentration, reaching about 80% at 500 µg/mL while pistachio oil reached about 65%. The ABTS results followed the same pattern: both oils were dose-dependent, and hazelnut oil again showed a greater inhibition percentage than pistachio oil, reaching about 85% at the highest concentration (500 µg/mL) against about 70% for pistachio oil.

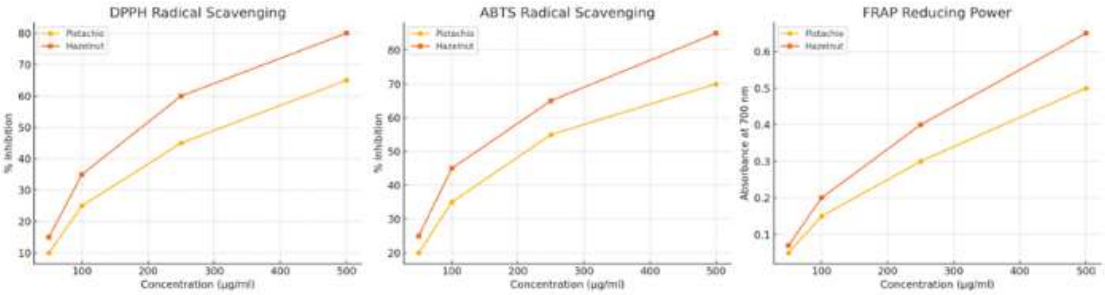

Fig 1. Percentage inhibition of DPPH and ABTS free radicals by pistachio and hazelnut oil.

Fig 2. IC_{50} values for the inhibition of DPPH and ABTS free radicals by pistachio and hazelnut oil. Letters a and b indicate a statistically significant difference between hazelnut and pistachio oil at $P < 0.05$.

3.1.4 Antimicrobial activity

The disk diffusion results (Table 2) showed that both pistachio and hazelnut oils inhibited the growth of the Gram-positive and Gram-negative bacteria studied. The largest inhibition zone belonged to Listeria monocytogenes, reaching 17 mm for pistachio oil and 15 mm for hazelnut oil. Staphylococcus aureus also produced a relatively large zone of inhibition (15 mm for pistachio oil and 13 mm for hazelnut oil). The smallest inhibitory effect for both oils was against Escherichia coli, which produced a zone of 9 mm for pistachio oil and 8 mm for hazelnut oil.

Table 2. Disk diffusion (inhibition zone, mm) of the pistachio and hazelnut oil samples against the test bacteria.

Bacterium	Pistachio oil (mm)	Hazelnut oil (mm)
Salmonella typhimurium	12 ± 1 c	10 ± 1 c
Staphylococcus aureus	15 ± 1 b	13 ± 1 b
Staphylococcus epidermidis	13 ± 1 c	11 ± 1 c
Escherichia coli	9 ± 1 d	8 ± 1 d
Listeria monocytogenes	17 ± 1 a	15 ± 1 a
Shigella dysenteriae	10 ± 1 cd	9 ± 1 cd

Values with different letters in each column indicate a statistically significant difference at $P < 0.05$.

According to Table 3, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) results showed that both oils had a considerable antibacterial effect, but pistachio oil was generally more active. Specifically, the lowest MIC for pistachio oil was against Listeria monocytogenes at 104 ± 8 µg/mL, and for hazelnut oil against Staphylococcus aureus at 151 ± 12 µg/mL. The highest MIC in both oils belonged to Escherichia coli — 264 ± 20 µg/mL for pistachio oil and 418 ± 33 µg/mL for hazelnut oil — indicating the greater resistance of this bacterium. For the MBC values, pistachio oil showed its lowest value against Listeria monocytogenes (381 ± 28 µg/mL) and its highest against Escherichia coli (947 ± 60 µg/mL); for hazelnut oil the lowest MBC was against Staphylococcus aureus (507 ± 38 µg/mL) and the highest against Escherichia coli (1084 ± 55 µg/mL). Statistical analysis showed significant differences between the MIC and MBC of the different bacteria within each oil ($P < 0.05$), with the MIC and MBC values of pistachio oil significantly lower than those of hazelnut oil.

3.1.5 Morphology of the pathogens

In the control sample (Figure 3a), untreated Escherichia coli cells had a uniform rod shape with a smooth, undamaged surface, reflecting the normal, intact state of the bacterial cell wall. In samples b and c, corresponding

Table 3. MIC and MBC values (µg/mL) of the pistachio and hazelnut oil samples against the test bacteria.

Bacterium	MIC pistachio oil	MIC hazelnut oil	MBC pistachio oil	MBC hazelnut oil
Salmonella typhimurium	201 ± 10 c	234 ± 18 c	673 ± 45 b	803 ± 50 c
Staphylococcus aureus	110 ± 5 e	151 ± 12 d	439 ± 35 c	507 ± 38 d
Staphylococcus epidermidis	123 ± 5 d	231 ± 19 c	465 ± 32 c	552 ± 41 d
Escherichia coli	264 ± 20 a	418 ± 33 a	947 ± 60 a	1084 ± 55 a
Listeria monocytogenes	104 ± 8 f	156 ± 13 d	381 ± 28 d	534 ± 40 d
Shigella dysenteriae	235 ± 10 b	309 ± 25 b	853 ± 55 b	941 ± 48 b

Values with different letters in each column indicate a statistically significant difference at P < 0.05.

to cells exposed to pistachio and hazelnut oil, the treated cells showed clear changes in cell-wall structure: membrane shrinkage, deformation and, in some cells, rupture. This morphological damage indicates a loss of cell-membrane integrity and probable leakage of intracellular contents; in some cells the shrinkage was more severe and the wall rupture more evident.

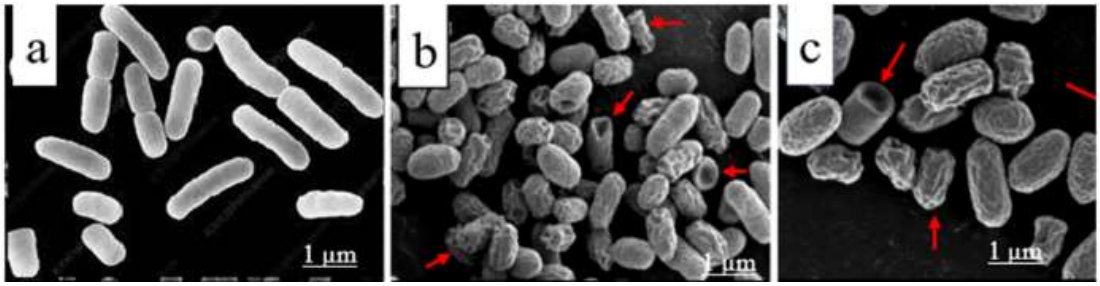

Fig 3. SEM images of (a) untreated Escherichia coli, (b) Escherichia coli treated with pistachio oil and (c) Escherichia coli treated with hazelnut oil.

3.2. Evaluation of the Kefir Samples

3.2.1 pH

As shown in Table 4, the pH of the kefir samples decreased significantly from day 1 to day 20 of storage (P < 0.05). On day 1 the P2H2 and P2 treatments had the highest pH (4.55), which indicates the effect of the vegetable oils in raising the initial pH of the product, whereas the control (C) showed the lowest day-1 pH (4.48). A declining trend was observed in all treatments during storage as a result of continued microbial activity and acid production. On day 20 the pH of the control fell to 4.27 ± 0.01, lower than all other treatments, reflecting greater acid production in the oil-free kefir. The oil-containing treatments, particularly P2 and H2, maintained a higher pH than the control on day 20 (4.35 ± 0.01), which is attributable to the role of the oil in buffering the medium and slowing acidification.

3.2.2 Titratable acidity

Table 5 shows that titratable acidity (% lactic acid) increased significantly in all treatments during storage (P < 0.05). On day 1 the control had the highest acidity (0.75 ± 0.01) and P2 the lowest (0.72).

Table 4. Changes in the pH of the enriched kefir samples over 1, 10 and 20 days of storage (4 °C).

Treatment	Day 1	Day 10	Day 20
C	4.48 ± 0.01 bA	4.37 ± 0.01 cB	4.27 ± 0.01 cC
P1	4.52 ± 0.02 aA	4.43 ± 0.01 bB	4.31 ± 0.01 bC
P2	4.55 ± 0.02 aA	4.46 ± 0.01 aB	4.35 ± 0.01 aC
H1	4.51 ± 0.02 aA	4.41 ± 0.01 bB	4.31 ± 0.01 bC
H2	4.53 ± 0.02 aA	4.46 ± 0.01 aB	4.35 ± 0.01 aC
P1H1	4.51 ± 0.02 aA	4.42 ± 0.01 bB	4.30 ± 0.01 bC
P2H2	4.55 ± 0.02 aA	4.44 ± 0.01 aB	4.34 ± 0.01 aC

Letters a–g indicate a significant difference within each column (between treatments) and letters A–C a significant difference within each row (between storage days).

At the end of storage (day 20) the acidity of all treatments had risen appreciably; the control reached the highest value (0.96 ± 0.01), while oil-containing treatments such as P2 (0.90 ± 0.01) and P2H2 (0.90 ± 0.01) had a lower acidity than the control. These results indicate that adding pistachio and hazelnut oils slows the acidification of kefir during storage, which may be due to the buffering capacity capacity of the fats and possibly to an effect on the growth of the kefir microflora.

Table 5. Changes in the titratable acidity (% lactic acid) of the enriched kefir samples over 1, 10 and 20 days of storage (4 °C).

Treatment	Day 1	Day 10	Day 20
C	0.75 ± 0.01 aC	0.87 ± 0.01 aB	0.96 ± 0.01 aA
P1	0.74 ± 0.01 aC	0.84 ± 0.01 bB	0.93 ± 0.01 bA
P2	0.72 ± 0.02 aC	0.82 ± 0.01 bB	0.90 ± 0.01 cA
H1	0.74 ± 0.01 aC	0.84 ± 0.01 bB	0.93 ± 0.01 bA
H2	0.73 ± 0.01 aC	0.83 ± 0.01 bB	0.93 ± 0.01 bA
P1H1	0.74 ± 0.01 aC	0.84 ± 0.01 bB	0.92 ± 0.01 cA
P2H2	0.73 ± 0.01 aC	0.83 ± 0.01 bB	0.90 ± 0.01 cA

Letters a–g indicate a significant difference within each column (between treatments) and letters A–C a significant difference within each row (between storage days).

3.2.3 Polyphenol content

According to Table 6, the polyphenol content of all treatments decreased significantly over time (P < 0.05). The control (C) showed the lowest total phenolic content on all three days, falling from 15.20 on day 1 to 13.52 mg

gallic acid per 100 g, which reflects the natural degradation of phenolic compounds during storage. The oil-enriched treatments, particularly those with the higher oil percentages (P2 and P2H2), contained considerably more polyphenols. In the P2H2 treatment, for example, the total phenolic content fell from 48.60 on day 1 to 44.18 mg gallic acid per 100 g on day 20. Because they contained both oils, the combined P1H1 and P2H2 treatments had higher polyphenol values than the single-oil treatments on every day.

Table 6. Changes in the polyphenol content (mg gallic acid per 100 g) of the enriched kefir samples over 1, 10 and 20 days of storage (4 °C).

Treatment	Day 1	Day 10	Day 20
C	15.20 ± 0.25 gA	14.35 ± 0.22 gB	13.52 ± 0.20 gC
P1	27.85 ± 0.30 eA	26.43 ± 0.28 eB	25.12 ± 0.25 eC
P2	42.10 ± 0.35 bA	40.35 ± 0.32 bB	38.67 ± 0.30 bC
H1	25.90 ± 0.28 fA	24.65 ± 0.26 fB	23.41 ± 0.24 fC
H2	39.80 ± 0.35 cA	37.95 ± 0.32 cB	36.18 ± 0.30 cC
P1H1	31.75 ± 0.32 dA	30.05 ± 0.30 dB	28.44 ± 0.28 dC
P2H2	48.60 ± 0.38 aA	46.35 ± 0.35 aB	44.18 ± 0.32 aC

Letters a–g indicate a significant difference within each column (between treatments) and letters A–C a significant difference within each row (between storage days).

3.2.4 Antioxidant activity

According to Table 7, antioxidant activity followed a similar downward trend in all treatments over time. The control (C) had the lowest antioxidant activity on every day (18.45% on day 1 to 16.28% on day 20). The highest antioxidant activity belonged to the P2H2 treatment, which fell from 60.45% on day 1 to 55.68% on day 20. The decrease was significant in all treatments ($P < 0.05$), but the oil-enriched treatments clearly retained a higher level of antioxidant activity.

Table 7. Changes in the antioxidant activity (% free radical inhibition) of the enriched kefir samples over 1, 10 and 20 days of storage (4 °C).

Treatment	Day 1	Day 10	Day 20
C	18.45 ± 0.20 gA	17.32 ± 0.18 gB	16.28 ± 0.17 gC
P1	35.80 ± 0.25 eA	34.22 ± 0.23 eB	32.74 ± 0.22 eC
P2	53.55 ± 0.30 bA	51.22 ± 0.28 bB	48.98 ± 0.26 bC
H1	33.25 ± 0.24 fA	31.65 ± 0.22 fB	30.05 ± 0.21 fC
H2	50.10 ± 0.28 cA	47.85 ± 0.26 cB	45.63 ± 0.25 cC
P1H1	40.15 ± 0.26 dA	38.47 ± 0.24 dB	36.83 ± 0.23 dC
P2H2	60.45 ± 0.32 aA	58.02 ± 0.30 aB	55.68 ± 0.28 aC

Letters a–g indicate a significant difference within each column (between treatments) and letters A–C a significant difference within each row (between storage days).

3.2.5 Probiotic viability

The counts of lactic acid bacteria and yeasts are presented in Figure 4. Lactobacillus, Lactococcus, Bifidobacterium and yeast counts were evaluated in the fermented kefir enriched with pistachio and hazelnut oils over the 20-day storage period.

3.2.6 Lactobacillus spp.

For all four microbial groups the counts declined during storage, but the decline was consistently smaller in the oil-containing treatments than in the control (Figure 4). Lactobacillus was the most abundant group; on day 20 its count in the control fell to 7.55 log CFU/mL, whereas P2H2 retained the highest value (8.1 log CFU/mL), indicating better stability at the higher oil concentration.

3.2.7 Lactococcus spp.

Lactococcus followed the same pattern, with P2H2 and P2 retaining more viable cells on day 20 (7.9 and 7.8 log CFU/mL) than the control (7.3 log CFU/mL).

3.2.8 Bifidobacterium spp.

Bifidobacterium, the most oxygen-sensitive group, showed the clearest benefit: its day-20 count was 6.75 log CFU/mL in the control against 7.4 log CFU/mL in P2H2.

3.2.9 Yeast

Yeast counts behaved similarly, the control reaching the lowest value on day 20 (5.85 log CFU/mL) and P2H2 the highest (6.4 log CFU/mL).

Overall, the treatments enriched with pistachio and hazelnut oil, and particularly the combined treatment with the higher oil concentration (P2H2), provided better survival for the probiotic bacterial and yeast populations during refrigerated storage than the control group.

3.2.10 Cell-surface hydrophobicity

The cell-surface hydrophobicity of the Lactobacillus, Lactococcus and Bifidobacterium isolates from the kefir samples is presented in Figure 5.

3.2.11 Lactobacillus spp.

Hydrophobicity was higher than the control (C) in all oil-containing treatments, and especially in P2H2 and P2. The P2H2 treatment had the highest hydrophobicity, 72% on day 1, decreasing to 66.8% by day 20 but still remaining the highest value. The oil-free treatment (C) showed a marked drop in hydrophobicity, from 60.5% to 54.2%.

3.2.12 Lactococcus spp.

Hydrophobicity was higher than the control in all oil treatments. P2H2 had the highest value on both day 1 (63.5%) and day 20 (59%), while treatment C fell from 55.8% on day 1 to 49% on day 20.

3.2.13 Bifidobacterium spp.

The P2H2 and P2 treatments had the highest hydrophobicity on day 1, at 50% and 49% respectively, and these values remained higher than the other treatments on day 20 (45% and 44.5% respectively). By contrast, the control (C) declined considerably, from 42% to 35%.

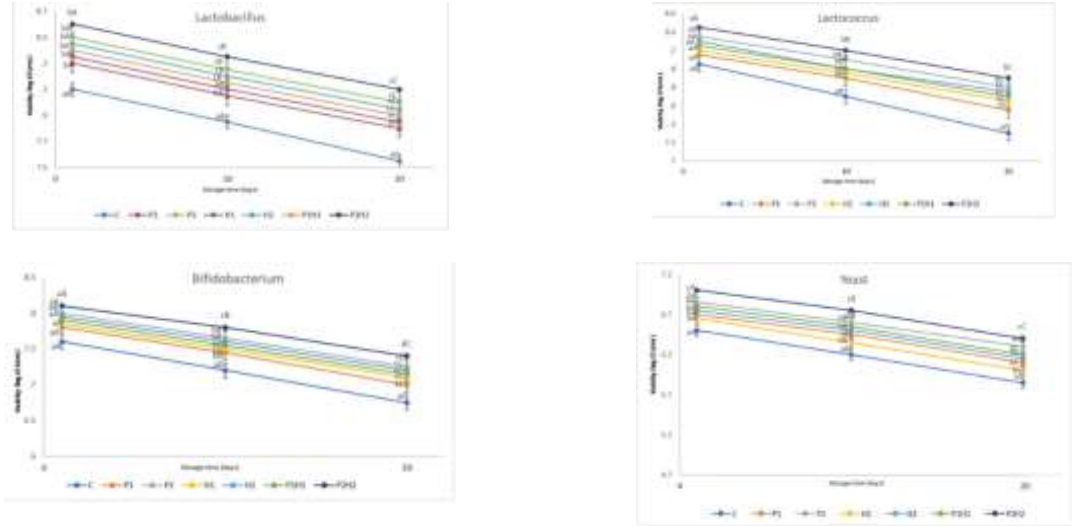

Fig 4. Counts of lactic acid bacteria and yeasts (log CFU/mL) in the kefir samples on days 1, 10 and 20 of storage. Letters a–g indicate a significant difference between treatments and letters A–C a significant difference between storage days.

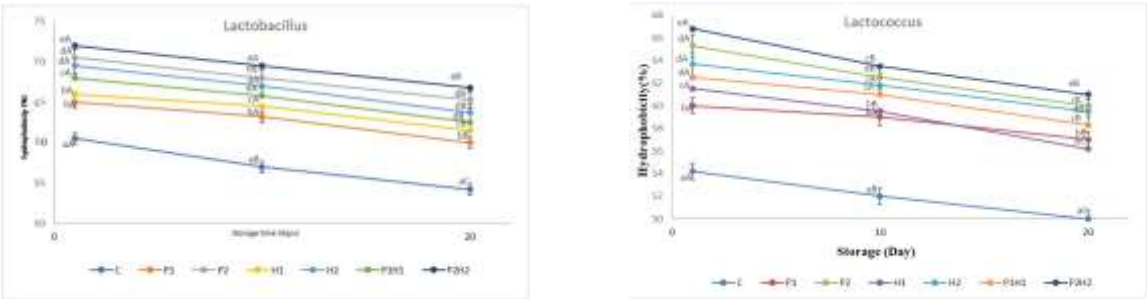

Fig 5. Hydrophobicity (%) of the Lactobacillus, Lactococcus and Bifidobacterium isolates of the kefir samples during storage. Letters a–g indicate a significant difference between treatments and letters A–C a significant difference between storage days.

3.2.14 Sensory evaluation

The sensory evaluation results are presented in Figure 6. The P1H1 treatment showed the best appearance on day 1 with a full score of 5 and declined only slightly to 4 by day 20. In contrast, the P2H2 treatment showed the greatest decline, from 4 on day 1 to 3.1 on day 20, which is attributed to the high oil concentration and the possibility of fat-phase separation or sedimentation. In the P1H1 treatment, flavour was a full 5 on day 1 and remained higher than most treatments even on day 20 at 3.7; the treatments with a higher oil content, P2 and P2H2, experienced a greater loss of flavour, reaching 3.1 and 2.8 respectively on day 20. Consistency followed the same pattern: P1H1 retained the best consistency over time (5 → 3.9), whereas P2H2 declined more (4 → 3). For odour, P1H1 again performed steadily and best (5 → 3.6), while the treatments with a high oil concentration (P2 and P2H2) declined more sharply, probably because of early fat oxidation. For overall acceptance, P1H1 remained higher than the others at all three time points (5, 4.5 and 3.8), while the overall acceptance of the control fell from 4.2 to 3 and P2H2 dropped to the lowest level of 3.

3.2.15 FTIR spectroscopy

The FTIR spectra of the fermented kefir samples containing pistachio and hazelnut oil on days 1 and 20 are shown in Figure 7. In all samples a broad band at about 3300 cm^{-1} was observed, corresponding to the stretching vibration of the hydroxyl group (O–H) and reflecting the extensive presence of water and alcoholic groups in the samples.

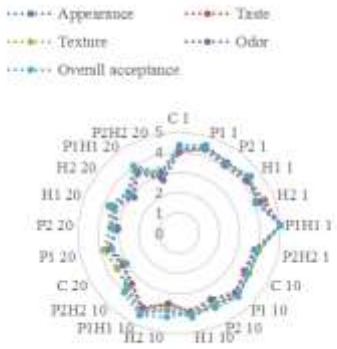

Fig 6. Sensory evaluation of the kefir samples for odour, consistency, flavour, appearance and overall acceptance over the 20-day storage period.

The intensity of this band was lower in the oil-containing samples, particularly P2, H2 and P2H2, than in the control (C), which may indicate an interaction between lipids and water or a reduced moisture content resulting from oil addition. The peaks corresponding to C–H stretching vibrations at around 2920 cm^{-1} were strengthened in all oil-containing samples, especially in the treatments with a higher oil percentage (P2 and H2), confirming the increased fat content of these samples; the same increase was also seen in the combined P1H1 and P2H2 treatments. The absorption band at around 1740 cm^{-1}, associated with carbonyl groups (C=O), became more prominent in the oil-containing treatments, particularly on day 20, which may result from an increase in lipid oxidation products or from protein–lipid interactions during storage. The peak at around 1640 cm^{-1}, generally attributed to the bending band of water or to the amide I band of proteins, decreased in intensity on day 20 relative to day 1, which may be due to structural changes in the proteins or to a reduction in free water in the product matrix. In the 1300–1000 cm^{-1} region, associated with C–O–C stretching vibrations, significant differences were seen between treatments; the stronger presence of this peak in the samples containing the combined oils (P1H1 and P2H2) is probably related to an increase in ester compounds and complex carbohydrates.

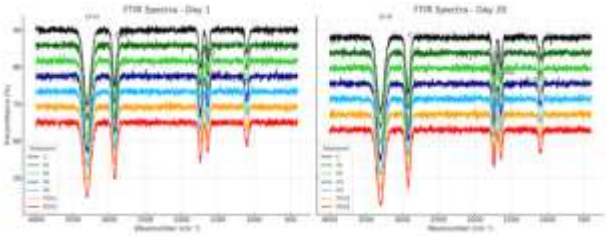

Fig 7. FTIR spectra of the kefir samples on days 1 and 20 of storage.

4. Discussion

4.1 Oil quality: acidity and peroxide value

The acidity of pistachio oil (0.29%) was appreciably lower than that of hazelnut oil (0.7%). A lower acidity usually indicates better oil quality, because it shows that less hydrolysis of the fats has occurred and that off-flavours and spoilage are less likely (Sena-Moreno et al., 2016). The peroxide value of pistachio oil was also far lower than that of hazelnut oil; a lower peroxide value indicates better oxidative stability and freshness, whereas higher peroxide values can lead to spoilage and quality loss (Ghirardello et al., 2014). The values obtained here are consistent with the ranges reported in the literature: the peroxide index of pistachio oil is usually between 0.14 and 0.33 meq O_2/kg (Ryan et al., 2006), and the peroxide value of hazelnut oil may be undetectable initially but can rise to about 2.15–3.39 meq O_2/kg after several months of storage (Ghirardello et al., 2014; Król et al., 2021). On both criteria, therefore, pistachio oil showed better quality than hazelnut oil. This can be attributed to its fatty acid composition — pistachio oil has a higher content of monounsaturated fatty acids, especially oleic acid, which is known for oxidative stability, whereas hazelnut oil contains a considerable amount of polyunsaturated fatty acids such as linoleic acid that are more prone to oxidation — and to its natural antioxidants, including tocopherols and vitamin E, which help prevent oxidation (Miraliakbari & Shahidi, 2008).

2.2 Total phenolic and flavonoid content

The total phenol and flavonoid contents of pistachio and hazelnut oils have been studied widely, and considerable differences are seen between studies. The total phenol content of pistachio oil has been reported to be relatively low: one study identified only 57.6 mg/kg in wild pistachio oil (Givianrad et al., 2013), while another reported 1359–4507 mg/kg in pistachio kernels depending on the cultivar (Ojeda-Amador et al., 2019). In an earlier study a total phenol content of approximately 512.9 mg gallic acid equivalent per 100 g dry matter was obtained for pistachio oil (Borisova, 2022), and a total flavonoid content of approximately 12 mg per 100 g was found (Chen & Blumberg, 2008), both of which are close to the values obtained in the present work. For hazelnut oil, the total phenol content varies considerably between cultivars, and flavonoid values of 6.50 quercetin equivalent per 100 g have been reported (Balik, 2021). In pistachio kernels several classes of phenolic compound have been identified, including flavanols (90% of total phenols), flavonols, anthocyanins and gallotannins (Ojeda-Amador et al., 2019), while hazelnut oil contains phenolic compounds such as quercetin and rutin that are known for their antioxidant properties (Altaee et al., 2024). The variation in phenol and flavonoid content between studies can be attributed to cultivar differences, environmental conditions and processing methods (Alasalvar & Bolling, 2015).

4.3 Antioxidant activity

The IC_{50} value represents the concentration of a sample able to inhibit 50% of free radicals; the lower the IC_{50}, the stronger the antioxidant activity. The IC_{50} values of hazelnut oil in both the DPPH and the ABTS assay were lower than those of pistachio oil, which indicates that hazelnut oil acted as the stronger antioxidant. In a study comparing several nut oils, pistachio oil showed an antioxidant activity of between 51.28% and 65.58% depending on the extraction method (cold pressing versus Soxhlet extraction) (Al Juhaimi et al., 2018), suggesting that although pistachio oil has appreciable antioxidant properties it may not rank highest among nut

oils. Hazelnut oil has shown stronger antioxidant activity in some studies, reaching 73.58% under particular conditions. Other oils, such as walnut oil, also show considerable antioxidant activity, with values of around 65.10% (Orhan et al., 2011), and argan oil has been reported to have a high antioxidant capacity comparable with pistachio oil (Taribak et al., 2013). The antioxidant capacity of these oils is influenced by the fatty acid composition and by the presence of bioactive compounds such as tocopherols and polyphenols (Arranz et al., 2008); hazelnut oil, for example, has been shown to contain appreciable levels of α-tocopherol, which reinforces its antioxidant properties (Maguire et al., 2004). The extraction method also matters: cold-pressed oils generally retain more bioactive compounds than solvent-extracted oils, and roasting can raise the total phenol content and antioxidant capacity of pistachio oil while reducing the levels of some tocopherols (Ling et al., 2016; Al Juhaimi et al., 2018).

4.4 Antimicrobial activity

The findings of this study showed that pistachio and hazelnut oils had considerable antibacterial activity against the pathogenic bacteria tested. Overall, pistachio oil had a stronger inhibitory and bactericidal effect than hazelnut oil, evident in its lower MIC and MBC values, particularly against Listeria monocytogenes and Staphylococcus aureus. This finding is consistent with El-Atki et al. (2020), who reported that vegetable oils containing phenolic and terpenoid compounds are able to inhibit the growth of pathogenic bacteria such as Staphylococcus aureus and Escherichia coli significantly, and who noted that the volatile and antioxidant compounds in such oils can destroy the bacterial cell wall and thereby kill the cells. In the study of Ghazanfari et al. (2020), the lowest MIC values were reported for Listeria monocytogenes at below 200 µg/mL, which agrees with the values obtained here for pistachio oil and indicates the high sensitivity of this strain to the active compounds of vegetable oils. The highest MIC and MBC values in our study were obtained for Escherichia coli, indicating the greater resistance of this bacterium; this agrees with El-Atki et al. (2020), who explained that Gram-negative bacteria such as E. coli show higher resistance to the antibacterial compounds of vegetable oils because of their lipopolysaccharide outer membrane. The resistance of Gram-negative strains is generally attributed to their more complex cell-wall structure, which makes the penetration of antimicrobial compounds more difficult (Silveira et al., 2012), while oils rich in phenolic compounds are particularly effective against Gram-positive pathogens, which are more susceptible because of their simpler cell-wall structure (Man et al., 2019). Research also shows that pistachio oil generally has lower MIC and MBC values than hazelnut oil (Dris et al., 2024). Several studies support the antimicrobial potential of these materials: essential oil from Turkish pistachio gum, rich in monoterpenes with α-pinene as the most abundant compound, showed considerable activity against a range of pathogenic bacteria and yeasts (Alma et al., 2004); polyphenol-rich compounds from raw and roasted pistachios showed antimicrobial properties against various Gram-positive and Gram-negative bacteria, with extracts of raw pistachios more effective than those of roasted ones (Bisignano et al., 2013); and essential oils from a wide range of plants are documented to have antibacterial properties against foodborne pathogens (Burt, 2004; Mith et al., 2014).

4.5 Morphological damage to the pathogens

The antimicrobial effects of these oils are usually attributed to their ability to disrupt the bacterial cell membrane and inhibit growth, which varies with the chemical composition of the oil. As the SEM images show, the mechanism involves destruction of the pathogen cell wall. Pistachio extracts have considerable antimicrobial properties against Gram-positive bacteria including Staphylococcus aureus and Listeria monocytogenes, and raw unpeeled pistachio extracts are more effective than roasted and salted ones, which shows that processing method can affect antimicrobial efficacy (Bisignano et al., 2013). Pistachio essential oil has shown a particularly strong inhibitory effect against Salmonella enterica (Ellahi et al., 2019). Fewer studies exist on the antimicrobial properties of hazelnut oil; one study found that refined oils, including hazelnut, did not have appreciable antimicrobial effects (Karaosmanoglu et al., 2010).

4.6 pH and acidity of the kefir samples

Adding vegetable oils to kefir raised the initial pH and slowed acid production during storage, and the treatments with the higher oil percentage (P2 and P2H2) had the greatest effect. The higher initial pH in the oil-containing treatments suggests that these oils may have a buffering property that helps keep the pH more stable as fermentation proceeds. This is particularly important in dairy products, because excessive acidity can lead to undesirable flavour and texture. The fall in pH and rise in acidity over time follow the natural course of fermentation, in which lactic acid bacteria convert lactose into lactic acid and produce the sour taste of kefir; the lower acidity of the oil-containing treatments at the end of storage suggests that the oils may affect the metabolic activity of the kefir microflora and slow the rate of acid production. Similar buffering effects of oils have been reported by Tsisaryk et al. (2022) in kefir, and the addition of milk fat globule membrane to whey-permeate-based products led to a more stable pH and lower acidity during fermentation (McGovern et al.,

2024). Slower acid production in the presence of fat is also consistent with the observation that fat can alter the metabolic pathways of lactic acid bacteria (Kwak et al., 1996), and with reports that additives change acidity and flavour profile in fermented dairy products (Anwar et al., 2021).

4.7 Polyphenols and antioxidant activity of the kefir samples

The decrease in total polyphenols over time is consistent with findings that polyphenols are prone to degradation during storage through contact with oxygen, light and temperature, which breaks these compounds down and reduces their antioxidant effect (Gutiérrez-del-Río et al., 2021; Ullah et al., 2022). The oil-enriched treatments, and particularly those with a higher oil percentage (P2 and P2H2), retained a higher level of polyphenols than the control. Milk and its derivatives themselves contain antioxidant compounds such as lactoferrin, β-lactoglobulin and bovine serum albumin, and the polysaccharide produced during fermentation (mainly kefiran), together with the lactic acid bacteria, carries distinct functional groups such as –COOH, –OH and –CO that can react with and neutralise free radicals. The higher antioxidant activity of the oil-enriched treatments shows that, besides preserving the polyphenols, the oils themselves have antioxidant properties (Pérez-Lamela et al., 2021). The marked decline in antioxidant activity over time, despite a high initial level, underlines the importance of treatment type and storage conditions in preserving food quality.

4.8 Probiotic viability

In the present study the higher-oil treatments, and especially P2H2, preserved significantly more viable cells on day 20 than the control, and the effect was largest for the oxygen-sensitive Bifidobacterium. This oil-dependent protection is consistent with earlier reports that plant extracts, fat compounds and oil emulsions improve probiotic survival in fermented dairy (Ekinci et al., 2008; Soukoulis et al., 2014; Oliveira et al., 2017; Moghanjougi et al., 2020). Three mechanisms may explain the effect observed here, although they were not tested directly: the fat droplets may act as a physical barrier against acid and oxygen; the unsaturated fatty acids may serve as an energy source or growth factor for some strains; and the phenolic compounds of the oils may lower oxidative stress, which would most benefit oxygen-sensitive organisms such as Bifidobacterium, in agreement with the greater protection observed for that group.

4.9 Cell-surface hydrophobicity

The assessment of the hydrophobicity of the Lactobacillus, Lactococcus and Bifidobacterium isolates from the oil-containing kefir samples provides notable insight into the effect of vegetable oils on probiotic adhesion properties, a characteristic that is important for adhesion to the intestinal wall and hence for gut health. The increase in hydrophobicity observed in the oil-containing treatments may arise from the interaction between the lipophilic compounds of the oils and the cell membrane of the probiotics. This interaction may facilitate adhesion to the intestinal mucosa and thereby increase probiotic survival and efficacy in the digestive tract; earlier research has likewise shown that the presence of lipids can help the stability and viability of probiotics under gastrointestinal conditions (Markowiak & Śliżewska, 2017). The consistent performance of the oil treatments confirms the role of vegetable oils in preserving the hydrophobicity of Lactococcus, which matters for their function in fermented products (Kök-Taş et al., 2013), and in maintaining the adhesion capacity of Bifidobacterium, which is critical for its probiotic function (Moghimani et al., 2023). Probiotics with a greater adhesion capacity act more effectively, so this finding suggests better health outcomes, particularly for gut health (Yilmaz et al., 2022).

4.10 Sensory acceptance

The P1H1 treatment initially scored highly for appearance (5) and fell only slightly to 4 by day 20. This stability is consistent with findings that the formulation of dairy products can significantly affect their visual appeal, an important factor in consumer acceptance (Cutrim et al., 2023). By contrast, the marked drop in the appearance score of P2H2 (from 4 to 3.1) may result from the high oil concentration and phase separation. Flavour in P1H1 fell from 5 to 3.7, whereas P2H2 experienced a considerable loss; this agrees with research showing that higher fat levels can reduce flavour quality through oxidation, which is particularly relevant for dairy products (Lethuaut et al., 2004). The consistent flavour profile of P1H1 suggests that its formulation may effectively mitigate the effects of oxidation, a strategy also used in other successful dairy formulations (Ghanbari et al., 2017). The overall acceptance scores of P1H1 were better than those of the other treatments; this trend is supported by studies emphasising the importance of sensory attributes in consumer preference, since products that retain their desirable sensory characteristics over time are more likely to be favoured (Celeghin et al., 2016). The stability of the sensory attributes of P1H1 over time also suggests that its formulation may limit syneresis, a common problem in dairy desserts (Staffolo et al., 2007). These results show that formulation plays a critical role in maintaining sensory quality over time, and that an optimal formulation balancing functional viability with high consumer appeal is achievable.

4.11 FTIR analysis

The infrared spectroscopy results showed that adding pistachio and hazelnut oil, and their combination, produces notable changes in the molecular structure of fermented kefir milk, and that changes in the FTIR bands occur over the 20-day storage period that may reflect fermentation, oxidation and biochemical interactions between the components of the product. The reduced intensity of the O–H stretching band at 3300 cm^{-1} in the samples containing nut oils suggests that the oils may cause interactions that reduce the availability of free hydroxyl groups, which could indicate a change in water structure or a reduction in moisture — both relevant to the texture and mouthfeel of kefir. Such changes may also affect the bioavailability of certain nutrients and bioactive compounds and thereby alter the health-promoting characteristics of kefir (Johnson & Steele, 2007). The distinct carbonyl absorption at 1740 cm^{-1} in the oil-containing treatments, especially on day 20, may indicate lipid oxidation or the formation of protein–lipid complexes; these interactions can lead to off-flavours or altered nutritional quality over time, so understanding them matters for optimising storage conditions and extending the shelf life of kefir products (Beltrán-Barrientos et al., 2016). The reduced peak intensity at around 1640 cm^{-1} suggests that structural changes in the proteins may occur during fermentation and storage, which could affect functional properties such as emulsification and foaming that are vital to the texture and stability of kefir.

5. Conclusion

This study showed that using pistachio and hazelnut oils as natural bioactive and prebiotic compounds significantly improves the quality and biological performance of the kefir beverage. The two oils are not only a rich source of unsaturated fatty acids, phenolic compounds, flavonoids and natural antioxidants; they also significantly increased the viability of the probiotic microorganisms during fermentation and storage. Both oils had a low peroxide value, a high phenol and flavonoid content and strong antioxidant activity, which makes them suitable for use in functional food products. Adding the oils increased the phenolic content and antioxidant activity of the kefir, kept the pH higher and the titratable acidity lower than the oil-free control throughout storage, and maintained higher counts of Lactobacillus, Lactococcus, Bifidobacterium and yeasts on day 20, the combined P2H2 treatment being the most effective. The oils also strengthened the adhesion potential of the probiotics by increasing their cell-surface hydrophobicity, while the milder combined level (P1H1) optimised the sensory indices and gained the highest overall acceptance. Synbiotic kefir enriched with pistachio and hazelnut oils therefore represents an innovative, healthy and stable dairy product with notable potential in the functional food market.

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