Research Square

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Quantitative characterization of biochemical and functional property differentiation in Kefir as influenced by targeted yeast supplementation

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Abstract

This study systematically characterized the biochemical and functional properties of kefir manufactured using a commercial starter culture supplemented with selected yeast strains. The yeast species evaluated were *Debaryomyces hansenii* LAF 3, *Kluyveromyces marxianus* LAF 4, *Candida colliculosa* LAF 7, *Yarrowia lipolytica* NCAIM Y00591, *Saccharomyces cerevisiae*, and *Kluyveromyces lactis* PYCC 4356. Proteolytic activity, free fatty acid profiles, antioxidant capacity, and organic acid composition were quantitatively determined to assess strain-dependent variations in kefir biochemical characteristics during storage. Kefir samples containing *Y. lipolytica*, *S. cerevisiae*, and *K. marxianus* exhibited significantly elevated proteolytic activity, indicating enhanced protein hydrolysis patterns. Increased free fatty acid accumulation was measured in samples containing *K. marxianus*, *K. lactis*, and *Y. lipolytica*, reflecting strain-specific lipolytic behavior. Antioxidant capacity measurements revealed distinct enhancement in kefir produced with *C. colliculosa* and *S. cerevisiae*, suggesting differential contributions to redox-active metabolite formation. Organic acid profiling demonstrated higher lactic and oxalic acid concentrations in kefir containing *Y. lipolytica*, whereas citric acid was detected exclusively in the *K. lactis*-containing sample at the end of storage, highlighting its specific metabolic role in citrate transformation. Overall, quantitative characterization demonstrated that yeast supplementation significantly modifies measured biochemical parameters of kefir, influencing proteolysis, lipid hydrolysis, antioxidant potential, and organic acid distribution. These findings provide measurable evidence of strain-dependent functional differentiation and contribute to improved analytical understanding of yeast-driven property modulation in fermented dairy systems.

Introduction

Fermented dairy products are produced by fermenting milk with specific microorganisms, primarily lactic acid bacteria (LAB), resulting in products with distinct structures, appearances, and flavors. A variety of fermented dairy products are commercially produced worldwide. Among these products, kefir is largely manufactured and marketed by commercial companies. With increasing public awareness of the health-promoting properties and nutritional value of kefir, along with the growing number of promotional media advertisements, kefir consumption has risen steadily in recent years [1, 2]. Today, kefir is produced using industrial methods and is available in major retail markets in plain, light, and fruit-flavored varieties. However, the number of commercial producers that still utilize traditional production methods remains limited [3, 4].

It is well known that the lyophilized kefir cultures commercially marketed for industrial kefir production contain very low levels of yeast or, in some cases, no yeast at all. This is commonly attributed to the fact that carbon dioxide produced by yeast fermentation can cause swelling or bloating of the product packaging, which consumers may perceive as spoilage [5]. For this reason, it is often stated that commercially produced kefir cultures exclude yeasts. Consequently, commercial kefir cultures typically contain only certain LAB, and the resulting products lack the characteristic foamy texture of traditional kefir and contain markedly lower levels of ethanol. It is generally recommended that commercially available kefir should contain yeast counts of at least 4 Log CFU/mL [5, 6]. Traditionally produced kefir is preferred by consumers over industrially produced kefir. Therefore, research in this area has focused on developing modern techniques and approaches to obtain industrial kefir products that more closely resemble traditional kefir in their properties. This difference in consumer preference is largely attributed to the distinct bacterial community and the presence of diverse yeast species in kefir produced using grains. In industrial kefir production, yeasts are seldom incorporated into the starter cultures [7].

Yeasts, commonly found in milk and dairy products, are a significant source of contamination in low-pH fermented milk products such as yogurt and sour milk, and are among the largest contributors to spoilage in these products. *Kluyveromyces marxianus* and *Saccharomyces* species are the yeast species most frequently associated with spoilage through fermentation, especially in sweetened and fruit-flavored yogurts [8]. In kefir grains, yeasts and LAB establish a symbiotic relationship based on metabolic cross-feeding, where sugars and growth factors are mutually exchanged. LAB acidify the environment while yeasts produce vitamins and ethanol, collectively maintaining microbial stability and contributing to the characteristic structure and flavor of kefir. Despite this, yeasts contribute to the fermentation process in dairy products such as kefir and koumiss. However, yeasts have been reported to play an important role in establishing microbial symbiosis within kefir grains. They also significantly influence the development of kefir's characteristic flavor and aroma, as well as the production of CO_2 [9, 10].

The distinctiveness of kefir compared with other fermented dairy products arises from the simultaneous occurrence of lactic acid and alcoholic fermentations carried out by the microorganisms present in kefir grains [11]. The production of organic acids and ethanol in kefir contributes to the development of its characteristic aroma [12, 13]. As a result of the combined fermentation activities of LAB and yeasts, kefir contains lactic acid, acetic acid, trace amounts of carbon dioxide, ethanol, and various aromatic compounds that conferred organoleptic properties different from those of other fermented dairy products [13, 14]. Organic acids produced during fermentation—such as lactic, succinic, pyruvic, α-ketoglutaric, and oxalic acids—provide kefir with its refreshing and acidic taste, while secondary metabolites including carbonyl compounds, volatile fatty acids, and alcohols contribute to its characteristic aroma. The flavor and aroma profile of kefir is shaped by components such as ethanol, diacetyl, formic acid, succinic acid, acetaldehyde, propionic acid, acetone, and lactic acid. Among the organic acids formed during fermentation, lactic acid is present at the highest concentration [15].

Studies on kefir have generally focused on comparing kefir produced using traditional grains with that produced by industrial methods, evaluating the use of various prebiotic ingredients in kefir [16, 17], and assessing the effects of raw milk fat content on product quality [18, 19]. In addition, numerous investigations have examined the functional and microbiological properties of kefir, as well as its organic acid composition Literature reviews have shown that there is insufficient research on how different yeast species and strains used as monocultures in kefir production affect product characteristics, how these effects change during storage, and how they relate to shelf life [13, 20, 21]. The aim of the present study was to investigate the effects of incorporating different yeast species into kefir on selected physicochemical and functional properties.

Materials and methods

Materials

For kefir production, Ultra High Temperature (UHT) milk obtained from Mis Süt A.Ş. was used. The milk contained 12.01% dry matter, 2.80% fat, 5.68% total carbohydrates, 2.88% protein, and 0.65% ash. The pH of the milk was 6.70, and its titratable acidity was 0.135% lactic acid. The kefir starter culture was supplied by Referans Gida (MicroMilk Srl.), while the yeast species and strains were obtained from the Microbiology Laboratory of the Department of Dairy Technology, Faculty of Agriculture, Ege University, as well as from various commercial culture suppliers. The KFA/A1 starter culture containing *Streptococcus thermophilus*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, and *Leuconostoc mesenteroides* subsp. *cremoris* was procured from Referans Gıda. The yeast species and strains used in the study included: *Debaryomyces hansenii* LAF 3 (Chr. Hansen), *Kluyveromyces marxianus* LAF 4 (Chr. Hansen), *Candida colliculosa* LAF 7 (Chr. Hansen), *Yarrowia lipolytica* NCAIM Y00591, *Saccharomyces cerevisiae* (Yuvam Maya), and *Kluyveromyces lactis* PYCC 4356. The 200-mL packages used for storage of kefir samples were obtained from Coveris™ Rigid, Turkey.

Methods

The kefirs were produced at the Microbiology Laboratory of the Department of Dairy Technology, Faculty of Agriculture, Ege University. The starter culture was weighed according to the manufacturer's instructions and activated in UHT milk for approximately 4 hours. Yeast strains were activated in Tryptic Soy Broth (Merck, Darmstadt, Germany) for 48–72 h, and their optical densities were adjusted to 0.5 McFarland at OD_{600}. The turbidity of a 0.5 McFarland standard is approximately equivalent to 1.5 x 10^8 CFU/mL bacterial suspension. The standardized yeast suspensions were inoculated into kefir milk at a rate of 0.5%. Commercial kefir starter culture commonly used in industrial production was employed in combination with different yeast strains. The yeast strains were added simultaneously with the starter culture during inoculation. The yeast population was monitored throughout the kefir fermentation process, and it was confirmed that a level of 4 log CFU/mL was achieved at the beginning of storage. The six yeast species described in the Materials section were individually combined with the kefir starter culture, resulting in six different kefir formulations. The kefir was stored in a dark, cold storage room at 4 degrees Celsius throughout the storage period. Proteolytic activity, color parameters, acidity index, antioxidant and phenolic activities, and organic acid profiles of the produced kefirs were evaluated throughout the storage period. The production steps for kefir are presented in detail in Fig. 1.

Proteolytic activity by the OPA (O-Phthaldialdehyde) method

The proteolytic activity of the kefir samples was determined using the OPA (o-phthaldialdehyde; Sigma-Aldrich, St. Louis, MO, USA) method, which is based on the quantification of free NH_3 groups. 2.5 mL of the sample was mixed with 5 mL of 0.75% trichloroacetic acid (TCA) and then filtered through a 0.45 µm cellulose membrane filter [22]. From the clear filtrate, 0.1 mL aliquots were transferred into cuvettes, followed by the addition of 2 mL of freshly prepared OPA reagent [19]. Absorbance was measured at 340 nm using a Thermo Scientific Multiskan Sky microplate reader equipped with Multiskan Sky Stack Software 5.0. Results were expressed as comparative absorbance values, and the degree of hydrolysis (mg serine / L) was calculated from the standard curve (R^2 = 0. 995).

Color measurement

The color parameters of the kefir samples were determined using a Konica Minolta CR-5 Chromameter by measuring the L*, a*, and b* values. For accurate measurement, the instrument was calibrated using the standard calibration plate supplied for light and color definition. The samples were placed in quartz cuvettes prior to analysis [21].

Antioxidant activity

The free radical scavenging activity of the kefir samples was determined using the method of Pavithra & Vadivukkarasi [23] with minor modifications. 10 mL of kefir sample was transferred to a Falcon tube and 10 mL of methanol was added. The solution, mixed in a vortex container, was kept in an ultrasonic water bath for 5 minutes and then centrifuged at 9000 rpm for 10 minutes. A total of 100 µL of extract was mixed with 100 µL of 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich). After incubation in the dark at room temperature for 30 min, absorbance was measured at 517 nm. Antioxidant activity was expressed as mg ascorbic acid equivalents per gram of sample, calculated using the standard curve (R^2 = 0.988).

Total phenolic activity

Total phenolic activity was determined according to the Folin–Ciocalteu method (Sigma-Aldrich), based on the procedure of Budak et al. [24] with some modifications. 10 mL of kefir was vortexed after adding 10 mL of methanol. Then, centrifugation was performed at 8602 x g, 4° C for 30 minutes, and the supernatant were filtered through Whatman No:41 filter paper. The resulting filtrates were used for total phenolic activity analysis. Filtrated samples were placed into cuvettes and incubated with Folin reagent for 5 min. Subsequently, 7.5% Na_2CO_3 solution was added, and the mixture was incubated in the dark at room temperature for 1 h. Absorbance was then measured at 760 nm using a spectrophotometer. Absorbance values were plotted against gallic acid concentrations to construct a standard curve. Linear regression analysis was performed to obtain the gallic acid (Sigma-Aldrich) calibration equation. The phenolic content of the samples was calculated by inserting their absorbance values into the regression equation and expressed as mg of gallic acid equivalents (GAE) per mL (R^2 = 0. 989).

Organic acid determination

The quantification of organic acids (acetic acid, fumaric acid, lactic acid, malic acid, citric acid, tartaric acid, quinic acid, formic acid, and oxalic acid) was performed at TÜBİTAK-MAM (Gebze, Turkiye) using an internal laboratory method (D.05.G176) based on HPLC-UV analysis. Organic acid profiles were determined using high-performance liquid chromatography (HPLC) with a Shimadzu Nexera XR 20 AD system. Chromatographic separation was achieved on an Avantor ACE C18 column (250 × 4 mm, 5 µm particle size) maintained at 35°C. The mobile phase consisted of a phosphate buffer prepared by dissolving 27.2 g KH_2PO_4 in 950 mL of distilled water, adjusting the pH to 2.4 with 85% H_3PO_4, and subsequently diluting to a final volume of 1 L. The analysis was performed under isocratic conditions at a flow rate of 0.8 mL/min, with an injection volume of 10 µL. Detection was carried out using a UV detector set at 214 nm. Quantification of organic acids was conducted using external standards, including acetic, formic, fumaric, lactic, oxalic, propionic, citric, and tartaric acids (Sigma-Aldrich). Prior to analysis, samples were appropriately diluted and filtered through a 0.45 µm membrane filter to remove particulate matter. Subsequently, 10 µL of the prepared filtrate was injected into the HPLC system for chromatographic analysis.

Statistical analysis

All experimental data are expressed as mean ± standard deviation (SD) obtained from triplicate and parallel analysis results (n = 3). One-way analysis of variance (ANOVA) was performed to determine differences among the properties of the kefir samples and to evaluate the effects of storage days. Statistical analyses were conducted using SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA). When the ANOVA indicated significant differences, Duncan's multiple range test was applied at a significant level of $p < 0.05$.

Results and discussion

Proteolytic activity

Proteolytic activity refers to the hydrolysis of proteins by proteolytic enzymes secreted by microorganisms, a process that is essential both for the acidification function of starter cultures and for the sensory attributes of the final product. OPA method is employed to determine the degree of protein hydrolysis by quantifying the free amino groups present in the product, thereby enabling the assessment of the proteolytic activity of starter cultures and other microorganisms within the matrix [25]. The degree of protein hydrolysis in the kefir samples was interpreted based on the amount of serine (mg/L), and changes in hydrolysis rates during storage are presented in Table 1. Table 1 indicates that the proteolytic activity (degree of hydrolysis) in kefir samples generally increased during storage. In all samples, proteolytic activity values increased throughout the storage period. The proteolytic activity results varied depending on the yeast species used. While similar levels were observed among all yeast species at the initial stage (day 1), the highest proteolysis value (0.92 mmol NH_2 serine/L) was recorded on day 28 in the sample containing *S. cerevisiae* (SC). This was followed by *Y. lipolytica* (YL) and *K. marxianus* (KM). In contrast, *D. hansenii* (DH) and *K. lactis* (KL) exhibited relatively lower proteolytic activity. The statistically significant differences ($p < 0.05$) demonstrate that both storage duration and yeast species play a critical role in proteolysis. These findings suggest that certain yeast species can markedly enhance protein hydrolysis, thereby significantly influencing the biochemical maturation of kefir.

In fermented dairy products, the presence of yeast species enriches the microbial ecosystem and significantly influences proteolytic activity. Yeasts contribute to the breakdown of milk proteins either by directly secreting proteinase and peptidase enzymes or by engaging in symbiotic interactions with bacteria [20, 26]. As mentioned in the studies of Ozturkoglu-Budak et al. [27] and Belloch et al. [28], species such as *K. marxianus*, *S. cerevisiae*, *D. hansenii* and *Y. lipolytica* possess proteolytic systems that play an active role in the hydrolysis of caseins. Through both extracellular protease production and continued enzymatic activity during storage, these yeasts alter the maturation dynamics of the product.

Differences in proteolytic activity among yeast species are influenced by several factors, including the genetic makeup of the species, cell wall permeability, metabolic profile, and fermentation conditions such as pH, temperature, substrate type, and oxygen availability. Certain yeast species—particularly *K. marxianus* — possess high proteolytic capacity and efficiently hydrolyze the β-fraction of caseins [29, 30], whereas the proteolytic activity of *S. cerevisiae* appears more variable. The combined use of yeasts with bacterial starter cultures creates a synergistic effect, increasing the levels of free amino acids and bioactive peptides, thereby enhancing the aroma, texture, and functional properties of the final product [31, 32].

In the study conducted by El et al. [22], the degree of protein hydrolysis in kefir produced from goat milk was reported as 26.1 %, and this rate was found to increase throughout the storage period. In this context, our findings are consistent with previous research indicating that the proteolytic activity of kefir samples increases during storage [33, 34]. Uzundag et al. [35] also reported that different yeast species may exhibit varying levels of proteolytic and lipolytic activity, which can contribute to cheese ripening. Proteolytic activity in fermented dairy products plays a decisive role in product maturation, texture development, flavor formation, and nutritional quality. The proteolytic system consists of extracellular and intracellular proteinases and peptidases secreted by starter cultures and adjunct microorganisms. These enzymes hydrolyze casein fractions (particularly α_{s1}-, α_{s2}-, β-, and κ-casein) into smaller peptides and free amino acids. The resulting compounds not only support microbial growth but also enrich the taste and aroma profile of the product. The presence of different yeast species increases the diversity of the proteolytic system and contributes to the formation of bioactive peptides (e.g., antihypertensive, antimicrobial, and antioxidant), which is significant [26, 36]. Yeast selection is therefore a key parameter influencing both the level and direction of proteolytic activity in fermented dairy products. Consequently, the use of appropriate yeast–bacteria combinations is critical for improving sensory quality and enhancing the production of functional peptides.

Color properties

The L*, a*, and b* values obtained from color measurements of the kefir samples during the 28-day storage period are presented in Figure 2. In these measurements, L* values indicate lightness (L* = 0 for black and L* = 100 for white), a* values represent the red–green axis (+a* for red and –a* for green),

and b* values represent the yellow–blue axis (+b* for yellow and –b* for blue). The L* values of all kefir samples exhibited changes throughout the 28-day storage period, and these changes were found to be statistically significant ($p < 0.05$). Similarly, when the L* values on days 1, 14 and 28 of storage were evaluated, significant differences were observed among the samples. Only on day 14 of storage were the L* values of all samples found to be similar. Nonetheless, day 14 was reported as the day on which the highest lightness values were recorded. b* values are very similar for each sample on day 1 and day 28, yet they are completely different on day 14. This can be explained by fermentation kinetics, yeast, and LAB metabolites.

The a* values of the kefir samples were found to vary within a relatively wide range (from −0.16 to −0.75). The changes observed in all samples throughout storage were statistically significant ($p < 0.05$), and significant differences ($p < 0.05$) were also detected among the samples on each storage day. In both cases, the a* values of the KM and KL samples were consistently lower than those of the other samples. This finding may indicate that *K. marxianus* and *K. lactis* contribute more extensively to red color formation than the other yeast species.

Moreover, there are reports in the literature evaluating the pigment-producing capabilities of various microbial species [37, 38]. Microorganisms with such properties may be utilized as secondary flora in certain dairy products—such as specific aromatic cheeses and ice creams—to exploit their pigment-production potential.

Microbial diversity during the ripening process of cheese has a significant effect on the colour, aroma, flavour and texture of the cheese. Particularly in surface-ripened cheeses*, D. hansenii, K. marxianus, Y. lipolytica* and *Geo. candidum* colonise the cheese surface and metabolise lactic acid during the early stages of ripening. This metabolism causes an increase in the pH of the cheese surface, promoting the growth of acid-sensitive bacteria and potentially affecting the pigmentation of other bacteria present in the flora. Not only this metabolism of yeasts, but also other factors such as light, pH, temperature, and salt concentration can affect pigmentation [39].

The b* values ranged between 7.83 and 11.61, with positive b* values indicating an increased intensity of yellow coloration. Significant differences ($p < 0.05$) were observed among the samples on each storage day, and the changes occurring within each sample during storage were also statistically significant ($p < 0.05$). The highest b* values were recorded on day 14 of storage, which corresponds to the pattern observed for the L* values, indicating a parallel trend. As with the L* value, the b* values on days 1 and 28 were very similar for each sample but were determined to be completely different on day 14. This situation can be explained by fermentation kinetics, yeast, and LAB metabolites.

During fermentation, the biochemical reactions occurring in milk—primarily driven by yeast activity—not only contribute to flavor formation in the final product but also influence its physicochemical characteristics. Color parameters represent only one aspect of these quality attributes. Statistical analyses demonstrated that different yeast species significantly affect the color properties of the final product. These findings provide insights not only for kefir production but also for the use of yeasts as adjunct cultures in other dairy products, highlighting potential changes in final product quality that may arise from their inclusion.

The presence of yeasts in fermented dairy products can influence product color through both direct metabolic activity and indirect biochemical interactions. Yeasts such as *Saccharomyces* and *Kluyveromyces* species metabolize carbohydrates during fermentation, producing organic acids, ethanol, CO_2, and various secondary metabolites. These metabolic processes lead to pH changes that alter the structure of casein micelles and light scattering properties, thereby affecting the visual appearance and opacity of the product. In addition, certain yeast species are capable of synthesizing pigments such as riboflavin or carotenoid-like compounds, which may directly contribute to shifts in color. Furthermore, yeast–lactic acid bacteria interactions can enhance proteolysis and lipolysis, resulting in the release of free amino acids and fatty acids that may undergo oxidative reactions, ultimately impacting color stability. Consequently, yeast composition and metabolic activity play a significant role in modulating color parameters (L*, a*, b*) in fermented dairy systems through both physicochemical and biochemical mechanisms [40].

Gursoy et al. [21] evaluated several physicochemical and rheological properties of commercial kefir samples collected from the Turkish market. The reported L*, a*, and b* values were 84.21–86.68, –2.87 to –2.40, and 4.80–6.03, respectively. When these data—obtained from commercial kefir samples that do not contain yeast—are compared with the findings of our study, the L* values were reported to be relatively lower. Both a* and b* values were higher in the kefir samples produced in this study compared to yeast-free commercial kefirs. This indicates that yeasts actively involved in the fermentation process enhance the perception of green and yellow coloration. As is well known, pigments responsible for yellow-to-red hues are defined as carotenoids, and many microorganisms, including yeasts, possess the ability to produce carotenoids. The use of yeasts as adjunct cultures in kefir production therefore explains the increased carotenoid-related color perception observed in the final product.

Antioxidant and phenolic activity

The data on antioxidant capacity and phenolic content of the kefir samples are presented in Table 2. Changes in antioxidant capacity were found to be statistically significant ($p < 0.05$) both throughout the storage period and among the samples on the same storage day. On the first day of storage, antioxidant activity ranged from 17.12 to 42.65, and it increased to 213.35–373.51 by the end of the 28-day storage period. This increase can be attributed to the formation of components during kefir production and the ongoing fermentation processes throughout storage that support antioxidant capacity. Proteins in milk and dairy products, primarily caseins and whey proteins, contribute to antioxidant capacity through peptides and amino acids released during fermentation. Additionally, certain vitamins and carotenoids also act as antioxidant components [41].

When evaluating total antioxidant capacity, kefir samples containing *S. cerevisiae* exhibited higher values compared to samples produced with other yeast species. The phenolic activity values of the kefir samples ranged from 0.45 to 0.59, with the highest phenolic activity observed in the CC sample containing *C. colliculosa*. All kefir samples exhibited similar phenolic activity values, and these values increased during storage.

In fermented dairy products, yeast species have a decisive impact on the antioxidant capacity and phenolic compound profile of the product. This effect is mediated through the enzymatic systems of yeasts, particularly β-glucosidase, esterase, protease, and lipase. These enzymes facilitate the release of antioxidant peptides from milk proteins and the biotransformation of existing phenolic compounds (e.g., conversion of phenolic glycosides to aglycones). Some yeast species — including *K. marxianus*, *Y. lipolytica* and *D. hansenii* — may contribute to enhanced antioxidant capacity and increased levels of bioactive compounds (e.g. phenolic-type molecules or yeast-derived metabolites such as glutathione or organic acids) in fermented products, likely through their enzymatic and metabolic activity [42, 43].

Y. lipolytica typically enhances volatile phenolic derivatives through its lipolytic and esterase activities, while *K. marxianus* can induce phenolic transformations via lactose metabolism and certain β-glucosidases. However, the outcomes depend on the yeast species and strain used. Therefore, yeast selection plays a critical role in enhancing antioxidant potential in fermented dairy products, and the choice of appropriate species or strains represents an effective strategy for improving both the functional properties and biological value of the final product.

Organic acid composition

As reported in the literature, it has long been known that significant amounts of organic acids are produced by various LAB during the fermentation of many foods, including vegetables, meat, and milk. These acids lower the pH of foods and possess inhibitory properties against certain harmful microorganisms that may be present [44, 45]. As consumer preferences drive the production of tailored food products, additives are increasingly incorporated to extend shelf life. In this context, biological control methods using natural preservatives are recommended, with fermentation technology being one of these approaches [46,47]. Pathogenic or spoilage-causing microorganisms can be inhibited in products using fermentation methods due to the production of organic acids. Lactic acid, acetic acid, propionic acid, and butyric acid are among the organic acids produced by LAB [48]. The sensory properties of kefir are determined not only by its content of lactic acid, oxalic acid, α-ketoglutaric acid, and certain volatile fatty acids but also by small amounts of CO_2, alcohol, and other aromatic compounds (e.g., acetaldehyde and acetone) released during fermentation by LAB and yeasts. In addition, organic acids which also function as natural preservatives, play a role in influencing the sensory characteristics of kefir [49]. Formic, fumaric, propionic, and tartaric acids were not detected in any of the kefir samples throughout the storage period. Citric acid was observed only on day 28, exclusively in the sample fermented with *K. lactis*. The quantified levels of lactic, oxalic, and acetic acids in the samples are provided in Table 3.

Among the organic acids present in kefir, lactic acid is found at the highest concentration. Kefir typically contains approximately 1 % lactic acid. Leite et al. [50] reported that lactic acid levels in their kefir samples increased over a 28-day storage period. The observed increase in lactic acid content can be attributed to the activity of LAB, as lactic acid is the primary end product of their fermentation. These acids naturally occur in foods or are produced via microbial fermentation in products such as yogurt, cheese, and milk. Lactic acid is predominantly produced by LAB, including *Lactobacillus*, *Streptococcus*, *Leuconostoc*, and *Enterococcus* species [51].

In a study by Akin [52] on acidophilus milk and yogurt, the lactic acid content in acidophilus milk produced from cow's milk was determined as 9060 µg/g, acetic acid as 104 µg/g, and citric acid as 625 µg/g. In yogurt, the citric acid content was 497 µg/g, lactic acid 8538 µg/g, and acetic acid 65 µg/g.

The acetic acid content of the kefir samples ranged from 646 to 981 mg/L. In kefir, acetic acid is typically present at concentrations of approximately 0.02 – 0.08% (200–800 mg/L), where it contributes positively to flavor complexity and microbial stability; however, levels exceeding ~0.08 – 0.10 % (> 800–1000 mg/L) lead to the development of a pronounced vinegar-like off-flavor, negatively affecting sensory quality and consumer acceptance, indicating the importance of controlling fermentation and storage conditions to maintain product quality. In the DH, KM, CC, and YL samples, acetic acid exhibited a consistent decrease, whereas in the other samples, fluctuations were observed. When comparing acetic acid levels at the beginning and end of storage, an overall increase was noted in these samples. Acetic acid, is widely used in foods to inhibit or reduce the viability of Gram-positive and Gram-negative bacteria, molds, and yeasts. Generally, a 0.2 % concentration inhibits bacterial growth, while 0.3 % is bactericidal. It is particularly effective against Gram-negative bacteria, and its antimicrobial activity is pH-dependent, being more effective at lower pH levels. Acetic acid is commonly added as an antimicrobial agent to salad dressings and mayonnaise and is also approved for use in carcass washing [48].

In the food industry, combinations of heat, acetic acid, and salt are studied for their inactivation effect on *Escherichia coli* O157:H7 [49]. These approaches are widely applied as hurdle technologies to control the growth of foodborne pathogens and spoilage microorganisms [48]. Compared with other organic acids, acetic acid exhibits stronger inhibitory effects on *Listeria monocytogenes* growth at pH 4.4 – 5.2, with the order of effectiveness being acetic acid > lactic acid > HCl. Acetic acid is also known to have greater antimicrobial activity against *Listeria monocytogenes* and *Staphylococcus aureus* compared to lactic acid. There are no established restrictions on daily intake of acetic acid and its salts. Acetic acid is commonly used in brined meat, fish, vegetables, mustard, salad dressings, garnishes, mayonnaise, ketchup, and certain infant foods [50, 51].

Gul et al. [52] reported that the acetic acid levels in their kefir samples exhibited fluctuations throughout a 21-day storage period. In contrast, Ender [33] found that acetic acid concentrations in samples produced with kefir grains and starter cultures increased over a 30-day storage period. Guzel-Seydim et al. [49] were unable to detect acetic acid production in samples with varying amounts of kefir grains. Nevertheless, Gul et al. [52] indicated that the use of starters or kefir grains significantly influenced acetic acid formation, with starter cultures producing higher levels of acetic acid.

Oxalic acid is a minor organic acid naturally present in kefir, primarily originating from microbial metabolism of amino acids and plant-derived substrates in the milk. Although typically found at low concentrations, oxalic acid can influence mineral bioavailability, particularly calcium, by forming insoluble complexes, and may contribute subtly to the overall acidity and flavor profile of kefir. Its presence is therefore relevant both for nutritional considerations and for the modulation of kefir's sensory characteristics. The oxalic acid content of the kefir samples generally followed a fluctuating trend. A decrease in oxalic acid was observed in the KM, YL, and SC samples during storage, whereas increases and decreases were noted in the other samples. Turker et al. [53] reported that kefir produced from cow's milk contained oxalic acid at 169.15 mg/L, lactic acid at 12,695.65 mg/L, acetic acid at 11,848.55 mg/L, and citric

acid at 762.24 mg/L. Although a decrease in oxalic acid levels was observed on day 28 in the DH, KM, and CC samples, the measured values were lower than those reported by Turker et al. [53]. In addition, oxalic acid has been associated with the formation of kidney stones.

Citric acid was detected only in the KL sample at the end of the storage period, at a concentration of 41 mg/L. Guzel-Seydim et al. [49] investigated changes in organic acids (citric, lactic, acetic) during kefir storage and reported an initial citrate concentration of 1438 µg/g on day 0, while acetic and propionic acids were not detected throughout storage. In another study, kefir produced from kefir grains was stored at 4 °C and sampled on days 0, 7, 14, and 21 to examine changes in organic acids. Lactic acid content increased significantly during storage, while citric acid concentrations showed only slight increases.

The use of yeast species in fermented dairy products has a significant impact on the organic acid profile, which in turn affects acidity balance. Organic acids are produced as end products of carbohydrate, protein, and lipid metabolism during fermentation and are key determinants of both sensory and biochemical characteristics of the product. Yeasts influence the quantity and distribution of these acids through their capacity to metabolize carbon sources and their enzymatic systems [54, 55].

Species such as *K. marxianus*, *Y. lipolytica*, *D. hansenii*, and *S. cerevisiae* contribute to the production of compounds including lactic, acetic, succinic, pyruvic, citric, and formic acids via different metabolic pathways. Specifically, lactose-fermenting *K. marxianus* increases total acidity through high production of lactic and acetic acids, whereas the highly lipolytic *Y. lipolytica* can enhance succinic and citric acid levels associated with fatty acid metabolism. *D. hansenii* contributes to the acid profile in salted and matured products, promoting a mildly acidic but balanced taste. In kefir fermentation, the co-culture of yeasts and bacterial starter strains markedly influences fermentation kinetics by modulating pH trajectories, enhancing acidification rates, and restructuring the organic acid profile through metabolic cross-feeding and complementary biochemical pathways. These synergistic interactions promote the formation of microbial stability and safety, and contribute to the desirable biochemical and physicochemical characteristics that define kefir maturation [56, 57].

In conclusion, yeast selection plays a pivotal role in shaping the organic acid composition of fermented dairy products. Strategic combinations of yeasts and bacterial starter cultures provide an effective means of modulating fermentation metabolism, thereby enabling the production of high-quality dairy products with targeted sensory profiles and optimized biochemical balance.

Conclusion and future recommendations

In recent years, there has been increasing interest in developing starter cultures to produce kefir beverages similar to traditional kefir made with grains. However, due to the specific characteristics of the yeasts used, not all yeast species can be employed, or their use is limited. In kefir, yeast–lactic acid bacteria interactions are generally positive, as yeasts can provide growth factors such as vitamins and amino acids that stimulate LAB activity, while LAB produce organic acids and metabolites that enhance yeast growth. This mutualistic relationship contributes to balanced acidification, improved flavor complexity, and desirable texture in the final product. However, under certain conditions, excessive yeast activity may lead to overproduction of ethanol or CO_2, potentially negatively affecting sensory quality and product stability. In this study, which investigated kefir production using different yeast species, it was observed that the yeasts included in the starter culture composition significantly influenced the properties of the final product. Overall, the results demonstrated that yeast species affected the organic acid composition and proteolytic activity of the kefir. Notably, *Kluyveromyces lactis* was the only species contributing to citric acid presence at the end of storage, while other samples lacked it. Proteolytic activity was highest in kefir containing *Saccharomyces cerevisiae* at the end of storage, but an increasing trend was observed in all kefir samples throughout the storage period. For future studies, it is recommended to investigate the effects of combining these yeast species in pairs to further optimize kefir composition and functionality.

Declarations

Acknowledgements

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.

CRediT authorship contribution statement

Oktay Yerlikaya: Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Formal analysis, Conceptualization.

Asli Akpinar, Derya Saygili: Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization.

Yigithan Balta: Writing – original draft, Formal analysis, Data curation.

Ethics declarations

Not applicable

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Availability of data and materials / Data availability

The data that has been used is confidential. The datasets generated or analyzed during the experimental study can be obtained from the corresponding author upon request.

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Tables

Table 1. Changes in proteolytic activity (hydrolysis rates) in kefir beverages (mmol- NH_2 serine /L)

Kefir samples				
Storage days	**DH**	**KM**	**CC**	**YL**
1	0.67 ± 0.02^{CK}	0.78 ± 0.04^{CX}	$0.70\pm0,04^{CZ}$	0.68 ± 0.01^{CT}
14	0.70 ± 0.00^{BK}	0.81 ± 0.07^{BX}	$0.74\pm0,01^{BYZ}$	0.71 ± 0.02^{BT}
28	0.81 ± 0.03^{AT}	0.85 ± 0.06^{AY}	$0.82\pm0,03^{AZ}$	0.84 ± 0.02^{AYZ}

DH: *Debaryomyces hansenii* LAF 3 **KM:** *Kluyveromyces marxianus* LAF 4, **CC:** *Candida colliculosa* LAF 7 **YL:** *Yarrowia lipolytica* N

^{A,B,C} (↓):The difference between means showing different letters is statistically significant ($p < 0.05$)

^{X,Y,Z,T,K,L} (→):The difference between means showing different letters is statistically significant ($p < 0.05$)

Table 2. Antioxidant and phenolic activity properties determined in kefir beverages

	Kefir samples					
Storage days	**DH**	**KM**	**CC**	**YL**	**SC**	**KL**
	Antioxidant Activity (mg ascorbic acid / g)					
1	28.66 ± 2.04^{BY}	26.04 ± 2.22^{CY}	48.90 ± 3.18^{BX}	28.10 ± 2.33^{BY}	42.49 ± 2.77^{BX}	17.12 ± 1.76^{CZ}
14	28.45 ± 0.34^{BT}	39.80 ± 1.84^{BZ}	56.20 ± 0.78^{BX}	37.35 ± 6.35^{BZT}	53.36 ± 6.07^{BY}	29.66 ± 5.67^{BT}
28	265.77 ± 6.36^{AY}	247.69 ± 5.58^{AZ}	213.35 ± 3.99^{AT}	221.88 ± 1.31^{AT}	373.51 ± 7.67^{AX}	258.75 ± 0.73^{AYZ}
	Phenolic Activity (mg gallic acid equivalents (GAE) / mL					
1	0.46 ± 0.01^{BYZ}	0.48 ± 0.01^{BY}	0.45 ± 0.00^{BZ}	0.52 ± 0.03^{X}	0.46 ± 0.01^{YZ}	0.45 ± 0.01^{Z}
14	0.51 ± 0.00^{A}	0.49 ± 0.00^{B}	0.56 ± 0.01^{A}	0.54±0.01	0.48±0.03	0.51±0.04
28	0.52 ± 0.01^{A}	0.55 ± 0.01^{A}	0.59 ± 0.06^{A}	0.58±0.02	0.53±0.01	0.53±0.02

DH: *Debaryomyces hansenii* LAF 3 **KM:** *Kluyveromyces marxianus* LAF 4, **CC:** *Candida colliculosa* LAF 7 **YL:** *Yarrowia lipolytica* NCAIM Y00591, **SC:** *Saccharomyces cerevisia* **KL:** *Kluyveromyces lactis* PYCC 4356

^{A,B,C} (↓):The difference between means showing different letters is statistically significant ($p < 0.05$).

^{X,Y,Z,T,K,L} (→):The difference between means showing different letters is statistically significant ($p < 0.05$).

Table 3. Organic acids determined in kefir beverages (mg/L)

Storage days	DH	KM	CC	YL
	Lactic acid			
1	17945.49±4.41^{AX}	16767.88±1.68^{BK}	16804.38±2.17^{AT}	16864.83±1.83^{CZ}
14	16797.06±5.81^{BK}	17015.76±2.91^{AT}	16776.60±2.00^{BL}	17061.90±1.42^{BY}
28	15097.44±0.81^{CL}	16533.73±0.98^{CZ}	16268.91±0.01^{CK}	17114.70±0.67^{AX}
	Acetic acid			
1	958.47±3.23b^{AYZ}	968.68±3.49^{AXY}	965.50±2.25^{AXY}	969.46±2.03^{AX}
14	935.86±3.80^{BTK}	967.53±2.02^{AY}	948.17±5.37^{BZ}	942.51±4.17^{BZT}
28	775.64±2.67^{CK}	887.85±1.23^{BZ}	830.40±3.15^{CT}	646.83±2.31^{CL}
	Oxalic acid			
1	95.28±0.37^{BK}	108.13±0.15^{AY}	107.75±0.44^{AY}	130.56±0.15^{AX}
14	107.67±0.00^{AY}	107.95±0.36^{AY}	94.29±0.11^{CK}	110.91±0.10^{BX}
28	82.92±0.12^{CK}	95.41±0.26^{BZ}	101.81±0.43^{BY}	109.94±0.11^{CX}
	Citric acid			
1	ND	ND	ND	ND
14	ND	ND	ND	ND
28	ND	ND	ND	ND

DH: *Debaryomyces hansenii* LAF 3 **KM:** *Kluyveromyces marxianus* LAF 4, **CC:** *Candida colliculosa* LAF 7 **YL:** *Yarrowia lipolytica* NCAIM Y00591, **SC:** *Saccharom*

ND: Not determined

^{A,B,C} (↓):The difference between means showing different letters is statistically significant ($p < 0.05$)

^{X,Y,Z,T,K,L} (→):The difference between means showing different letters is statistically significant ($p < 0.05$)

Figures

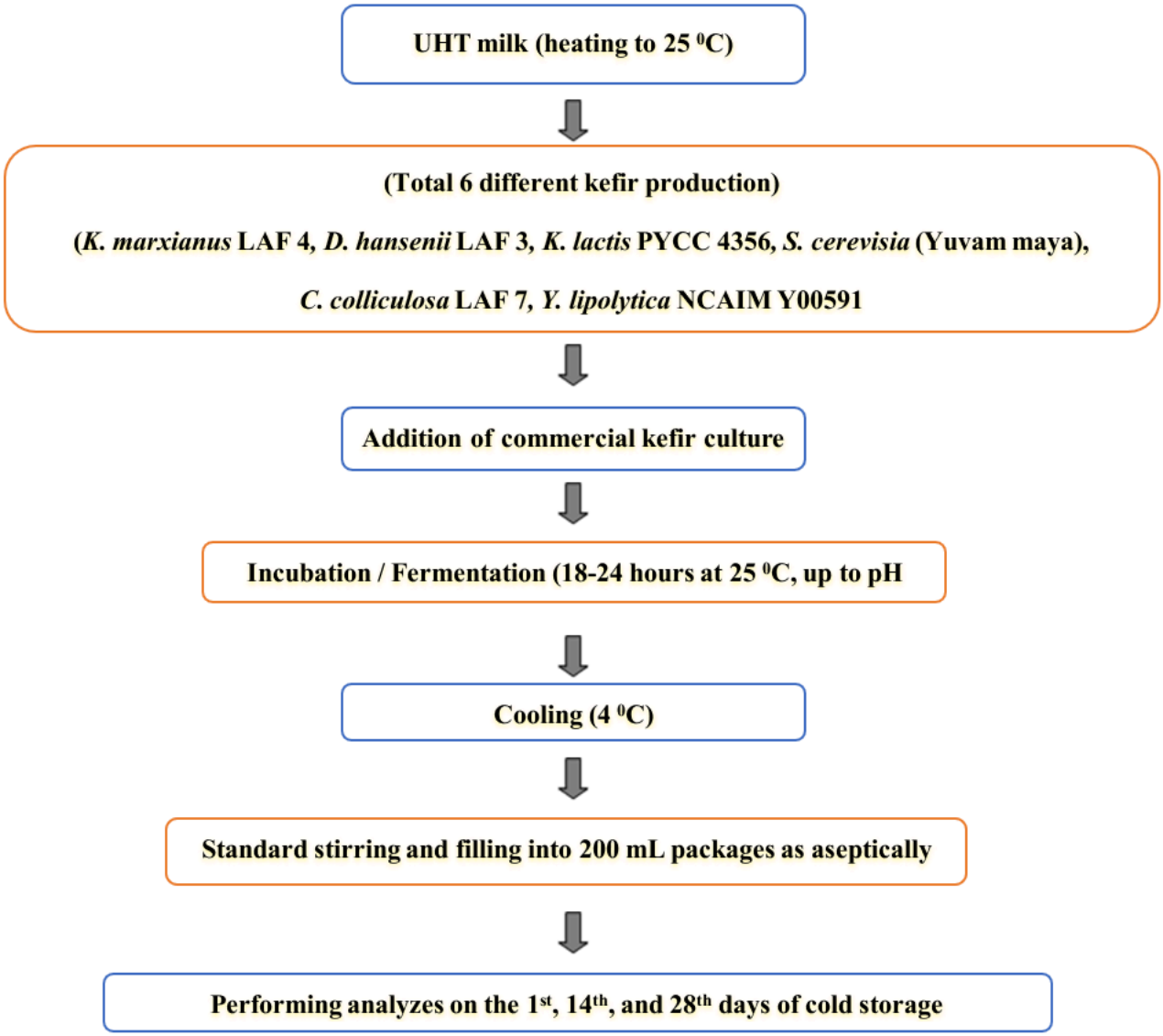
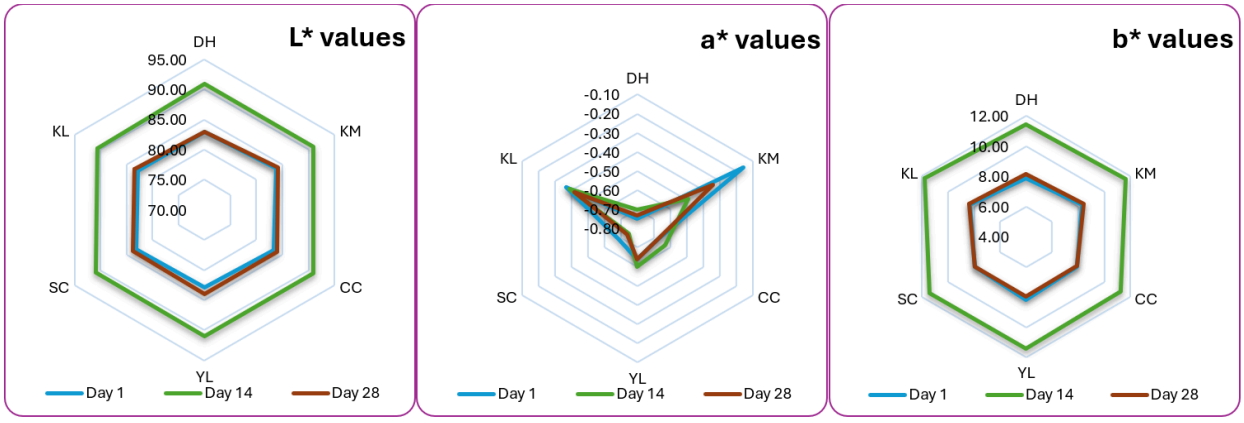


Figure 1

Kefir production flow diagram



DH: *Debaryomyces hansenii* LAF3 **KM:** *Kluyveromyces marxianus* LAF4, **CC:** *Candida colliculosa* LAF7 **YL:** *Yarrowia lipolytica* NCAIM Y00591, **SB:** *Saccharomyces bouardii* CNCM I-745®, **SC:** *Saccharomyces cerevisia* **KL:** *Kluyveromyces lactis* PYCC 4356, **GC:** *Geotricum candidum* GEO

Figure 2

Color values of kefir beverages (L*, a* and b*)