Research Article Open-Access

Black seed oil impact on authentic kefir microbiota

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Submission Date: August 21st, 2023; **Acceptance Date**: October 6th, 2023; **Publication Date**: October 30th, 2023

Please cite this article as: Nixon J.E., Guzel-Seydim Z.B., Seydim A.C., Jiang X., Bridges W.C., Greene A.K. Black Seed Oil Impact on Authentic Kefir Microbiota. Functional Food Science 2023; 3(10): 217-227. DOI: https://www.doi.org/10.31989/ffs.v3i10.1210

ABSTRACT

Introduction: Authentic kefir originates from the Caucasus mountains of Eastern Europe and is a fermented milk product made from kefir grains. Authentic kefir contains lactic acid bacteria, acetic acid bacteria and yeasts which provides kefir with numerous health benefits such as anticarcinogenic, antimutagenic and anti-allergic properties. *Nigella sativa* is a plant known by many regional names such as black cumin and black carraway. Seeds from the plant are processed to produce black seed oil. Black seed oil has many potential health benefits such as antibacterial and antifungal capabilities. In countries including Turkey and India, black seed oil is commonly added to kefir or yogurt. The purpose of this study was to determine if different concentrations of black seed oil would negatively impact beneficial kefir microorganisms when consumers mix black seed oil into kefir.

Results: Black seed oil concentrations of 0%, 0.1%, 1% and 5% were added to milk with kefir grains and incubated. Each mixture was tested for pH, *Lactobacillus* spp., *Lactococcus* spp., yeast, and coliform microbial counts. Results indicated that the pH and microbial counts of the control (0%) and 0.1% black seed oil samples were not significantly different (P

> 0.05). The pH and microbial count results of 1% black seed oil in kefir indicated slight although not significant inhibition (P > 0.05) as compared to the control and 0.1% black seed oil. The pH and microbial counts of 5% black seed oil were significantly different (P < 0.05) from the other samples indicating inhibition of the kefir microorganisms.

Conclusion: Black seed oil inhibited kefir microorganism when added at the rate of 5%.

Key Words: Kefir, Black Seed Oil, *Lactobacillus* spp., *Lactococcus* spp., Yeast, Probiotic

INTRODUCTION

Authentic kefir is a unique dairy product with a long history of use due to its intrinsic probiotic content. It contains a unique and diverse microbiota of bacteria and yeasts with well-documented functional food properties. Studies on the health benefits of authentic kefir have observed antibacterial and cholesterol-lowering effects, anticarcinogenic and antimutagenic properties, positive impacts on dental health, positive effects on blood sugar, effects against renal failure, lactose intolerance reduction, lowered cholesterol, and stimulation of the digestive and immune systems [1-9]. As a fermented milk product, kefir contains a variety of fermentation by-products dominated by lactic acid and ethanol [10].

Kefir originated in the Caucasus mountains of Eastern Europe and is widely consumed in Turkey, Russia, and Southeastern Asia [11]. Authentic kefir is produced by fermenting milk with kefir grains. The resulting

beverage contains unique kefir microorganisms and their fermentation products which confer the health benefits of authentic kefir [12-13].

In Eastern Europe, cow, goat, or sheep milk is traditionally used to prepare kefir [11]. Kefir grains are small, white to yellow-white, and granular in physical appearance. The grains have a semi-solid consistency and can vary in size ranging from 0.3 to 3.5 cm in diameter. Kefir grains have a polysaccharide matrix that contains a microbiota of acetic acid bacteria (approximately 10^5 cfu/g), lactic acid bacteria (approximately 10^8 cfu/g), and yeast (approximately 10^6–10^7 cfu/g) [14]. The microbiota of bacteria and yeast within kefir grains maintains a synergistic population that produces metabolites and is known to promote and inhibit various types of microbial growth [2, 11, 15].

Black seed oil is generated by processing the seeds of the *Nigella sativa* plant. *N. sativa* can be found in numerous regions around the globe, including Africa, Central Asia, Europe, and the Middle East [16] and is known also as black cumin, blessed seed, black caraway, and a number of other regional names. Research describes several potential health benefits of the seeds and the extracted oil to the digestive, cardiovascular, immune, and urinary systems [17]. Black seed oil components bind free radicals *in vitro* [18] and increase white blood cell activity and antibody production in the presence of abnormal cells [19]. Thymoquinone has been isolated from black seed oil and reportedly inhibits inflammation associated with arthritis and ulcerative colitis [20-21] and may inhibit cancer cell proliferation [22]. Black seed oil also has antifungal and antibacterial properties. In studies, black seed oil inhibited *Staphylococcus, Shigella, Salmonella, Listeria monocytogenes* and *Staphylococcus aureus* in fresh produce [23-24].

In many countries, consumers commonly mix black seed oil into kefir. Since black seed oil has known antibacterial and antifungal properties which hypothetically could inhibit the probiotic microorganisms in kefir, this study was conducted to determine if different concentrations of black seed oil could negatively impact beneficial kefir microorganisms when consumers mix black seed oil into kefir. To understand if black seed oil could impact kefir during fermentation and then subsequent refrigerated storage, this study examined microbial populations in the product with and without added levels of black seed oil.

METHODS

Kefir grain rehydration and activation: Freeze dried kefir grains (Endanem™) were obtained from Danem Dairy and Dairy Products Ltd. (Suleyman Demirel University, Technopark, Isparta, Turkey) and rehydrated and activated according to label instructions by adding 1 packet to 1 liter of pasteurized whole milk (Parmalat™ UHT processed, Lactalis American Group, Inc., Buffalo, NY).

Kefir fermentation: Rehydrated, activated kefir grains were inoculated into 1 L whole milk (Parmalat™ UHT processed, Lactalis American Group, Inc., Buffalo, NY) at the rate of 2 g/L. The milk was fermented for 22 h at 25°C on a VWR Standard Analog Shaker (Product Number 89032-096, VWR International LLC, Radnor, PA) set at 90 RPM. After the pH reached 4.61 (at approximately 22 hours), the kefir grains were aseptically removed using a sterile stainless-steel sieve and transferred to a sterile glass beaker. The remaining fluid kefir was used at the rate of 2% as the starter culture for the experiment [26]. The pH of each kefir sample was measured using a Thermo Orion 2 Star pH meter (Thermo Fisher Scientific,

Inc., Beverly, MA) and the kefir culture was stored at 4℃ until use.

Kefir and black seed oil samples: To prepare the four treatments, the first step was to add kefir culture to whole milk (Parmalat™ UHT processed, Lactalis American Group, Inc., Buffalo, NY) in eight sterile glass dilution bottles as the eight experimental units for the study. Then 0, 0.1, 1.0, or 5.0 ml of the black seed oil (BSO) (Amazing Herbs, Buford, GA) was added to two of the bottles on separate days as follows:

CTRL: 2 mL of kefir culture, 0 mL BSO, 100 mL of whole milk

KBS01: 2 mL of kefir culture, 0.1 mL BSO, 100 mL of whole milk

KBS1: 2 mL of kefir culture, 1.0 mL BSO, 100 mL of whole milk

KBS5: 2 mL of kefir culture, 5.0 mL BSO, 100 mL of whole milk

This created four separate treatments with two replications, each in a completely randomized design. Each bottle was vortexed (Labline 1290 Super Mixer/Vortex, Lab-Line Instruments, Inc., Melrose Park, IL) for 2 min. The pH of each sample was measured prior to incubation using 10 mL of sample aseptically collected and transferred to a glass beaker. Sample pH was measured in duplicate and recorded as Day 0.

The control and sample bottles were placed in a shaking water bath (Precision Shaking Water Bath Model 25, Chicago, IL) at 25℃. After 22 h of incubation, the bottles were removed. Sample pH was measured in duplicate as above and recorded as Day 1.

Microbial count of bacteria and yeast: Prior to incubation, samples were collected from the uninoculated whole milk (UCTRL) and each inoculated treatment with added black seed oil (CTRL, KBS01, KBS1, KBS5) for microbiological counts and designated as Day 0 samples. After incubation, samples were immediately collected from each bottle for microbiological counts and designated as Day 1 samples. Bottles were stored at 4℃ for subsequent collection of Day 7 and Day 14 samples for pH and microbiological counts (resulting in a completely randomized design with repeated measures).

Day 0, Day 1, Day 7 and Day 14 samples were plated using serial dilutions in sterile Class O phosphate buffer with magnesium chloride [25] on MRS agar (110660, Merck KGaA, Darmstadt, Germany) to enumerate *Lactobacilli*, M17 agar (115108, Merck KGaA) to enumerate *Lactococci*, Potato Dextrose Agar (110130, Merck KGaA) to enumerate yeast, and Violet Red Bile Agar (101406, Merck KGaA) to enumerate coliform bacteria. All MRS and M17 plates were incubated at 35ºC for 48 h, all PDA plates were incubated at 25℃ for 120 h and all VRBA plates were overlaid and incubated at 37ºC for 24 h.

STATISTICAL ANALYSIS

All statistical analyses were performed using the JMP Pro 14.1.0 computer software (SAS Institute Inc., Cary, N.C.). A two-way repeated measures ANOVA was used to test for differences in the means among the four treatments, the four days, and the treatment and day combinations. This was followed by the Tukey-Kramer HSD test to determine specific differences among the specific treatments and days. Results were considered significantly different when $P < 0.05$.

RESULTS AND DISCUSSION

pH and fermentation: The mean pH measurements of fermented kefir samples at Day 0, Day 1, Day 7 and Day 14 are shown in Figure 1.

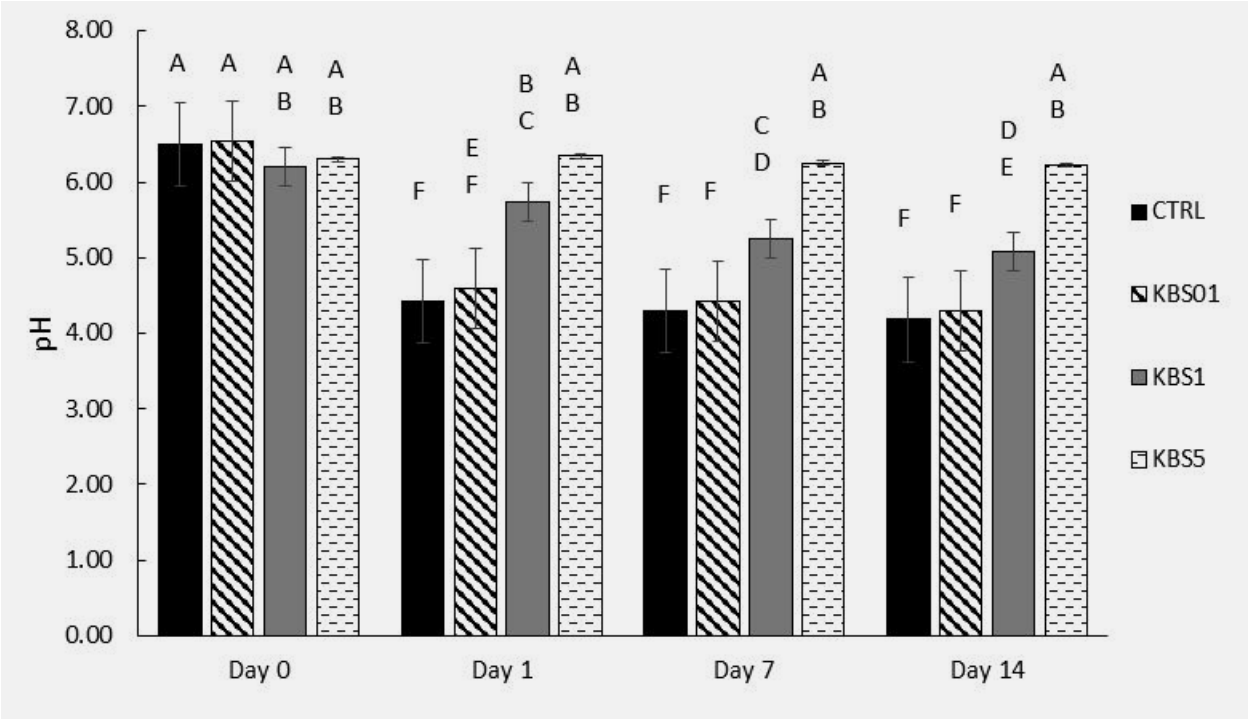

Figure 1: Mean pH of Kefir with Black Seed Oil on Day 0, Day 1, Day 7 and Day 14. [A,B,C] Different letters indicate means were statistically different ($P < 0.05$). Day 0 was pre-fermentation. Day 0 to Day 1 was the fermentation period. After Day 1, samples were collected, and all samples were placed in refrigerated storage thereafter. KEY: CTRL (kefir + whole milk control), KBS01 (kefir + 0.1 ml BSO + whole milk), KBS1 (kefir + 1.0 ml BSO + whole milk), KBS5 (kefir + 5.0 ml BSO + whole milk).

The average pH of the UHT whole milk (UCTRL) prior to inoculation (Day 0) was 6.82. The average pH of the inoculated CTRL at Day 0 was 6.50 and significantly decreased ($P < 0.05$) to 4.42 on Day 1 and thereafter during refrigerated storage there was no significant change in pH between Day 1, Day 7 and Day 14 (Figure 1). The mean pH of KBS01 at Day 0 was 6.54 and significantly decreased ($P < 0.05$) to 4.59 on Day 1 and during refrigerated storage there was no significant change in pH observed between Day 1, Day 7 and Day 14.

The mean pH of KBS1 at Day 0 was 6.21 and, although it decreased slightly to pH 5.74, there was no significant decrease ($P < 0.05$) observed at Day 1. During refrigerated storage there was a significant decrease ($P < 0.05$) between Day 1 and Day 14 for KBS1. Although there was a slight decrease in mean pH between Day 1 and Day 7 of KBS1 there was no significant difference. Similarly, between Day 7 and Day 14 there was a slight decrease in mean pH of KBS1, but it was not significantly different. However, there was a significant difference in mean pH of KBS1 between Day 0 and Day 7 ($P < 0.05$). Comparing results of KBS1 to the CTRL and KBS01 indicated the 1% black seed oil negatively impacted fermentation.

The mean pH of KBS5 at Day 0, Day 1, Day 7 and Day 14 were 6.30, 6.34. 6.25, and 6.22, respectively, and there was no significant difference ($P > 0.05$) observed between days. As compared to the CTRL, the addition of 5% black seed oil inhibited fermentation and pH reduction.

Enumeration of bacteria and yeast: The bacteria and yeast count of kefir and black seed oil samples were recorded on Day 0, Day 1, Day 7, and Day 14. Results of the microbial plate counts are presented in Figures 2-4. Uppercase letters represent comparisons both within and across all days.

The mean *Lactobacillus* spp. count of the CTRL on Day 0 was 7.99 log cfu/ml and significantly increased ($P <$

0.05) to 10.21 log cfu/ml on Day 1 (Figure 2). From Day 1 to Day 7 and on Day 14 of refrigerated storage there was a slight decrease in mean *Lactobacillus* spp. count of CTRL; however, the decrease was not significant.

The mean *Lactobacillus* spp. count of KBS01 on Day 0 was 7.91 log cfu/ml and significantly increased ($P < 0.05$) to 9.98 log cfu/ml on Day 1. There was a slight increase in mean *Lactobacilus* spp. count of KBS01 from Day 1 to Day 7 to Day 14 during refrigerated storage; however, these increases were not significant. Although the KBS01 *Lactobacillus* spp. count during refrigerated storage for Day 7 and Day 14 was slightly higher than CTRL *Lactobacillus* spp. count on these same days, these counts were not significantly different. Results indicated that 0.1% black seed oil did not inhibit *Lactobacillus* spp.

The mean *Lactobacillus* spp. count of KBS1 on Day 0 was 7.79 log cfu/ml and significantly increased ($P < 0.05$) to log 9.03 cfu/ml on Day 1. Although there was an increase in *Lactobacillus* spp. count of KBS1 from Day 1 on Day 7 during refrigerated storage the increase was not significant. Additionally, there was no significant difference between the *Lactobacillus* spp. count for KBS1 Day 7 and Day 14 during refrigerated storage.

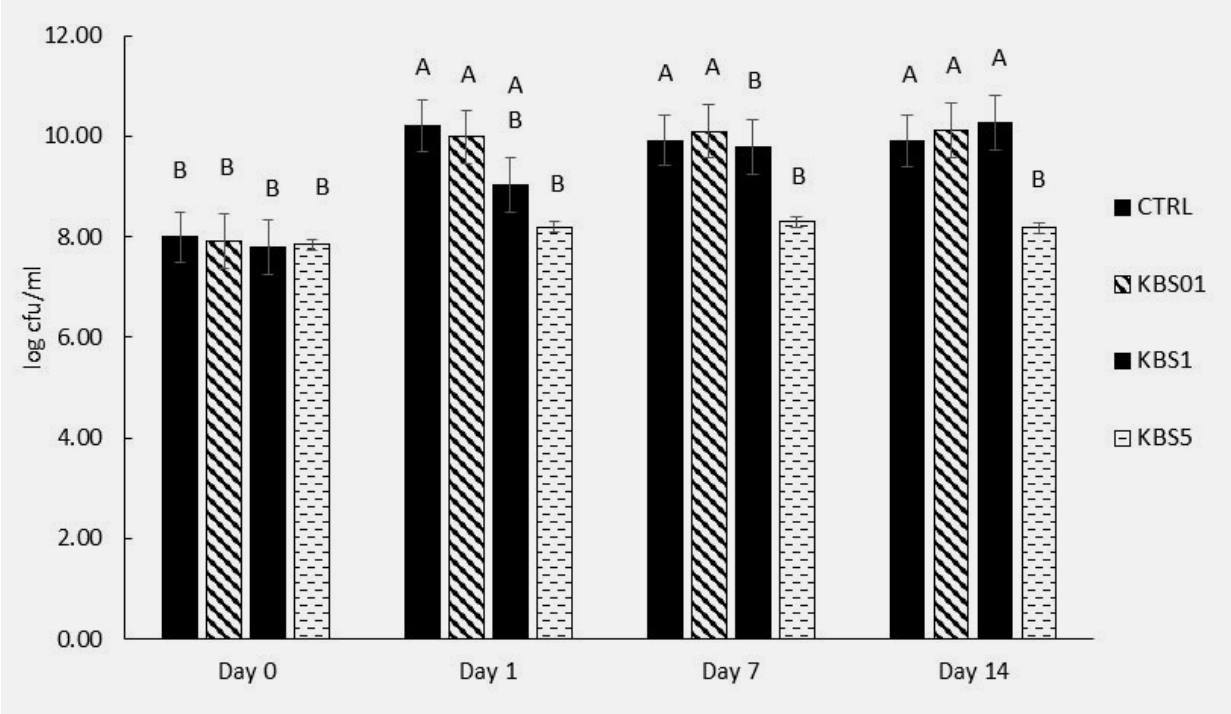

Figure 2: Mean *Lactobacilus* spp. on Day 0, Day 1, Day 7, and Day 14**.** [A,B,C] Different letters indicate results were statistically different ($P < 0.05$). Day 0 was pre-fermentation. Day 0 to Day 1 was the fermentation period. After Day 1 samples were collected, all samples were placed in refrigerated storage and plated on Day 7 and Day 14. KEY: CTRL (kefir + whole milk control), KBS01 (kefir + 0.1 ml BSO + whole milk), KBS1 (kefir + 1.0 ml BSO + whole milk), KBS5 (kefir + 5.0 ml BSO + whole milk)

It was noted that the post-fermentation *Lactobacillus* spp. count of KBS1 on Day 1 was less although not significantly different than CTRL and KBS01 on Day 1. In comparing *Lactobacillus* spp. counts of KBS1 to CTRL and KBS01, black seed oil slightly impacted *Lactobacillus* spp. counts and fermentation. The mean *Lactobacillus* counts of KBS5 at Day 0, Day 1, Day 7 and Day 14 were 7.84, 8.20, 8.30 and 8.17 log cfu/ml, respectively, and there was no significant difference observed on any of these days. However, KBS5 was

significantly lower ($P < 0.05$) than CTRL and KBS01 on Day 1 and significantly lower ($P < 0.05$) than all other samples on Day 7 and Day 14 indicating that 5% black seed oil inhibited *Lactobacillus* spp.

On Day 0, the mean CTRL *Lactococcus* spp. count was 8.01 log cfu/ml and significantly increased ($P < 0.05$) to 10.20 log cfu/ml on Day 1 (Figure 3). During refrigerated storage there was a slight increase in the population of *Lactococcus* spp. on Day 7 and a decrease on Day 14 although the changes were not significant.

The mean *Lactococcus* spp. count of KBS01 on Day 0 was 7.91 log cfu/ml and significantly increased ($P < 0.05$) to 9.98 log cfu/ml on Day 1. During refrigerated storage of KBS01 there was a slight increase in the population of *Lactococcus* spp. on Day 7 and a decrease on Day 14 although the changes were not significant.

On Day 0, the mean KBS1 *Lactococcus* spp. count was 7.81 log cfu/ml and significantly increased ($P < 0.05$) to 9.40 log cfu/ml on Day 1. During refrigerated storage of KBS1, the *Lactococcus* spp. count on Day 7 was not significantly higher than Day 1 and although there was a slight increase on Day 14, it was not significantly different from Day 7.

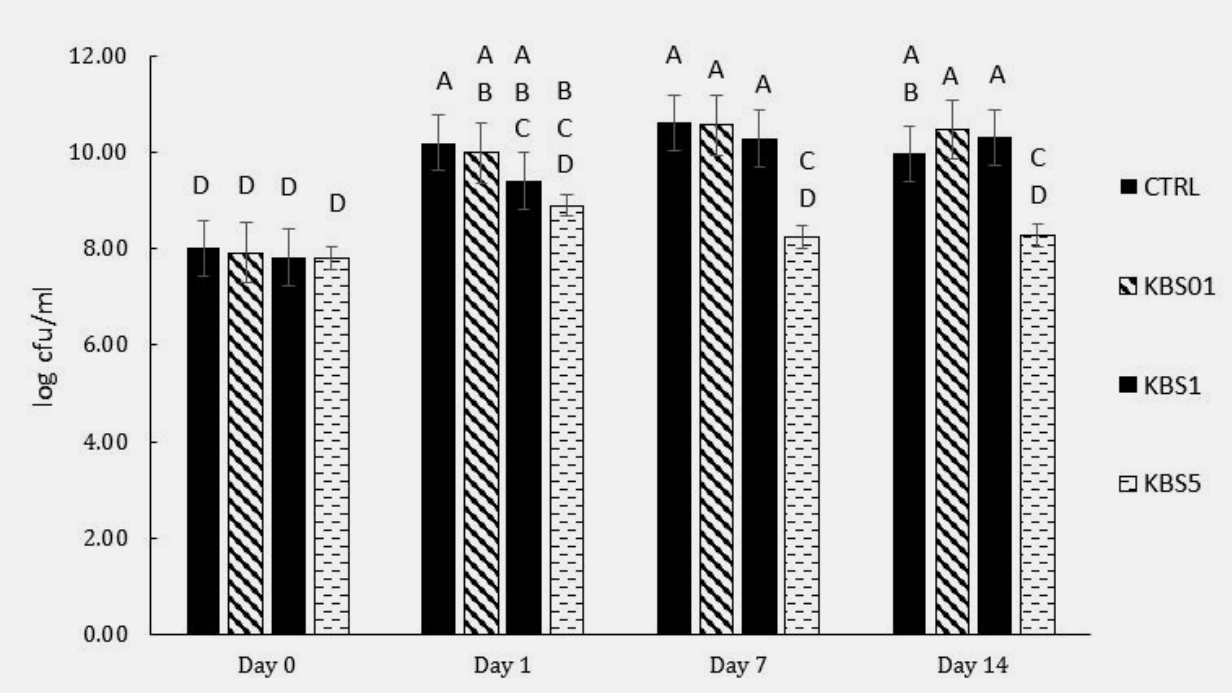

Figure 3: Mean *Lactococcus* spp. on Day 0, Day 1, Day 7, and Day 14. [A,B,C] Different letters indicate results were statistically different ($P < 0.05$). Day 0 was pre-fermentation. Day 0 to Day 1 was the fermentation period. After Day 1 samples were collected, all samples were placed in refrigerated storage and plated on Day 7 and Day 14. KEY: CTRL (kefir + whole milk control), KBS01 (kefir + 0.1 ml BSO + whole milk), KBS1 (kefir + 1.0 ml BSO + whole milk), KBS5 (kefir + 5.0 ml BSO + whole milk)

Results indicate that the 1% black seed oil in KBS1 samples either slowed or inhibited *Lactococcus* spp. slightly but by Day 7 that inhibition had been overcome and the populations began to increase even during refrigerated storage although not significantly different. On Day 1, KBS1 *Lactococcus* spp. counts were observed to be lower than CTRL and KBS01 although not significantly. On Day 1, Day 7 and Day 14, it was noted there was no significant difference between CTRL, KBS01 and KBS1.

The mean *Lactococcus* spp. counts of KBS5 at Day 0, Day 1, Day 7 and Day 14 were 7.80, 8.90, 8.24 and 8.27 log cfu/ml, respectively, and there was no significant difference observed across all four days. Based on the comparison of KBS5 to the remaining three samples, 5% black seed oil inhibited *Lactococcus* spp.

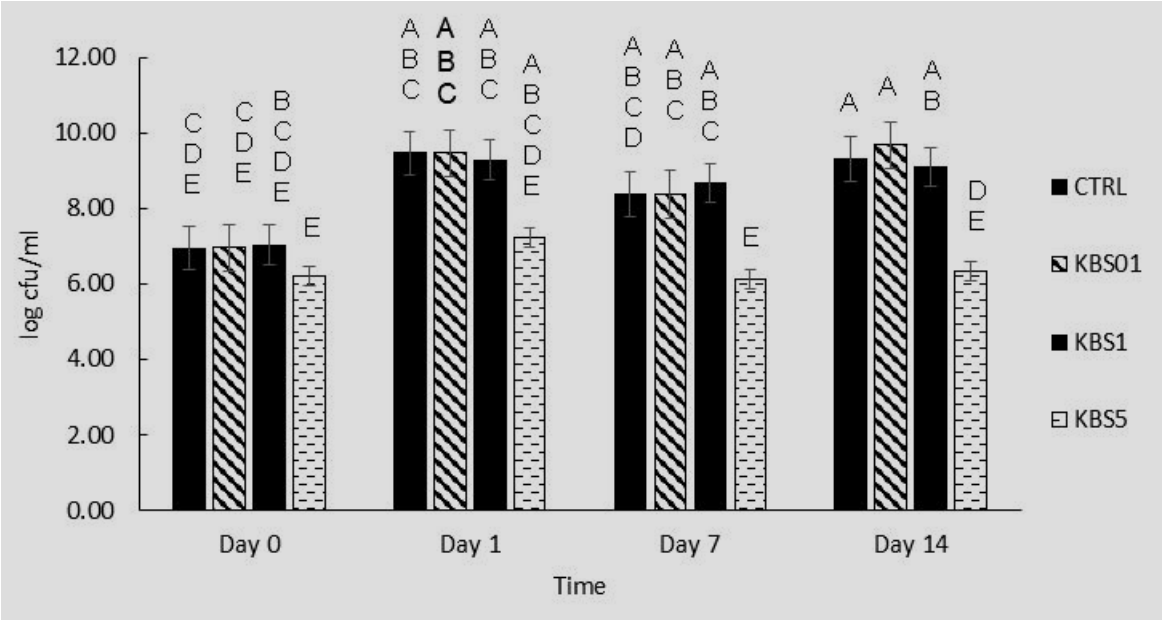

Figure 4: Mean Yeast on Day 0, Day 1, Day 7, and Day 14. [A,B,C] Different letters indicate results were statistically different ($P < 0.05$). Day 0 was pre-fermentation. Day 0 to Day 1 was the fermentation period. After Day 1 samples were collected, all samples were placed in refrigerated storage and plated on Day 7 and Day 14. KEY: CTRL (kefir + whole milk control), KBS01 (kefir + 0.1 ml BSO + whole milk), KBS1 (kefir + 1.0 ml BSO + whole milk), KBS5 (kefir + 5.0 ml BSO + whole milk)

On Day 0, the mean CTRL yeast count was 6.94 log cfu/ml and increased ($P > 0.05$) to 9.46 log cfu/ml on Day 1 (Figure 4). During refrigerated storage the mean CTRL yeast count decreased on Day 7 but not significantly and increased on Day 14 but not significantly.

The mean KBS01 yeast count on Day 0 was 6.95 log cfu/ml and increased ($P > 0.05$) to 9.46 log cfu/ml on Day 1. KBS01 mean yeast count on Day 7 decreased but not significantly while counts on Day 14 increased but not significantly.

On Day 0, the mean KBS1 yeast count was 7.03 log cfu/ml and increased but not significantly to 9.28 log cfu/ml on Day 1. During refrigerated storage of KBS1, the yeast count on Day 7 was lower than Day 1 although it was not significant. The KBS1 count from Day 7 to Day 14 increased but was not significant. There was no significant difference between mean yeast counts of CTRL, KBS01 and KBS1 across Day 1, Day 7 and Day 14, indicating that 0.1% and 1% black seed oil did not inhibit these organisms. CTRL, KBS01, and KBS1 mean yeast counts increased from Day 7 to Day 14 although not significantly.

The mean KBS5 yeast count on Day 0 was 6.20 log cfu/ml and increased on Day 1 to 7.23 log cfu/ml although not significantly. On Day 0, the mean KBS5 yeast was not significantly different from the other three samples while the mean KBS5 yeast was significantly lower than all other samples on Day 7 and Day 14. Since Day 0 to Day 1 was the fermentation period, the increase in mean yeast indicated that 5% black seed oil did not

completely inhibit the yeasts, but comparison to the other treatments indicated that this level of black seed oil did slow the population increase. On Day 7, the mean KBS5 yeast count decreased to 6.13 log cfu/ml while slightly increasing to 6.35 log cfu/ml on Day 14 although the changes were not significant. The refrigerated storage results indicated that the yeast may have partially overcome the antimicrobial action of 5% blackseed oil although the total yeast counts in KBS5 samples were significantly lower ($P < 0.05$) than in CTRL, KBS01 and KBS1 samples.

As pH values decreased over time, the yeast counts increased. Research by Turgut and Cakmakci indicated that yeast counts can increase in yogurt during refrigerated storage [26]. This may suggest that during refrigeration a reduction in bacteria competitiveness allowed yeast counts to increase as yeasts are generally acid tolerant.

The limit of detection across all samples and all days for the coliform count was 10 log cfu/ml. Samples from all days had no colonies of coliform bacteria within the range of detection.

CONCLUSIONS

It is common practice in Turkey and other countries for consumers to add the functional food black seed oil to the functional food kefir. It is known that several components of black seed including thymoquinone, p-cymene, a-thujene, thymohydro-quinone and longifolene are antimicrobial [27]. This study was designed to determine if black seed oil would inhibit the microorganisms in kefir resulting in a potential impact to functional food properties of the kefir. Results of this study indicated that black seed oil had a dose-dependent effect on acid production as measured by pH and by growth of microorganisms from authentic kefir grains. Since the probiotic microorganisms in kefir are beneficial for health, making kefir a valuable functional food, inhibition of these organisms would not be desirable [28].

Results indicated that 0.1% black seed oil did not significantly inhibit any of the microorganisms in the kefir. This corresponds to research by Hassanien et al. which indicated that 0.1% black seed oil did not impact lactic acid bacteria in cheesemaking [29]. At 1% and 5%, black seed oil appeared to slow the growth of *Lactobacillus* spp., *Lactococcus* spp. and yeast after incubation. It appears that the yeasts were the most sensitive to black seed oil. Also, it appears during refrigerated storage that yeast could overcome the antimicrobial action of black seed oil and continue increasing in population.

The results of this study indicate that if black seed oil is added to kefir beverage, the concentration should be limited to 1% black seed oil or less to not inhibit the beneficial microorganisms and not impact the functional properties of authentic kefir.

List of abbreviations (if any): Black seed oil (BSO)

Competing interests: The authors declare no competing interests.

Author Contributions: XJ submitted the grant for research funding. JEN, ZGS and ACS prepared the kefir and carried out the analyses. JEN and WCB conducted the statistical analyses. JEN, ZGS, ACS, XJ, WCB and AKG drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgments: Kefir grains were provided by Dr. Zeynep B. Guzel-Seydim and Dr. Atif C. Seydim of Danem Dairy and Dairy Products Ltd. (Suleyman Demirel University, Technopark, Isparta, Turkey). Authors ZGS

and ACS were supported by Suleyman Demirel University and as visiting professors at Clemson University. Authors JEN, XJ, WCB, AKG were supported by Clemson University. Technical Contribution No. 6987 of the Clemson University Experiment Station.

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