Antibacterial Activity of Different Kefir Types Against Various Plant Pathogenic Bacteria

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ARTICLE INFO
Research Article
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Received: 20 December 2018, Received in Revised Form: 04 February 2019, Accepted: 05 May 2019

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ABSTRACT
Kefir is a probiotic, dairy product produced by the fermentative activity of a diverse range of lactic acid bacteria, acetic acid bacteria, and yeast. In this study, we revealed the antimicrobial spectra of five types of kefir supernatants (EG, AN, KF, KY and SD) from different regions of Turkey fermented for 24 and 48 h against seven plant pathogenic bacteria and one bacterial biocontrol agent in vitro and in vivo for the first time. In vitro, antibacterial activity was investigated by the disk diffusion agar method. Their antibacterial potencies varied according to the type of kefir and the fermentation time. Also, we showed that the antimicrobial activity of kefir could be attributable to antimicrobial substances in supernatants rather than the low pH. In vivo, studies using the most potent kefir type on cucumber and common bean with their pathogenic bacteria in the climate chamber showed no remarkable decrease in diseases but revealed an increase in some plant growth parameters. The application resulted in an increase of 22% in shoot fresh weight, 20% in shoot dry weight, 79% in root fresh weight and 113% in root dry weight in common bean, on the other hand, 25% in shoot fresh weight, 34% in root fresh weight and 30% in shoot dry weight in cucumber.

Keywords: Kefir; Probiotic; Plant pathogens; Biological control

1. Introduction

Bacteria cause diseases in plants which are generally characterized by morphological symptoms such as galls and overgrowths, wilts, leaf spots, specks and blights, soft rots, as well as scabs and cankers. In general, the most common and devastating plant pathogenic bacteria belong to genera Erwinia, Pectobacterium, Agrobacterium, Pseudomonas,Ralstonia, Burkholderia, Acidovorax, Xanthomonas, Clavibacter, Streptomyces, Xylella, Spiroplasma, and Phytoplasma (Bull et al 2010). The diseases caused by bacteria in essential crop plants cause financial problems on a global scale. For example, The U.S. government spent US$1 billion to eradicate citrus canker disease during 1995-2005, while the Indian government spent more than INR20 million to combat bacterial blight diseases in pomegranate during 2003-2008. These and other examples indicate the importance of controlling bacterial plant diseases throughout the world (Borkar & Yumlembam 2017).

There are many approaches to controlling the plant bacterial diseases but the most commonly used one is chemical control (Kannan & Bastas 2015). The chemical management of individual bacterial diseases has been largely driven by the use of copper compounds and the antibiotics (Sundin et al 2016). However, as a result of extensive use of these compounds, bacteria have started to evolve resistance either through mutation or the acquisition of a resistance gene(s) ( Förster et al 2015; Sundin & Wang 2018). Also, the use of antibiotics in plant disease control is banned in European
Union because of potential impacts on human health and the transfer of antibiotic resistance into clinical pathogens (Sundin et al 2016; Sundin & Wang 2018).

Kefir is a fermented milk product that contains both mainly lactic acid bacteria (Lactobacillus, Lactococcus spp., Leuconostoc spp., Aceto bacter spp. and Streptococcus spp.) and yeasts (Kluyveromyces spp., Torula spp., Candida spp. and Saccharomyces spp.) (Güzel-Seydim et al 2011; Fiorda et al 2017). Various functional properties of kefirs such as cholesterol-lowering effects (Liu et al 2006) and the antitumor activities (Renner & Munzner 1991; Liu et al 2005; Güzel-Seydim et al 2006) were reported. There are also studies focusing on the effects of different kefir products. Jeong et al (2017) showed the antimicrobial activities of the exopolysaccharide (EPS) produced by a Lactobacillus kefiranofaciens strain which is a member of the microflora of kefir granules against Listeria monocytogenes and Salmonella enteritidis in their study.

Rodrigues et al (2005) conducted a study to examine the antimicrobial activity of the kefir and kefiran which is the polysaccharide extracted from kefir beads on various types of human pathogenic bacteria. Ulusoy et al (2007) studied the antibacterial effect of 24 h and 48 h fermented kefir produced from commercial starter culture against several food pathogens. Kim et al (2016) reported a study to elucidate the optimal fermentation time and conditions for antimicrobial activities of kefirs from different origins against food-borne pathogens. They found that the spectra and potencies of kefirs varied according to the type of kefir and the fermentation time. In addition to those, Marquina et al (2002) reported that the consumption of kefir caused to decrease in Enterobacteriaceae and Clostridia population in the mucosa of mice bowel. Furthermore, other than the main ones mentioned above various functional properties such as improving lactose tolerance, stimulation of immune system and control of irritable bowel symptoms are exhibited by kefir (Güzel-Seydim et al 2011).

Although kefir has been shown to have an antibacterial effect on human and food pathogens in several studies, there is no evaluation of such application for plant pathogenic bacteria, to the best of our knowledge. We investigated the in vitro antimicrobial activities of kefirs from different origins fermented for 24 and 48 h against seven plant pathogenic strains and one bacterial biocontrol agent. Additionally, we studied the effects of the kefir product on common bean and cucumber diseases and plant growth parameters in a climate chamber. This study aimed to elucidate the antimicrobial activities of kefirs against plant pathogenic bacterial strains and evaluate the potential of this dairy product to be used in plant pathology for the first time.

2. Material and Methods

2.1. Kefir and supernatant preparation

Five types of kefir grains, i.e., SD, AN, EG, KY and KF, were used in this study. Among them, kefir SD is the one which is being sold commercially and the others were collected from private households in a different region of Turkey. Starter grains (5 g) were continuously cultured in pasteurized, low-fat milk for 15 days prior to experiments. The medium was changed at 24 h intervals and the grains washed with sterile water. For antibacterial activity tests, viable kefir grains, 10% w/v, were inoculated in pasteurized, low-fat milk and cultured at 25 °C for 24 and 48 h. At the end of the fermentation process, the grains and milk were separated using a sterilized plastic filter (2-mm pore size).

For antimicrobial activity tests, kefir milk was centrifuged at 6,000 g for 10 min and the supernatant was sterilized by filtration using a 0.45-μm pore-size syringe filter (Millipore Co., USA). The pH of the filtered kefir supernatant was determined with a pH meter.

2.2. Bacterial strains

All bacterial strains were provided by the bacteriology laboratory in the Department of Plant Protection, Faculty of Agriculture, Van Yuzuncu Yil University. The pathogens; Pseudomonas syringae pv. syringae (Pss), Pseudomonas syringae pv. lachrymans (Psl), Pseudomonas syringae pv. tomato (Pst), Xanthomonas axonopodis pv. phaseoli (Xap), Xanthomonas euvesicatoria (Xe), Erwinia amylovora (Ea), Clavibacter michiganensis spp. michiganensis (Cmm) and the biocontrol agent of plant diseases, Bacillus spp. 66/3 (66/3), were used as the test strains in this study. All strain cultures were grown either in Nutrient Broth (NB) broth (Difco, Detroit, MI, USA) or on Nutrient Broth agar plates at 25 °C.
2.3. In vitro antibacterial activity

Antibacterial activity of kefir supernatants was evaluated using the disk diffusion method as described by the National Committee for Clinical Laboratory Standards (NCCLS 2002). Cell suspensions were prepared with the spectrophotometric method and the final bacterial cell concentration applied on the agar surface was approximated to $10^6$ CFU. Sterile test disks (Bioanalyse Co., Ltd., Ankara, Turkey) were applied to the agar surface previously inoculated with 0.1 mL bacterial suspension. 20 μL of each kefir supernatant was directly dropped onto the surface of disks. The plates were incubated for 48 h at 25 °C, and the inhibition zones were measured. All experiments were done in nine replicates. The presence of a clear zone around the disks was considered as inhibition.

To see whether the antimicrobial activity of kefirs could be attributable to low pH value or not, we conducted a control experiment. For this, lactic acid solution (Sigma-Aldrich, USA), acetic acid solution (Merck, Germany), and absolute ethyl alcohol (JT Baker, USA) were diluted with sterilized distilled water. The pH of the diluent was adjusted to 3.5 for both acid solutions. Ethyl alcohol was diluted to 2.0% v/v. All solutions were sterilized by filtration using a 0.45-μm pore-size syringe filter before use. Antibacterial activity of control solutions was evaluated as mentioned above.

2.4. In vivo antibacterial activity

In this part of the study, kefir AN fermented for 48 h which was one of the most potent kefir types on Psl and Xap in in vitro tests were investigated in climate room. Bacterial blight disease of common bean (Phaseolus vulgaris cv. Gina) caused by Xap and angular leaf spot disease of cucumber (Cucumis sativus cv. Gordion F1) caused by Psl were tested in a climate chamber.

2.5. Cultivation of plants, application of pathogens and kefir supernatant

Pesticides free cucumber and bean seeds were planted in 250 mL volume containers filled with sterile peat and left in a climate room at 24±2 °C with 60% humidity and 14-h light conditions. During the study, nutrition required by seedlings was provided as recommended by Akköprü & Özaktan (2018).

The supernatant was diluted with sterile distilled water at a rate of 1 to 3 just before application to prevent phytotoxicity due to its low pH. The supernatant was applied to the plants three times in two different ways. First, the suspension was applied twice to the seedlings by drenching method with 10 mL plant$^{-1}$ and 20 mL plant$^{-1}$ 48 h prior to the pathogen application. The second, the suspension was sprayed onto plants 24 h prior to the pathogen application.

For the application of pathogens, 48-h Psl and Xap cultures grown on Nutrient Broth medium were prepared in suspension with $10^6$ cfu mL$^{-1}$ and 0.01% Tween 80 was added as a surfactant. Pathogens were inoculated with a hand sprayer 24 h after last application of kefir supernatant. The Psl suspension was applied to the cucumber seedlings when the second true leaves began to open (Akköprü & Özaktan 2018). The Xap culture was applied to the bean seedlings during the trifoliate leaf period (Akköprü et al 2018). Immediately after pathogen application, the plants were left in high relative humidity for 48 hours.

2.6. Determination of disease severity and plant development parameters

The diseases severity ratings were based on the infected leaf area. Three weeks after Xap application, disease symptoms on common bean plants were assessed by scale 1-5 (1: no disease symptoms, 2: a few necrotic spots or ≤ 5%, 3: 6-25%, 4: 26-50%, 5: ≥ 50% and defoliation) (Abbasi et al 2002). 14 days after Psl application, disease symptoms on cucumber plants were assessed by scale 1-6 (1: no disease symptoms, 2: a few necrotic spots or < 10%, 3: 10-25%, 4: 26-50%, 5: 51-75%, 6: ≥ 76% or fallen or dead leaves) (Akköprü & Özaktan 2018). The disease index (1) and % efficacy (2) were calculated using the following formulas:

\[
\text{Disease index} = \frac{\sum (\text{Rating number} \times \text{Number of leaves in the rating})}{\text{Total number of leaves} \times \text{Highest rating}} \times 100 \tag{1}
\]

\[
\text{Efficacy} \% = \frac{\text{Control value} - \text{treatment value}}{\text{Control value}} \times 100 \tag{2}
\]
The effect of Kefir supernatant on cucumber and common bean were determined in the 15th and 21st day, respectively. After the root parts of the seedlings cut from the root collar were washed, roots and shoots were weighed separately to determine fresh weight. Then they were dried at 65 °C for 72 h and weighed again to obtain dry weights.

2.7. Statistical analysis

All in vitro experiments were performed in nine replicates. In vivo experiments were set up according to completely randomized with ten replicates. SPSS 17.0 package was used for statistical analysis.

3. Result and Discussion

3.1. In vitro antibacterial activity

Antimicrobial spectra of the kefir supernatants for 24 h and 48 h against the plant pathogens are presented in Table 1 and 2, respectively. Antimicrobial activity generally increased along with prolonged fermentation time in all types of kefirs; this effect was evaluated by using Student’s t-test and found statistically important (Table 3). This test indicated that all kefir types showed higher antibacterial activity against Pss and Xe after 48 h fermentation compared with 24 h fermentation. On the other hand, not all kefir types showed a statistically significant increase in antibacterial activity with time against all bacterial strains. For example, the antibacterial activity of kefir types increased with prolonged fermentation time against Cmm, except for kefir KF. It did not exhibit a statistically significant increase in antibacterial activity with time against this pathogen. The similar situation was also observed for Ea, Pst, Psl, Xap and 66/3 (Table 3).

Table 1- Antibacterial spectrum of five types of kefir supernatants fermented for 24 h against eight bacterial strains.

| Kefir Types | EA | PST | PSL | XAP | XE | 66/3 | CMM | PSS |
|-------------|----|-----|-----|-----|----|------|-----|-----|
| EG          | 9.78±0.15ab | 8.33±0.24a | 9.33±0.26a | 10.20±0.15a | 7.00±0.11c | 6.40±0.18ac | 3.33±0.24a | 8.00±0.29ac |
| KY          | 9.89±0.11ab | 7.11±0.26c | 9.78±0.15c | 9.50±0.33b | 6.11±0.33d | 5.67±0.24c | 3.33±0.41b | 7.33±0.67c |
| AN          | 10.67±0.75a | 10.11±0.39a | 11.00±0.65b | 9.56±0.50b | 7.67±0.17c | 6.44±0.44c | 6.11±0.48a | 8.22±0.70c |
| KF          | 8.60±0.71b | 8.89±0.35b | 11.33±0.67c | 5.00±0.01b | 13.02±0.99b | 8.50±0.90b | 16.20±0.28a | 11.40±0.97c |
| SD          | 9.78±0.40ab | 10.9±0.35c | 10.00±0.17bc | 9.22±0.49bc | 9.67±0.33b | 7.56±0.80b | 6.44±0.18c | 9.89±0.26bc |

*Mean values followed by the same letter were not significantly different based on the Duncan’s Multiple Range Test at P< 0.05 significance level.

Table 2- Antibacterial spectrum of five types of kefir supernatants fermented for 48 h against bacterial strains.

| Kefir Types | EA | PST | PSL | XAP | XE | 66/3 | CMM | PSS |
|-------------|----|-----|-----|-----|----|------|-----|-----|
| EG          | 10.00±0.65a | 9.33±0.26a | 12.00±0.69d | 8.00±0.26ed | 16.89±0.89d | 6.56±0.24a | 15.78±0.68b | 8.67±0.37bd |
| KY          | 10.00±0.17c | 10.3±0.50c | 10.22±0.37d | 9.00±0.17c | 12.78±0.70b | 9.44±0.50c | 10.11±0.35c | 10.78±0.64c |
| AN          | 15.44±0.40b | 15.67±1.66b | 18.22±0.87b | 16.11±0.79b | 15.33±0.53b | 13.89±0.98c | 7.89±0.26a | 13.56±1.04d |
| KF          | 13.33±0.60a | 12.67±1.00b | 15.89±0.62b | 6.67±0.37d | 16.17±1.05a | 11.78±0.43b | 18.78±0.36a | 16.22±0.88a |
| SD          | 12.89±0.56b | 15.33±0.28a | 14.22±0.41b | 13.44±0.44b | 17.44±1.02a | 12.11±0.35b | 10.11±0.72c | 14.89±0.48ab |

*Mean values followed by the same letter were not significantly different based on the Duncan’s Multiple Range Test at P< 0.05 significance level.

Our results are consistent with those of the study conducted by Kim et al (2016). They found that the spectra and potencies of kefirs against several food pathogens varied according to the type of kefir and the fermentation time. Also, Silva et al (2009) reported that the antimicrobial activities of kefirs generally increased with prolonged fermentation times. However, Ulusoy et al (2007) showed no difference in the antimicrobial activities of kefirs fermented for 24 or 48 h against some food-borne pathogens in vitro.

In general, all kefir types showed a remarkable antibacterial effect against the bacteria we used. Kefir AN was the most potent one on Ea, Pst, Psl, Xap, and Xe. Xe was inhibited by all kefir types strongly except kefir KY which gave a slightly smaller zone compared to others. On the other hand, kefir KF gave the highest activity against Cmm, Pss, and Xe (Table 2). Those results suggest that kefirs from different origins have different antimicrobial spectra against a particular
strain. Also, the results indicate that the same kefir type may have different antibacterial potencies against different species and even subspecies.

Table 3- Comparison of fermentation time (24 and 48 hours) on antibacterial activities of kefir supernatants against bacterial strains using Student’s t-test

| Strain/Kefir | EG     | KY     | SD     | KF     | AN     |
|-------------|--------|--------|--------|--------|--------|
| EA          | -0.34±0.741 | -0.56±0.588 | -4.49±0.001 | -4.80±0.001 | -4.27±0.001 |
| PST         | -1.38±0.001 | -10.42±0.001 | -9.77±0.001 | -7.80±0.001 | -8.22±0.001 |
| PSL         | -3.67±0.002 | -1.13±0.283 | -9.73±0.0001 | -4.82±0.001 | -6.70±0.001 |
| XAP         | 8.06±0.0001 | 1.41±0.179 | -6.36±0.001 | -4.47±0.0001 | -4.16±0.001 |
| XE          | -13.24±0.001 | -6.47±0.001 | -5.44±0.001 | 2.36±0.034 | -6.27±0.001 |
| 66/3        | 1.48±0.163 | -10.51±0.001 | -3.47±0.003 | -13.18±0.001 | -2.80±0.015 |
| CMM         | -9.30±0.001 | -4.24±0.001 | -10.68±0.001 | 0.05±0.961 | -12.88±0.001 |
| PSS         | -1.41±0.176 | -3.73±0.002 | -9.09±0.001 | -3.67±0.002 | -4.24±0.001 |

One-way variance analysis was carried out using Student’s t-test (P<0.05) to detect a significant difference between variables; *, means no statistically significant effect of prolonged fermentation time on antibacterial activities of kefir.

The microbial composition of kefir varies according to kefir origin, the substrate used in the fermentation process and the storage conditions also (Prado et al. 2015). Pintado et al. (1996) found different microbiological diversity in terms of yeast and Lactobacilli species content in Portuguese kefir grains compared to some other kefir grains such as Russian, Yugoslavian and Bulgarian grains. Also, it was shown that the composition of Tibetan kefir differs from that of others such as Russian, Taiwan or Turkey kefir. (Gao et al. 2012; Altay et al. 2013). Since the substrate used in the fermentation, storage and handling conditions were all the same for each kefir grain in our study, it can be concluded that different kefir types have variable antibacterial spectra against the bacterial strains due to their different origins.

The probiotic species, especially lactobacilli, are known to produce a wide range of antimicrobial compounds such as organic acids (lactic and acetic acids), hydrogen peroxide, ethanol, and bacteriocins (Guzel-Seydim et al. 2011; Kim et al. 2015). In this study, during the fermentation process, the pH gradually decreased in all kefir samples (Table 4). However, our overall data suggested that the antimicrobial activity of kefirs could not be attributable to low pH value simply. To demonstrate this, the antimicrobial activities of lactic and acetic acid solutions against the test strains were also investigated. All strains were resistant to both acid solutions at pH 3.5. The growth of test strains was inhibited by kefir supernatants, although the lowest pH value of kefirs in our study was 3.67. Additionally, since some yeast strains produce ethyl alcohol during kefir fermentation, we wanted to use ethyl alcohol solution (2% v/v) to see the antibacterial activity possibly interact with each other to enhance or antagonize their antimicrobial effects.

Table 4- pH values of five different kefir supernatants fermented for 24 and 48 hours. Measurements were performed in supernatants at temperatures of 24±2 °C

| Kefir types | pH value according to fermentation times |
|------------|----------------------------------------|
|            | 24 h       | 48 h       |
| EG         | 3.80       | 3.70       |
| KY         | 3.95       | 3.75       |
| AN         | 3.91       | 3.67       |
| KF         | 4.50       | 4.20       |
| SD         | 3.82       | 3.70       |

It could be postulated that the antimicrobial effect may be caused by the antagonistic action of metabolites and inhibitory compounds synthesized and released into the supernatant by various microorganisms present in kefir, and possibly interact with each other to enhance or antagonize their antimicrobial effects.

3.2. In planta studies

The effect of using Kefir AN, one of the most potent kefir types, on Psl and Xap were also investigated in the climate...
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room. Xap causes common bacterial blight disease in bean and Psl causes angular leaf spot disease in cucumber.

The application of kefir supernatant to bean seedlings resulted in a statistically significant increase in all plant growth parameters. It resulted in an increase of 22% in shoot fresh weight, 20% in shoot dry weight, 79% in root fresh weight and 113% in root dry weight. However, no effect on plant growth parameters was observed under disease pressure (Figure 1).

On the other hand, in cucumber experiments, kefir supernatant application significantly increased at the rate of 34% in root fresh weight, 25% in shoot fresh weight and 30% in dry weight but did not cause an increase in root dry weight. However, no effect on plant growth parameters was observed under disease pressure (Figure 1).

Also, the application did not show any inhibitory effect on common bacterial leaf blight disease symptoms in bean and angular leaf spot disease symptoms in cucumber (Figure 2).

The positive effect of kefir supernatant on plant development parameters may be because of the presence of many organic and bioactive compounds in the supernatant. These compounds might have been used by plant cells and plant-associated bacteria in many ways, such as carbon and other plant nutrients sources. Also, many undefined bioactive
compounds of kefir supernatant might have affected metabolic pathways of plant cells or plant-associated bacteria which resulted in an increase of those parameters we measured.

On the other hand, we did not observe any inhibitory effect of kefir supernatant on common bacterial leaf blight disease of bean caused by Xap and angular leaf spot disease of cucumber caused by Psl. At first, we used supernatant without any dilution on plants, however; it produced slightly phytotoxicity most probably due to its low pH. Buffering the supernatant to increase the pH caused strong precipitation. Therefore, we diluted the supernatant with a 1/3 ratio with sterile distilled water before use. Phytotoxicity was prevented by this way but we could not observe any inhibitory effect on plants infected with Xap or Psl. It can be speculated that inhibitory compounds of the supernatant might have been degraded by plants itself or natural microflora on them. The other possible explanation is that dilution of the supernatant might have prevented the inhibitory compounds to exert their activities on the pathogens due to their low concentration.

Future studies should be followed to determine whether a single compound is responsible for antimicrobial properties or this was a result of antagonistic effects of various metabolites in kefir. Also, it should be created more knowledge about the key antimicrobial compounds against each pathogenic bacterium and their mechanisms. By this way, they can be used more effectively as a single compound against plant bacterial diseases without affected by undesirable conditions like low pH or dilution effect. In addition, after the isolation and identification of each microorganism in the microflora of kefir, they should be investigated as biological agents against plant pathogens.

4. Conclusions

In conclusion, our results revealed different antibacterial potencies according to the type of kefir and the fermentation time. Although we could have not shown this effect in planta studies, some plant growth parameters increased significantly. There is an urgent need for the development of novel antimicrobial agents against pathogenic strains in agriculture. In parallel with the recent developments in the new generation technologies, natural product research has also gained positive momentum. With these technologies, the potential of organisms to produce natural products can be revealed. In principle, such compounds may also find applications in medicine where the antibiotic-resistance problem is also increasing. New narrow-spectrum antimicrobials like bacteriocins may contribute to agriculture’s need for more sustainable and effective strategies for plant disease control.

Acknowledgements

This work was supported by the Scientific Research Project Units of Van Yuzuncu Yil University (Project number: FYL-2017-6263).

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