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Determination of The Antifungal Capacity of Kefir Components Against Spoilage Fungi

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Abstract

The purpose of this study is to investigate the potential alternative use of kefir and its derived components in the biocontrol of fungi that cause economic losses during the harvesting and storage of fruits. The antifungal properties of kefir and its derived components (the non-protein liquid fraction of kefir, kefiran, and yeast cells isolated from kefir) were examined using the disk diffusion method, and the minimum inhibitory concentration (MIC) against naturally occurring fungi during the storage of hazelnuts, oranges, and apples were determined. Fungal isolates obtained from different fruits were identified as Aspergillus niger, Penicillium digitatum, and Penicillium expansum through 18S rRNA Polymerase Chain Reaction. The inhibition zones of A. niger, P. digitatum, and P. expansum were evaluated using the disk diffusion method. The minimum inhibitory concentrations (MICs) for kefir against A. niger, P. digitatum, and P. italicum were found to be 0.4, 0.2, and 0.25 µg mL^{-1}, respectively. The effectiveness of these applications was analyzed using a technique known as TOPSIS (Technique for Order Preference by Similarity to an Ideal Solution). With the development of the results of this study, alternative biocontrol applications using kefir and its components could be developed to prevent fungi-related microbial spoilage, providing an alternative to chemical treatments.

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Kefir Bileşenlerinin Bozulma Küflerine Karşı Antifungal Kapasitesi

Öz

Bu çalışmanın amacı, kefir ve ondan elde edilen bileşenlerin, meyvelerin hasat ve depolanması sırasında ekonomik kayıplara yol açan küflerin biyokontrolünde alternatif kullanım potansiyelini araştırmaktır. Kefir ve kefirden elde edilen bileşenler (kefirin protein olmayan sıvısı, kefiran ve kefirden izole edilen maya hücreleri) antifungal özelliklerinin etkisi disk difüzyon ile araştırılmış ve fındık, portakal ve elmanın depolanması sırasında doğal olarak üreyen küflere karşı minimum inhibitör konsantrasyon belirlenmiştir. Farklı meyvelerden elde edilen küf izolatları 18S RNA Polimeraz Zincir Reaksiyonu ile Aspergillus niger, Penicillium digitatum ve Penicillium expansum olarak tanımlanmıştır. A.niger, P. digitatum ve P. expansum küflerin inhibisyon zonları disk difüzyon yöntemi ile değerlendirilmiştir. Kefirin A. niger, P. digitatum ve P. italicum için minimum inhibitör konsantrasyonları (MIC) sırasıyla 0.4, 0.2 ve 0.25 µg mL^{-1} olmuştur. Bu uygulamaların etkinliği, TOPSIS (Technique for Order Preference by Similarity to an Ideal Solution) analizi olarak bilinen teknik kullanılarak gerçekleştirilmiştir. Bu çalışmanın sonuçlarının geliştirilmesi ile, küf kaynaklı mikrobiyal bozulmaların önlenmesi için kefir ve kefirden elde edilen bileşikler kullanılarak kimyasal uygulamalara alternatif biyokontrol uygulamaları geliştirilmesi önerilir.

Anahtar Kelimeler

Aspergillus niger
Kefir
Kefiran
Penicillium digitatum
Penicillium expansum

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Introduction

Kefir is a fermented milk product that contains a complex microbial community, including yeasts and lactic acid bacteria. It has been reported to possess antimicrobial properties, including antifungal activity against various pathogenic fungi (Kim et al., 2016). Apart from containing beneficial bacteria and yeast, it is abundant in amino acids, vitamins, minerals, and enzymes. Kefir has been approved to have antibacterial, antifungal and anti tumoural activities in addition to other beneficial attributes (Lakshmi et al., 2017; Sharifi et al., 2017; Azizkhani et al., 2021; González-Orozco et al., 2022).

Microbial decay is a significant challenge for the food industry, and it can lead to significant economic losses due to spoilage and food waste. According to some estimates, microbial spoilage accounts for up to 25% of total food losses worldwide. Microbial decay can occur due to a variety of factors, including contamination during processing, transportation, and storage, as well as inadequate preservation methods (Wang et al., 2018). The fungi such as *Penicillium expansum, Fusarium* spp., *Penicillium* spp., *Aspergillus* spp., etc. can be developed on fruits which have high economic values and cause deterioration (Gamba et al., 2016; Moure et al., 2023). Over the past few decades, there has been a rising awareness regarding the necessity to substitute chemical control substances like fungicides and insecticides. This shift is motivated by the detrimental impact these chemicals have on the environment and the health of both humans and animals. The proposed solution is to replace them with natural products. The use of biological control agents, such as microbial antagonists and natural products, does not result in toxic residues on the fruit, unlike the application of fungicides. (Bazioli et al., 2019). Biological control is also harmless and healthier for the people who handle the products. Antagonists can be isolated from wide variety of sources such as soil, fruits, leaves, etc. (González-Estrada et al., 2018).

Recently, kefir has been used as a biocontol agent in several investigations targeting the food industry. Kefir is certainly very difficult to use in the industry, however, it will be possible to develop components for industrial use only by determining the substance(s) effective on spoilage organisms. Gamba et al. (2016) tested antifungal activity of kefir on corn areas in order to improve shelf life and its antibacterial activity has been exhaustively demonstrated. Fouad et al. (2015) reported that kefir has antimicrobial activity on several fungus which grown on fruits including lemon, pomegranate. Zhimo et al. (2020) documented numerous specific bacterial and yeast species found in kefir grains that exhibit biocontrol properties against *Penicillium* infections in apples and grapefruits. The antifungal properties of water kefir, in addition to the components of milk kefir, have been evaluated in various studies. Gonda et al. (2019) investigated the potential of microorganisms derived from water kefir (WK) to control the growth of *Aspergillus flavus* in aerobic-phase ensiled sorghum grains and suggested that kefir could be a viable alternative for disease management. A range of isolated bacteria, including *Pseudomonas cepacia, Pseudomonas syringae,* and *Bacillus subtilis,* as well as yeasts such as *Candida sake, Rhodotorula glutinis,* and *Debaryomyces hansenii,* were assessed for their potential to combat postharvest fruit fungi. These fungi included *Penicillium digitatum, Penicillium italicum, Alternaria, Aspergillus, Botrytis, Fusarium, Geotrichum, Gloeosporium, Monilinia, Mucor, Colletotrichum,* and *Rhizopus*. Both in vitro and in vivo tests demonstrated their significant potential, as reported by González-Estrada et al. (2018).

The antifungal effects of kefir have been investigated by many studies and its inhibitory activities are well demonstrated against a wide variety of pathogens (Kim et al., 2016; Taheur et al., 2020). Erdogan et al. (2019) reported that all fungus disappeared on the 3rd day in the stools of mice fed kefir. It has also been determined in different studies that kefir has antifungal properties This research was planned to investigate the antifungal properties of kefir. As is known, kefir contains bacteria and yeasts. Does the yeast of kefir provide the inhibition of fungus or is it the protein-free component? Or is component of polysaccharide from microorganism? Or is the complex of kefir the most effective? The purpose of this study is to investigate alternative methods which may provide biocontrol of fungi that are grown during harvesting and spreading during storage of fruits. These fungi can cause serious economic loss. The effects of kefir and components from kefir on the fungi isolates (*A. niger*, *P. digitatum*, *P. expansum*) are evaluated *in vitro* tests.

Kefiran is a type of exopolysaccharide produced by lactic acid bacteria during kefir fermentation. Research suggests that kefiran has potential health benefits, including antioxidant, anti-inflammatory, and cholesterol-lowering effects. However, its antibacterial and antifungal properties are still a matter of debate. Some studies have reported antibacterial activity of kefiran against various bacteria, such as *Staphylococcus aureus*, *Escherichia coli*, and *Listeria monocytogenes* (Moradi and Kalanpour, 2019). Similarly, the antifungal properties of kefiran are also not well-established. Some studies have reported that kefiran has antifungal activity against *Candida albicans* (Al-Mohammadi et al., 2021), while others have found no significant antifungal activity. Additional research is necessary to fully understand the antifungal

properties of kefiran. Overall, while there is some evidence to suggest that kefiran may have antifungal properties, more research is needed to fully confirm these effects. The antifungal effect of kefir on fungus was made for the first time in this study.

Material and Method

Fungal source

Hazelnut, orange, apple and lime fruits which were purchased from a local market were placed in separate sterile closed pots with controlled humidity (70%) at room temperature (25 °C) for 10 days, and fungi growth was observed.

Fungal isolation and cultivation, identification of fungus

The fungi from decayed hazelnut, orange and apple fruits were transferred in the YPD (Yeast Extract-Peptone-Dextrose (YPD broth), Lab M, England) broth. It was inoculated at 25 °C for 3 days. Activated fungi were grown to Potato Dextrose Agar (PDA) (Biolife, Italy) at 25 °C 5 days for two times. A single colony was transferred to a YPD broth for further activation. In addition, all fungi were adjusted to approximately 6 log CFU/mL to be used in the disc method and for enumeration. Also, single colonies were analyzed using 18S RNA polymerase chain reaction by REFGEN Biotechnology Company in Ankara, Turkey. It was amplified via PCR using primer pair ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Halıcı and Güllü, 2024).

Preparation of kefir and the components from kefir (CFS-Kefiran-Yeast)

In this study, kefir and three different components from kefir that can also be used as an ingredient in a variety of foods were investigated on some spoilage fungi. Kefir (Kefirzadem 250 mL) was obtained from Danem Co. (Isparta, Turkey). This Kefir is produced by the fermentation of milk with kefir grains. The first component of kefir used in the research; non-protein kefir liquid (CFS-pH 4.4) was obtained by centrifuging kefir at 4500 RPM. The protein-free portion of kefir, also known as kefir water or kefir whey, is a yellowish translucent liquid that separates from the kefir grains during the fermentation process. This liquid contains a complex mixture of organic acids, vitamins, minerals, and other bioactive compounds, including polysaccharides and peptides. The second components of kefir; kefiran was obtained by applying according to the method of Koçak et al. (2021). The third component of kefir (yeast) was obtained by taking and isolating the colonies of kefir growing on PDA medium.

Organic acid profile analysis of CFS and kefir

In this analysis, organic acids in kefiran were not examined because kefiran predominantly consists of polysaccharide structures in terms of concentration/dominance. It is responsible for the polysaccharide structure of kefir. To determine the presence of organic acids, 2 grams of CFS and kefir weremixed with double-distilled water and homogenized for 180 seconds at 4000 rpm. Next, 10 mL of the mixture was mixed with 12.5 mL of 0.01 N H_2SO_4 and vortexed for 1 minute. The top layer was separated and centrifuged at 10,000 x g for 5 minutes. The identification of organic acids was done by comparing their retention times at 210 nm with those of pure standards. Linear equations were calculated using standard organic acid solutions (lactic, acetic, citric and formic acid standards) with high correlation coefficients (R^2=0.9991, 0.9998, 0.9992 and 0.9988, respectively) to obtain the results (Duran et al., 2022). After the centrifugation process, the resulting supernatant, which is the clear liquid remaining above the precipitated material, was passed through a nitrocellulose membrane with a pore size of 0.22 µm (Sigma-Aldrich, St. Louis, USA) to remove any impurities and ensure sterility. The filtered supernatant was then stored at a temperature of -20°C until it was used for the antifungal activity assays (Gamba et al.,2016).

Sugar profile analysis of kefiran and kefir

In this analysis, the sugar profile of CFS, a component of kefir, was not examined. CFS has a high organic acid content and reflects the organic acid content of kefir. The fermented milk samples were tested for their

glucose, galactose, and lactose content using the same pre-extraction process. The analysis was carried out using High-Performance Liquid Chromatography (HPLC) with a refractive index detector (RID) and a Transgenomic CARBOSep COREGEL-87P column. Linear equations were calculated using standard sugar solutions to obtain the results, with high correlation coefficients (R^2=0.9936, 0.9914, and 0.9984 for glucose, lactose, and galactose, respectively) (Koçak et al., 2021).

Isolation and cultivation of yeast from kefir

The third components of kefir; isolation of yeasts from kefir was obtained by taking all colonies growing on PDA with lactic acid (1.4%) as supplement. For activation of kefir yeasts, colonies were inoculated into YPD medium and incubated at 25 °C for 5 days. All the colonies were inoculated in a YPD broth and activated at 25 °C for 5 days. Activated yeast cells were centrifuged by being removed from the media and stored at 4 °C for further use. The yeasts obtained from kefir were propagated in broth medium and used to test their effect against fungi.

Antifungal capacity of kefir and components from kefir by disk methods

Activated each fungus isolates (*A. niger*, *P. digitatum*, *P. expansum*) was inoculated by using the spread plate method in the PDA. Sterilized filter disks were embedded with 50 µl kefir, 50 µl CFS, 50 µl yeast, 50 µl kefiran and 0.1 ppm natamycin (Danisco, Denmark) as positive control in 5 minutes. Disks which include kefir and kefir-formed compounds were placed on the petri plates. Single disks containing kefir, and components from kefir samples were also placed on separate plates. These studies were carried out in duplicates. Subsequently, the samples were incubated for 72 hour at 25 °C. Since the surface is covered by the conidia produced by the fungus, the measurement is obscured. Hence 5 days was not fulfilled. Following incubation, zones of inhibition around the filter paper discs were measured and recorded using a caliper (Rocaltrol, Germany).

Minimum inhibitory concentration testing of the kefir and components from kefir (CFS-Kefiran-Yeast)

Four different samples, were prepared and were tested against three different fungi using the well diffusion plate method. Determination of minimum inhibitory concentration (MIC) of the tested kefir and kefir- components was carried out using agar dilution methods. Then serial dilutions 10, 5, 2, 1, 0.7, 0.5, 0.4, 0.3, 0.25, 0.2, 0.1, 0.05 µl/mL were prepared. Minimum inhibitory concentrations (MICs) were measured as the lowest concentration of treatments inhibiting the visible growth of the pathogen on the agar dish.

TOPSIS multi-index comprehensive evaluation

The TOPSIS method, which was developed by Talebanpour and Javadi (2015) as a means of ranking multi-attribute decision-making analysis, has been utilized in this study to perform a comprehensive evaluation of various indicators for tofu prepared under different treatment conditions. The objective of the evaluation was to identify the optimal treatment technique.

Results and Discussion

In this study, we conducted a comparison between the isolates generated here and sequences available in the GenBank database using the BLAST search tool, accessible at http://www.ncbi.nlm.nih.gov/BLAST. *A. niger P. digitatum, P. expansum* were identified from hazelnut, orange, and apple, respectively.

Detection of Organic acids of CFS and kefir

The Table 1. shows the concentration of four organic acids (lactic acid, acetic acid, citric acid, and formic acid) in two different samples, CFS (cell-free supernatant) and kefir. CFS has a high organic acid content and reflects the organic acid content of kefir. According to the Table 1, the concentration of lactic acid in CFS and kefir is similar (0.699±0.050 g/100mL and 0.709±0.033 g/100mL, respectively). Table 1. was shown that the amounts of total organic acids are >0.5%. Overall, the table suggests that lactic acid is the

most abundant organic acid in both samples, and kefir has slightly higher concentrations of acetic acid and formic acid than CFS. The concentration of citric acid is very low in both samples. However, it's important to note that the table only shows the concentration of four organic acids, and there may be other organic acids or compounds that are present in different amounts in these samples. The amount of lactic acid needed to inhibit fungal growth can vary depending on the type of fungus, the pH of the environment, and the concentration of other organic acids present. Generally, a pH of 4.0 or lower can inhibit fungal growth, and lactic acid is often used in combination with other organic acids to create a synergistic effect. In terms of specific amounts, it would depend on the application and the specific fungus being targeted. In food preservation, for example, the use of lactic acid bacteria in fermentation can result in the production of lactic acid at levels ranging from 0.5% to 2.5% (w/v) in the final product. However, in other applications such as medical treatments, higher concentrations may be required. Therefore, the appropriate amount of lactic acid to use would need to be determined based on the specific application and the intended effect. Although the organic acid values are close when kefir and CFS are compared, it is interpreted that CFS is appropriate in practice. This is thought to be the reason why they recommend CFS in the literature. Taheur et al. (2020) reported that the antifungal activity of the cell-free supernatant was found to be affected by the pH. Additional research is needed to determine the bioactive compounds present in kefir that contribute to the observed antifungal activity against mycotoxigenic strains. Further studies are also required to understand the mechanism involved in the reduction of mycotoxin levels.

Table 1. Contents organic acid of CFS and kefir (g/100 mL).

	Lactic acid	Acetic acid	Citric acid	Formic acid
CFS	0.699±0.050	0.705±0.054	0.046±0.002	0.106±0.007^{b}
Kefir	0.709±0.033	0.728±0.037	0.047±0.001^{d}	0.127±0.005^{ab}

Detection of Sugar profiles of kefiran and kefir

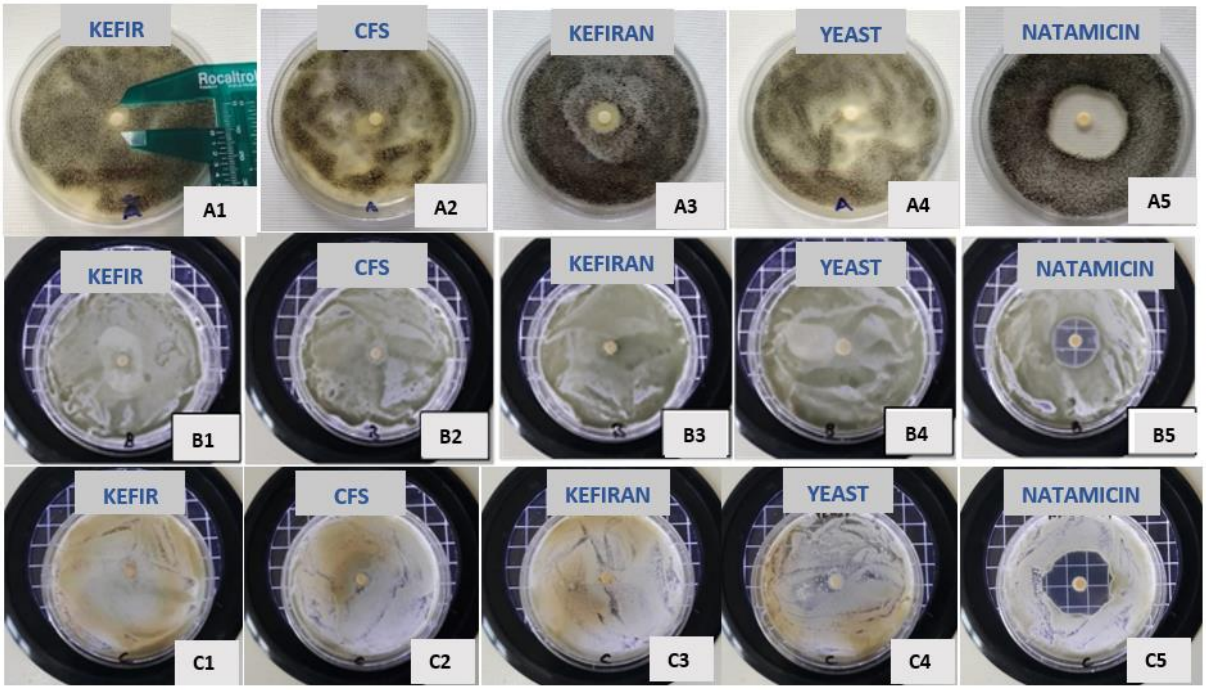
Kefiran is a polysaccharide produced by LAB during kefir fermentation, while kefir is a fermented milk product. kefiran predominantly consists of polysaccharide structures in terms of concentration/dominance. It is responsible for the polysaccharide structure of kefir. Both samples contained significant amounts of glucose, with kefir containing more than kefiran (Table 2). Galactose was also present in both samples, but in a lower concentration than glucose.

Table 2. Contents sugar of kefiran and kefir (g/100 mL).

	Glucose	Galactose	Xylose
Kefiran	0.150±0.02	0.121±0.001	0.345±0.022
Kefir	0.413±0.028	0.132±0.007	0.277±0.001

Effects on fungi with treatments of kefir and components from kefir (CFS-Kefiran-Yeast)

The study evaluated the antifungal activities of kefir and components from kefir against *Aspergillus niger* using inhibition zone assays (Figure 1). Natamycin treatment was a positive control. As can be seen in the images, the zones of kefir and kefir components were not as clear as the positive control sample. The sizes of inhibition zones was are presented in Table 3. The results showed that yeast treatment resulted in the strongest antifungal activity with an inhibition zone of 19 mm, followed by kefiran treatment with an inhibition zone of 16 mm. Kefir and CFS treatments showed weaker antifungal activity with inhibition zones of 11 mm and 16 mm, respectively. The size of the inhibition zone is related to the level of antifungal activity present in the sample, and a larger inhibition zone usually means that the antimicrobial is more potent. The comparison of the inhibition zones to the control positive for natamycin showed that yeast and kefiran treatments were comparable in their antifungal activity, while kefir and CFS treatments were less effective (Figure 1).



1: Kefir, 2: CFS, 3: Kefiran, 4: Yeast, 5: Natamycin; A: *Aspergillus niger*, B: *Penicillium digitatum*, C: *Penicillum expansum*.

Figure 1. View of inhibition zone formed by kefir and compounds from kefir against *Aspergillus niger*, *Penicillium digitatum*, *Penicillum expansum*.

Table 3. Inhibition zone diameter formed by kefir and components from kefir against *Aspergillus niger*, *Penicillium digitatum, Penicillum expansum*.

Fungi	Inhibition zone diameter (mm)				
	Kefir	**CFS**	**Kefiran**	**Yeast**	**Natamycin**
Aspergillus niger	11±0.25	16±0.19	16±0.28	19±0.24	35±0.09
Penicillium digitatum	20±0.11	7±0.29	0.5±0.02	7±0.14	25±0.08
Penicillum expansum	10±0.18	9±0.17	0.5±0.02	7±0.21	27±0.09
	Percentage (%) Values of Data Based on Natamycin				
Aspergillus niger	31.42	45.71	45.71	54.28	100
Penicillium digitatum	80.00	28.00	2.00	28.00	100
Penicillum expansum	37.03	33.33	1.85	25.92	100

When the MIC values of kefir and components obtained from kefir are examined, it is seen in Table 3. that the effects of kefir yeasts, serum and kefir are higher, respectively, when we compare them to natamycin. It is effective in kefir, but others are more effective on this fungus (Table 4). In a study conducted by Purutoglu et al. (2020) the antifungal activities of thirty kefir isolates were tested against five different fungus species, namely *P. chrysogenum, A. parasiticus, B. cinerea, F. oxysporum,* and *A. niger*. The inhibition levels of these fungi species varied, with *A. niger* being the most lower by the kefir isolates. In addition, non- protein of kefir fermented with kefir grains showed antifungal activity against *A. flavus, P. crustosum, Trichoderma longibrachiatum* and *Rhizopus macrospores* (Londero et al., 2014). Previous investigations involving water kefir, a beverage produced through the fermentation of sugary solutions using kefir grains, have demonstrated its inhibitory effects on *A. ochraceus*, as evidenced by Caro and Leon (2014). Recently, abacteriocin obtained from *Lactobacillus paracasei*, isolated from kefir grains, inhibited *A. flavus, A. niger and P. glaucum* (Miao et al., 2014).

A study by Koç et al. (2018) evaluated the applicability of vinegar obtained from hazelnut shells against the growth of *A. niger* and found the vinegar useful for the prevention of fungi. None of the treatments applied above for the control of *A. niger* presented complete inhibition of the fungi.

The inhibition zone formed by kefir and components from kefir against *Penicillium digitatum* is shown in Figure 1. The size of the inhibition zone is presented in Table 3. The size of the inhibition zone formed by kefir and kefir-derived compounds against *P. digitatum* is measured between 0 and 20 mm. Compared to control

positive for natamycin, the most efficient but unclear 20 mm inhibition zone against *P. digitatum* was obtained by kefir treatment followed by CFS and yeast treatments, respectively. Results indicated that the treatment of kefiran against this fungi is not potent.

P. digitatum, which is the fungus tested, is a pathogenic fungus that can cause fruit decay. There is no literature on the effect of kefir on this mould species.

The study likely aimed to identify potential natural antifungal agents that could be used as alternatives to synthetic fungicides. It's important to note that the results of this study should be interpreted within the context of the specific experimental conditions used. The effectiveness of these substances against *P. digitatum* may vary depending on factors such as concentration, application method, and the specific strain of the fungus being tested. Additionally, further research would be needed to determine the safety and efficacy of using these substances in practical applications, such as in agricultural settings. Overall, the results suggest that kefir, kefir-derived compounds, and yeast may have potential as natural antifungal agents against *P. digitatum,* although natamycin remains the most effective substance tested in this study.

According to Table 3, you provided, kefir was tested and found to have an inhibition zone (20±0.11 mm) against *P. digitatum*, which suggests that kefir has antifungal properties and may be effective in inhibiting the growth of this particular fungus. However, it's important to note that this result was obtained under specific experimental conditions, and further research would be needed to determine the optimal concentration and application method for using kefir as an antifungal agent in practical settings (Table 4).

Table 4. Kefir and components from kefir MIC analysis on the *Aspergillus niger, Penicillium digitatum, Penicillium expansum*.

		MIC Values (µg mL^{-1})											
		10	5	2	1	0.7	0.5	0.4	0.3	0.25	0.2	0.1	0.05
Aspergillus niger	Natamicin	+	+	+	+	+	+	+	+	+	+	+	-
	Kefir	+	+	+	+	+	+	+	_	_	_	_	_
	CFS	+	+	+	+	+	+	+	+	-	-	-	-
	Kefiran	+	+	+	+	+	+	+	+	-	-	-	-
	Yeast	+	+	+	+	+	+	+	+	-	-	-	-
Penicillium digitatum	Natamicin	+	+	+	+	+	+	+	+	+	+	+	-
	Kefir	+	+	+	+	+	+	+	+	+	+	-	-
	CFS	+	+	+	+	+	+	-	-	-	-	-	-
	Kefiran	+	-	-	-	-	-	-	-	-	-	-	-
	Yeast	+	+	+	+	+	-	-	-	-	-	-	-
Penicillum expansum	Natamicin	+	+	+	+	+	+	+	+	+	+	+	-
	Kefir	+	+	+	+	+	+	+	+	+	-	-	-
	CFS	+	+	+	+	+	+	+	+	+	-	-	-
	Kefiran	+	+	-	-	-	-	-	-	-	-	-	-
	Yeast	+	+	+	+	-	-	-	-	-	-	-	-

It's important to note that the effectiveness of kefir and other substances against *P. digitatum* may vary depending on factors such as concentration, application method, and the specific strain of the fungus being tested. Additionally, further research would be needed to determine the safety and efficacy of using kefir as an antifungal agent in practical applications.

Bazioli et al. (2019) indicated that decay reduction of 41.7% and 19.8% was observed by the *Saccharomycopsis crataegensis* and sodium bicarbonate, respectively.

Blue fungi caused by *P. expansum* is one of the most crucial postharvest diseases in apple and synthetic fungicides are the most common method of combating it (Guerrero et al. 2014). Alternatives such as oxidative treatments, volatiles, heat applications, microorganisms and etc. to chemical control are still under investigation and some of them are proven to be as effective as chemical fungicides. The inhibition zones formed by kefir and kefir-derived compounds against *Penicillium expansum* are shown in Figure 1. The size of the inhibition zone is presented in Table 3. The sizes of the inhibition zone formed by kefir and kefir kefir-derived compounds against *P. expansum* are between 0 and 10 mm. The most efficient inhibition

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zone which is 10 mm in diameter was obtained by kefir treatment compared to control positive for natamycin. Yeast treatment was found to be also effective against the growth of *P. expansum* fungi. Results of the study have shown that the treatment of kefiran against this fungi is not potent.

Table 3. shows the inhibition zone diameter in millimeters (mm) formed by different substances against *P. expansum,* which is a type of fungi that can cause postharvest decay in fruit. The substances tested include kefir, kefir-derived compounds (CFS and kefiran), yeast, and natamycin. The results indicate that all of the substances tested were able to inhibit the growth of *P. expansum* to some extent. The largest inhibition zone diameter was observed for natamycin, which was 27±0.09 mm. The inhibition zone diameter for kefir was 10±0.18 mm, which is 37.03% of the inhibition observed for natamycin. The components from kefir (CFS and kefiran) and yeast had smaller inhibition zone diameters, at 9±0.17 mm and 7±0.21 mm, respectively. However, these values were still significant compared to the control (no substance added).

Overall, the results suggest that kefir, components from kefir, and yeast may have potential as natural antifungal agents against *P. expansum*, although natamycin remains the most effective substance tested in this study (Table 4.).

Mesías et al. (2021) presented that BIO 126 strain of *Metschnikowia pulcherrima* is very effective as a biocontrol agent in the control of *P. expansum*. Several volatile compounds obtained from plants were evaluated by Buonsenso et al. (2023) for their effectiveness against *P. expansum* in *in vivo* and *in vitro* tests. Among the compounds tested, trans-2-hexenal vapor treatments showed the best control against *P. expansum*. Oxidative treatments such as sodium hypochlorite and hydrogen peroxide were applied by Cerioni et al. (2013) in the control of *P. expansum* and the results showed they can be alternative treatments for apple postharvest diseases. Guerrero et al. (2014) showed that in control of postharvest diseases on apple caused by *P expansum,* strains of *C. oleophila* performed similar to those of synthetic fungicide.

Among the treatments applied against *A. niger,* two samples have reduced the growth of the fungus and some of them completely inhibited *A. niger* (Figure 1). A total inhibition (100%) was obtained with the kefir on *A. niger* . Figure 1. presents fungi count results showing the effects of kefir and components from kefir obtained during the activation stage of *A. niger* (6.21 log cfu/mL), *P. digitatum* (6.09 log cfu/mL) and *P. expansum* (6.35 log cfu/mL). Erdogan et al. (2019) according to the study report, all fungi were inhibited in the stools of mice fed kefir. Taheur et al. (2020) reported that kefir inhibited the growth of *A. favus* and *A. carbonarius* by 93.88% and 100%, respectively.

Kefir grains house a complex community of microorganisms, including lactic acid bacteria (such as Lactobacilli, Lactococci, and Leuconostocs), acetic acid bacteria, and yeast, which coexist symbiotically within a polysaccharide matrix, as described by Oliveira et al. in 2019. This microbiota is responsible for generating a range of compounds, including amino acids, peptides, ethanol, acetaldehyde, bacteriocins, exopolysaccharides, acetoin, calcium, folic acid, acetic acid, and various vitamins. Furthermore, the inhibitory effects of kefir against different fungi have been documented in previous studies (Londero et al., 2014; Gamba et al., 2016; Al-Mohammadi et al., 2021). For instance, Al-Mohammadi et al. (2021) demonstrated the inhibitory impact of kefir on the growth of *A. favus*. Our findings align with prior research involving the use of CFS from whey fermented with CIDCA AGK10 grains, which demonstrated a reduction in the germination of conidia from *Rhizopus* sp., *A. fumigatus, A. terreus, A. flavus,* and *A. parasiticus*, as reported by Londero et al. (2014). Similarly, analogous CFS obtained from kefir products exhibited antifungal properties against *A. flavus AH3* and *Fusarium graminearum* CZ, as indicated by Al-Mohammadi et al. (2021). Furthermore, Caro and León (2014) demonstrated that CFS derived from 'panela' (a product obtained from unrefined sugarcane) fermented with water kefir grains led to a decrease in the growth parameters of *A. ochraceus* AFUNL9.

Multi-index comprehensive evaluation of TOPSIS on effective of treatments

In this study, the impact of four different treatments on the growth of fungal pathogens, specifically *A. niger, P. digitatum,* and *P. expansum*, were compared. To determine the optimal treatment conditions, the

TOPSIS method was employed, which is a well-established approach for resolving issues associated with multi-property decision analysis with finite alternatives, as outlined by Chen (2019). Before to ranking the treatments, the original data were normalized via the TOPSIS method to address differences in property dimensions and units of measurement. The antifungal effects of kefir and kefir-derived compounds on three different fungal species were evaluated using the TOPSIS method, and the results are presented in Table 5., indicated that kefir treatment demonstrated the highest Ci value, suggesting that it is the most effective treatment for improving pathogen preservation. The TOPSIS method is increasingly being utilized in the field of food processing due to its simplicity, ease of understanding, and ability to integrate with other methods, such as food safety evaluations. For instance, Gul et al. (2018) utilized the TOPSIS method to ascertain the ideal hazelnut cake concentration and HPH (High-Pressure Homogenization) conditions for the production of hazelnut milk. Similarly, Ansarifar et al. (2015) applied the TOPSIS method to assess the impact of chitosan on deep-fried Kurdish cheese nuggets and to optimize the deep-frying parameters. Consequently, the TOPSIS method has significant potential for utilization within the food processing sector.

Table 5. The antifungal effects of kefir and components from kefir on three different fungihave been investigated by TOPSIS method.

	D^+	D^-	C_i	Ranking
Natamisin	0	0.307450486	1	1
Kefir	0.188659336	0.164432835	0.465693801	2
CFS	0.220844167	0.08789481	0.284689712	4
Kefiran	0.297973461	0.025829534	0.079769286	5
Yeast	0.220454316	0.091283945	0.292822397	3

Note: D^+ was the distance between each evaluation index and the positive ideal solution; D^- was the distance between each evaluation index and the negative ideal solution; C_i represented the relative proximity.

Conclusion

In conclusion, the health benefits of kefir are well known and studies regarding antifungal effects are still in progress. In this study, the antifungal effects of kefir and kefir-derived compounds on three different fungi have been investigated. The study can not suggest the most influential treatment on for preventing fungi growth, because every treatment has specific effects on the fungus tested. Based on the results of the study, kefir and kefir-derived compounds exhibit biocontrol activity against isolated fungi from selected fruits. Results suggest that kefir obtained from kefir grains could be used against *A. niger*, *P. digitatum* and *P. expansum*. The findings demonstrated a notable level of antifungal effectiveness, suggesting the potential utility of kefir in biocontrolling *P. digitatum*. Moreover, both CFS and yeast strains isolated from kefir grains hold promise for inhibiting the growth of *P. expansum*. In a commercially viable and effective alternative control method, specific fungus that can cause deteriorations in products must be identified and an than application that has positive inhibitory effects against fungus must be studied *in vitro* and tested on the products.

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Author Contributions

Tuğba Kök Taş wrote the first draft of the manuscript. Tuğba Kök Taş, Kübra Küçüksoku, Nilüfer Sena Aydoğdu and Meryem Ateş made substantial scientific contributions. All the authors approved the final version of the manuscript, which has been revised by a professional native English translator/editor.

Conflict of Interest

As the authors of this study, we declare that we do not have any conflict of interest statement.

Ethics Committee Approval

As the authors of this study, we confirm that we do not have any ethics committee approval.

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