Artisanal Kefir: Antimicrobial Activity and Bacterial Populations

A Dissertation

Presented in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

with a

Major in Food Science

in the

College of Graduate Studies

University of Idaho

by

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December 2020

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Abstract

Food products can be contaminated by a range of pathogenic and spoilage bacteria. Contamination of foods results in food spoilage and foodborne illnesses and leads to economic losses in the food industry. Similar to chemical antimicrobials, natural preservatives (biopreservatives) can be used to improve food quality and safety. Biologically based preservation (biopreservation) uses lactic acid bacteria (LAB), their bacteriocins, bacteriophages and bacteriophage-encoded enzymes to ensure food safety and quality for foods that are not fermented. Among biopreservatives, bacteriocins are ribosomally synthesized small antimicrobial proteins, secreted by bacteria to inhibit the growth of other, usually closely related bacteria. Bacteriocins inhibit select pathogenic and/or spoilage bacteria without changing the chemical and physical characteristics of food.

Kefir is a fermented dairy product made using kefir grains, which are composed of LAB and yeast in a protein-polysaccharide matrix called kefiran. Kefir has antimicrobial activity due to many metabolic products, including bacteriocins produced by LAB. For this study, it was hypothesized that international artisanal kefirs have diverse microflora, generating distinctive bacteriocin content, resulting in varied levels of antimicrobial activities. The objectives of this work were: 1) compare the antimicrobial activity of artisanal kefirs from Fusion Tea (Amazon, USA), Britain, the Caucasus region, Ireland, Lithuania, and South Korea against select foodborne pathogens, 2) examine whether the antimicrobial effect is due to bacteriocin production or other antimicrobials present in kefir, and 3) reveal bacterial populations and elucidate the diversity and abundance of LAB species in artisanal kefirs.

This dissertation is comprised of two interconnected studies. In the first study, the antimicrobial activities of artisanal kefirs from Fusion Tea (A), Britain (B), Ireland (I), Lithuania (L), the Caucasus region (C), and South Korea (K) were investigated against select foodborne pathogens. *Listeria monocytogenes* CWD 1198, *Salmonella enterica* serovar Enteritidis ATCC 13076, *Staphylococcus aureus* ATCC 25923, and *Bacillus cereus* ATCC 14579 were inhibited by artisanal kefirs made with kefir grains from diverse origins. Kefirs A, B, and I inhibited all bacterial indicator strains examined at varying levels, except *Escherichia coli* ATCC 12435 (non-pathogenic, negative control). Kefirs K, L, and C inhibited all indicator strains, except *S. aureus* ATCC 25923 and *E. coli* ATCC 12435. Bacteriocins present in artisanal kefirs were determined to be the main antimicrobials in all kefirs examined. Kefir-based antimicrobials are being proposed as promising natural biopreservatives as per the results of the study.

A typical kefir microbial community includes LAB, acetic acid bacteria, and yeast among other species in a symbiotic matrix. In the second study, the 16S rRNA gene sequencing was used to reveal bacterial populations and elucidate the diversity and abundance of LAB species in international artisanal kefirs from Fusion Tea, Britain, the Caucasus region, Ireland, Lithuania, and South Korea. Bacterial species found in high abundance in most artisanal kefirs included *Lactobacillus kefiranofaciens*, *Lentilactobacillus kefiri*, *Lactobacillus ultunensis*, *Lactobacillus apis*, *Lactobacillus gigeriorum*, *Gluconobacter morbifer*, *Acetobacter orleanensis*, *Acetobacter pasteurianus*, *Acidocella aluminiidurans*, and *Lactobacillus helveticus*. Some of these bacterial species are LAB that have been reported for their bacteriocin production capabilities and/or health promoting properties.

Acknowledgements

I would like to thank Saudi Arabian Cultural Mission for their financial support. I also would like to thank Dr. Gülhan Ünlü for all of her academic advice, efforts, hard work and support. I would like to thank Mrs. Barbara Nielsen, my committee members, Drs. Ahmadzadeh, Michael, Rasco, and Zhu, and my lab partners for their time and support.

Dedication

I would like to dedicate my dissertation to my parents Halah Ajabnoor and Atif Sindi, my husband Ibrahem Aljuhani and to all my family and friends.

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Abbreviations list

Amazon Fusion Tea (A)

Baird Parker agar (BPA)

Britain (B)

DGGE (Denaturing gradient gel electrophoresis)

Didehydroalanine (Dha)

Disability-adjusted life year (DALY)

Distilled water (DI)

Food and Agriculture Organization (FAO)

Generally Recognized as Safe (GRAS)

Hierarchical clustering (HC)

Ireland (I)

Kefir product from Fusion Tea (AP)

Kefir grains from Fusion Tea (AG)

Kefir product from Britain (BP)

Kefir grains from Britain (BG)

Kefir product from the Caucasus region (CP)

Kefir grains from the Caucasus region (CG)

Kefir product from Ireland (IP)

Kefir grains from Ireland (IG)

Kefir product from South Korea (KP)

Kefir grains from South Korea (KG)

Kefir product from Lithuania (LP)

Kefir grains from Lithuania (LG)

Lactic acid bacteria (LAB)

Lithuania (L)

Mannitol Salt Agar (MSA)

Mannitol Yolk Polymyxin (MYP)

Methyllanthionine (MeLan)

National Institute of Food and Agriculture (NIFA)

Ready-to-eat (RTE)

South Korea (K)

The Caucasus region (C)

The Centers for Disease Control and Prevention (CDC)

The Food and Drug Administration (FDA)

The United States Department of Agriculture (USDA)

The World Health Organization (WHO)

Toxic Shock Syndrome (TSS)

Tryptic soy agar (TSA)

Tryptic soy broth (TSB)

Whole genome sequencing (WGS)

Xylose Lysine Deoxycholate agar (XLD)

Microbial Genera Abbreviations

A. flavus (Aspergillus flavus)

A. malorum (Acetobacter malorum)

A. niger (Aspergillus niger)

A. orientalis (Acetobacter orientalis)

A. orleanensis (Acetobacter orleanensis)

A. pasteurianus (Acetobacter pasteurianus)

Acid. aluminiidurans (Acidocella aluminiidurans)

B. cereus (Bacillus cereus)

B. subtilis (Bacillus subtilis)

C. perfringens (Clostridium perfringens)

C. sakazakii (Cronobacter sakazakii)

E. faecalis (Enterococcus faecalis)

G. morbifer (Gluconobacter morbifer)

G. frateuii (Gluconobacter frateuii)

G. kondonii (Gluconobacter kondonii)

L. acidophilus (*Lactobacillus acidophilus)*

L. apis (*Lactobacillus apis)*

L. crispatus (*Lactobacillus crispatus)*

L. gigeriorum (*Lactobacillus gigeriorum)*

L. helveticus (*Lactobacillus helveticus)*

L. innocua (Listeria innocua)

L. lactis (Lactobacillus lactis)

L. monocytogenes (Listeria monocytogenes)

L. otakiensis (*Lactobacillus otakiensis)*

L. ultunensis (*Lactobacillus ultunensis)*

Lac. lactis (Lactococcus lactis)

Lacti. casei (*Lacticaseibacillus casei)*

Lacti. paracasei (*Lacticaseibacillus paracasei*)

Lacti. plantarum (*Lactiplantibacillus plantarum*)

Lacti. rhamnosus (*Lacticaseibacillus rhamnosus*)

Lenti. diolivorans (*Lentilactobacillus diolivorans*)

Lenti. kefiranofaciens (Lentilactobacillus kefiranofaciens)

Lenti. kefiri (*Lentilactobacillus kefiri*)

Leu. citreum (Leuconostoc citreum)

Leu. mesenteroides (Leuconostoc mesenteroides)

P. aeruginosa (Pseudomonas aeruginosa)

S. aureus (Staphylococcus aureus)

S. cerevisiae (*Saccharomyces cerevisiae*)

S. enterica (Salmonella enterica)

S. Enteritidis *(Salmonella enterica* serovar Enteritidis*)*

Sh. dysenteriae (Shigella dysenteriae)

Strep. thermophilus (Streptococcus thermophilus)

Statement of Contribution

Manuscript I:

Author Contributions: Conceptualization, G.Ü.; Methodology, A.S., M.B.B., B.N., and G.Ü; Formal Analysis, A.S., M.B.B., and G.Ü; Investigation, A.S., B.N., and G.Ü; Resources, A.S., M.B.B., B.N., and G.Ü; Writing—Original Draft Preparation, A.S.; Writing—Review and Editing, A.S., M.B.B., B.N., and G.Ü; Supervision, G.Ü; Project Administration, G.Ü.; Funding Acquisition, A.S. and G.Ü.

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Chapter 1: Introduction

Introduction

1.1 Kefir: An Introduction

Kefir, a fermented dairy product that originated thousands of years ago in the Caucasus Mountains between Europe and Russia, is one of the oldest milk ferments in existence [1]. Kefir is an acidic, viscous milk beverage with a small percentage of alcohol and a strong flavor and aroma that is similar to that of a yeasty buttermilk [1,2]. However, kefir has different fermentation requirements than buttermilk and yogurt. Kefir fermentation requires inoculating milk with the entire kefir grain. Kefir grains contain 30-50 species of bacteria and yeast embedded in a soft, insoluble protein polysaccharide matrix, known as kefiran. Kefiran is produced by some of the lactobacilli embedded in the matrix [1,2].

Kefir grains vary in size from a couple of millimeters to a few centimeters. The grains have irregular shapes and uneven surfaces, giving them a cauliflower-like appearance [2]. The grains' typical chemical composition on a percent weight by weight basis is: water (89–90%), lipids (0.2%), proteins (3.0%), carbohydrate (6.0%), and ash (0.7%) [3]. Kefir grains start as a thin layer structure and develop over time to form a more complicated structure with a flat and rough-sided sheets folding themselves into scrolls [2]. Earlier studies of kefir showed that non-lactose-fermenting yeast are more abundant than lactose-fermenting yeast in the deeper layers of the kefir grain [4]. Although some studies show that the interior and exterior surfaces of the grains are occupied by microorganisms [5], it is hard to observe bacteria on the exterior surfaces of the grains, as bacteria are only embedded in the fibrillar matrix near the surface [6,7].

Kefir contains vitamins, minerals and essential amino acids that are beneficial [8]. Kefir contains vitamins B1, B2, and B5 at levels that are influenced by milk type and

microflora. Kefir is a good source of calcium, magnesium and phosphorus. The macro elements found in kefir on a percent weight by weight basis are potassium (1.65%), calcium (0.86%), phosphorus (1.45%) and magnesium (0.30%) while the micro elements found are copper, zinc, iron, manganese, cobalt, and molybdenum [9]. Kefir contains higher levels of threonine, serine, alanine, and lysine than milk. It also contains other amino acids such as valine, isoleucine, methionine, lysine, phenylalanine and tryptophan [9].

Kefirs originating from various regions of the world have their unique flavor and aroma. Microorganisms isolated from kefir grains have been shown to produce end products which contribute to kefir's aroma and flavor. The lactic acid bacteria (LAB) such as *Lactobacillus kefiranofaciens* (*Lentilactobacillus kefiranofaciens*), *Lactobacillus acidophilus*, *Lactobacillus helveticus* and *Lactococcus lactis* produce lactic acid which gives acidic flavor to kefir [10]. *Acetobacter pasteurianus* and *Lactococcus lactis* subsp. *cremoris* have been reported to give vinegar, green, fruity, sour flavor and aroma. *Leuconostoc mesenteroides* has been reported to produce diacetyl which gives buttery flavor. *Candida guilliermondii* produces diacetyl which provides kefir with the creamy aroma. *Saccharomyces cerevisiae* produces nonanal, octanal, ethyl acetate and 3-methyl-butanol which gives kefir the green, citrus, soapy, fruity and wine-like flavor and aroma [10].

Kefir has been recognized for its beneficial effects on health. *In vitro* and *in vivo* studies have demonstrated the health promoting effects of kefir such as improving digestion and tolerance to lactose [11], antibacterial effect, hypocholesterolaemic effect [12], control of plasma glucose, anti-hypertensive effect, anti-inflammatory effect, antioxidant activity, anti-carcinogenic activity, anti-allergenic activity and wound healing effects [13].

1.2 Kefir Production

Traditional kefir is produced by the fermentation of pasteurized milk by kefir grains, which can be filtered from kefir product and used to inoculate a new batch of milk. Commonly, cow's milk is used for kefir fermentation, but it can be substituted with other milks such as sheep's milk and goat's milk, as well as plant-based milks such as coconut milk. A greater fat content usually results in better quality kefir [1,2]. Kefir grains are usually inoculated, at a level of 1:10 (w/v), into whole milk and the inoculated milk is incubated at 22-25ºC for 24 hours [14,15]. Kefir product is stored at 4℃ to reduce microbial fermentation. In contrast, commercial kefir is produced by homogenizing the milk and heating it at 90-95℃ for 5-10 minutes. Then, the milk is cooled to18-24℃ and inoculated with kefir cultures (2-8%) such as *Lactobacillus lactis, Lactobacillus rhamnosus* (*Lacticaseibacillus rhamnosus*), *Lac. lactis, Streptococcus diacetylactis, Lactobacillus delbrueckii, Lactobacillus plantarum* (*Lactiplantibacillus plantarum*), *Lactobacillus casei* (*Lacticaseibacillus casei*), *Lactobacillus paracasei* (*Lacticaseibacillus paracasei*), *L. acidophilus, Saccharomyces florentinus, Leuconostoc cremoris, Bifidobacterium longum, Bifidobacterium breve* and *Bifidobacterium lactis* for the fermentation process to take place in 18-24 hours. The product is then recovered and distributed into bottles and stored at 4℃ [16].

1.3 Kefir Microbial Profile

Multiple microorganisms sharing symbiotic relationships have been isolated from kefir, including yeasts (*Kluyveromyces, Candida, Saccharomyces* and *Pichia*), LAB (*Lactobacillus, Lactococcus*, *Leuconostoc,* and *Streptococcus*), and some acetic acid bacteria

(*Acetobacter* and *Gluconobacter*) [3,17]. These symbiotic relationships among the microorganisms allow bacteria and yeast to survive and share their bioproducts as growth factors [18]. *Acetobacter* species have an important role in maintaining viscosity and the symbiosis in kefir [19, 4]. Homofermentative and heterofermentative *Lactobacillus* species account for about 65-80% of the total microbial content. The rest are *Lactococcus* species and different species of LAB, acetic acid bacteria, as well as lactose-fermenting and non-lactose-fermenting yeast (about 5%) [20].

1.3.1 Identification of Microbial Populations in Artisanal Kefir

Both culture-dependent and culture-independent methods have been used to identify microbial populations in artisanal kefirs. Differences exist among microbial populations identified by these methods. For example, Kesmen and Kacmaz (2011) identified *Lac. lactis*, *Leu. mesenteroides* and *Lactobacillus kefiri* (*Lentilactobacillus kefiri*) as the most abundant bacterial species with culture-dependent methods. Using the culture-independent method of PCR denaturing gradient gel electrophoresis (DGGE), they identified *Lenti. kefiranofaciens* and *Lac. lactis* as the most abundant bacterial species [20]. The LAB found in kefir require specific nutrients and conditions for growth [2], so selected LAB in kefir may have been undetected via culture-dependent methods. Some bacteria are present in kefir at low levels, which makes them difficult-to-detect using classical tools of microbiology [2]. Thus, culture-independent methods are highly recommended for more accurate identification of kefir microbiota.

1.3.2 Regional Difference in Kefir Microbial Community

Kefir microbial community and the most abundant bacterial species in kefir can vary depending on geographical regions. For example, a kefir sample originating from Taiwan showed *Lenti. kefiranofaciens* as the most dominant bacterium while a kefir sample originating from China showed *Lacti. casei* as the most dominant bacterium when culture-independent methods were used [21, 22]. Table 1 is a compilation table that includes bacterial species isolated from kefirs originating from multiple regions of the world and the methods used for their isolation and/or identification.

Table 1: Bacterial species of artisanal kefirs*

Species	**Bacterial identification method**	**Country**	**Reference**
Lactobacillus diolivorans (Lentilactobacillus diolivorans) *Lactobacillus kefiri* *Lactobacillus kefiranofaciens* *Lactobacillus lactis* *Lactobacillus otakiensis* *Lactobacillus paracasei (Lacticaseibacillus paracasei)* *Enterococcus durans* *Acetobacter fabarum* *Acetobacter okinawensis* *Acetobacter orientalis*	16S rRNA sequencing	Turkey	[23]
Lactobacillus kefiranofaciens *Lactobacillus kefiri* *Lactobacillus buchneri* *Lactobacillus sunkii* *Lactobacillus otakiensis* *Lactobacillus kefiri* *Enterococcus hirae* *Enterococcus villorum* *Enterococcus ratti* *Enterococcus faecium*	PCR-DGGE and pyrosequencing	Italy	[24]

Enterococcus thailandicus *Enterococcus durans* *Enterococcus sanguinicola* *Enterococcus lactis* *Lactococcus lactis* *Acetobacter fabarum* *Acetobacter orientalis* *Lactobacillus crispatus* *Lactobacillus intestinalis* *Porphyromonas* sp. *Streptococcus thermophilus* *Bacillus* spp.			
Lactobacillus kefiranofaciens *Acetobacter fabarum* *Leuconostoc mesenteroides* *Lactobacillus kefir* *Lactobacillus lactis* *Lactobacillus crispatus* *Lactobacillus farraginis* *Lactobacillus psittaci* *Tanticharoenia aidae*	Next generation sequencing	South Korea	[25]
Lactobacillus kefiranofaciens *Lactobacillus kefiri* *Lactobacillus parakefiri* *Lactococcus lactis* *Leuconostoc* spp.	Culture dependent methods	Russia	[26]
Lactobacillus kefiranofaciens *Lactobacillus kefiri* *Lactobacillus buchneri* *Lactobacillus sunkii* *Lactobacillus otakiensis* *Lactococcus lactis* *Leuconostoc mesenteroides*	PCR-DGGE and culture dependent methods	Turkey	[20]
Pseudomonas spp. *Leuconostoc mesenteroides* *Lactobacillus helveticus* *Lactobacillus kefiranofaciens* *Lactobacillus lactis* *Lactobacillus kefiri* *Lactobacillus casei*	PCR-DGGE	China	[27]
Lactobacillus kefir *Lactobacillus kefiranofaciens* *Lactobacillus paracasei* *Lactobacillus plantarum*	Culture-dependent and culture-independent methods	Argentina	[28]

Lactococcus lactis ssp. *lactis* *Lactobacillus parakefiri* *Leuconostoc mesenteroides* *Acetobacter* sp. *Lactococcus lactis* ssp. *lactis* *Lactobacillus kefiri* *Lactobacillus parakefiri*			
Lactobacillus kefiranofaciens *Lactobacillus kefiri* *Lactobacillus parabuchneri* *Lactobacillus kefiranofaciens* *Lactobacillus helveticus* *Lactobacillus acidophilus* *Lactobacillus parakefiri*	16S rRNA gene sequencing (using the V4 variable regions)	Ireland	[29]
Lactobacillus paracasei *Acetobacter lovaniensis* *Lactobacillus parabuchneri* *Lactobacillus kefir* *Lactococcus lactis*	Culture-dependent and culture-independent methods	Brazil	[30]

*The table is a compilation table based on the following references: 20, 23, 24, 25, 26, 27, 28, 29, and 30.

1.3.3 Probiotic Bacteria in Artisanal Kefir

Select LAB have been reported to have probiotic properties. Probiotic bacteria have been associated with immunomodulation, digestion benefits, production of short-chain fatty acids and essential vitamins, stability in the GI-tract, and inhibition of pathogenic microorganisms [31, 32]. *Lactobacillus* species with probiotic potential have been reported in kefir. *Lenti. kefiranofaciens, Lenti. diolivorans, L. acidophilus, Lacti. plantarum, Lacti. casei, Lacti. rhamnosus* and *Lenti. kefiri* were identified as probiotic bacteria isolated from kefir originating from different regions [33, 34, 35]. For example, Malaysian kefir was reported to contain *L. harbinensis, Lacti. paracasei,* and *Lacti. plantarum* which exhibit probiotic properties and antioxidant activities [36]. These probiotic properties included acid and bile

salt tolerances, adherence to the intestinal mucosa and antibiotic resistance [36]. In addition, these species showed high antioxidant activities including total phenolic content, total flavonoid content, and ferric reducing ability of plasma [36]. Several *Lenti. kefiranofaciens* strains, *Lenti. kefiranofaciens* M1, 10058, DN1, DD2, and KCTC 5075, were isolated from multiple kefirs and reported to carry probiotic properties such as anti-inflammatory characteristics, antimicrobial activity, antiallergenic effect and reducing cholesterol levels *in vitro* [37, 38, 39].

1.4 Kefir Antimicrobials

Kefir has been shown to contain a variety of natural antimicrobials, including bacteriocins, organic acids, hydrogen peroxide, and free fatty acids, produced by microorganisms present in kefir grains. For example, *Lacti. plantarum* ST8KF, isolated from kefir, generates a 3.5 kDa bacteriocin which inhibited the growth of *Lacti. casei, Lactobacillus salivarius, Lactobacillus curvatus* and *Listeria innocua* [40]. *Lacti. plantarum* strains CIDCA 83114 and CIDCA 8336 were isolated from kefir and studied for their protection of the vero cells from cytotoxicity by type-II shiga toxin from *Escherichia coli* O157:H7 and for their inhibitory activity against *Shigella* invasion by protecting human Hep-2 cells [41, 42]. These *Lacti. plantarum* strains were suggested to be used as probiotics due to their health promoting benefits [42].

1.4.1 The Antimicrobial Activity of Kefir Originating from Various Regions

Multiple studies demonstrated the antimicrobial activity of kefir originating from different countries. Joao et al. (2013) indicated that kefir from Brazil showed growth inhibition for *Staphylococcus aureus* ATCC 6538 (42.80-69.15%), *Escherichia coli* ATCC 11229 (30.73-59.89%), *Salmonella* Typhi ATCC 6539 (44.99-73.05%), *Listeria monocytogenes* ATCC 15313 (41.45-54.18% for) and *Bacillus cereus* RIBO 1222-173-S4 (70.38-86.80%) [15]. Another study extracted 46 peptides ranging from 703 to 1881 Da (6-17 amino acids) from Brazilian sheep milk kefir. This study reported that 13 of the 46 peptides were able to inhibit the growth of pathogenic microorganisms *Klebsiella pneumoniae* ATCC 29665, *Pseudomonas aeruginosa* ATCC 27853, *Enterobacter faecalis* ATCC 6057, *Bacillus cereus* ATCC 33019, *Bacillus subtilis* ATCC 6633 and *S. aureus* ATCC 6538 [43]. These studies suggested that Brazilian kefir can be used as an effective antimicrobial product.

Mariana et al. (2011) investigated the antimicrobial activity of Romanian kefir against *B. subtilis*, *S. aureus*, *E. coli*, *E. faecalis* and *S.* Enteritidis using the agar well diffusion method. Romanian kefir showed strong antibacterial activity against Gram-negative and Gram-positive indicator strains when compared with neomycin sulfate and ampicillin [44]. In a study from India, coconut milk kefir demonstrated the maximum zone of inhibition (39 mm) against *E. coli* and some inhibition (7 mm) against *S. cerevisiae* [45].

Kim et al. (2016) compared the antimicrobial spectra of four types of kefirs (A, L, M, and S) all from South Korea. South Korean kefir A inhibited the growth of *Bacillus cereus* ATCC 14579, *Escherichia coli* ATCC 25922, *Salmonella* Enteritidis (FDA), *Pseudomonas aeruginosa* ATCC15522, and *Cronobacter sakazakii* ATCC29544, while South Korean

kefirs L, M, and S inhibited *B. cereus, S. aureus* ATCC6538, *E. coli, S.* Enteritidis, *P. aeruginosa,* and *C. sakazakii. Listeria monocytogenes* ATCC51776 was only inhibited by kefir M [14]. This study indicates that kefirs originating from the same region can show differences in their antimicrobial activity against foodborne pathogens. Since kefir grains vary in their microbial composition, they show variation in their antimicrobial properties.

The antimicrobial spectra of five types of kefir supernatants from Turkish kefirs were reported against plant pathogenic bacteria *Pseudomonas syringae, Xanthomonas axonopodis, Xanthomonas euvesicatoria, Erwinia amylovora, Clavibacter michiganensis* and *Bacillus* spp. both *in vitro* and *in vivo* [46]. *In vitro,* antibacterial activity detection using the disc diffusion agar method revealed different antibacterial potencies based on the type of kefir and the fermentation time. *In vivo,* studies using kefir supernatants on cucumber and common bean challenged with the plant pathogenic bacteria in the climate chamber showed no significant decrease in diseases but revealed an increase in some plant growth parameters [46]. This was the first study to apply filter sterilized kefir supernatant *in vitro* on cucumber and beans.

1.4.2 Factors Affecting Kefir's Antimicrobial Activity

Various aspects of kefir fermentation can affect kefir's antimicrobial activity. The effects of type of milk, fermentation time, fermentation temperature, and stirring conditions on the antibacterial activity of kefir against *Shigella dysenteriae, S. aureus,* and *B. cereus* were investigated. Full-fat milk kefir incubated at 37°C and stirred kefir showed greater antibacterial activity when compared with nonfat milk kefir, kefir incubated at 25°C, and

non-stirred kefir. In addition, kefir fermented for 48 hours or more showed greater antibacterial activity when compared with kefir with shorter fermentation time [47].

1.4.3 The Antimicrobial Activity of Artisanal Kefir Compared to Yogurt and Commercial Kefir

The antimicrobial activity of Iranian kefir and probiotic yogurt produced from cow, camel, ewe, and goat milk on *S. aureus*, *E. coli*, *L. monocytogenes*, *S. enterica*, *Aspergillus niger* and *Fusarium* sp. was investigated. Results showed that kefir samples had stronger antifungal and antibacterial effect than yogurt samples. Kefir samples from ewe and cow milk showed the highest antimicrobial activity [48]. Particularly, this study suggested that kefir produced from cow milk had a stronger antimicrobial activity than probiotic yogurt produced from the same milk. This could be due to the diversity of microbial community in kefir compared to probiotic yogurt.

The antimicrobial activities of commercial and artisanal kefirs were investigated in Turkey. Artisanal kefir had a greater inhibitory effect on *Enterobacter cloacae* and *E. coli* than commercial kefir, while commercial kefir had a greater inhibitory effect on *E. faecalis* [49]. This result suggested that microbial diversity of artisanal kefir plays an important role on kefir antimicrobial activity. As expected, the starter cultures in commercial kefir affected kefir's antimicrobial properties.

1.4.4 Bacteriocin Producing Lactic Acid Bacteria

Bacteriocin producing LAB have been isolated from kefir. *Lenti. kefiri* was isolated from kefir and shown to inhibit *P. aeruginosa, Salmonella* species, *Sh. flexneri, L. monocytogenes, B. cereus, E. faecalis* and *S. aureus* at different levels depending on the strains used [50]. The bacteriocin FX-6 was isolated from cow milk-based Tibetan kefir and identified as a bacteriocin produced by *Lacti. paracasei* subsp. *tolerans* FX-6 [51]. The bacteriocin has a wide range of antimicrobial activity, strong heat stability and pH stability. It inhibited the growth of *S. aureus, B. thuringiensis, S. enterica, Sh. dysenteriae, A. flavus, A. niger, Rhizopus nigricans* and *Penicillium glaucum*. A partial reduction in antimicrobial activity was observed following treatment by pepsin and trypsin, confirming the proteinaceous nature of the bacteriocin [51].

Bacteriocin-producing *Lac. lactis* subsp. *lactis* strains BGKF8, BGKF17, BGKF26, BGKF49, and BGKF55 were isolated from kefir. The strains showed different plasmid profiles and no cross-inhibition among them was detected [52]. Another study tested the inhibitory effect of cell-free filtrates of *Lactobacillus bulgaricus, Lacti. casei, Lacti. plantarum, L. acidophilus, Lactobacillus brevis, Lactobacillus fermentum, L. lactis,* and *L. helveticus*. This study showed that some strains from the same species had antimicrobial activity against *S. aureus, E. coli,* and *Yersinia enterocolitica.* It was observed that the inhibition activity is due to bacteriocin-production [53].

2. Lactic Acid Bacteria, Bacteriocins, and Biopreservation

2.1 Lactic Acid Bacteria

LAB are a group of Gram-positive, non-spore-forming, facultatively anaerobic, cocci or rods. This diverse group of bacteria are grouped together because of their ability to produce lactic acid from various carbohydrates [54]. LAB can be divided into two groups based upon the products produced from the fermentation of glucose. Homofermentative LAB produce only lactic acid from glucose fermentation. Heterofermentative LAB ferment glucose to multiple end products, such as acetic acid, ethanol, formic acid, and CO_2 [55]. LAB can ferment food and perform an essential function in preserving and producing a wide range of foods including milk, brined vegetables, cereal and meats with added carbohydrates [56].

LAB have Generally Recognized as Safe (GRAS) status, therefore, they are approved by the FDA to be safe for consumption. Some LAB are probiotic organisms. There are many health benefits offered by these probiotic LAB such as enhanced lactose digestion, stimulation of the immune system, control of serum cholesterol levels and inhibition of foodborne pathogens [57].

2.2 Bacteriocin Classes and Mode of Action

Some LAB produces antimicrobials called bacteriocins. Bacteriocins are ribosomally synthesized proteins that are secreted by bacteria and they inhibit other closely related bacteria using different mechanisms [58]. Bacteriocins are divided into four major groups:

class I, class II, class III and class IV. The class I bacteriocins (lantibiotics) contain the first bacteriocin discovered from LAB, nisin [59].

Nisin was discovered by Rogers and coworkers in 1928. Nisin is synthesized by a lactic acid bacterium called *Lac. lactis* and is commercially produced in an industrial fermentation process utilizing the food-grade bacterium. The polypeptide nisin consists of 34 amino acids [59]. Nisin has uncommon amino acids, due to posttranslational alterations, such as lanthionine, methyllanthionine (MeLan) and didehydroalanine (Dha). Nisin is heat stable and has a decreased solubility at alkaline and neutral pH. Nisin exists in a stable dimer form of 3 kDa and often it is found as a tetramer form of 14 kDa. Nisin is also called lanitibiotic and its cyclic feature facilitates in membrane insertion and provides shielding against thermal denaturation [60]. The lactococci releasing nisin exist naturally in raw milk and cheese, indicating that it has likely been consumed by humans for centuries. Nisin is not toxic, and digestive enzymes can inactivate nisin at a quick rate, protecting the microflora of the GI track [60].

Nisin has at least two distinct forms, nisin A and Z. The difference between nisin Z and nisin A is the substitution of asparagine for histidine in nisin Z. Both have a similar antimicrobial activity [61]. Nisin targets two general groups of organisms in foods. First, it protects food products from spoilage caused by bacteria such as *Brochothrix thermosphacta* and *Clostridium thermosaccharolyticum* that can impact the color, flavor and texture of the products. Secondly, it destroys Gram-positive disease-causing pathogens such as *S. aureus, B. cereus, L. monocytogenes* and *Clostridium perfringens* [62].

Class II bacteriocins are the largest group of bacteriocins. This class is small, heat-stable, non-lanthionine peptides including pediocin or Listeria-active bacteriocins (class IIa), two-peptide bacteriocins (class IIb), and circular bacteriocins (class IIc) [63]. Pediocin, produced by *Pediococcus acidilactici*, has been named *Listeria*-active bacteriocin because of its inhibitory activity against *Listeria* species [63]. Class III bacteriocins consist of heat labile proteins with large molecular weight. Bacteriocins representing this group are helveticin I, which is produced by *L. helveticus*, and enterolysin, which is produced by *E. faecium* [64]. Class IV bacteriocins are formed by poorly characterized complex proteins, containing carbohydrate or lipid [65]. They consist of an N-terminal lyase, a central kinase, and a C-terminal cyclase domain [66].

Bacteriocins have different mechanisms to inhibit spoilage and pathogenic bacteria. The antimicrobial mechanisms of nisin are cell membrane pore formation and the inactivation of lipid II, which is a precursor molecule important for cell wall synthesis. Nisin binds to the target cell C-terminus and inserts into the membrane through its N-terminus, causing cell death [59]. For class II bacteriocins, the antimicrobial activity is receptor mediated by recognition of specific proteins on the membrane of target cells. Generally, class II act on disrupting the integrity of the membrane of target cells [67]. The antimicrobial mechanisms of pediocin include binding to the cell membrane, pore formation, insertion into the membrane and disrupting protein motive force [63].

2.3 Lactic Acid Bacteria and Biopreservation

Biopreservation has been defined as “the use of LAB, or their metabolic products, or both to improve or ensure the safety and quality of foods that are not fermented” for years [58]. Recently, biopreservation is defined as “a technique of food preservation in which antimicrobial potential of naturally occurring organisms and their metabolites are exploited” [68]. The biopreservation techniques use antimicrobial systems such as LAB and/or their bacteriocins, bacteriophages and bacteriophage-encoded enzymes. Bacteriophages are viruses that infect and destroy pathogenic bacteria by entering into bacterial cells, causing metabolism disturbance and cell lysis [69]. Bacteriophage-encoded enzymes endolysins are produced at the end of the phage lytic cycle to destroy bacterial cell wall to facilitate the release of the phages from bacteria cells [69].

Biopreservation is used to control the growth of select pathogenic and spoilage organisms. It is capable of increasing shelf life with keeping food quality and minimizing nutritional and organoleptic losses [68]. As discussed earlier, LAB produce antimicrobials such as bacteriocins and other antimicrobials. Bacteriocins produced by LAB can be used in food biopreservation, which can help to decrease the addition of chemical preservatives and the intensity of heat treatments in the food industry. The resulting foods can be more naturally preserved and richer in nutritional properties [70]. Heat treatment can be effective to reduce microbial load; however, it can change food properties and nutrition. It also requires high energy and built-up cost when compared with biopreservation.

2.4 Application of Lactic Acid Bacteria Producing Bacteriocins

The application of bacteriocin-producing LAB in unfermented food may induce undesirable sensory properties, such as changes in the color, aroma, and flavor of the products. The introduced LAB may not grow well in the product because of the complexity of the food matrix or the harsh processing conditions, resulting in the production of insufficient amounts of bacteriocin [71,72]. The use of cell-free supernatants that contain bacteriocins has been shown to be a better alternative to the use of LAB themselves. This is because bacteriocins are more resistant to food processing, do not change food properties and do not need to grow in the product like LAB [72]. The inhibition of foodborne pathogens in food systems by bacteriocin preparations has been reported [73, 74, 70].

Bacteriocins can be added to food directly or indirectly [75]. For example, nisin can be added directly to foods such as salad dressings and cheese. For the indirect application of bacteriocin, a food fermented by bacteriocin-producing LAB can be used as an ingredient in a second food product where it might ensure the safety and/or extend the shelf life of the food product. It is important to ensure a uniform dispersion of the bacteriocin during the application of nisin or another bacteriocin in a food product. Post-process contamination of foods can occur, therefore, it is suggested that bacteriocin should be added at the last stage of the process [75, 76].

3. Foodborne Illnesses

Biopreservation can be used to inhibit growth of pathogens in foods and thus reduce foodborne illness [68]. In this section, select pathogens that were used as pathogenic

indicator strains in this work (Chapter 2) and the illnesses they cause are discussed. The World Health Organization (WHO) estimates 600 million cases and 420,000 deaths globally from foodborne illness [77]. Foodborne illnesses in the United States account for 47.8 million cases yearly, resulting in 127,839 hospitalizations and 3,037 deaths. *Salmonella* sp., *S. aureus, B. cereus, L. monocytogenes* and *C. perfringens* continue to be the leading pathogens contributing to foodborne illnesses in the United States [77]. According to the WHO in their 2018 report, non-typhoidal *S. enterica* caused 230,000 deaths worldwide. The Centers for Disease Control and Prevention (CDC) estimates 1,600 cases of *L. monocytogenes* occur annually in the United States resulting in 250 deaths. Although the number of listeriosis cases is small when compared with some other foodborne illnesses, listeriosis is a major public health concern due to its high mortality rate of about 20% [78]. *S. aureus* related foodborne illness causes an estimated 240,000 cases per year in the United States [79, 80]. The CDC estimates that there is over 63,400 cases of *B. cereus* foodborne illness, leading to 20 hospitalizations yearly in the United States [79, 80]. In addition to the cost to human lives, foodborne pathogens have a significant impact on the economy. Annual economic cost from foodborne illness is estimated at over 15.6 billion dollars in the United States [81].

3.1 Foodborne Pathogens

Many biological agents can cause foodborne illness by contaminating food. Most foodborne illnesses are infections caused by consuming food contaminated with bacteria, viruses, or parasites. Other foodborne illnesses are caused by poisonings from harmful toxins or chemicals [82]. According to CDC, the top five microorganisms that cause illnesses from

food consumed in the United States are as follows: Norovirus, *Salmonella* species, *C. perfringens, Campylobacter jejuni* and *S. aureus.* Other microorganisms causing foodborne illness leading to hospitalization include *L. monocytogenes* [82]. Below is some general information about key foodborne pathogens used in the study (Chapter 2).

3.1.1 *Salmonella enterica*

Salmonella enterica is a Gram-negative rod-shaped facultatively anaerobic bacterium. A typical *S. enterica* bacterium measures about 1-5 microns and has peritrichous flagella for motility [83]. *S. enterica* can be subdivided into the subspecies *enterica, salamae, arizonae, diarizonae, houtenae,* and *indica* based on biochemical and genomic variations. *Salmonella* serovars which have been linked to recent outbreaks include Enteritidis, Typhimurium, Newport and Stanley [58].

As part of the standard protocol for the identification of *Salmonella*, the Food and Drug Administration (FDA) recommends that the sample preparation should include the standard culturing techniques to confirm the isolate. This culturing process may take weeks to be completed [84]. Biochemical tests may include testing for the inability to hydrolyze urea, decarboxylate lysine and ornithine, and to utilize citrate as a sole carbon source. The majority of *S. enterica* are hydrogen sulfide producers and lactose fermenters, as well as being catalase-negative and oxidase negative. *S. enterica* may also be characterized based on several non-culture techniques, with the most common ones including molecular assays (Real-time PCR and conventional PCR), rapid serologic tests for *Salmonella* antigens, and the Widal test [85].

Salmonella is naturally found in human, livestock, and poultry intestinal tracks, as well as in the intestinal tracks of reptiles, insects and wild birds. Most of *Salmonella* infections are associated with consumption of contaminated food of animal origin such as eggs, chicken, pork, etc. [85]. *Salmonella* infection can lead to many conditions such as enteric fever, uncomplicated enterocolitis and systemic infection by nontyphoid *Salmonella*. Enteric fever is associated with typhoid and paratyphoid strains [58]. The most common symptoms of *S. enterica* infections in humans include diarrhea, vomiting, and nausea, but in chronic cases, a person may develop aseptic reactive arthritis [83]. Incidents of foodborne salmonellosis tend to overshadow most other foodborne illnesses. For example, in 2015, an outbreak of *S. enterica* affected 94,625 people in Europe, where 126 of the cases were fatal [86]. In 2020, a rapid growing outbreak of *Salmonella* Newport infections has been linked to red onions in the USA. A total of 396 foodborne illnesses have been reported from 34 states linked to *Salmonella* Newport [87].

Salmonella can be serious for certain people including young children, seniors, and people with compromised immune system. The mainstay treatment method for severe *S. enterica* infection is the use of antimicrobial agents – where the first line of antimicrobials include chloramphenicol, amoxicillin, and trimethoprim-sulfamethoxazole. [85]. *Salmonella* infection can be dehydrating, most treatment focuses on replacing fluids and electrolytes.

The virulence factors involved in *Salmonella* foodborne illness include the attachment and invasion of intestinal cells, growth and survival within the host cells, virulence plasmids, siderophores, diarrheagenic enterotoxin and thermolabile cytotoxic protein production. [58, 88]. A potential problem with this pathogen is the development of antimicrobial resistance [89].

3.1.1.1 Foodborne Salmonellosis Prevention

According to WHO, prevention of foodborne salmonellosis requires control of all stages in the food chain, starting from the agricultural production, processing, and manufacturing to the preparation of foods [90]. Elimination of *Salmonella* in food and farm animals can be achieved by thermal treatment of food, chemical additives, biopreservatives, feed additives, such as organic acids, free fatty acids, prebiotics, probiotics, and essential oils [91]. At home, adhering to basic food hygiene practices as well as avoiding raw milk and undercooked food are recommended as preventive measures against salmonellosis [90].

Much research has concentrated on finding effective antimicrobials to inhibit *Salmonella* growth especially in chicken, which is one of the primary sources of infection. A study reported that *Lacti. casei*, *Lacti. plantarum*, *L. fermentum*, and *Lacti. paracasei* produce bacteriocins that can inhibit *S. enterica* [92]. The inhibitory effect of *L. reuteri* and its purified bacteriocin against *S. enterica* was confirmed in another study [93]. LAB can protect human cells from the infection caused by *S. enterica. L. acidophilus*, *L. rhamnosus* and *Lacti. casei* decreased *Salmonella enterica* serovar Javiana intestinal epithelial infection [94].

3.1.2 *Staphylococcus aureus*

S. aureus is a cocci-shaped Gram-positive bacterium. It is facultatively aerobic, and when cultured in 10% salt media, it forms golden to yellow colonies caused by a golden colored carotenoid pigment called staphyloxanthin [95]. This species has unique biochemical properties: it is novobiocin sensitive, catalase positive, mannitol fermentation positive, and

coagulase positive [95]. Classical identification of *S. aureus* may be achieved through such methods as DNase test, hemolysis on sheep blood agar, Gram stain, and growth on Mannitol Salt agar (MSA) [96,97].

S. aureus is found naturally in the environment and as commensal normal flora on human skin as well as the mucosa. However, *S. aureus* is pathogenic when it finds its way into the bloodstream or urinary tract [95]. *S. aureus* is known as the leading cause of soft tissue and skin infections such as abscesses, cellulitis, bloodstream, and respiratory tract infections. *S. aureus* foodborne illness is caused by consuming foods contaminated with *S. aureus* toxins [98]. This bacterium produces multiple toxins as the major virulence factor, including enterotoxins A-E as the major types. Enterotoxins G-J have been more recently discovered while enterotoxin F is associated with Toxic Shock Syndrome (TSS) [58, 98]. Other virulence factors, including environmental adaptation mechanisms and horizontal gene transfer, make *S. aureus* a resilient pathogen [99].

S. aureus foodborne illness has symptoms like nausea, vomiting, stomach cramps and diarrhea [95,100]. Poor hygiene, improper food handling, and improper food storage and distribution can lead to foodborne illness. Staphylococcal foodborne illness is usually observed when large amounts of food are prepared and served to large groups. For example, *S. aureus* affected 24 of the 42 customers who had dinner at a local restaurant in Umbria, Italy, with the customers exhibiting gastrointestinal symptoms in 2015 [101]. *S. aureus* caused an outbreak on a military unit lunch party in the United States in 2012 [102].

3.1.2.1 *Staphylococcus aureus* Foodborne Illness Prevention

S. aureus foodborne illness is self-limiting, in most cases it requires fluids to avoid dehydration. The treatment of severe illness can include antibiotics [100]. However, *S. aureus* is recognized for its ability to become resistant to antibiotics [103]. To control *S. aureus* foodborne illness, CDC recommends preventing food from being held at an unsafe temperature (40-140°F) for more than 2 hours. The CDC also recommends proper personal hygiene to eliminate the illness [104]. The main challenge in the food industry is to prevent the growth of the organism and inhibit enterotoxin production. Many preservation methods can be considered to prevent the growth of this bacteria due to potential post-production contamination. Some of those methods can make the environment less favorable for bacterial growth, by changing the pH, water activity or chemical composition [99]. The use of biopreservation is emerging to control this bacterium. In 2020, bacteriocin BM1157 was confirmed to cause cell wall and DNA damage in *S. aureus.* This bacteriocin reduced positively charged hydrophilic groups on cell surface, caused an increase in cell hydrophobicity and leakage of cytoplasmic materials [105]. Lacticin 3147 was shown to be effective against *S. aureus* also [106]. Pediocin AcH and enterocin AS-48 bacteriocins were tested in milk and dairy products and confirmed to protect those products from *S. aureus* growth [107].

3.1.3 *Bacillus cereus*

B. cereus is a rod-shaped, facultatively aerobic, Gram-positive, beta-hemolytic, motile, spore-forming bacterium. The bacterium produces several toxins that are responsible

for foodborne illness. These include the enterotoxins hemolysin HBL, non-hemolytic enterotoxin Nhe, enterotoxin FM, pore-forming enterotoxins, and cytotoxin K, all of which are produced in the small intestine of the host. The emetic toxin cereulide is an enzymatically synthesized peptide that preforms in foods contaminated with *B. cereus* [58, 108].

This species has unique biochemical properties. It is nitrate reduction positive, catalase positive, lysozyme resistant, and it can decompose tyrosine [109]. Based on FDA recommendations, samples suspected to be contaminated with *B. cereus* should be collected and transported under refrigerated conditions at temperatures not higher than 6°C [109]. To characterize *B. cereus*, the microbiological lab should scan for enterotoxins (BHL and NHe), conduct a genotyping test, and explore the characteristic low-temperature growth profile [110]. Psychrotrophic *B. cereus* strains can grow to hazardous concentrations at refrigeration temperatures ≤ 8°C. This bacterium may survive mild heat treatments and continue its growth and produce the emetic toxin cereulide at colder temperatures [111].

B. cereus is commonly found in vegetation, soil, and foods. *B. cereus* cause two types of gastrointestinal illness: the emetic syndrome and the diarrheal syndrome. The diarrheal syndrome includes symptoms such as diarrhea, nausea and abdominal pain. It emerges after consuming *B. cereus*-contaminated foods such as sauces, vegetables, soups, dairy and meat products. Generally, the diarrheal syndrome result from the production of one or more of the *B. cereus* enterotoxins in the GI tract [108]. On the other hand, the emetic syndrome includes symptoms such as vomiting and nausea resulting from the consumption of *B. cereus* contaminated foods like pasta and rice. This condition develops due to the release of the *B. cereus*' emetic toxin cereulide in food [108].

Outbreaks of *B. cereus* are common in mass catering facilities. According to the CDC, over 36,000 cases of foodborne illness due to *B. cereus* were reported in Canada in 2006 [112]. *B. cereus* illness is usually under reported due to fast recovery. Infections of *B. cereus* are usually self-limiting [113].

3.1.3.1 *Bacillus cereus* Control and Prevention

B. cereus is prevalent in the environment; control measures should be focused on inhibiting its growth and the formation of emetic toxin in foods. Similar to other foodborne pathogens, to prevent *B. cereus* foodborne illness it is recommended to store foods at temperatures lower than 40°F or higher than 140°F if stored longer than 2 hours. It is also recommended to ensure that the temperature reaches at least 165 °F when reheating foods [114]. Pasteurization generally kills vegetative cells of *B. cereus*; however, spores can survive the process, which allow them to germinate and outgrow at room temperature. Hurdle technology is widely used to control spore forming bacteria. In recent studies, LAB with bacteriocins have been used as part of hurdle technology to control the growth of *B. cereus* in fresh cheese. When *Lac. lactis* ssp. *lactis* and *Lac. lactis* ssp. *cremoris* were added to cheese matrix at a high starting dosage, they were effective against *B. cereus* vegetative cells and spores [115]. Another study reported that the use of plantaricin GZ1-27 effectively inhibits *B. cereus* AS1 at the molecular level [116]. Spore forming bacteria such as *B. cereus* is hard to control using heat treatments. Use of other food preservation techniques such as biopreservation can be used to inhibit microbial growth and sporulation.

3.1.4 *Listeria monocytogenes*

L. monocytogenes is a Gram-positive, rod-shaped bacterium that is a non-spore-forming facultative anaerobe [117]. Foodborne listeriosis is an infection caused after ingestion of food containing live *L. monocytogenes* cells. *L. monocytogenes* produces select toxins as virulence factors. The most important toxin is listeriolysin O, a cytolytic toxin that makes *L. monocytogenes* a virulent bacterium [118].

This species has unique biochemical properties. It is catalase positive, nitrate negative, oxidase negative, rhamnose positive, mannitol negative, xylose negative, methyl-d-mannoside positive, methyl-red positive and Voges Proskauer positive [117]. The FDA BAM protocol recommends standard and rapid methods for the isolation and detection of *L. monocytogenes* from environmental and food samples [119]. The species and its strains may be characterized using such techniques as antimicrobial susceptibility testing, genotyping through pulsed-field gel electrophoresis and PCR [120].

L. monocytogenes is ubiquitous in the environment: water, soil, plants, decaying plants, feces of healthy animals, feces of healthy and ill humans [120]. The CDC reported that listeriosis is the third leading cause of death from foodborne illness with about 260 deaths per year. The FDA estimated that listeriosis has a high mortality rate of 20 to 30 percent [121]. In recent years, several *L. monocytogenes* outbreaks have been linked to fresh produce, with an average outbreak size of hundreds of illnesses. For example, in 2018, an outbreak connected to cantaloupe melons imported from Australia were observed in Malaysia, Kuwait, Japan, Oman, the United States and Singapore [122]. The latest outbreak in the United States was a multistate outbreak linked to enoki mushrooms with a total of 36 cases and 4 deaths [123].

L. monocytogenes is a pathogen that is commonly infective to the internal organs (liver, spleen, gallbladder, brain, and placenta) of animals and humans. Symptoms caused by *L. monocytogenes* include flu-like infection in otherwise healthy adults. Listeriosis is not a typical foodborne illness in that it can cause meningitis, meningoencephalitis, septicemia, and stillbirth/abortion in high risk groups: the elderly, newborns, pregnant women, and immunocompromised adults [121]. The treatment of severe *L. monocytogenes* infections involves the use of antibiotics, especially gentamicin with ampicillin administration [124].

3.1.4.1 *Listeria monocytogenes* Prevention

According to the CDC, there are some general recommendations to prevent listeriosis at home: cooking meat thoroughly, washing raw vegetables thoroughly, keeping uncooked meats separate from ready to eat foods, avoiding raw milk, and washing knives and cutting boards thoroughly after handling uncooked foods [125].

Government agencies and the food industry have taken steps to reduce contamination of food by *L. monocytogenes*. Due to the severity of *L. monocytogenes* infection, the United States Department of Agriculture (USDA) and FDA enforces a zero-tolerance policy in ready to eat food [125].

Biopreservation can be successfully used against *L. monocytogenes*. The inhibitory effects of the pediocin PA-1 as a biopreservative was confirmed against *L. monocytogenes* in various food systems including ready-to-eat food. Pediocin PA-1 and the pediocin-producing strain *Pediococcus acidilactici* MCH14 itself were investigated against *L. monocytogenes* on frankfurters and fermented sausage. The growth of *L. monocytogenes* was reduced 2 logs in

frankfurters and fermented sausage [126]. Another study tested the effect of enterocins synthesized by *Enterococcus avium* DSMZ17511. The enterocins were added on agar edible films and applied as antimicrobial coatings on cheese matrix inoculated with *L. monocytogenes.* Anti-listeria effect was reported in all cheese samples [127].

4. Summary

Kefir, a fermented dairy product that originated thousands of years ago, has been shown to be an effective antimicrobial agent against spoilage and pathogenic bacteria. Kefir's antimicrobial activity depends on many factors. The origin of kefir grains could play an important role on kefir's antimicrobial activity due to the variation of microbial composition based on geographical regions. Differences among antimicrobial activity among kefirs are observed even when their grains originate from the same geographical region. This could be attributed to differences in the type of milk used, fermentation time, temperature, and stirring conditions. The antimicrobials produced by kefir, including bacteriocins, organic acids, hydrogen peroxide, and fatty acids, determine kefir's antimicrobial activity. In the study discussed in Chapter 2, it was hypothesized that international artisanal kefirs originating from various regions have distinctive bacteriocin content resulting in different levels of antimicrobial activities against *B. cereus, L. monocytogenes, S. aureus* and *S. enterica*. The hypothesis was proven.

Kefir microbial community includes LAB, acetic acid bacteria, and yeast among other species in a symbiotic protein polysaccharide matrix. Both culture-dependent and culture-independent methods can be used to identify kefir microbiota. Culture-independent

methods are used for more accurate identification. Using culture-independent methods, kefir microbial community and the most abundant bacterial species in kefir have been explored. Kefir microbiota have been shown to vary based on geographical regions and the kefir grains used in fermentation. Select LAB isolated from kefir have been reported to have probiotic properties and/or bacteriocin producing capabilities. In Chapter 3, it was hypothesized that international artisanal kefirs have diverse microflora containing LAB with known bacteriocin production capabilities and/or health promoting properties. This hypothesis was proven.

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Chapter 2: Antimicrobial Activity of Six International Artisanal Kefirs Against *Bacillus cereus, Listeria monocytogenes, Salmonella enterica* serovar Enteritidis, and *Staphylococcus aureus*

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Antimicrobial Activity of Six International Artisanal Kefirs Against *Bacillus cereus, Listeria monocytogenes, Salmonella enterica* serovar Enteritidis, and *Staphylococcus aureus*

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Received: 29 April 2020; Accepted: 2 June 2020; Published: 4 June 2020

Abstract: Kefir, a fermented dairy beverage, exhibits antimicrobial activity due to many metabolic products, including bacteriocins, generated by lactic acid bacteria. In this study, the antimicrobial activities of artisanal kefir products from Fusion Tea (A), Britain (B), Ireland (I), Lithuania (L), the Caucasus region (C), and South Korea (K) were investigated against select foodborne pathogens. *Listeria monocytogenes* CWD 1198, *Salmonella enterica* serovar Enteritidis ATCC 13076, *Staphylococcus aureus* ATCC 25923, and *Bacillus cereus* ATCC 14579 were inhibited by artisanal kefirs made with kefir grains from diverse origins. Kefirs A, B, and I inhibited all bacterial indicator strains examined at varying levels, except *Escherichia coli* ATCC 12435 (non-pathogenic, negative control). Kefirs K, L, and C

inhibited all indicator strains, except *S. aureus* ATCC 25923 and *E. coli* ATCC 12435. Bacteriocins present in artisanal kefirs were determined to be the main antimicrobials in all kefirs examined. Kefir-based antimicrobials are being proposed as promising natural biopreservatives as per the results of the study.

Keywords: Artisanal kefir; kefir product; kefir grain; natural antimicrobial; bacteriocin; *Listeria monocytogenes*; *Salmonella enterica* serovar Enteritidis; *Staphylococcus aureus*; *Bacillus cereus*

1. Introduction

Foodborne illnesses represent a significant public health challenge worldwide, with almost 1 in 10 people becoming sick and 33 million people dying [1]. Foodborne pathogens also have a huge impact on the economy. According to the World Bank Organization, the total productivity loss related to foodborne disease in low- and middle-income countries is valued at $95.2 billion per year, in addition to the annual cost of $15 billion used to treat affected individuals [2].

The Center for Disease Control and Prevention (CDC) estimates numbers for foodborne illness each year in the United States at 47.8 million cases, with 128,000 hospitalizations, 3,030 deaths, and $78 billion in cost, including the costs attributed to premature deaths, medical expenses, and loss of productivity. Unspecified pathogens cause 80% of the illnesses (38.4 million illnesses, 72,000 hospitalizations, and 1,686 deaths) [3]. Known pathogens are responsible for 20% of the illnesses, estimated at 9.4 million illnesses, 59,961 hospitalizations, and 1,351 deaths [4]. Of these, 90% have been linked to just seven microorganisms: *Campylobacter* sp., *Clostridium perfringens, Escherichia coli* (Serotype O157:H7), *Listeria*

monocytogenes, Salmonella (non-typhoidal), norovirus, and *Toxoplasma gondii*. Non-typhoidal *Salmonella* was determined to be the principal cause of hospitalization and death among these seven pathogens [5].

Disability-adjusted life year (DALY) is a measure developed by the World Health Organization [1]. The DALY pools data on premature mortality and morbidity from acute illness and long-term sequelae into a single statistic, which in turn synopsizes years of healthy life lost. Scallan et al. [5] explored the overall impact of foodborne illness caused by the seven leading foodborne pathogens in the United States using DALY. They defined health states (acute illness and long-term sequelae) for each foodborne pathogen and then estimated the average annual incidence of each health state using data from public health surveillance and previously published estimates. These seven foodborne pathogens caused about 112,000 DALYs on an annual basis due to foodborne illnesses acquired in the United States. Non-typhoidal *Salmonella* (32,900) and *Toxoplasma gondii* (32,700) caused the most DALYs, trailed by *Campylobacter* sp. (22,500), norovirus (9,900), *L. monocytogenes* (8,800), *C. perfringens* (4,000), and *E. coli* O157:H7 (1,200). Among all foodborne pathogenic bacteria that can cause foodborne illness, non-typhoidal *Salmonella* and *L. monocytogenes* are responsible for 42,900 DALYs total (37% of all DALYs). These two organisms are included as target organisms in the presented work. As a foodborne pathogen, *Bacillus cereus* is estimated to cause 63,623 foodborne disease cases per year in the United States [4,6]. Staphylococcal food poisoning accounts for about 241,994 foodborne disease cases per year in the United States [6]. *B. cereus* and *S. aureus* are also included in our work because foodborne illness caused by these organisms are highly underreported and underdiagnosed [6].

Reducing foodborne illness takes a great deal of time, effort, and collaboration. The ultimate goal for most public health and food safety officials worldwide is not just stopping foodborne illness outbreaks once they occur but also preventing them from happening in the first place. Long-term prevention of foodborne illness outbreaks takes the actions of countless partners in the food production chain stretching from farm to table: production, harvest, storage, processing, distribution, and preparation. Chemical preservation, biologically based preservation, and physical methods of food preservation are all used, individually and in combination, to inhibit foodborne pathogens in food processing.

Biologically based preservation methods are among the newer and emerging forms of food preservation. According to Matthews et al., "biopreservation is the use of lactic acid bacteria (LAB), their metabolic products, or both to improve or ensure the safety and quality of products that are not fermented" [7,8]. Some LAB produce antimicrobial peptides called bacteriocins which inhibit foodborne pathogenic bacteria. Bacteriocins are ribosomally synthesized proteins or peptides that are secreted by bacteria that inhibit other closely related bacteria using various mechanisms [8,9]. Bacteriocins are divided into four major groups: class I, class II, class III, and class IV. The class I bacteriocins (lantibiotics) contain the first bacteriocin discovered from LAB, nisin [10]. Bacteriocins such as nisin are safe for human consumption since they are natural proteins and peptides that are degraded by the digestive enzymes in the stomach [11]. Nisin has "generally recognized as safe" (GRAS) status in the United States, granted by the United States Food and Drug Administration (FDA), for several applications in the food industry. It has been added as a food safety measure to a variety of foods in the world market, including dairy products, canned foods, salad dressings, sauces, and baby food. Nisin

is effective against Gram-positive pathogens such as *S. aureus*, *B. cereus*, *L. monocytogenes*, and *C. perfringens* [12,13].

Kefir, a fermented dairy beverage produced by the actions of the microflora encased in the "kefir grain" on the carbohydrates in the milk, originated thousands of years ago in the Caucasus mountain region between Europe and Russia. Containing many bacterial species already known for their probiotic properties, it has long been popular in Eastern Europe for its purported health benefits, where it is routinely administered to patients in hospitals and recommended for infants and the infirm. More than 30–50 species of yeasts (*Saccharomyces* sp., *Kluyveromyces* sp., *Candida* sp., *Torulaspora* sp., *Cryptococcus* sp., *Pichia* sp., etc.) and LAB (*Lactobacillus* sp., *Lactococcus* sp., *Leuconostoc* sp., etc.) have been isolated and identified from kefir grains [14,15]. Kefir grains have been shown to have regional differences in microbial composition, producing variability in the kefirs produced, due in part to local LAB finding a niche in the grains [16].

Kefir has been shown to contain a variety of natural antimicrobials, including bacteriocins, organic acids, hydrogen peroxide, and fatty acids. A Brazilian kefir product showed growth inhibition, measured as percent inhibition, against *S. aureus* ATCC 6538 (42.80%–69.15%), *E. coli* ATCC 11229 (30.73%–59.89%), *S. typhi* ATCC 6539 (44.99%–73.05%), *L. monocytogenes* ATCC 15313 (41.45%–54.18%) and *B. cereus* RIBO 1222-173-S4 (70.38%–86.80%) [17]. Another study investigated the antimicrobial activity of a Romanian kefir product against *B. subtilis* spp. *spizizenii* ATCC 6633, *S. aureus* ATCC 6538, *E. coli* ATCC 8739, *Enterococcus faecalis* ATCC 29212 and *S. enteritidis* ATCC 13076. The Romanian kefir showed strong antibacterial activity against Gram-negative and Gram-positive indicator strains when compared to neomycin sulfate and ampicillin [18]. The antimicrobial spectra of four

types of kefirs (A, L, M, and S) from South Korea were determined in another study. With kefir A, *B. cereus* ATCC 14579, *E. coli* ATCC 25922, *S. enterica* serovar Enteritidis FDA, *Pseudomonas aeruginosa* ATCC 15522, and *Cronobacter sakazakii* ATCC 29544 were inhibited. *B. cereus* ATCC 14579, *S. aureus* ATCC 6538, *E. coli* ATCC 25922, *S. enterica* serovar Enteritidis FDA, *P. aeruginosa* ATCC 15522, and *C. sakazakii* ATCC 29544 were inhibited to different extents by kefirs L, M, and S. *L. monocytogenes* ATCC 51776 was only inhibited by kefir M [19].

To our knowledge, comparisons among international artisanal kefirs regarding their antimicrobial activities against select foodborne pathogens have not been reported. For this study, we hypothesized that international artisanal kefirs have diverse microflora, generating distinctive bacteriocin content, resulting in varied levels of antimicrobial activities. The objectives of our study were to 1) compare the antimicrobial activity of artisanal kefirs from Fusion Tea (A), Britain (B), the Caucasus region (C), Ireland (I), Lithuania (L), and South Korea (K) against select foodborne pathogens, and 2) examine whether the antimicrobial effect is due to bacteriocin production or other antimicrobials present in kefir.

2. Materials and Methods

2.1. Artisanal Kefir Preparation for Determining Kefir Antimicrobial Activity

Six types of artisanal kefir grains originating from Britain (B; Etsy Inc., Brooklyn, New York, NY, USA, Seattle, WA, USA), the Caucasus (C; Etsy Inc., Brooklyn, New York, NY, USA, Seattle, WA, USA), Ireland (I; Etsy Inc., Brooklyn, New York, NY, USA, Seattle, WA, USA), Lithuania (L; Etsy Inc., Brooklyn, New York, NY, USA, Seattle, WA, USA), South

Korea (K) [19], and a compilation of blended world-sourced grains (A; Fusion Tea, Amazon, Seattle, WA, USA) were used in this study. Kefir grains were examined to evaluate their similarities and differences in shape, appearance, texture, and size while kefir products were evaluated for their flavor, aroma, and texture.

Artisanal kefir products were prepared using traditional methods. Kefir grains were inoculated into pasteurized whole milk daily for at least one week before any experiment. Kefir grains were inoculated (10% (w/v)) into whole pasteurized milk and the resulting mixture was incubated at 22–24 °C for 24 h. The fermentation process was stopped when the pH reached 3.9–4.1 as measured by a calibrated PB Basic Meter (Denver Instrument, Bohemia, NY, USA). A clean plastic strainer with 1 mm pore size was used to separate the grains from kefir products. Designated plastic strainers were used for each kefir product/grains to avoid cross-contamination. Once separated, the kefir grains were inoculated (10% (w/v)) into a new batch of whole pasteurized milk to maintain their activity. The resulting kefir products were centrifuged at 10,000 *g* for 15 min at 4 °C using an Avanti J-17 high-speed centrifuge (Beckman Coulter, Inc., Palo Alto, CA, USA) to remove solids. Filtration through a Millex filter (Millipore Corporation, Bedford, MA, USA) with 45 µm pore size was used to sterilize the samples. The filter-sterilized kefir samples were tested for their antimicrobial activities immediately.

2.2. Protein Concentration Measurements

The total protein concentration was used as a measure to standardize artisanal kefir samples. A Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) was used to determine the total protein concentration in kefir samples. A standard procedure for

microtiter plates was used with bovine serum albumin (standard II). Absorbance was measured at 595 nm using a microtiter plate reader (SpectraMax 190, Molecular Devices, San Jose, CA, USA). Kefir samples were examined in triplicate to determine the total protein concentration.

2.3. Bacterial Strains, Microbiological Media, and Growth Conditions

Bacterial strains used in the study were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA), National Collection of Dairy Organisms (NCDO; now National Collection of Food Bacteria (NCFB); Scotland) and our in-house culture collection. *Lactobacillus plantarum* NCDO 995 and *Micrococcus luteus* ATCC 10420 were used as the non-pathogenic indicator strains. *M. luteus* was selected for its sensitivity to bacteriocins [20]. *Lb. plantarum* is also sensitive to bacteriocins produced by closely related LAB [11]. *E. coli* ATCC 12435 is not sensitive to bacteriocins from LAB and thus was used as a negative control. The pathogenic indicator strains included *S. enterica* serovar Enteritis ATCC 13076, *S. aureus* ATCC 25923, *B. cereus* ATCC 14579, and *L. monocytogenes* CWD 1198. Frozen stocks were maintained in sterile glycerol (25%) and tryptic soy broth (TSB, Criterion, Hardy Diagnostics, Santa Maria, CA, USA) or Lactobacillus MRS broth (Remel, Thermo Fisher Scientific, Lenexa, KS, USA) and kept at –80 °C. Prior to experiments, all indicator organisms were streaked onto tryptic soy agar (TSA) or Lactobacillus MRS agar (for *Lb. plantarum* NCDO 995) and incubated at their optimum growth temperature. The following selective media were used to streak the pathogenic indicators for isolation as needed: Xylose Lysine Deoxycholate agar (XLD) for *S. enterica* serovar Enteritis ATCC 13076, Mannitol Yolk Polymyxin (MYP) agar for *B. cereus* ATCC 14579, Baird Parker agar (BPA) for *S. aureus* ATCC 25923, and HardyCHROM Listeria for *L. monocytogenes*. XLD, MYP, BPA, and HardyCHROM Listeria

were obtained from Hardy Diagnostics (Santa Maria, CA, USA). The resulting single colonies on selective media were picked and inoculated into TSB and incubated for 24 h. The incubation temperature used for *Lb. plantarum* NCDO 995, *S. enterica* serovar Enteritis ATCC 13076, *S. aureus* ATCC 25923, and *L. monocytogenes* CWD 1198 was 35–37 °C. *B. cereus* ATCC 14579 and *M. luteus* ATCC 10420 cultures were incubated at 30 °C. *M. luteus* ATCC 10420 was incubated while shaking at 200 rpm in an orbital shaking incubator.

2.4. Detection of Antimicrobial Activity in Artisanal Kefirs

The agar-well-diffusion method [20] with some modifications was used to study the antimicrobial activity of filter-sterilized artisanal kefir samples. Soft Lactobacillus MRS and TSA, containing 0.75% agar, were inoculated (10^7–10^8 CFU/mL) with the indicator (non-pathogenic and pathogenic) organisms. Bacterial growth curves, generated for all indicator organisms, were used for determining accurate inoculum levels. Filter-sterilized kefir samples were obtained as described above. The wells generated with sterile plastic Pasteur pipettes in soft agar were filled with 100, 150, 200, and 250 µL of filter-sterilized kefir samples. Nisin (1000 IU/mg) and polylysine (1200 IU/mg), purchased from Zhengzhou Bainafo Bioengineering Co., Ltd. (Zhengzhou City, China), were used as positive bacteriocin controls at 220 IU and 200 IU, respectively, and in a volume of 100 µL. Sterilized distilled water (DI) was used as a negative control, also in a volume of 100 µL. Lactobacillus MRS and TSA plates with wells containing filter-sterilized kefir samples, bacteriocin controls, and sterile DI water were incubated for 24 h at 30 °C or 37 °C depending on the optimum growth temperature of the indicator strains. Following the incubation, the diameter of clear zones was measured (in

mm) using a ruler. Two independent experiments, each in duplicate, were performed for any given kefir sample.

2.5. Ruling-Out Any Antimicrobial Activity Due to Organic Acids, Hydrogen Peroxide, and Free Fatty Acids Produced in Artisanal Kefir

2.5.1. Artisanal Kefir Preparation

The Fusion Tea, Irish, and South Korean kefirs (A, I, and K) were selected for this study due to their high antibacterial activity observed in experiments using the agar-well-diffusion method. Artisanal kefir samples were prepared as mentioned above (2.1).

2.5.2. Bacterial Strains, Microbiological Media, and Growth Conditions

Bacterial strains, microbiological media, and growth conditions were the same as described above.

2.5.3. Detection of Antimicrobial Activity Due to Bacteriocin Production in Artisanal Kefir

The agar-well-diffusion method was used to detect antimicrobial activity due to bacteriocins present in artisanal kefir samples following the protocol by Dimitrieva-Moats and Ünlü (2011) and Ünlü et al. (2015) [20,21] with some modifications. Artisanal kefir samples were prepared as mentioned above, filter sterilized, and pH adjusted to 6.0 using NaOH (5M). Sterile bovine liver catalase (2000–5000 U/mg protein), *Aspergillus oryzae* lipase (≥ 100,000

U/g), and Proteinase K from *Tritirachium album* (≥ 30 units/mg), all of which were purchased from Sigma Aldrich (Saint Louis, MO, USA), were added to sterile kefir samples A, K, and I at a final concentration of 1 mg/mL. β-glycerophosphate (Sigma Aldrich, Saint Louis, MO, USA) was added to sterile kefir samples A, K, and I at 1% (w/v). These mixtures were incubated at 37 °C for 1 h. Sterile β-glycerophosphate was used to buffer artisanal kefir samples with pH adjusted to 6.0. Bovine liver catalase and lipase from *Aspergillus oryzae* were used to degrade hydrogen peroxide and free fatty acids, respectively. Proteinase K from *Tritirachium album* (≥ 30 units/mg) was used to break down bacteriocins, which are proteinaceous, and thus confirm their contribution to antimicrobial activity in kefir. Indicator strains (100 µl of cultures containing 10^8–10^9 CFU/mL) were spread onto soft TSA agar (0.75 % agar (w/v)). Several wells with a diameter of 7 mm were formed with sterile plastic Pasteur pipettes and the filter-sterilized kefir samples (100 µL), with and without treatments, were added to the wells. Nisin and polylysine were used as positive bacteriocin controls at 220 IU and 200 IU, respectively, and in a volume of 100 µl. Sterilized distilled water (DI) was used as a negative control in the volume of 100 µl. Plates were incubated overnight at 30 °C or 37 °C depending on the indicator strain. The diameter (in mm) of clear zones was measured using a ruler. Two independent experiments, each in duplicate, were performed for any given kefir sample.

2.6. Statistical Analysis

For the detection of the antimicrobial activity in artisanal kefirs, the experiment was a completely random design. A three-way ANOVA was used for the following: indicator organisms, kefir types, kefir volumes and their interactions, followed by Tukey's multiple comparison procedure using R (R Studio Inc., Boston, MA, USA). For ruling out any

antimicrobial activity due to organic acids, hydrogen peroxide and free fatty acids produced in kefir, a three-way ANOVA was used as well, followed by Tukey's multiple comparison procedure using R. The statistically significant difference was determined by cut-off for significance level at 5% (i.e., $p < 0.05$).

3. Results

3.1. Artisanal Kefir Products and Kefir Grains Description

Artisanal kefir products and grains were described in Table 1. The kefir grains sizes ranged from >1 mm to 50 mm depending on the origin. The smallest grains were from Ireland while the largest grains were from Britain. Kefir products differed in their flavor and aroma, as indicated in Table 2.

Table 2: Artisanal kefir origin, source, grains' description and products' description.

Kefir Origin	Source	Grains' Description	Products' Description
Lithuania	Etsy Inc.	Cauliflower-like appearance, off-white to pale yellow, medium size (1–10 mm) and firm grains	Mild, smooth, and not sour (sweet)
Ireland	Etsy Inc.	Soft, small size (>1 mm) grains	Mild, sweet and pleasant taste, smooth,

			sweet aroma, fresh, and cheesy
The Caucasus region	Etsy Inc.	Cauliflower-like appearance, off-white to pale yellow, size 2–10 mm, firm, rubbery with smooth grains	Earthy, cheesy aroma, and sour taste
South Korea	[19]	Soft, curling, size 2–10 mm	Earthy, cheesy aroma, and sour taste
Britain	Etsy Inc.	Cauliflower-like appearance, small to large size (2.5–50 mm), rubbery, firm, smooth grains	Creamy, earthy, cheesy aroma, slightly sour
Fusion Tea	Amazon	Cauliflower-like appearance, off-white to pale yellow, mixed sizes (2–7 mm), firm, rubbery textured grains	Smooth, mild sour, creamy, pleasant, and fresh, sweet, yeasty aroma

3.2. The Antimicrobial Activity Spectra of Filter-Sterilized Artisanal Kefirs

3.2.1. Protein Concentration

All artisanal kefir samples tested were standardized based on total protein content (0.5 mg/mL kefir).

3.2.2. The Antimicrobial Activity Spectra of Filter-Sterilized Artisanal Kefirs Determined by the Agar Well Diffusion Method

As anticipated, nisin and polylysine controls inhibited the growth of all pathogenic and non-pathogenic indicator organisms excluding *E. coli* ATCC 12435, a non-pathogenic indicator organism used as the negative control (Figure 1a–g). Nisin showed the largest inhibition zone (33 mm) against *Lb. plantarum* NCDO 995 (Figure 1b). Polylysine showed the largest inhibition zone (17 mm) against *M. luteus* ATCC 10420 (Figure 1c).

E. coli ATCC 12435, the non-pathogenic indicator organism used as the negative control, was not sensitive to any artisanal kefirs (Figure 1a). *M. luteus* and *Lb. plantarum*, non-pathogenic indicators, were sensitive to nisin, polylysine, and all artisanal kefirs (A, B, C, I, K, and L) used at a range of volumes (100–250 µL) (Figure 1b–c). *Lb. plantarum* inhibition zones observed with artisanal kefirs (250 µL) were 8.5–16 mm (Figure 1b). *M. luteus* inhibition zones observed with artisanal kefirs (250 µL) were 14–20.5 mm (Figure 1c). Results obtained with non-pathogenic indicators confirmed that experiments employing the agar well diffusion method worked well.

All artisanal kefirs, A, B, C, I, K, and L, showed significant ($p < 0.002$) antimicrobial activity against pathogenic indicators (Figure 1d–g). Kefirs A, B, and I inhibited all pathogenic indicators at different levels ($p < 0.001$) (Figure 1d–g). Kefirs C, K, and L inhibited all pathogenic indicators (Figure 1d–g), except *S. aureus* ATCC 25923 (Figure 1f), which was inhibited by kefirs A, B, and I, compared to the negative control *E. coli* ATCC 12435 ($p < 0.05$). *S. aureus* ATCC 25923 displayed inhibition zones with increasing kefir volumes (150–250 µL) with kefirs A, B, and I (Figure 1f). *S. enterica* serovar Enteritis ATCC 13076 was inhibited by all kefir types ($p < 0.05$), with inhibition zones of 7–10 mm at 250 µL volume. *L.*

monocytogenes CWD 1198 was inhibited the most by kefir I, K, and B ($p < 0.05$), with an inhibition zone of 12.5–14 mm, and at a volume of 250 µL (Figure 1e). The antilisterial effect of kefir I and kefir K (used at 250 µL) was equal to the antilisterial activity of nisin (100 µL = 220 IU) (Figure 1e). *L. monocytogenes* CWD 1198 displayed increasing inhibition zones with increasing kefir volumes (150–250 µL) with kefirs A, B, C, I, and K but not kefir L (Figure 1e). With kefir L, *L. monocytogenes* CWD 1198 inhibition zone was constant (7 mm) regardless of the kefir L volume applied (Figure 1e). *B. cereus* showed the same inhibition zone (7 mm) with all artisanal kefirs, regardless of the kefir volumes used (100–250 µL), and with both positive bacteriocin controls (100 µL each) (Figure 1d).

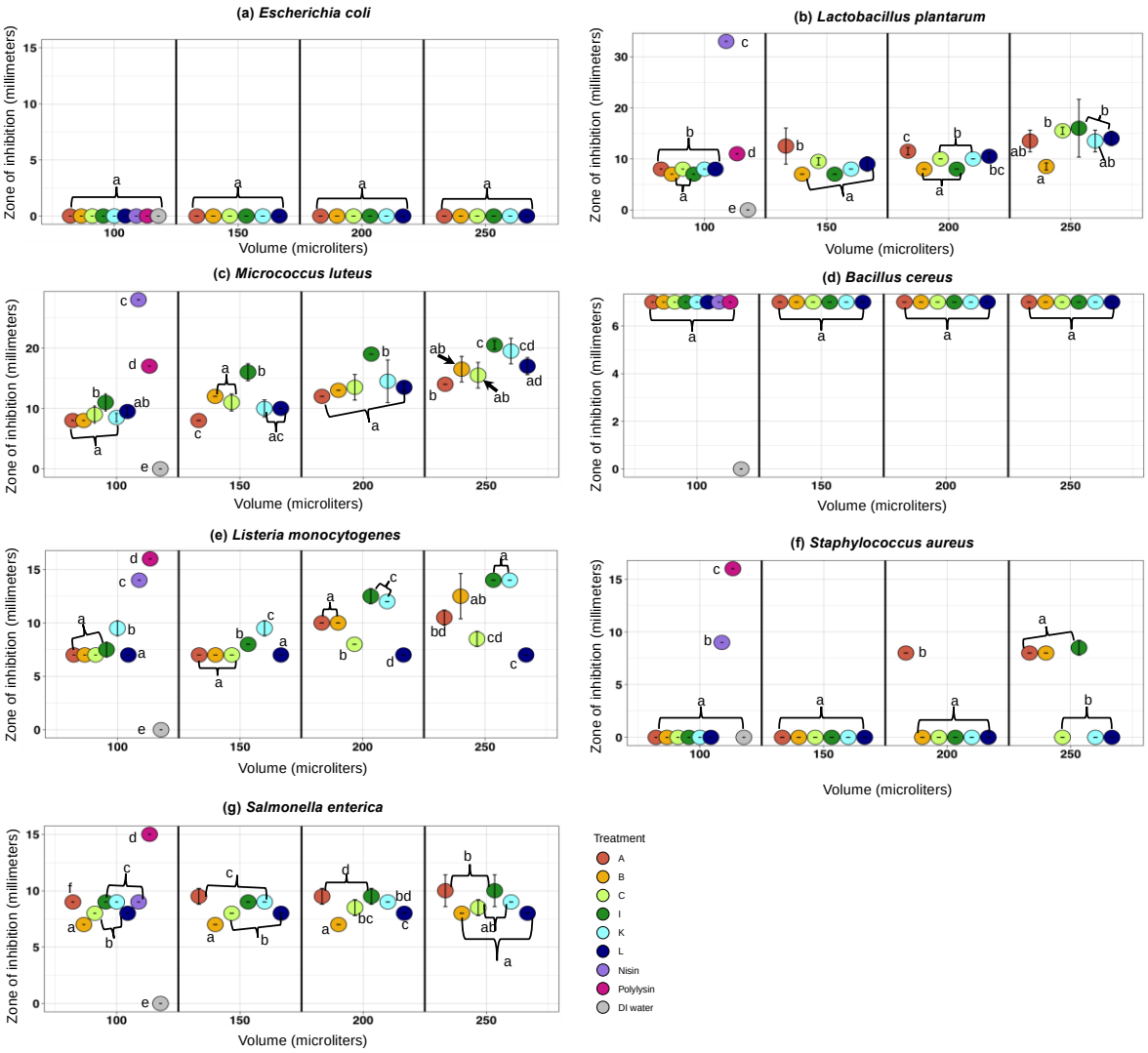

Figure 1: Comparison of the antimicrobial activity spectra of artisanal kefirs from Fusion Tea (A, Amazon); Britain (B); the Caucasus region (C); Ireland (I); South Korea (K); and Lithuania (L) against (**a**) *Escherichia coli* ATCC 12435, (**b**) *Lactobacillus plantarum* NCDO 995, (**c**) *Micrococcus luteus* ATCC 10420, (**d**) *Bacillus cereus* ATCC 14579, (**e**) *Listeria monocytogenes* CWD 1198, (**f**) *Staphylococcus aureus* ATCC 25923, and (**g**) *Salmonella enterica* serovar Enteritidis ATCC 13076 using the agar well diffusion method. A range of filter-sterilized artisanal kefir volumes (100 µL, 150 µL, 200 µL, and 250 µL) were placed inside the wells. Nisin (N; 220 IU) and polylysin (P; 200 IU) were used as positive controls in

a volume of 100 µL. Sterilized DI water (100 µL) was used as a negative control. The diameter of the inhibition zones was measured in mm. All experiments were conducted two independent times and each time in duplicate. Different letters (a–f) indicate statistical pairwise comparisons between the treatments within each volume performed by post-hoc Tukey's multiple comparison procedure. The same letter indicates no significant difference between the treatments within each volume.

3.3. Ruling-Out Any Antimicrobial Activity Due to Organic Acids, Hydrogen Peroxide, and Free Fatty Acids Produced in Artisanal Kefir

LAB are known to produce antibacterial metabolites, including bacteriocins, organic acids, H_2O_2, and fatty acids. Application of filter-sterilized artisanal kefir samples treated with proteinase K from *Tritirachium album*, β-glycerophosphate, bovine liver catalase, and lipase from *Aspergillus oryzae* in agar well diffusion experiments allowed us to rule out any antimicrobial activity due to these metabolites produced by LAB in kefir.

As anticipated, *E. coli* ATCC 12435, a non-pathogenic organism used as a negative control, was not sensitive to any artisanal kefir (A, I, and K), untreated or treated with proteinase K, β-glycerophosphate, catalase, and lipase. (Figure 2a–c). This confirms the antimicrobial activity results obtained for kefirs A, I, and K described in Section 3.2.2.

No bacterial inhibition zones were observed with *M. luteus* or any pathogenic indicators when proteinase K-treated kefirs A, I, and K were used ($p < 0.001$), confirming that the majority of the antibacterial activity observed was due to bacteriocins with proteinaceous nature (Figure 2a–c). As expected, proteinase K-treated nisin and polylysin, used as bacteriocin controls, did not show any inhibitory activity against *M. luteus* or any pathogenic indicators.

M. luteus, a non-pathogenic organism used as a positive control, was sensitive to all untreated kefirs (A, I, and K) and all catalase-, lipase-, and β-glycerophosphate-treated kefirs (A, I, and K) (Figure 2a–c). *M. luteus* inhibition zones observed with untreated kefir A were 9.5 mm while the catalase-, lipase-, and β-glycerophosphate-treated kefir A samples were 8, 8, and 11 mm, respectively, indicating that both H_2O_2 and free fatty acids made contributions to total antimicrobial activity (Figure 2a). The increase in antimicrobial activity with β-glycerophosphate-treated kefir A can be explained by an increase in bacteriocin(s) activity at the higher pH achieved with β-glycerophosphate addition to kefir A. While untreated kefir I resulted in an inhibition zone of 10.5 mm for *M. luteus*, the catalase-, lipase-, and β-glycerophosphate-treated kefir I samples resulted in an inhibition zone of 9.5 mm, which was not a significant difference ($p > 0.05$) (Figure 2b). *M. luteus* was inhibited by untreated kefir K, with an inhibition zone of 10 mm, as well as the catalase-, lipase-, and β-glycerophosphate-treated kefir K samples with inhibition zones of 8.5, 8, and 8 mm, respectively. These results show a very similar contribution to the total antimicrobial activity by H_2O_2, free fatty acids, and organic acids (Figure 2c).

The untreated and the catalase-, lipase-, and β-glycerophosphate-treated kefir A samples resulted in the inhibition zones of 8 and 7 mm for *S. aureus* and *B. cereus*, respectively, indicating that the bacteriocin activity is responsible for the antimicrobial activity against the two pathogenic indicator organisms (Figure 2a). While the untreated kefir A sample resulted in an inhibition zone of 10.5 mm against *L. monocytogenes*, the catalase-, lipase-, and β-glycerophosphate-treated kefirs resulted in the inhibition zones of 8, 8, and 11.5 mm, respectively (Figure 2a). These observations indicate that *L. monocytogenes* was inhibited mostly by bacteriocins in kefir A, but organic acids, free fatty acids, and H_2O_2 made

contributions to the total inhibition. The individual contributions of free fatty acids and H_2O_2 to the total antimicrobial activity in kefir A were identical as per these results.

The bacteriocin activity in Kefir I was solely responsible for the antimicrobial activity observed against *B. cereus* (Figure 2b). Because, the untreated and the catalase-, lipase-, and β-glycerophosphate-treated kefir I samples resulted in the identical inhibition zones of 7 mm against *B. cereus.* In the case of *S. aureus*, the catalase-, lipase-, and β-glycerophosphate-treated kefir I samples resulted in the inhibition zone of 8 mm, which is 1 mm less than the inhibition zone (9mm) observed with untreated kefir I (Figure 2b). With *L. monocytogenes*, the catalase-, lipase-, and β-glycerophosphate-treated kefir I samples resulted in the inhibition zones of 7.5–8 mm, which are smaller than the inhibition zone (10 mm) observed with untreated kefir I (Figure 2b). These results indicated that organic acids, free fatty acids, and H_2O_2 made identical contributions to the total inhibition of the two pathogens. An identical inhibition zone (8 mm) was observed with *S. enterica* serovar Enteritidis when using the untreated and β-glycerophosphate-treated kefir I, indicating that the total antimicrobial activity is due to bacteriocins in kefir I (Figure 2b). The *S. enterica* serovar Enteritidis inhibition zones of 8.25 and 8.75 mm, observed with the lipase- and catalase-treated kefir I respectively, were slightly larger than the 8 mm zone observed with untreated kefir (Figure 2b). The difference was not statistically significant.

The untreated and the catalase-, lipase-, and β-glycerophosphate-treated kefir K samples resulted in the inhibition zones of 8 and 7 mm for *S. enterica* serovar Enteritidis and *B. cereus,* respectively, illustrating that the bacteriocin activity in kefir K is solely responsible for the total antimicrobial activity against these two pathogens (Figure 2c). For *L. monocytogenes,* an inhibition zone of 8 mm was observed with the catalase-, lipase-, and β-glycerophosphate-

treated kefir K samples while the untreated kefir K sample resulted in an inhibition zone of 9 mm (Figure 2c). Based on the results, the individual contribution of organic acids, free fatty acids, and H_2O_2 to total antimicrobial activity is identical. The untreated and the catalase-, lipase-, and β-glycerophosphate-treated kefir K samples did not exhibit any anti-staphylococcal activity (Figure 2c), confirming the results described for kefir K in Section 3.2.2.

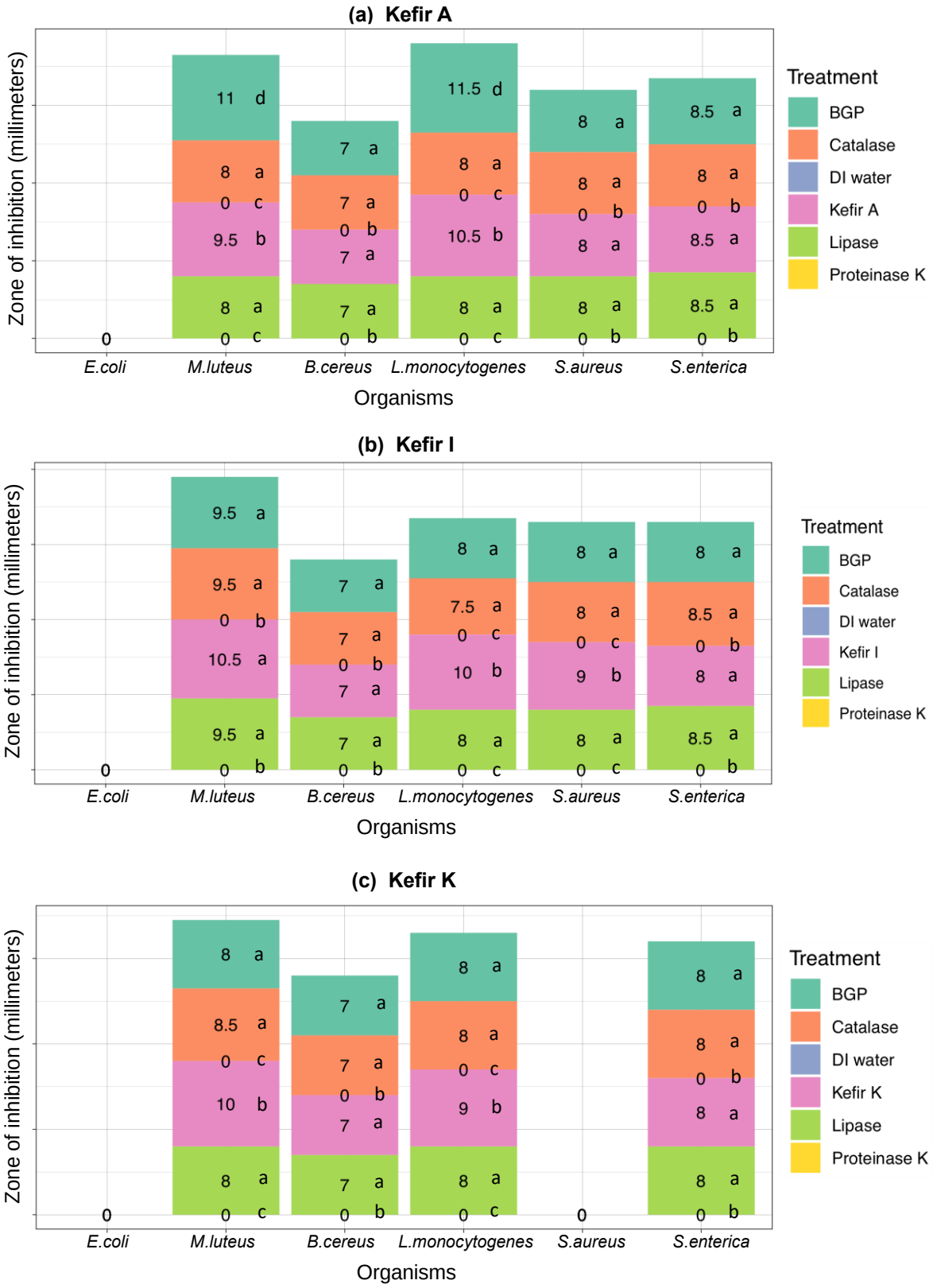
(a) Kefir A
Zone of inhibition (millimeters)
11 d
8 a
0 c
9.5 b
8 a
0 c
0
7 a
7 a
0 b
7 a
7 a
0 b
11.5 d
8 a
0 c
10.5 b
8 a
0 c
8 a
8 a
0 b
8 a
8 a
0 b
8.5 a
8 a
0 b
8.5 a
8.5 a
0 b
E.coli
M.luteus
B.cereus
L.monocytogenes
S.aureus
S.enterica
Organisms
Treatment
BGP
Catalase
DI water
Kefir A
Lipase
Proteinase K
(b) Kefir I
Zone of inhibition (millimeters)
9.5 a
9.5 a
0 b
10.5 a
9.5 a
0 b
0
7 a
7 a
0 b
7 a
7 a
0 b
8 a
7.5 a
0 c
10 b
8 a
0 c
8 a
8 a
0 c
9 b
8 a
0 c
8 a
8.5 a
0 b
8 a
8.5 a
0 b
E.coli
M.luteus
B.cereus
L.monocytogenes
S.aureus
S.enterica
Organisms
Treatment
BGP
Catalase
DI water
Kefir I
Lipase
Proteinase K
(c) Kefir K
Zone of inhibition (millimeters)
8 a
8.5 a
0 c
10 b
8 a
0 c
0
7 a
7 a
0 b
7 a
7 a
0 b
8 a
8 a
0 c
9 b
8 a
0 c
0
8 a
8 a
0 b
8 a
8 a
0 b
E.coli
M.luteus
B.cereus
L.monocytogenes
S.aureus
S.enterica
Organisms
Treatment
BGP
Catalase
DI water
Kefir K
Lipase
Proteinase K

Figure 2: Bacteriocin-based antimicrobial activity in filter-sterilized artisanal kefir samples from (**a**) Fusion Tea (Kefir A), (**b**) Ireland (Kefir I), and (**c**) South Korea (Kefir K) against select indicator strains using β-glycerophosphate (BGP, teal), bovine liver catalase (coral), lipase from *Aspergillus oryzae* (lime green), and proteinase K from *Tritirachium album* (yellow). The agar well diffusion method was used. Sterile DI water (blue) was used as the negative control. Wells generated in each plate contained filter-sterilized artisanal kefir product (100 µL) with or without the additives mentioned above. The diameter of the inhibition zones was measured in mm after incubation for 24h at 37 or 30 °C. Different letters (a–c) indicate statistical pairwise comparisons between the treatments for a given organism performed by post-hoc Tukey's multiple comparison procedure. The same letter indicates no significant difference between treatments for a given organism.

4. Discussion

In the presented work, we explored the antimicrobial activity of international artisanal kefirs from Fusion Tea, Britain, the Caucasus region, Ireland, Lithuania, and South Korea and made comparisons among these kefirs regarding their antimicrobial activity. Based on our findings, antimicrobials with proteinaceous nature (e.g., bacteriocins) are responsible for the majority of antibacterial activity observed against the foodborne pathogens tested. To our knowledge, this is the first report in the literature that deals with such comparisons among international artisanal kefirs.

Our work targeted select foodborne pathogens, including *L. monocytogenes*, *S. enterica* serovar Enteritidis, *S. aureus*, and *B. cereus*, all known to be hazardous to human health. One of these pathogens, *L. monocytogenes*, is considered to be a major challenge for the food

industry worldwide. In particular, refrigerated, ready-to-eat (RTE) foods pose a high listeriosis risk because refrigeration offers an environment in which *L. monocytogenes* can outcompete other mesophilic microorganisms. Another target pathogen, *B. cereus*, especially psychrotrophic strains, are problematic in dairy foods for various reasons: (1) *B. cereus* readily spread from healthy and decaying plants and soil to the cows and raw milk; (2) hydrophobic *B. cereus* spores attach to surfaces in dairy plants; *B. cereus* spores survive milk pasteurization, germinate in the absence of competitive microflora, and cause problems in milk products. Therefore, additional food preservation approaches need to be in place to ensure the safety of refrigerated foods. Biologically based preservation, through the use of LAB, can be used to enhance microbial food safety of refrigerated foods without modifying them. LAB are accepted by many countries in the world as "GRAS". LAB are also perceived by consumers in the world as "natural," "healthy," and "health-promoting".

LAB have been shown to produce bacteriocins that inhibit foodborne pathogens such as *L. monocytogenes* [22]. Kefir contains many LAB species known for their bacteriocin production and probiotic benefits [16,23,24]. Joao et al. tested kefir products from Brazil against *L. monocytogenes* ATCC 15313 and found 54.18% inhibition when compared to that of the untreated control [17]. Our research findings are in agreement with that of Joao et al. in that kefir has antilisterial activity. In our work, all artisanal kefirs (A, B, C, I, K, and L) exhibited varying levels of inhibition against *L. monocytogenes*.

Artisanal kefir has been shown to exhibit various antimicrobial activities against foodborne pathogens and spoilage organisms [17–19]. Coconut milk inoculated with kefir grains from India showed antimicrobial and antifungal activity against *E. coli*, *S. typhi*, *S. aureus*, *Saccharomyces cerevisiae,* and *Aspergillus niger* [25]. Kefir originating from

Argentina inhibited the growth of *E. coli* ATCC 11229, *S. enterica* serovar Enteritidis CIDCA 101, and *B. cereus* ATCC 10876 [26]. In our study, artisanal kefirs originating from six different regions of the world showed antimicrobial activity against select foodborne pathogens. Kim et al. compared the antimicrobial spectra of four types of kefirs from South Korea, which showed inhibition against select strains of *B. cereus*, *S. enterica* serovar Enteritidis, *P. aeruginosa*, *C. sakazakii*, and *L. monocytogenes* [19]. We showed that the same South Korean kefir (K) inhibited all pathogenic indicator strains tested except *S. aureus* ($p < 0.05$). Our study supports the findings of Kim et al. in that South Korean kefir has a wide antimicrobial activity spectrum [19]. When compared to other kefirs in this study, the kefir from South Korea (K) showed the highest antimicrobial activity against *L. monocytogenes* when using the smallest volume (100 ul) of kefir product. The kefirs from Ireland, Britain, and Fusion Tea inhibited *S. aureus* ($p < 0.05$), in contrast to the South Korean kefir.

In our study, kefir K exhibited antimicrobial activity against all foodborne pathogenic indicators, except *S. aureus*. Consequently, our goal is to carry out additional research on kefir K with emphasis on isolation and characterization of LAB and their bacteriocins and application of these bacteriocins as natural antimicrobials against *L. monocytogenes*, *B. cereus*, and *S. enterica*. In addition, kefir A and I showed the highest inhibition zones against *S. aureus*. Therefore, we are interested in further studying kefir A and kefir I as sources of natural antimicrobials against *S. aureus.*

5. Conclusions

Consumers demand natural, health-promoting, and nutritious food. Chemical additives have been extensively used in food preservation but their safety and impact on human health

continue to be under discussion. The food industry desires to replace chemical preservatives with natural biopreservatives. Kefir has a natural antimicrobial activity due to the presence of LAB with bacteriocin production capability. In this study of artisanal kefirs from different countries, we have elucidated that bacteriocin production is the main reason for these kefirs' antimicrobial activity against select foodborne pathogens. Based on our findings in this study, kefir-based antimicrobials are being explored in our laboratory as promising natural biopreservatives in model food systems.

Author Contributions: Conceptualization, G.Ü.; Methodology, A.S., M.B.B., B.N., and G.Ü; Formal Analysis, A.S., M.B.B., and G.Ü; Investigation, A.S., B.N., and G.Ü; Resources, A.S., M.B.B., B.N., and G.Ü; Writing—Original Draft Preparation, A.S.; Writing—Review and Editing, A.S., M.B.B., B.N., and G.Ü; Supervision, G.Ü; Project Administration, G.Ü.; Funding Acquisition, A.S. and G.Ü.; All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the United States Department of Agriculture (USDA) National Institute of Food and Agriculture (NIFA) Hatch project [1015890)] and the BUILD Dairy Program of Utah State University (17597). The APC was funded by the BUILD Dairy Program of Utah State University (17597).

Acknowledgments: We thank Saudi Arabian Cultural Mission for their support of Abrar Sindi. We thank Glenn Grout and Samantha Mamer for their assistance in the lab. We express our appreciation to Mete Yüksel for his assistance with the figures.

Conflicts of Interest: The authors declare no conflict of interest. The sponsors had no role in the design, execution, interpretation, or writing of the study.

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Chapter 3: Bacterial Populations in International Artisanal Kefirs

microorganisms

MDPI

Article

Bacterial Populations in International Artisanal Kefirs

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Received: 18 July 2020; Accepted: 27 August 2020; Published: 29 August 2020

Abstract: Artisanal kefir is a traditional fermented dairy product made using kefir grains. Kefir has documented natural antimicrobial activity and health benefits. A typical kefir microbial community includes lactic acid bacteria (LAB), acetic acid bacteria, and yeast among other species in a symbiotic matrix. In the presented work, the 16S rRNA gene sequencing was used to reveal bacterial populations and elucidate the diversity and abundance of LAB species in international artisanal kefirs from Fusion Tea, Britain, the Caucasus region, Ireland, Lithuania, and South Korea. Bacterial species found in high abundance in most artisanal kefirs included *Lactobacillus kefiranofaciens*, *Lentilactobacillus kefiri*, *Lactobacillus ultunensis*, *Lactobacillus apis*, *Lactobacillus gigeriorum*, *Gluconobacter morbifer*, *Acetobacter orleanensis*, *Acetobacter pasteurianus*, *Acidocella aluminiidurans*,

and *Lactobacillus helveticus*. Some of these bacterial species are LAB that have been reported for their bacteriocin production capabilities and/or health promoting properties.

Keywords: fermented food; fermented beverage; dairy fermentation; artisanal kefir; artisanal kefir grain; lactic acid bacteria (LAB), acetic acid bacteria; 16S rRNA gene sequencing; microbial population; microbiome; probiotic; health benefits; bacteriocin; safe food

1. Introduction

Artisanal kefir is an ancient fermented beverage obtained via fermentation of milk by kefir grains [1]. Kefir grains are a combination of yeast, bacteria, and bacterial polysaccharides [2]. Up to 50 different bacterial and yeast species have been identified in artisanal kefirs [1,3,4]. Numerous combinations of these microorganisms at the species level lead to artisanal kefirs with unique characteristics. It is important to determine the specific microbial compositions of artisanal kefirs and their grains from different origins [5] to obtain a better understanding of kefir as a functional dairy product. Early attempts to isolate kefir-associated microorganisms were obstructed by these microorganisms' fastidious nature. The lactic acid bacteria (LAB) found in kefir require specific nutrients and conditions for growth, so select LAB in kefir may have been undetected via culture-dependent methods [6]. Culture-independent methods are commonly used to identify microbial diversity in fermented foods and beverages. Among these methods, the 16S rRNA gene sequencing has been suggested as a suitable method for identification of bacteria at the species level [7].

As previously mentioned, a typical kefir microbial community includes LAB, acetic acid bacteria, and yeast among other species in a symbiotic matrix [8]. The 16S rRNA gene sequencing has been successfully used to identify bacterial species in kefir [9,10]. For example,

Gulitz et al. identified *Lactobacillus nagelii* (*Liquorilactobacillus nagelii*), *Lactobacillus hordei* (*Liquorilactobacillus hordei*), *Bifidobacterium psychraerophilum, Lactobacillus hilgardii* (*Lentilactobacillus hilgardii*), *Lactobacillus satsumensis* (*Liquorilactobacillus satsumensis*), *Acetobacter orientalis, Clostridium tyrobutyricum,* and *Leuconostoc citreum* from kefir originated from Germany using the 16S rRNA gene sequencing of the V1 to V4 hypervariable regions [10]. Another study identified *Lactobacillus kefiranofaciens, Lactobacillus acidophilus,* and *Lactobacillus sunkii* (*Lentilactobacillus sunkii*) with abundance of 77–78%, 10–11%, and 2–4%, respectively, in two Turkish kefir grains using whole genome and 16S rRNA shotgun sequencing [2].

LAB are known to produce one or more of the following products with antimicrobial properties: organic acids, free fatty acids, diacetyl, hydrogen peroxide, and bacteriocins. Bacteriocins are ribosomally synthesized proteins or peptides that are secreted by bacteria and inhibit closely related Gram-positive and some Gram-negative bacteria using various mechanisms of action [11]. Kefir contains LAB which are known to produce bacteriocins. For example, Lacticin 3147, a bacteriocin produced by *Lactococcus lactis* DPC3147 isolated from Brazilian kefir grains, inhibited the growth of *Escherichia coli, Listeria monocytogenes, Salmonella* Typhimurium, and *Salmonella* Enteritidis [12].

FAO/WHO (Food and Agriculture Organization and the World Health Organization) define probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit to the host” [13]. Recently, The International Scientific Association for Probiotics and Prebiotics recommended that “the term probiotic be used only on products that deliver live microorganisms with a suitable viable count of well-defined strains with a reasonable expectation of delivering benefits for the wellbeing of the host” [14]. Kefir, reported

as a natural probiotic beverage by some researchers [1], contains LAB species with documented beneficial properties. For example, *L. kefiranofaciens* XL10, which is a homofermentative LAB that produces lactic acid as the main product, was reported to have probiotic properties in both *in vitro* and *in vivo* studies [15,16]. *Lactobacillus kefiri* (*Lentilactobacillus kefiri*) is a heterofermentative LAB that produces lactic acid, acetic acid, ethanol, and carbon dioxide, and was reported, in both *in vivo* and *in vitro* studies, to have probiotic properties such as adherence to mucus extracted from the small intestine and colon, strong cholesterol assimilation abilities, and lowering the secretion of IL-8 caused by *Salmonella enterica* infection [17,18].

In our recently published work [19], the antimicrobial activities of artisanal kefir products from Fusion Tea, Britain, the Caucasus region, Ireland, Lithuania, and South Korea were investigated against select foodborne pathogens. It was confirmed that bacteriocin production is the main reason for these kefirs' antimicrobial activity [19]. In the presented work, the 16S rRNA gene sequencing was used to reveal bacterial populations and elucidate the diversity and abundance of LAB species in these international artisanal kefirs. Several LAB species identified in the presented work have been reported by other researchers to have bacteriocin production capabilities and/or health promoting properties. Based on our findings, bacteriocin-producing and/or potentially beneficial LAB are being isolated from select artisanal kefirs and being characterized in our laboratory.

2. Materials and Methods

2.1. Genetic Approaches for Identification of Bacteria in Kefir

2.1.1. Kefir Preparation

Six artisanal kefir grains originating from South Korea (K; [20]), Ireland (I; Etsy Inc.), Lithuania (L; Etsy Inc.), Britain (B; Etsy Inc.), the Caucasus (C; Etsy Inc.), and a compilation of world-sourced grains blended (A; Fusion Tea, Amazon) were used in this study. Kefir grains were inoculated (10% (w/v)) into whole pasteurized milk and incubated at 22–24 °C for 24 h. At the end of the fermentation process (pH 3.9–4.1), clean plastic strainers with 1-mm pore size was used to separate kefir grains from kefir products. Designated plastic strainers were used for each kefir product/grains to avoid cross contamination. Kefir products were centrifuged (Eppendorf Microcentrifuge 5415D, Hauppauge, NY, USA) at 16,000 *g* for 10 min at room temperature to remove the lipid layer. Kefir grains separated were washed three times with sterilized DI water. Kefir grains were mixed with sterile DI water (3 mL), homogenized using Stomacher 400 (Seward Limited, Worthing, West Sussex, UK) for 60–120 s at high speed, and then centrifuged at 16,000 *g* for 10 min at room temperature to remove the lipid layer. The resulting pellets were used for DNA extractions.

2.1.2. DNA Extraction

The E.Z.N.A Universal Pathogen Kit (Omega Biotech, Norcross, GA, USA) was used for DNA extraction from all international artisanal kefirs and their grains. The kit's user guide was followed with two additional washing steps with the DNA washing buffers included in the kit. The concentration of DNA preparations was determined by ultraviolet spectrophotometry (Spectra MAX 190). Absorbance 260/280 values between 1.8 and 2.0 were considered

acceptable. The quality of DNA preparations was determined by running DNA samples on 1.2% (w/v) agarose gels with TAE (1X) buffer and using PowerPac 200 submerged horizontal gel electrophoresis systems from Bio-Rad (Hercules, CA, USA). Two DNA samples, one representing a given kefir product and another representing the kefir grains in that product, were sent out to Omega Bioservices (Norcross, GA, USA) for the 16S rRNA gene sequencing. With two DNA samples representing a given artisanal kefir, a total of 12 DNA samples representing six international artisanal kefirs were sequenced by Omega Bioservices.

Commercial kefirs, made with known cultures indicated on their labels, were purchased from local (Moscow, ID, USA) grocery stores and used as controls: Lifeway (plain), The Greek Gods (plain), Wallaby (organic plain), and Maple Hill (organic plain). DNA from the commercial kefir samples was extracted by Omega Bioservices using their E.Z.N.A Universal Pathogen Kit.

2.1.3. The16S rRNA Gene Sequencing

Illumina MiSeq Sequencing was used for the 16S rRNA gene sequencing (≈200K reads per sample) by Omega Bioservices. Both the V1–V3 and the V3–V4 primer sets were used for PCR amplification by Omega Bioservices. The V1 and V2 regions have been historically used for identification of LAB [21]. All variable regions in the 16S rRNA gene have been reported to be effective in identifying bacterial communities in kefir through the 16S rRNA gene sequencing [22]. Library preparation type was KAPA HiFi PCR as per Omega Bioservices. Data was delivered via Illumina BaseSpace web site.

2.2. The 16S rRNA Gene Sequencing Analyses

The 16S rRNA gene sequencing reads were obtained from Omega Bioservices for the V1–V3 and the V3–V4 regions. The total number of reads (often referred to as read depth) were converted to relative abundance and rounded to 0.1%. The percent relative abundance was calculated by dividing the number of reads for each phylum, genera, or species with the total number of reads for each kefir and multiplying the outcome by 100. Pie charts were created for the relative abundances of bacterial phyla in kefir products and their grains based on the V1–V3 and V3–V4 regions of the 16S rRNA genes. Stacked bar charts were created for the average abundance of the 21 most abundant bacterial genera, based on the V1–V3 and V3–V4 regions of the 16S rRNA genes, in kefir products and their grains. Additional stacked bar charts were created for the average relative abundance of the 10 most abundant bacterial species, based on the maximum percentage between the V1–V3 and the V3–V4 regions of the 16S rRNA genes, and scaling (0–100) species contribution. Stacked bar charts were also created for the percent species contribution of LAB found in kefir samples and known to produce bacteriocins, based on the maximum percentage between the V1–V3 and the V3–V4 regions of the 16S rRNA genes, and scaling (0–40) species contribution. Canonical correlations were performed using the aggregated lists of taxa to examine relationships among kefir products and their grains. Correlation plots were used to understand whether and how strongly kefir products and their grains are related. The Pearson's correlation coefficient (*r*) provides information about the strength and direction of a relationship between a given kefir product and its grains. The regression line describes how kefir products change as kefir grains change. Heatmaps were created using Pearson correlations among the kefir products and their grains. The results of hierarchical clustering (HC) using complete linkage distance method represented by a

dendrogram was included to show taxonomic relationships among international artisanal kefirs. The Pearson's correlation coefficients, regression lines, and dendrogram were generated by R-3.5.1 programming (R Studio Inc., Boston, MA, USA).

3. Results

3.1. Genetic Approaches for Identification of Bacteria in Kefir Samples

3.1.1. Bacterial Phyla and Genera Present in Artisanal Kefirs and Their Grains

Based on the 16S rRNA sequencing data generated in this work, a diverse group of bacteria were determined to be present in artisanal kefir products and their grains from different regions of the world. Specifically, relative abundances of bacterial phyla and bacterial genera based on the V1–V3 and the V3–V4 regions of the 16S rRNA genes were determined for the kefir product from Lithuania (LP), kefir grains from Lithuania (LG), kefir product from South Korea (KP), kefir grains from South Korea (KG), kefir product from Ireland (IP), kefir grains from Ireland (IG), kefir product from the Caucasus region (CP), kefir grains from the Caucasus region (CG), kefir product from Britain (BP), kefir grains from Britain (BG), kefir product from Fusion Tea (AP, Amazon), and kefir grains from Fusion Tea (AG, Amazon) (Figure 3). The *Firmicutes* phylum was the most abundant in all artisanal kefir products, followed by the phyla *Proteobacteria, Actinobacteria, Verrucomicrobia, Planctomycetes,* and *Nitrospirae* (Figure3). The phylum *Firmicutes* was the most abundant phylum in kefir grains, especially for CG, with 97.9% (based on V1–V3) and 97.7% (based on V3–V4) (Figure 3), followed by BG, LG, AG, KG, and IG for the V1–V3 regions (Figure3). Based on the V3–V4 regions, *Firmicutes* was the most abundant phylum in CG followed by LG, BG, AG, KG, and IG

(Figure3). The following genera were identified as the major genera in artisanal kefirs: *Lactobacillus*, *Lentilactobacillus*, *Lacticaseibacillus*, *Acetobacter*, *Swaminathania*, *Gluconobacter*, *Streptococcus*, *Pediococcus*, *Pseudomonas*, *Acidocella*, *Cohnella*, *Peptoniphilus*, *Saccharopolyspora*, *Thermodesulfovibrio*, *Singulisphaera*, *Chthoniobacter*, *Paenibacillus*, *Knoellia*, *Leuconostoc*, *Bifidobacterium*, and *Lactococcus* (Figure 3).

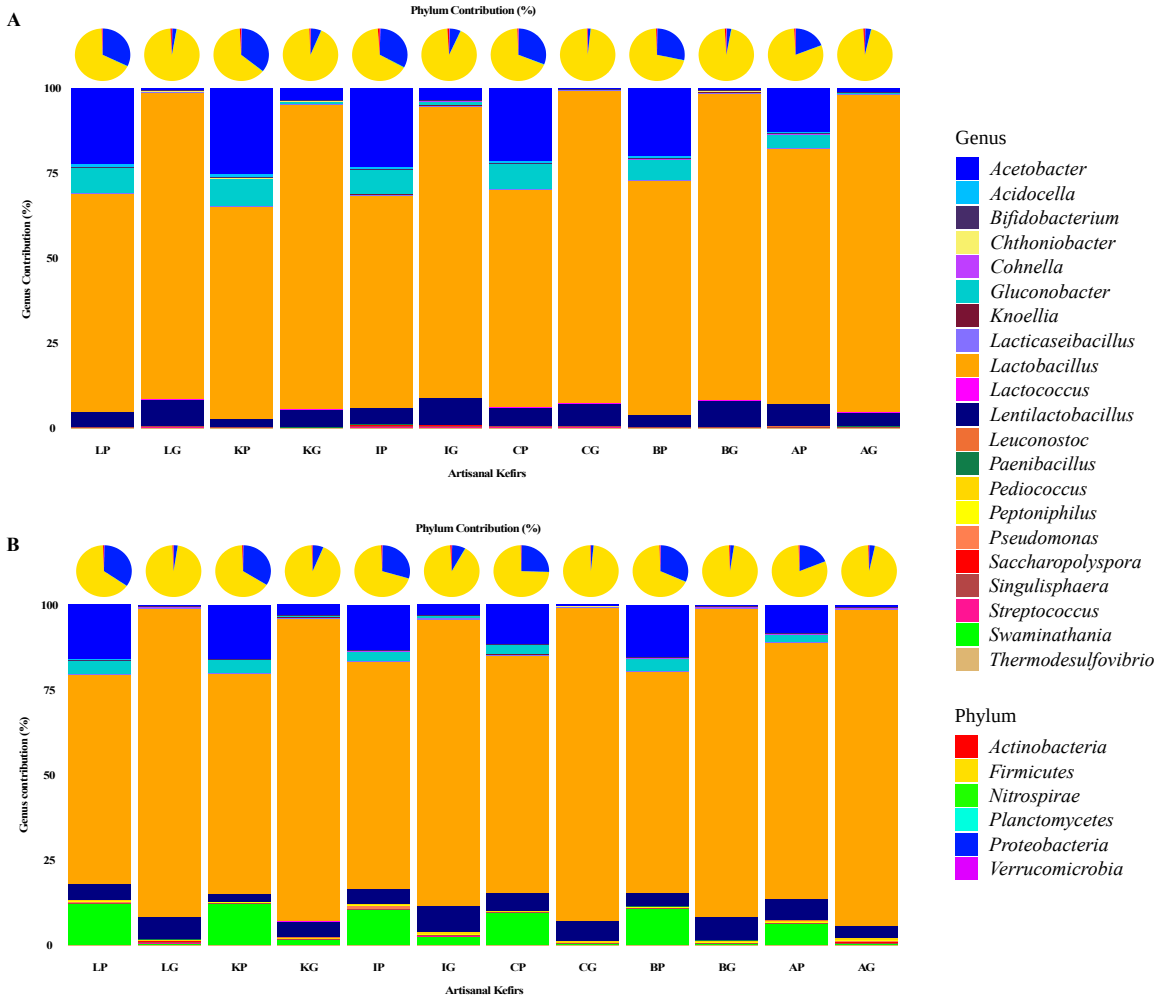

Figure3. Relative abundances of bacterial phyla (pie charts) and an aggregation of the 21 most abundant bacterial genera (column charts) for the V1–V3 (**A**) and V3–V4

(**B**) regions of the 16S rRNA genes. Kefir product from Lithuania (LP), kefir grains from Lithuania (LG), kefir product from South Korea (KP), kefir grains from South Korea (KG), kefir product from Ireland (IP), kefir grains from Ireland (IG), kefir product from the Caucasus region (CP), kefir grains from the Caucasus region (CG), kefir product from Britain (BP), kefir grains from Britain (BG), kefir product from Fusion Tea (AP, Amazon), kefir grains from Fusion Tea (AG, Amazon).

The 16S rRNA gene sequencing targeting the V1–V3 and the V3–V4 regions resulted in various relative abundance percentages for the phylum and genus level designations in all artisanal kefirs (Figure3). For example, LP has 67% *Firmicutes* based on the V1–V3 region, while it has 64.6% *Firmicutes* based on the V3–V4 region. The IP has 65.6% and 69.7% *Firmicutes* based on the V1–V3 and the V3–V4 regions, respectively. The AP has the highest relative abundance for *Lactobacillus* species, from 75% (based on V1–V3) to 75.5 % (based on V3–V4) while LP has the lowest abundance for *Lactobacillus* species from 61.6% (based on V3–V4) to 64.2% (based on V1–V3). The genus *Lentilactobacillus* was found to be in the highest abundance in IG, from 7.7% (based on V1–V3) to 7.4% (based on V3–V3), when compared to all other kefir grains and products. It appears that the combination of both the V1–V3 and V3–V4 regions to identify bacteria at the phylum and genus levels in kefir products and their grains worked well. As an exception, the V3–V4 region identified the genus *Swaminathania* in all kefir products and in all kefir grains (Figure 3B), while it was not identified by the V1–V3 region (Figure 3A).

Commercial kefirs are defined, prepared using a starter culture of LAB and yeast species as indicated on the packaging. Therefore, the 16S rRNA gene sequencing was performed on commercial kefirs as controls to test the accuracy of the identification. Commercial kefirs used

in the study were determined to have the exact probiotic bacteria, both at the genus and species level as listed on their labels (Appendix A).

3.1.2. Bacterial Species Present in Artisanal Kefirs and Their Grains

The 10 most abundant bacterial species found in artisanal kefirs included *L. kefiranofaciens*, *Lent. kefiri*, *Lactobacillus ultunensis*, *Lactobacillus apis*, *Lactobacillus gigeriorum*, *Gluconobacter morbifer*, *Acetobacter orleanensis*, *Acetobacter pasteurianus*, *Acidocella aluminiidurans*, and *Lactobacillus helveticus* (Figure 4). *L. kefiranofaciens* was determined to be the most abundant bacterium in all artisanal kefirs with relative abundance between 48.22% and 93.76% (Figure 4). The relative abundance for *Lent. kefiri* varied between 2.94% (KP) and 7.3% (AP) among kefir products and between 3.7% (AG) and 7.86% (IG) among kefir grains (Figure 4). *L. ultunensis* was found to be more abundant in KP and KG when compared to all other artisanal kefirs and their grains. *A. pasteurianu*s was more abundant (17.1%) in KP while *G. morbifer* was more abundant (5.8%) in LP when compared to their relative abundance in all other artisanal kefirs.

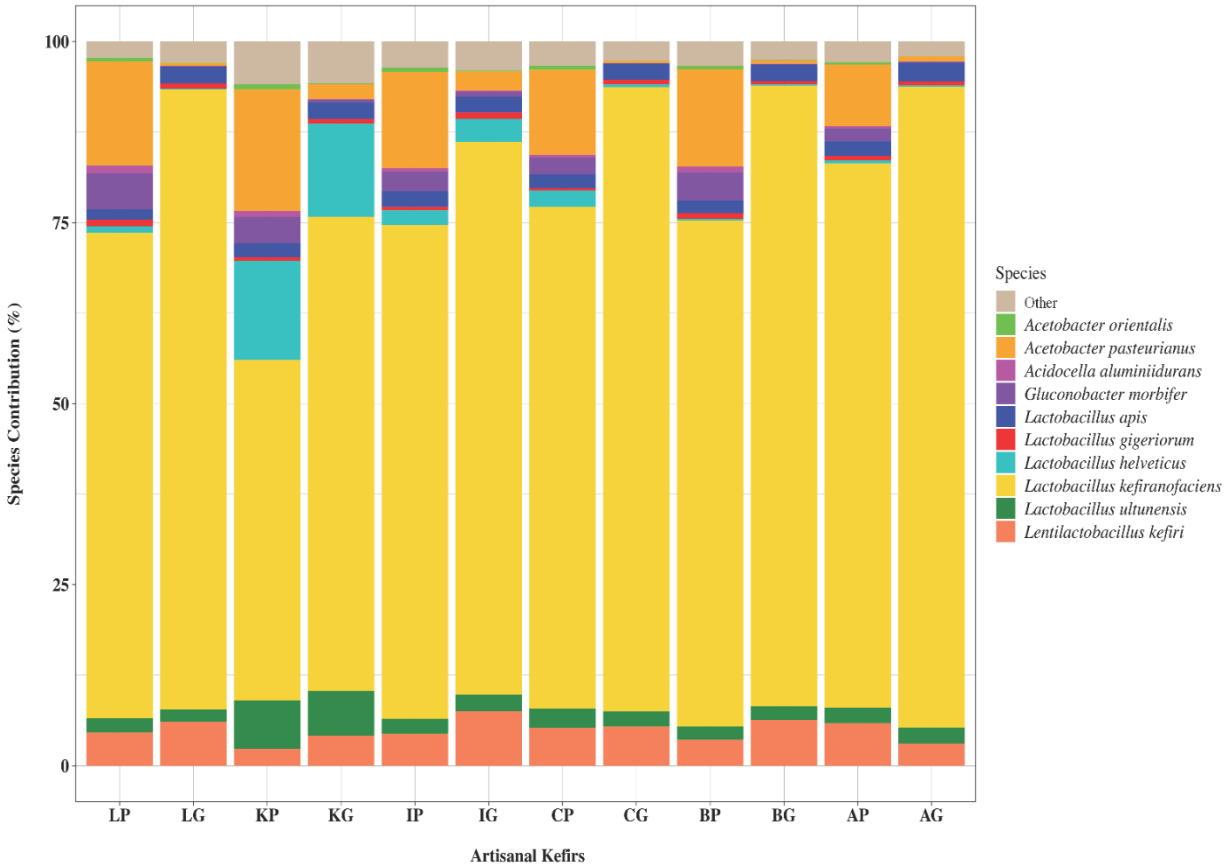

Figure 4. An aggregation of the 10 most abundant bacterial species, using the calculated relative abundance and scaling (0–100) species contribution. Kefir product from Lithuania (LP), kefir grains from Lithuania (LG), kefir product from South Korea (KP), kefir grains from South Korea (KG), kefir product from Ireland (IP), kefir grains from Ireland (IG), kefir product from the Caucasus region (CP), kefir grains from the Caucasus region (CG), kefir product from Britain (BP), kefir grains from Britain (BG), kefir product from Fusion Tea (AP, Amazon), kefir grains from Fusion Tea (AG, Amazon).

Differences exist in artisanal kefirs regarding microorganisms present in kefir products versus their grains. For example, *L. kefiranofaciens* exhibits a higher relative abundance in all kefir grains when compared to their corresponding products (Figure 4). Similarly, *Lent.*

kefiri exhibits a higher relative abundance in kefir grains B, I, K, and L when compared to their corresponding products (Figure 4). On the other hand, *Lent. kefiri* exhibits a lower relative abundance (3%) in AG when compared to that (6%) of in AP (Figure 4).

3.1.3. Bacteriocinogenic and Beneficial Bacteria in Artisanal Kefirs

Several LAB species recognized as bacteriocin producers in the current literature were identified in all artisanal kefirs subjected to this work, albeit in various relative abundance (Figure 5). The following bacteriocinogenic species were found in artisanal kefirs: *Lent. kefiri, L. helveticus, Lactobacillus delbrueckii, Lacticaseibacillus paracasei (Lactobacillus paracasei), Lacticaseibacillus casei (Lactobacillus casei), Lacticaseibacillus rhamnosus (Lactobacillus rhamnosus), L. apis, Lactobacillus crispatus, Lactobacillus acidophilus, Streptococcus thermophilus, Leuconostoc mesenteroides,* and *Lactococcus lactis* (Figure 5). All artisanal kefirs, except kefir K, were determined to contain *Lent. kefiri* as the most abundant LAB with known bacteriocin production capability. *Lent. kefiri* was the third most abundant LAB with known bacteriocin production in Kefir K (Figure 5).

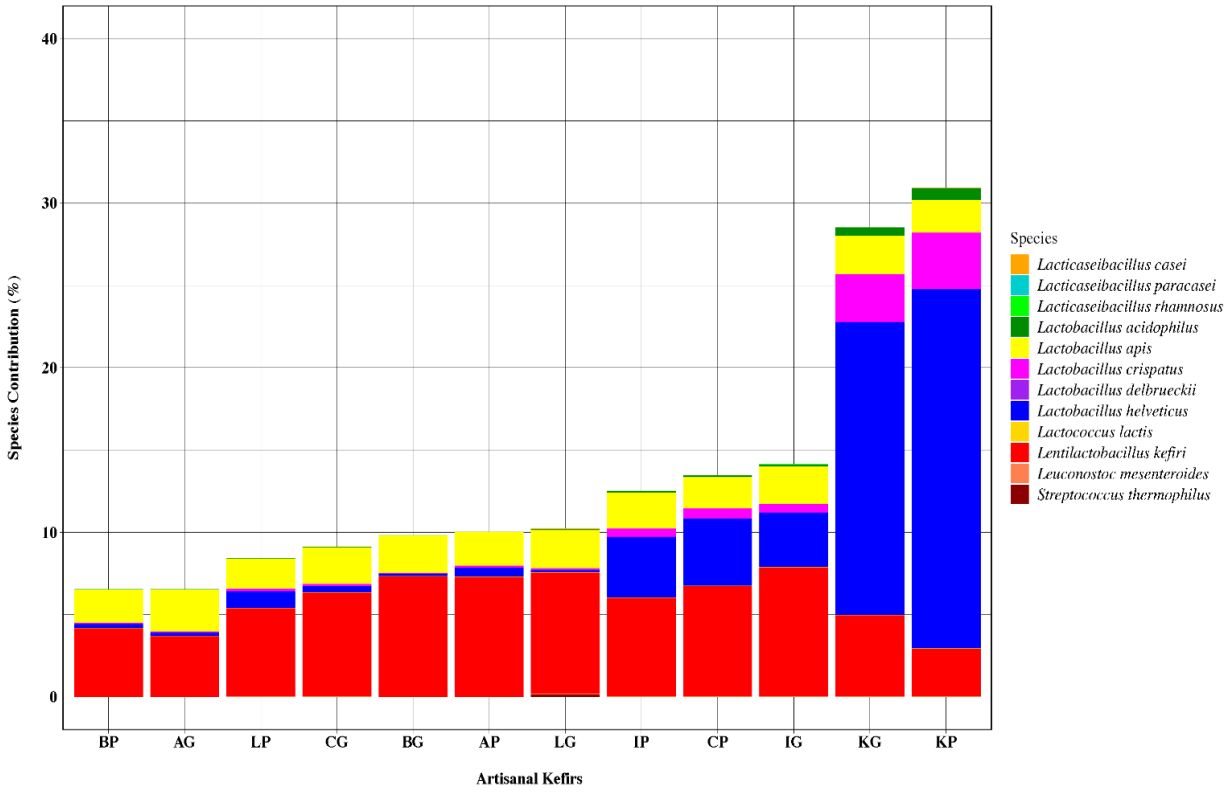

Figure 5. The 16S rRNA gene sequencing results showing percent species contribution of lactic acid bacteria (LAB). The IP, and IG share the same ranking with CP: *L. apis*, *L. helveticus*, *L. acidophilus*, and *L. crispatus.* The CG shares with IP, IG, and CP the same ranking for *L. crispatus* and *L. acidophilus* only. The LG has the highest relative abundance for *S. thermophilus* and the lowest relative abundance for *L. helveticus* when compared to LP as well as other artisanal kefirs.

Kefir K was a stand-alone kefir product with respect to the ranking of species with known bacteriocin production: *L. helveticus, L. crispatus, Lent. kefiri, L. apis,* and *L. acidophilus*. The most abundant LAB with known bacteriocin production capability was identified to be *L. helveticus* in KP with relative abundance of 21.8% and in KG with relative abundance of 17.8% (Figure 5). *L. helveticus* was found in other artisanal kefir products with relative abundance

between 0.26% (BP) and 4.11% (CP) and in artisanal kefir grains with relative abundance between 0.13% (LG) and 3.34% (IG).

Lactobacillus crispatus, another *Lactobacillus* species with known bacteriocin production, followed *L. helveticus* in KP and KG with relative abundance of 3.44% and 2.93%, respectively (Figure5). The relative abundance for *L. crispatus* in other artisanal kefir products and kefir grains varied between 0.07% (BP) and 0.6% (CP) and between 0.045% (BG) and 0.51% (IG), respectively.

Lactobacillus apis, a lesser known *Lactobacillus* species with reported bacteriocin production capability, was determined to be present in all artisanal kefir products and their grains. The relative abundance of *L. apis* was in the range of 1.79% (LP) to 2.17% (IP) in kefir products and 2.21% (CG) to 2.54% (AG) in kefir grains (Figure 5). *L. apis* was determined to have the second highest and the third highest relative abundance among other bacteriocin producing LAB in kefir products A, B, I, and L and kefir product C, respectively (Figure 5). The organism had the second highest abundance ranking among other bacteriocin producing LAB in kefir grains A, B, C, L, while the ranking was the third highest abundance in kefir grain I and the fourth highest abundance in kefir K (Figure 5).

Lactobacillus acidophilus, an organism with reported bacteriocin production capability, ranked fifth most abundant bacteriocinogenic LAB species in all artisanal kefir products. *L. acidophilus* was present in KP with relative abundance of 0.7% and in KG with relative abundance of 0.5% (Figure 5). All other artisanal kefirs (A, B, C, I, and L), both products and their grains, were determined to contain much less *L. acidophilus* with relative abundance of 0.01%–0.1% (Figure 5).

Some LAB species with known bacteriocin production capability were absent in select kefir products plus their grains (Figure5): *S. thermophilus* in kefir A; *L. delbrueckii* in kefir A; *Lacti. paracasei* in kefirs A and B; *Lacti. casei* in kefirs A, B, C, I, and K; *Lacti. rhamnosus* in kefir K; *Lac. lactis* in kefirs B, C, and K; and *Leu. mesenteroides* in kefirs A, B, I, and L. *Lacti. casei* was determined to be present, albeit at a low relative abundance, in kefir L grains only (Figure 5).

3.1.4. Correlation of Artisanal Kefir Products to Their Grains and Taxonomic Relationships Among International Artisanal Kefirs

Kefir grains showed a high correlation to their products in their species content (Figure 6A–C). Kefir A presented the highest correlation between its grains and the product ($r = 0.994$), followed by kefirs I, C, B, and L with the following r values, respectively: $r = 0.986$, 0.986, 0.981, and 0.976. The lowest correlation was observed in kefir K ($r = 0.948$), (Figure 6B). The heatmap (Figure 6C) created confirmed that KG and KP had the lowest correlation with each other and the lowest correlation with all other kefirs (Figure 6C). The AP, on the other hand, appeared to have the highest correlation with all kefir grains ($r = 1$) except for KG. The AG was not highly correlated with other kefir products (Figure 6C).

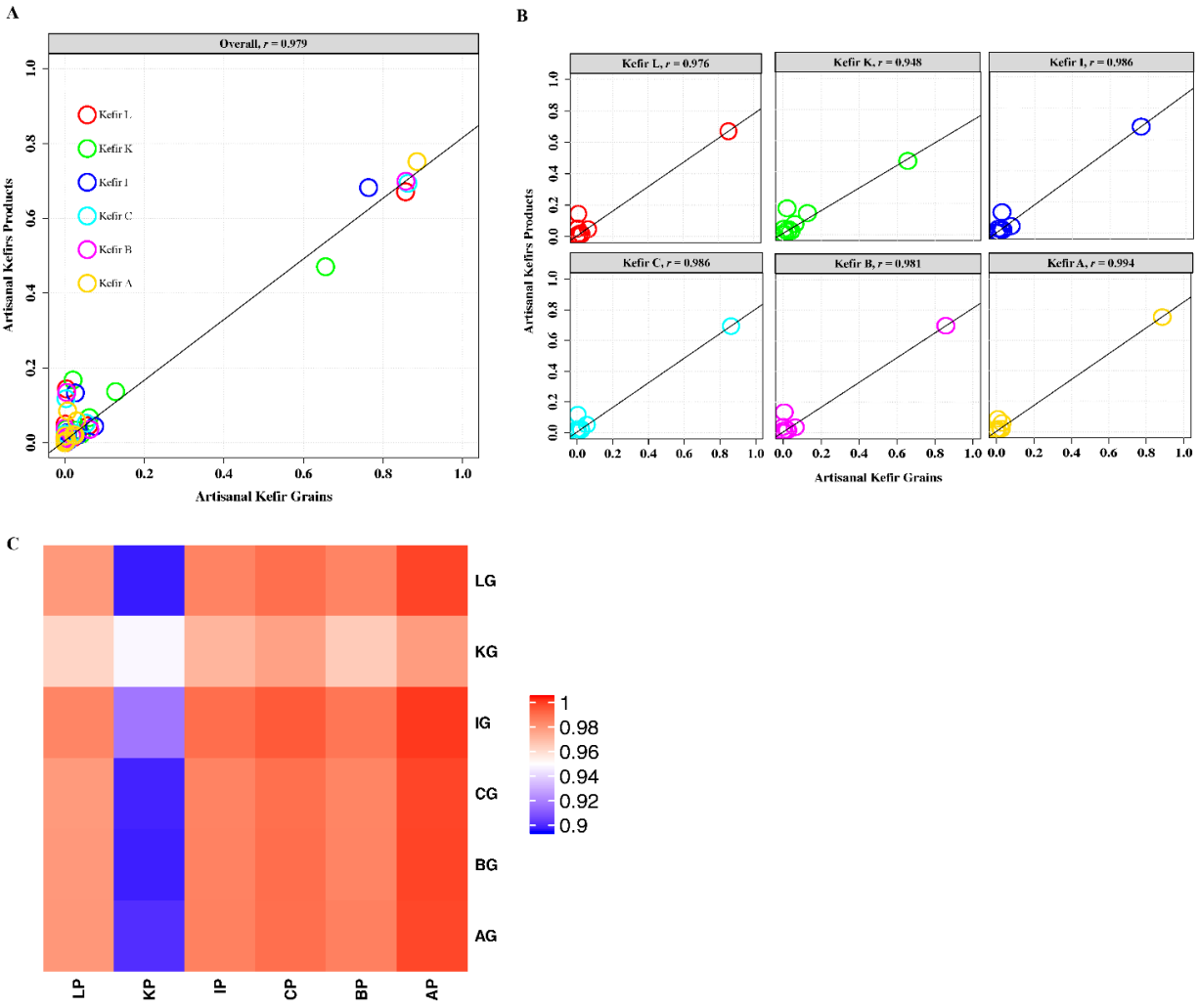

Figure 6. (**A**) Canonical correlation plot with a regression line between all kefir grains (x-axis for each plot) from Lithuania (LG) and South Korea (KG), Ireland (IG), the Caucasus region (CG), Britain (BG), and Fusion Tea (AG, Amazon), and all kefir products (y-axis for each plot) from Lithuania (LP) and South Korea (KP), Ireland (IP), the Caucasus region (CP), Britain (BP), and Fusion Tea (AP, Amazon) for species level. (**B**) Correlation plots with a regression line between each kefir grains and their products. The *r* indicates the Pearson's correlation coefficient for each plot. (**C**) Heatmap showing Pearson correlation between each kefir grains and their products.

Hierarchical Clustering (HC) dendrogram (Figure 7) shows taxonomic relationships among international artisanal kefirs. KP appears to be a stand-alone kefir product with respect to species- level taxonomy. KP has its own highest cluster, while AP is the most related kefir to KP when compared to all other kefir products. In addition, IP and CP are related with respect to species-level taxonomy similar to LP and BP which are also related to each other (Figure 7).

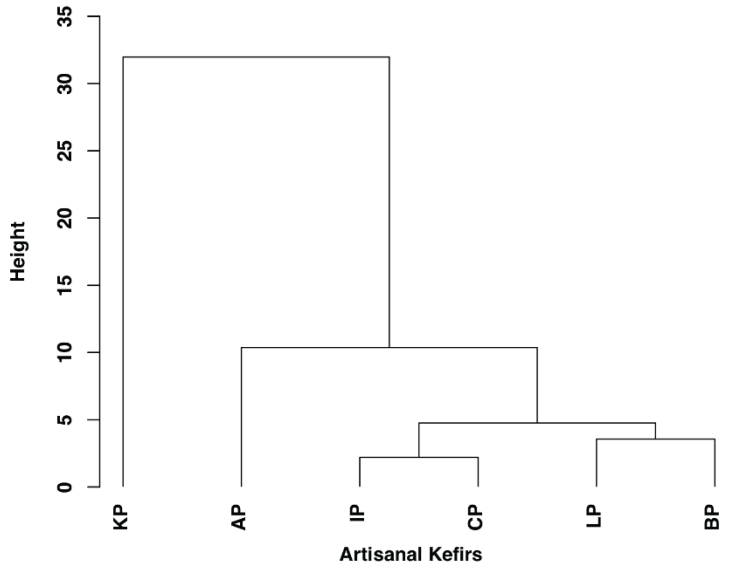

Figure 7. The hierarchical clustering (HC) based dendrogram showing species level taxonomy relatedness for all kefir products (X-axis) from Lithuania (LP) and South Korea (KP), Ireland (IP), the Caucasus region (CP), Britain (BP), and Fusion Tea (AP, Amazon). The complete linkage method used for computing distance between clusters to determine similar clusters.

4. Discussion

In the presented work, the 16S rRNA gene sequencing was successfully applied to six international artisanal kefirs and revealed microbial populations at the phyla, genera, and species levels in international artisanal kefirs. A total of six phyla were identified, *Firmicutes Proteobacteria, Actinobacteria, Verrucomicrobia, Planctomycetes,* and *Nitrospirae*, in artisanal kefirs studied. To our knowledge, the presence of a combination of six phyla has not been reported for any artisanal kefir. This could be due to the complexity of the artisanal kefirs examined in our work and/or the effectiveness of the 16S rRNA gene sequencing and the sequence analyses. The phylum *Firmicutes* was the most abundant phylum among all other phyla in kefir grains and their products, which is a commonly found phylum in artisanal kefir. *Lactobacillus* was the most abundant genus with the highest relative abundance in AG and the lowest relative abundance in IP. This aligns well with relative abundance of lactobacilli in artisanal kefirs described in the literature. The genus *Lactobacillus* has been recently reclassified into 25 new genera including *Lactobacillus, Lentilactobacillus*, and *Lacticaseibacillus* [23]. This reclassification was fully considered in our work.

Our goal was to utilize multiple variable regions of the 16S rRNA gene to successfully identify all genera and species present in international artisanal kefirs. One interesting outcome of our study was the identification of the genus *Swaminathania* through the use of the V3–V4 region of the 16S rRNA gene but not through the use of the V1–V3 region. Since the 16S rRNA gene sequencing of four commercial kefirs, used as controls to test the accuracy of the identification, determined the exact probiotic bacteria listed on their labels at the species level, we are confident with the results of the 16S rRNA gene sequencing. The interesting outcome can be attributed to the fact that different variable regions in the 16S rRNA gene can be less or

more suitable to identify bacteria at the genus or species level. Perhaps the V1–V3 region is less suitable for identification of the genus *Swaminathania* than the V3–V4 region.

A study by Marsh et al. for kefirs sourced from the United Kingdom, Canada, and the United States reported that the 16S rRNA reads for the V4–V5 region identified three bacterial phyla: *Actinobacteria, Firmicutes,* and *Proteobacteria* [24]. Both *Proteobacteria* and *Firmicutes* were verified to be the most abundant phyla. The *Proteobacteria* phylum was greater in abundance, in general, in the grains than the products for all kefirs studied. The phylum *Firmicutes* was in higher abundance in the product than in the grains. The abundance of *Actinobacteria* was low, in general, in both grains and products. Marsh et al. found that the most dominant genus was *Zymomonas* with a relative abundance of 87–49% followed by *Lactobacillus,* ranging in relative abundance from 38.8% to 12% [24]. Differences observed between our work and that of Marsh et al. can be attributed to differences among the kefirs used and the regions that were targeted in the 16S rRNA sequencing.

Lentilactobacillus kefiranofaciens was the most abundant lactobacilli in all artisanal kefirs. Our findings align with that of Wang et al., who reported *L. kefiranofaciens* as the sole dominant and stable species in Tibetan kefir [25]. *L. kefiranofaciens* has been reported to have probiotic properties and health benefits [26,27]. A study with both *in vivo* and *in vitro* components suggested *L. kefiranofaciens* M1 to be applied in fermented dairy products as an alternative therapy for intestinal disorders [26]. An increase in the production of regulatory T-cell cytokines was observed when *L. kefiranofaciens* M1 was cocultured with spleen cells [27].

Other highly abundant bacterial species identified in artisanal kefirs in the presented work included the following organisms*: Lenti. kefiri, L. ultunensis, L. apis, L. gigeriorum, G. morbifer, A. orleanensis, A. pasteurianus, Acid. aluminiidurans,* and *L. helveticus.* Out of

these, *L. ultunensis* and *L. apis* were reported to be present in kefir grains from different regions of Turkey [28]. Based on our knowledge, *L. gigeriorum* has not been reported in kefir until the presented work, however, the organism has been reported to be closely related to *L. acidophilus* [29]. In addition, our work is the first study reporting *Acid. aluminiidurans* occurrence in kefir. *Acid. aluminiidurans* is an aluminum-, acid-, and sulfate-tolerant bacterium, which was originally isolated from a waterweed in Vietnam [30].

Lentilactobacillus kefiri was found in higher relative abundance in kefir grains B, I, K, and L when compared to their corresponding products. Perhaps *Lenti. kefir* is imbedded in the outer layers of the kefir A grains and immigrate, during kefir fermentation, more into the kefir product than in kefirs B, I, K, and L. A study by Korsak et al. evaluated the microbiota of kefir samples from Belgium using 16S pyrosequencing revealed the presence of *L. kefiranofaciens, Lactococcus lactis* ssp. *cremoris, Gluconobacter frateurii, Lenti. kefiri, A. orientalis, Leu. mesenteroides,* and *Acetobacter lovaniensis* [31]. In that study, some samples showed *L. kefiranofaciens* to be the most abundant—similar to our work—while other samples showed *Lac. lactis* as the most abundant, constituting ≈80% of the bacterial population. Korsak et al. samples [31] did not report the occurrence of *L. ultunensis, L. apis, L. gigeriorum, G. morbifer, A. orleanensis, A. pasteurianus, Acid. aluminiidurans,* and *L. helveticus*, which are being reported in the presented study. As expected, similarities and differences exist among international artisanal kefirs regarding their bacterial populations at the species level.

Acetic acid bacteria have been reported to contribute to exopolysaccharide formation and increase in kefir grain biomass, without negatively affecting the sensory properties and other microflora of kefir [32]. *Acetobacter fabarum, A. lovaniensis,* and *A. orientalis* were identified in kefir originated from Italy [33]. *A. orleanensis, A. orientalis, Acetobacter malorum,* and *A.*

pasteurianus were found in all artisanal kefirs in the presented work. Therefore, it appears that different acetic acid bacteria species occupy different artisanal kefirs. The genus *Gluconobacter* is a member of the acetic acid bacteria family. Research has indicated a symbiotic relationship between LAB isolated from kefir and *Gluconobacter* spp. [34]. *G. frateuii* was identified in kefir originated from Belgium [31]. *G. morbifer* and *Gluconobacter kondonii* were identified in all artisanal kefirs examined in the presented work.

Kefir has been reported to be a health-promoting beverage. Numerous studies have suggested kefir's health benefits in terms of improving lactose digestion [35], protecting against foodborne pathogens, anticancer effects [36], immunomodulatory effects [37], and probiotic activity [5]. These health benefits appear to be related to the kefir microbiota and/or their metabolites [5].

Potential probiotics need to exhibit functional properties such as viability and persistence in the GI-tract, immunomodulation, antagonistic and antimutagenic properties [38]. The antagonistic abilities of probiotics include aggregation and coaggregation, adhesion to the intestine, inhibiting pathogenic bacterial adhesion to the intestine as well as production of antimicrobial substances such as bacteriocins [39]. Occurrence of LAB with probiotic potential in kefir has been reported. *L. acidophilus* LA15, *Lactobacillus plantarum* (*Lactiplantibacillus plantarum*) and *L. kefiri* (*Lentilactobacillus kefiri*) D17 isolated from Tibetan kefir were proposed as beneficial probiotics [17]. *L. rhamnosus* (*Lacticaseibacillus rhamnosus*) is reported to be a probiotic organism found in kefir [40]. *L. paracasei* (*Lacticaseibacillus paracasei*) MRS59 displayed significant antioxidant activity and adhesion to Caco-2 cells, which indicated its probiotic potential [41]. *L. kefiranofaciens* 8U, *Lactobacillus diolivorans* (*Lentilactobacillus diolivorans*) 1Z, and *L. casei* (*Lacticaseibacillus casei*) 17U isolated from

Brazilian kefir were reported as potential probiotics [5]. Moreover, 11 *Lac. lactis* strains isolated from Brazilian kefir were reported to show probiotic properties such as antagonistic activity and antioxidative activity [41]. *L. acidophilus* Z1L, *L. helveticus* Z5L, and *L. casei* (*Lacticaseibacillus casei*) Z7L, isolated from Turkish homemade kefirs, were reported to have probiotic activities [42]. *L. helveticus* was identified in all artisanal kefirs in the presented work. Our results indicated that *L. helveticus* in kefir K product and kefir K grains was 5–84 times and 5–137 times more abundant than other artisanal kefirs and artisanal kefir grains, respectively. In this study, all bacteria found in kefir with known bacteriocin producing capabilities were reported to have beneficial properties, except *L. apis*. To our knowledge, *L. apis* has not been reported as a beneficial bacterium. *L. plantarum* Lp27, isolated from Tibetan kefir, exhibited efficient cholesterol-reducing ability [43]. *L. plantarum* was not found in any kefir grains or products in the current study.

The 16S rRNA gene sequencing described in the presented work has allowed us to determine LAB that might be responsible for the production of bacteriocins which were linked to the inhibition of foodborne pathogenic bacteria in our previously published study [19]. *Lenti. kefiri* is a bacterium that has been isolated from kefir and shown to inhibit both Gram (+) and Gram (-) pathogens [44,45]. *Lenti. kefiri* was the second most abundant lactobacilli in all artisanal kefirs except kefir K. In Taiwanese kefir grains, *Lenti. kefiri* was determined to be the most abundant *Lactobacillus* species [46,47]. *L. helveticus*, a species known to produce bacteriocins, was found to be the most abundant lactobacilli in kefir K. This bacterium was reported to produce heat-labile, large molecular mass (> 30 kDa) peptides lysostaphin, enterolysin A, and helveticin J with antimicrobial activities [48,49]. *L. acidophilus* was reported to affect the membrane permeability and cell wall formation of its target organisms

by producing acidocin B, entereocin P, and reuterin 6 peptides [50,51]. *L. crispatus* was reported in both *in vivo* and clinical studies to have antimicrobial activity against bacterial vaginosis and uropathogenic *Escherichia coli* [52,53]. In the presented work, *L. crispatus* was found to be 5.7–49 times and 5.7–65 times more abundant in KP and KG, respectively, when compared to other artisanal kefir products and their grains. *Leu. mesenteroides* was found in kefirs K and C in our work. The organism was reported to produce Leucocyclicin Q, a novel cyclic bacteriocin which shows antimicrobial activity against Gram-positive bacteria such as *Bacillus coagulans* [54]. *Lacti. casei*, which was found only in kefir L in the presented work, was reported to be effective against *Lis. monocytogenes, Listeria innocua, Corynebacterium difterium,* and *Bacillus cereus*. *Lacti. rhamnosus* was found in small abundance in kefir C, I, and L in our work. This organism was reported to inhibit *Staphylococcus aureus, Lis. monocytogenes, Lis. innocua, C. diphtheriae,* and *B. cereus* [55].

A research project that focused on the microbial diversity of Tibetan kefir grains from different origins did not detect *Lac. lactis* in kefir grains examined but in kefir products [56,57]. Another published study found that *Lac. lactis* and *Strep. thermophilus* were dominant microorganisms accounting for 53–65% of the total microflora of Tibetan kefir grains and accounting for 74–86% of the total microflora of kefir products [58]. The artisanal kefirs tested in our work exhibit very low relative abundance for these organisms and thus they do not resemble the Tibetan kefir with dominant *Lac. lactis* and *Strep. thermophilus*. The variation in bacterial distribution in kefir products versus their grains can be attributed to temperature increase created by active fermentation or where these bacteria exist in the kefir grain [4] among other factors.

Owing to the fact that kefir A is a mixture of grains from various geographical regions, it is not surprising that it is related to kefir K and to all other kefirs in species level taxonomy. An interesting result for the species level taxonomy is that the Irish kefir is not closely related to the British kefir—even though Ireland is geographically close to Britain—but it is related to the Caucasus kefir. The British kefir is found to be closely related to the Lithuanian kefir. Kefir K, on the other hand, appears to be the most unique kefir in terms of its species-level taxonomy and its composition comprised of LAB reported to produce bacteriocins. In our former work [19], kefir K exhibited antimicrobial activity against a diverse group of foodborne pathogenic indicators. Due to kefir K's robust antimicrobial activity and its unique species-level taxonomy, our goal is to carry out additional research on kefir K with emphasis on isolation and characterization of LAB and their bacteriocins as well as application of these bacteriocins as natural, clean-label biopreservatives for shelf life protection and assurance of microbial food safety.

5. Conclusions

Geographical origins of kefir grains and kefir production methods affect the microbial composition of artisanal kefirs. Types of milk, incubation temperatures, incubation times, and the ratios of kefir grains to milk play important roles on kefir's microbial composition [1,59,60]. Kefir grains have been shown to exhibit regional differences in microbial composition due in part to local LAB finding a niche in the grains [6]. The culture-independent method employed in our work, the 16S rRNA gene sequencing, successfully revealed the microbial populations in six international artisanal kefirs and demonstrated the diversity and abundance of LAB found in each kefir tested, many with reported capability of producing

bacteriocins and potential health benefits. Species found in high relative abundance in most artisanal kefirs included *L. kefiranofaciens, Lenti. kefiri, L. ultunensis, L. apis, L. gigeriorum, G. morbifer, A. orleanensis, A. pasteurianus, Acid. aluminiidurans,* and *L. helveticus*. LAB with documented bacteriocin production capabilities, *Strep. thermophilus, Lenti. kefiri, L. helveticus, L. delbrueckii, Lacti. paracasei, Lacti. casei, Lacti. rhamnosus, L. crispatus, Leu. mesenteroides, L. acidophilus,* and *Lac. lactis*, were found in diverse relative abundances in the artisanal kefirs examined in this study. LAB species with documented health benefits in the literature and identified in the artisanal kefirs tested in this work were *Lent. kefiri, L. helveticus, L. delbrueckii, Lacti. paracasei, Lacti. casei, Lacti. rhamnosus, L. crispatus, L. acidophilus, S. thermophilus, Leu. mesenteroides,* and *Lac. lactis.*

Supplementary Materials: Figure S1 illustrates relative abundances of bacterial phyla and an aggregation of the 21 most abundant bacterial genera for the V1-V3 and V3-V4 regions of the 16S rRNA genes in four commercial kefir controls.

Author Contributions: Conceptualization, G.Ü.; methodology, A.S., M.B.B., and G.Ü; formal analysis, A.S., M.B.B., and G.Ü; investigation, A.S., M.B.B., and G.Ü; resources, A.S., M.B.B., and G.Ü; writing—original draft preparation, A.S. and M.B.B.; writing—review and editing, A.S., M.B.B., and G.Ü; supervision, G.Ü; project administration, G.Ü.; funding acquisition, A.S. and G.Ü.; All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the United States Department of Agriculture (USDA) National Institute of Food and Agriculture (NIFA) Hatch project [1015890)] and the BUILD Dairy Program of Utah State University (17597). The APC was funded by the BUILD Dairy Program of Utah State University (17597).

Acknowledgments: We thank Saudi Arabian Cultural Mission for their support of Abrar Sindi. We express our appreciation to Mete Yüksel for his assistance with the figures.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Chapter 4: Contributions and Recommendations for Future Work

Contributions:

Pathogens can readily contaminate food. Chemical and natural antimicrobials can successfully be used to eliminate foodborne pathogens that might be present in minimally processed food. Biopreservation is a newer and reliable method that has been used to inhibit foodborne pathogens such as *Listeria monocytogenes*, *Salmonella enterica* serovar Enteritidis, *Staphylococcus aureus*, and *Bacillus cereus.* LAB and/or their metabolites, such as bacteriocins, can be used as biopreservatives as long as they do not change the properties of foods. The use of bacteriocins as natural antimicrobials is emerging in the area of biopreservation. Bacteriocins can be isolated from fermented foods where they are produced by LAB. Artisanal kefir is a fermented dairy product that is rich in LAB and their antimicrobials, including bacteriocins.

This dissertation compared the antimicrobial activity of artisanal kefirs from Fusion Tea, Britain, Ireland, Lithuania, the Caucasus region, and South Korea against select foodborne pathogens. Artisanal kefirs successfully inhibited the growth of *L. monocytogenes, S. enterica* serovar Enteritidis, *S. aureus*, and *B. cereus*. Bacteriocin production was determined to be the main reason for these kefirs' antimicrobial activity. Kefir-based antimicrobials are being proposed as promising natural biopreservatives as per the results of the study.

This dissertation compared multiple kefir grains and their products for their microbial diversity. The 16S rRNA sequencing successfully revealed the microbial populations in six international artisanal kefirs and demonstrated the diversity and abundance of LAB found in each kefir tested, many with probiotic potentials and reported capability of producing bacteriocins. LAB species found in high relative abundance in all artisanal kefirs included: *L.*

kefiranofaciens, Lenti. kefiri, L. ultunensis, L. apis, L. gigeriorum, G. morbifer, A. orleanensis, A. pasteurianus, Acid. aluminiidurans, and *L. helveticus.* LAB with documented bacteriocin production capabilities and probiotic activity, *Strep. thermophilus, Lenti. kefiri, L. helveticus, L. delbrueckii, Lacti. paracasei, Lacti. casei, Lacti. rhamnosus, L. crispatus, Leu. mesenteroides* were found in diverse relative abundance in the artisanal kefirs examined in this study. The 16S rRNA gene sequencing revealed LAB that might be responsible for the production of bacteriocins linked to the inhibition of foodborne pathogenic bacteria, including *L. monocytogenes*, *S. enterica* serovar Enteritidis, *S. aureus*, and *B. cereus*.

This dissertation offers several contributions to the study of kefir. This dissertation (1) compared, for the very first time, the antimicrobial activity of artisanal kefirs, originating from different countries of the world, against *S. enterica, S. aureus, B. cereus,* and *L. monocytogenes*; (2) determined that the antimicrobial effect in artisanal kefirs is mainly due to bacteriocin production but not due to other antimicrobials; (3) identified the bacterial populations in six artisanal kefirs by sequencing the two variable regions (V1-V3 and V3-V4) of the 16S rRNA gene unlike prior published research; (4) reported bacterial genera/species with known health-promoting benefits and bacterial genera/species known for their bacteriocin production capabilities in all kefir types; (5) used the new taxonomy for *Lactobacillus,* which differentiates *Lentilactobacillus* and *Lacticaseibacillus* from the genus *Lactobacillus;* (6) reported the occurrence of *L. gigeriorum* and *Acid. aluminiidurans* in kefir for the first time.

Recommendations

Kefir-based antimicrobials are being explored in our laboratory as promising natural biopreservatives. Further work is needed to study the application of kefir supernatants, freeze-dried kefir preparations, and kefir-based bacteriocins themselves against select foodborne pathogens in food matrix such as cottage cheese, RTE meat, etc. These proposed studies need to consider the effect of the addition of bacteriocin on food properties such as color, structure, rheology, and composition. It is important to find out the best mechanism for application (how it is added) and the time (when it is added) in the process. As mentioned in the first chapter (literature review), some bacteriocins are heat resistant, while others are not. In addition, pH can affect bacteriocin activity, and consequently impact the process of bacterial inhibition. Further work is needed to study the antimicrobial activities of kefir supernatants, freeze-dried kefir preparations, and kefir-based bacteriocins after heat treatment or pH alteration.

The concentration of filter-sterilized artisanal kefir samples is highly recommended. I tried to concentrate artisanal kefir using ultrafiltration tubes with centrifugation (Amicon Ultra-15 Centrifugal Filter Unit, 3-100 kDa, Millipore Sigma) at 4,000 or 5,000 *g* for 15–60 minutes, but the effort was not successful. The antimicrobial activity of the concentrated products did not show larger inhibition zones but showed reduced inhibition zones instead. This could be due to the potential inactivation of proteinaceous bacteriocins due to temperature increase during long-term centrifugation. I recommend using other concentration methods for kefir supernatants such as freeze-drying.

Future research should also study bacteriocins' arbitrary units for the proposed application of kefir as a natural antimicrobial. In our study, we standardized kefir using the

final pH and total protein content. However, when kefir-based biopreservatives are added into food, it is recommended to calculate the arbitrary units before application to standardize the procedure and to determine the specific affective arbitrary units which inhibit foodborne pathogens. By definition, the arbitrary unit of a bacteriocin preparation is the highest dilution showing a clear zone of inhibition of an indicator strain of choice.

Future research should also deal with the isolation of LAB with known bacteriocin production capabilities from artisanal kefirs of interest. Some of these bacteriocins might be novel bacteriocins that can be subjected to isolation, purification, and characterization at the biochemical and molecular levels. These novel bacteriocins can then find applications in biopreservation of foods, RTE foods, in particular.

In this study DNA extraction from kefir grains and products was challenging. Some traditional methods, which were intensively mentioned in the literature, were used. Liquid nitrogen was used to freeze kefir grains and their products, but this process led to low quality DNA. Modern DNA extraction kits (QIAGEN's DNeasy Mericon Food Kit, DNeasy Stool kit, DNeasy Soil Kit, OMEGA's E.Z.N.A Soil DNA Kit, Food DNA Kit, Bacterial DNA Kit, MicroElute Genomic DNA Kit) were used also, but most of them did not work well. Specifically, no DNA could be extracted, or the quality or quantity of the DNA obtained was unsatisfactory. Future research should deal with new DNA extraction kits promising better extraction yields and DNA quality. In addition, the use of multiple washing steps (more than what is usually recommended by the manufacturer of the kits) is recommended to improve DNA extraction processes.

The 16S rRNA gene sequencing is a good tool to study bacterial community in kefir. Whole genome sequencing (WGS) can simultaneously identify yeast and bacterial

communities. The WGS was not needed in our study because we focused on LAB. Future studies can include yeast identification in international artisanal kefirs. Although yeast does not produce bacteriocins, some yeast is considered probiotic. Kefir is an example of a complex symbiotic relationship between yeast and bacterial species. Learning more about microbe-microbe interactions in kefir upon identification of microbial abundance would be particularly useful for future research in kefir.

Although commercial kefir is known as a probiotic dairy product, artisanal kefir has a more diverse microbial community. In this study, LAB species with reported probiotic activities were identified in the international artisanal kefirs of interest. Therefore, future research on the isolation/characterization of the probiotic bacteria from artisanal kefirs is highly recommended.

Appendix A. Relative abundances of bacterial phylum (Pie charts) and an aggregation of the 21 most abundant bacterial genera (column charts) for the V1–V3 (A) and V3–V4 (B) regions of 16S rRNA genes. Commercial kefir controls: Lifeway (plain), The Greek Gods (plain), Wallaby (organic plain), and Maple Hill (organic plain).

Supplementary Materials