ANTI-SALMONELLA PROPERTIES OF KEFIR AND KEFIR YEAST ISOLATES: POTENTIAL APPLICATION IN INFECTION CONTROL AND PREVENTION

A thesis submitted in fulfilment of the requirements of the degree of

Doctor of Philosophy

By

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This thesis is dedicated to my parents; my beloved father late Gut Jok Yuot and loving mother Nyabol Ngong (Chinkok) Chol.

ABSTRACT

The rise of antibiotic resistance has increased the need for alternative ways of controlling and preventing enteric bacterial infections. Various probiotic bacteria have been used in animals and humans prophylactically and therapeutically. Kefir is an acidic and low alcoholic beverage produced by fermentation of milk, fruit juice, or sugary water with kefir grains and its consumption is associated with prophylactic and therapeutic properties conferred by probiotics components. There is scarce research conducted on kefir and kefir yeast isolates despite claimed potential preventative and curative effect on enteric bacterial pathogens. This thesis investigates traditional kefir and kefir yeast isolates for potential application in the prevention and control of *Salmonella* in *in vitro* experiments. *Kluyveromyces lactis* and *Saccharomyces unisporus*, *Lactobacillus kefiri,* and *Lactococcus lactis* were isolated from kefir and identified using 26s rDNA, ITS region sequencing and MALDI-TOF for yeasts and bacteria respectively. Kefir made from two traditional kefir grains rapidly killed *Salmonella Arizoniae* and *Salmonella* Typhimurium possibly due to the action of lactic acid as kefir cell-free supernatant analysis showed high concentration of lactic acid ranging from 83.59 to 229.92 mM. Other compounds with recognized antibacterial activities including carbonyl compounds, histone, cathelicidin, and various peptides were also detected using shotgun proteomics. Kefir yeast isolates showed some potential probiotic properties including survival in the gastrointestinal tract, auto-aggregation, hydrophobicity and lack of hydrolytic enzymes production. These probiotic characteristics were comparable to *Saccharomyces boulardii* strains used as controls in the study. Adhesion and sedimentation slide agglutination, microscopy, and turbidimetry showed that *Salmonella* adhered onto yeast cells, which resulted in growth inhibition. Furthermore, yeast-fermented

killer toxin medium showed *Salmonella* growth inhibition likely due to antimicrobial metabolites such as cathelicidin detected by shotgun proteomics in the cell-free supernatant. In conclusion, kefir and kefir yeast isolates may have the potential to control and prevent *Salmonella* infection.

Certificate

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This is to certify that the thesis titled “Anti-salmonella properties of kefir and kefir yeast isolates*:* potential infection control and prevention” submitted by Abraham Majak Gut to Victoria University in partial fulfilment of the requirement for the award of Doctor of Philosophy with specialization in microbiology, is a record of a bonafide research work carried out by him under my personal guidance and supervision and that the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Dr. Osaana N. Donkor

(Principal Supervisor)

Date: 16/11/21

DECLARATION

"I, Abraham Majak Gut, declare that the PhD thesis entitled "Anti-salmonella properties of kefir and kefir yeast isolates*:* potential infection control and prevention" is no more than 80,000 words in length including quotes, tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work. I have conducted my research in alignment with the Australian code for the responsible conduct of research and Victoria University's higher degree by research policy and procedures".

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Abraham Majak Gut

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Chapter No.	Publication Title	Publication Status • Published • Accepted for publication • In revised and resubmit stage • Under review • Manuscript ready for submission	Publication Details • Citation, if published • Title, Journal, Date of acceptance letter and Corresponding editor's email address • Title, Journal, Date of submission
3	Salmonella infection–prevention and treatment by antibiotics and probiotic yeasts: a review	Published	Microbiology Society, SJR Q2; published 23/08/2018; citations: 62.
4	Kefir characteristics and ant bacterial properties-potential applications in control of enteric bacterial infection.	Published	International Dairy Journal, SJR Q1; published 25/02/2021; citations: 2.
5	Antimicrobial properties of traditional kefir: an in vitro screening for antagonistic effect on Salmonella Typhimurium and Salmonella Arizonae	Published	International Dairy Journal, SJR Q1; published 13/08/2021
6	Characterization of yeasts isolated from traditional kefir grains for potential probiotic properties	Published	Journal of Functional Foods, SJR Q1; published 30/04/2019; citations: 26.
7	Anti-salmonella properties of kefir yeast isolates: an in vitro screening for potential infection control	Published	Saudi Journal of Biological Sciences, SJR Q1; published on line 20/09/2021

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LIST OF ABBREVIATIONS

AAD	Antibiotic-associated diarrhoea
ACE	Angiotensin-converting enzyme
AGA1	A-agglutinin anchorage subunit
AP	Activator protein
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
ATP	Adenosine triphosphate
B cells	Lymphocytes from bone marrow
BAX	BCL2-Associated X Protein
Bcl-2	B-cell lymphoma 2
$CaCl_2$	Calcium chloride
Caco-2	Human colonic epithelial cell lines
cAMP	Adenosine 3′, 5′-cyclic monophosphate
CD	Cluster of differentiation
CED	Cell death abnormality protein
CFS	Cell free supernatant
CFU	Colony Forming Unit
CorA	Magnesium transport protein
CR3	Complement receptor 3
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
EPS	Extracellular polysaccharide

ERK 1/2	Extracellular signal-regulated kinases
FAO	Food and Agriculture Organization
FIG2	Factor-induced gene 2 protein
fliC	*Salmonella* flagellin gene
FLO	Flocculation protein
GC	Gas chromatography
GCMS	Gas chromatography mass spectrometer
G-CSF	Granulocyte colony-stimulating factor
GIT	Gastrointestinal tract
GPR	G protein-coupled receptors
h	Hour
H antigen	Flagella antigen
HCL	Hydrochloric acid
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HSK	Kefir originated from Kazakhstan
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
INOS	Inducible nitric oxide synthase
IPEC	Intestinal epithelial cell lines
ITS	Internal transcribed spacer
JNK	c-Jun N-terminal kinases
K. lactis	*Kluyveromyces lactis*

kb	Kilobase
KCFS	Kefir cell-free supernatant
KCl	Potassium Chloride
Kg	Kilo gram
KH_2PO_4	Potassium dihydrogen phosphate
KTM	killer toxin medium
L	Litter
LAB	Lactic Acid Bacteria
LAB	Lactic acid bacteria
LC-MS	Liquid chromatography–mass spectrometry
Lg-FLO1	Gene encoding flocculin
Lpf	Long polar fimbriae
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionisation - Time of Flight
MAMP	Microbe-associated Molecular Pattern
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MEL	Mannosylerthritol lipids
$MgCl_2 \cdot 6H_2O$	Magnesium chloride hexahydrate
MgtA	Magnesium-transporting ATPase, P-type 1 for *S.* Typhimurium
MgtB	Magnesium-transporting ATPase, P-type 1 for E. coli
µL	Microliter

mL	Milliliter
MRS	De Man, Rogosa and Sharpe
MRS	De Man, Rogosa and Sharpe
MRS-NNLP	nalidixic acid, neomycin sulfate, lithium chloride, and paramomycin sulfate
MSK1	Mitogen- and stress-activated protein kinase-1
MUC1	Mucin-like protein
NaCl	Sodium Chloride
NADH	Nicotinamide adenine dinucleotide hydrogenase
NAFLD	Non-alcoholic fatty liver disease
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NATA	National Association of Testing Authorities
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
$(NH_4)_2CO_3$	Ammonium carbonate
NNDSS	National Notifiable Diseases Surveillance System
NTS	Non-typhoidal *Salmonella*
O antigen	Capsular antigen
OD	Optical density
PBS	Phosphate buffered saline
pH	Potential hydrogen
PqsA	Pseudomonas quinolone signal gene A
PRR	pattern recognition receptors

RBCA	Rose-Bengal chloramphenicol agar
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
RNA	Ribonucleic acid
S	Second
S. Dublin	*Salmonella* enterica serovar Dublin
S. Enteritidis	*Salmonella* enterica serovar Enteritidis
S. Heidelberg	*Salmonella* enterica serovar Heidelberg
S. Newport	*Salmonella* enterica serovar Newport
S. paratyphi	*Salmonella* enterica serovar Paratyphi
S. Typhi	*Salmonella* enterica serovar Typhi
S. Typhimuriym	*Salmonella* enterica serovar Typhimurium
S. unisporus	*Saccharomyces unisporus*
SAIF	*S. boulardii* anti-inflammatory factor
SAPK	stress-activated protein kinase
SB48 MYA-796	*Saccharomyces boulardii* strain SB48/MYA-796
SB49 MYA-797	*Saccharomyces boulardii* strain SB49/ MYA-797
SCARF1	scavenger receptor class F member 1
SD	standard deviation
SDA	Sabouraud dextrose agar
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SPI	*Salmonella* pathogenicity island

SPSS	Statistical Package for the Social Sciences
SREC	scavenger receptor from endothelial cells
T cells	Lymphocytes that mature in thymus
T3SS	Type III secretion system
Tafi	Thin aggregative fimbriae
TCA	Tricarboxylic acid cycle
TcdA	*C. difficile* toxin B
TcdB	*C. difficile* toxin B
TLR	Toll-like receptor
TMP-SMX	Trimethoprim/sulfamethoxazole
TNF	Tumour necrosis factor
Trk	Potassium uptake protein
tviA	Virulence polysaccharide biosynthesis protein for S. paratyphi
tviB	Virulence polysaccharide biosynthesis protein for *S.* Typhi
TVR	Kefir originated from Russia
Ty21a	Attenuated live *S.* Typhi vaccine
UHT	Ultra-high temperature
V/V	Volume per volume
Vi	Capsular polysaccharide vaccine for typhoid fever
Vi-rEPA	Recombinant exoprotein A of Pseudomonas aeruginosa (Vi-rEPA)/S. Typhi vaccine
WHO	World Health Organization

XLD	Xylose Lysine Deoxycholate
YEPD	Yeast extract peptone dextrose
ZnuABC	zinc import ATP-binding protein

Chapter 1: Introduction

This chapter introduces thesis by giving brief background information, aim and specific objectives, and structure of the thesis.

1.1. Introduction

The burden of morbidity and mortality from human enteropathogenic bacteria, including *Salmonella* species, globally is immense despite the presence of antibiotic drugs. It is estimated that *Salmonella* infection causes 2.8 billion cases of diarrhea annually worldwide. *Salmonella enterica* serovar Typhi *(S.* Typhi), the bacteria responsible for typhoid fever, is reported to cause 16 - 33 million cases with an estimated 500,000 to 600,000 deaths, whereas non-typhoidal *Salmonella* (NTS) infections is responsible for 90 million cases and 155,000 deaths worldwide annually (Bula-Rudas, Rathore, & Maraqa, 2015).

Salmonella is a Gram-negative non-spore-forming rod-shaped and facultative anaerobic bacterium from the *Enterobacteriaceae* family. Members of the *Salmonella* genus are motile by means of a peritrichous flagellum and measure 2–5 μm long by 0.5–1.5 μm wide in cell size, and genome ranges from 4,460 to 4,857 kb. *Salmonella* was first identified in a veterinary laboratory in the 19th century in the USA. Members of *Salmonella* genus are lactose fermenters (some sub-species), generally hydrogen sulfite producer, oxidase-negative, and catalase-positive. *Salmonella* hydrolyses urea, utilizes citrate, and decarboxylates lysine as its sole carbon source (Andino & Hanning, 2015; Feasey, Dougan, Kingsley, Heyderman, & Gordon, 2012). *Salmonella* is classed into two *Salmonella enterica* and *Salmonella bongori*. Based on biochemical and genomic characteristics, *Salmonella enterica* is further divided into *enterica, salamae, arizonae, diarizonae, houtenae,* and *indica* subspecies (Andino & Hanning, 2015; Brenner, Villar, Angulo, Tauxe, & Swaminathan, 2000; Tindall, Grimont, Garrity, & Euzeby, 2005). Clinically important *Salmonella* species are classified

under *Salmonella enterica*, which is again divided into more than 2,579 serovars on the basis of their antigenicity (Andino & Hanning, 2015; Monte & De Santos, 2012). Infections caused by *S.* Typhi and *S.* Paratyphi may involve life-threatening complications and need treatment with antibiotics such as cefixime, chloramphenicol, amoxicillin, trimethoprim-sulfamethoxazole (TMP-SMX), azithromycin, aztreonam, cefotaxime, or ceftriaxone to prevent death (Kumar & Kumar, 2017). Resistance of *Salmonella* species to antibiotic drugs is emerging (Crump, Sjölund-Karlsson, Gordon, & Parry, 2015) as exemplified by a report in Malawi where 7% of *S.* Typhi infection cases were multi-drug resistant in 2010, but in 2014, the resistance percentage increased to 97% (Feasey et al., 2015; Wong, Baker, Pickard, & Parkhill, 2015). In the USA, *S.* Enteritidis accounted for 50% of ciprofloxacin-resistant infections, while *S.* Newport, *S.* Typhimurium, and *S.* Heidelberg were reported to be responsible for 75% of the antibiotic-resistant infections, because of their resistance to ceftriaxone and ampicillin. This resistance coupled with the effect of antibiotics on normal gut microbiota, and antibiotic-associated diarrhea, bring a growing need for alternative treatments, including the use of probiotic microorganisms. Probiotics are defined by the World Health Organization (WHO) and Food and Agriculture Organization (FAO) as 'live microorganisms which when administered in adequate amount confer a health benefit on the host' (FAO/WHO, 2002).

Probiotic bacteria found in *Lactobacillus, Lacticaseibacillus, Ligilactobacillus, Lactiplantibacillus, Limosilactobacillus, Enterococcus, Bifidobacterium*, *Pediococcus, E.coli, Streptococcus*, and *Leuconostoc* genera naturally exist in human GIT, or are introduced into GIT via probiotic products ingestion (Zheng et al., 2020; Priyodip, Prakash, & Balaji, 2017). Probiotics are natural components of products such as kefir

or are artificially included in popular fermented functional foods such as yogurt, milk, cheese, soybean, fruits, sourdough, and vegetable products making their consumption easier and enjoyable while at the same time providing health benefits (Plessas et al., 2016; Prado et al., 2015; Priyodip et al., 2017; Saarela, Mogensen, Fondén, Mättö, & Mattila-Sandholm, 2000).

Kefir is a low alcoholic, and acidic product made from kefir grain. Kefir grain is a consortium of exopolysaccharides and many microorganisms (Plessas et al., 2016; Prado et al., 2015). Bacterial components of kefir grains including *Lactobacillus, Lactococcus, Leuconostoc,* and *Streptococcus* genera are known to possess probiotic properties (Plessas et al., 2016). The yeast components include *Kluyveromyces, Candida, Saccharomyces, and Pichia* (Plessas et al., 2016). Kefir is widely consumed in the Caucasus Mountains, Europe, Asia, South and North America due to its health benefits conferred by the microbial components (Plessas et al., 2016). Kefir consumption has been linked with health benefits in the management and treatment of gastrointestinal problems, hypertension, allergies, cancers, and ischemic heart disease. Furthermore, antibacterial properties against pathogenic bacteria have been reported (Bourrie et al., 2016; Zavala et al., 2016).

1.2. Research aim and objectives

This research overall aim is to investigate the anti-salmonella properties of two traditional kefir drinks and their yeast isolates in an *in vitro* laboratory experiments. This aim will be achieved through the following key objectives:

1. Assess anti-salmonella properties of two traditional kefir drinks against *Salmonella* Arizonae and *Salmonella* Typhimurium.

2. Isolate, identify and screen kefir grains yeast isolates for basic probiotic properties.
3. Assess adherence of *Salmonella* Arizonae and *Salmonella* Typhimurium onto yeast cell wall as a potential way of infection control and prevention.
4. Assess kefir yeast isolates for anti-salmonella metabolites production.

1.3. Thesis outline

Chapter 1 introduces *Salmonella* infection by giving brief background. It also provides the aim and objectives of the research project and thesis outline. Chapter 2 supplements literature review covered in chapter 3 and 4 by focusing on probiotic properties. It briefly highlights the general health benefits of probiotics and probiotic products consumption; however, antimicrobial properties of probiotics are covered in more detail.

Chapter 3 provides a critical review of *Salmonella* infections, the current treatment options of *Salmonella* infection. It seeks to understand the prophylactic and therapeutic potentials of probiotic microorganisms, their mechanisms of action in preventing and treating *Salmonella* and other enteric pathogens infections. In particular, the chapter focuses on probiotic yeasts; however, probiotic bacteria are also briefly discussed. Chapter 4 is a literature review, which focuses on the prophylactic and therapeutic potential of kefir. Antibacterial properties of kefir on enteric bacterial pathogens are comprehensively discussed while other health benefits of kefir consumption on immunity and gastrointestinal improvement are briefly highlighted.

Chapter 5 focuses on the analysis of two different traditional kefir drinks made from uncharacterized kefir grains. The antimicrobial effect of these drinks on *Salmonella enterica* serovar Arizonae and *Salmonella enterica* serovar Typhimurium were investigated. Antimicrobial molecules in kefir were determined. Chapter 6 concentrates on

isolating, identifying, and characterizing yeasts isolated from two traditional kefir grains for general probiotic properties. Chapter 7 focuses on screening kefir yeast isolates for potential application in *Salmonella* infection control. *Salmonella* adhesion onto kefir isolates as well as growth inhibition due to antibacterial metabolites was analyzed in *in vitro* experiments in comparison to *Saccharomyces boulardii* strains. Chapter 8 summarizes the finding of the research. It highlights the significance of the study and recommends future studies.

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Chapter 2. Supplementary Literature review: probiotics

Chapter 2 supplements the literature review covered in chapter 3 and 4 by focusing on probiotic properties. It briefly highlights the general health benefits of probiotics and probiotic products consumption; however, antimicrobial properties of probiotics are covered in more detail.

2.1. Introduction

Probiotics are defined by the World Health Organization (WHO) and Food and Agriculture Organization (FAO) as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). Probiotics are commonly included or exist naturally in popular fermented functional foods such as yogurt, milk, cheese, soybean, fruits, sourdough, kefir, and vegetable products (Prado et al., 2015; Priyodip, Prakash, & Balaji, 2017; Saarela, Mogensen, Fondén, Mättö, & Mattila-Sandholm, 2000). Probiotic products can be lyophilized as in capsules or powders or in aqueous solutions (Martins, Veloso, Arantes, & Nicoli, 2009). Furthermore, probiotic bacterial genera such as *Lactobacillus, Lacticaseibacillus, Ligilactobacillus, Lactiplantibacillus, Limosilactobacillus Enterococcus, Bifidobacterium*, *Pediococcus, E. coli, Streptococcus*, and *Leuconostoc* are found in the human gastrointestinal tract (GIT) where they form part of the normal microbiota or are introduced into GIT via probiotic products ingestion (Zheng et al., 2020; Priyodip et al., 2017).

To qualify as effective probiotic, microorganisms are required to meet specific criteria. These include adherence to host cells in the GIT, ability to exclude or reduce adherence of pathogens to the GIT, an ability to persist and multiply in the GIT (that is being able to withstand acidic gastric juice, basic pancreatic juice, lysozyme, and bile salts exposure), and ability to modulate immunity. Furthermore, other criteria include the ability to produce acids, hydrogen peroxide, and bacteriocins that are antagonistic to the pathogens; ability to co-aggregate or auto-aggregate to form a normal sustaining microbiota. They must possess some, but not all these properties to qualify as probiotics. Demonstration of health benefits in randomized clinical trial is required for microorganism to be classified as probiotic (Morelli, 2000). Moreover, probiotic microorganisms should be non-carcinogenic and non-pathogenic (FAO/WHO, 2002; Kaur,

Chopra, & Saini, 2002; Perez-Sotelo et al., 2005). These probiotic selection criteria are strain-specific. Therefore, full identification and characterization of potential probiotic microorganisms are also required (Kesenkaş, Gürsoy, & Özbaş, 2017). Methods including molecular, phenotypes/biochemical, and Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) are used in the identification of probiotics (Angelakis, Million, Henry, & Raoult, 2011; Yadav & Shukla, 2017).

Many health benefits of probiotic organisms have been reported and these include antimicrobial properties, immunomodulation, alleviation of symptoms of lactose intolerance, treatment of diarrhea, anti-carcinogenicity, reduction of blood cholesterol, antihypertensive properties, and biotransformation of isoflavone phytoestrogen to improve hormonal balance in post-menopausal women (Kesen & Aiyegoro, 2018; Lye, Kuan, Ewe, Fung, & Liong, 2009).

Probiotic microorganisms' mode of action against pathogens includes modulation of both innate and acquired immunity, direct action on the pathogens in the GIT, and production of antimicrobial molecules (Oelschlaeger, 2010). These mechanisms of action are influenced by probiotics metabolism, cell surface molecules, and the ability to secrete antibacterial molecules (Oelschlaeger, 2010).

Lactobacillus, Bifidobacterium and *Saccharomyces boulardii* (*S. boulardii*) are the most commonly used probiotics in the prevention and treatment of many diseases including infectious diseases such as antibiotic-associated and travelers' diarrheas. However, other microorganisms, including *Streptococcus thermophilus, Enterococcus faecium*, *Leuconostoc* species*, Escherichia coli* (*E. coli*) Nissle 1917 strain, and *Bacillus* species, are being researched *in vitro* or in animals and human trials; or they are used in humans for prophylaxis or therapeutic purposes (Bakken, 2014; Bekar, Yilmaz,

& Gulten, 2011; Khodadad, Farahmand, Najafi, & Shoaran, 2013; Nami et al., 2015). Figure 1 summarizes examples of probiotics and potential probiotic microorganisms.

This supplementary chapter focuses on probiotic properties. It briefly highlights the general health benefits of probiotics and consumption of probiotic products; however, the antimicrobial properties of probiotics are covered in more detail.

2.2.1. Probiotic microorganisms

Traditionally, lactic acid bacteria (LAB) were perceived to be the only microorganisms with probiotic properties. However, other microorganisms, which are not members of LAB, have probiotic properties as shown in Figure 1 below. LAB are defined as facultative anaerobes, fastidious, acid-tolerant, and fermentative Gram-positive rods or cocci. LAB lack cytochrome C and therefore, are catalase-negative. Based on physiology and biochemical properties, LAB include *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus*, *Weissella*, and *Bifidobacterium* genera. However, *Bifidobacterium* is phylogenetically different from the rest of LAB as it contain higher guanine + cytosine (>55 mol %) and are classified as part of *Actinomycetes* phylum (Salminen et al., 1998; Wessels et al., 2004). Furthermore, LAB are classified based on carbohydrate catabolism into homo-fermentative and hetero-fermentative. *Lactococcus*, *Pediococcus*, *Enterococcus*, *Streptococcus* main product of catabolism is lactic acid and therefore classified as homo-fermentative while LAB such *Leuconostoc*, *Weissella* and some lactobacilli fermentation products are heterogeneous and include lactate, CO_2, ethanol or acetate; hence referred to as hetero-fermentative LAB (Gänzle, 2015; Klein, Pack, Bonaparte, & Reuter, 1998).

While bacteria genera have been the focus of studies with respect to probiotic properties, yeasts especially *Saccharomyces boulardii* has been proven as probiotics. Yeasts are classified into ascomycetes, and basidiomycetes (Walker, 1998; Watkinson, Boddy, & Money, 2015). The ascomycetes division contains yeast species with probiotic potentials including *Saccharomyces, Schizosaccharomyces, Kluveromyces, Zygosaccharomyces*, and *Devaryomyces* genera (Walker, 1998). Studies have indicated that different yeast species can be efficaciously used in treatment and diseases prevention. However, *Saccharomyces boulardii* is the only yeast strain characterized and being used as a probiotic in humans (Kelesidis & Pothoulakis, 2012; Rajkowska & Kunicka-Styczyńska, 2012; Tomičić et al., 2016), while other yeast species and strains have been proven to be efficacious in *in vitro* and in animals' trials (Palma et al., 2015).

De Man, Rogosa and Sharpe (MRS) agar (Charalampopoulos, Pandiella, & Webb, 2002), M17 medium (Kimoto-Nira, Kobayashi, Nomura, Sasaki, & Suzuki, 2009) can be used for isolation and enumeration of *Lactobacillus* and *Lactococcus* respectively with growth temperature of 35-40 °C for 2-3 days depending on target species. Furthermore, MRS agar can be supplemented with bile and pH adjusted to about 5.2 to make it selective for lactobacilli. MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride, and paramomycin sulfate) is a selective medium for the isolation and enumeration of *Bifidobacterium* species (Van de Casteele et al., 2006) with anaerobic incubation at 37 °C for 3 days. Many microbiological media are used in the isolation and enumeration of fungi including yeasts. Rose-Bengal chloramphenicol agar can be used in isolation and enumeration of yeasts with incubation at 25 °C for up to 5 days (Garofalo et al. 2015). Other media that can be used for the isolation of potential

probiotic yeasts include dichloran rose-bengal chloramphenicol, dichloran-glycerol, potato dextrose agar and oxytetracyclin-glucose-yeast media (Pitt et al., 1986).

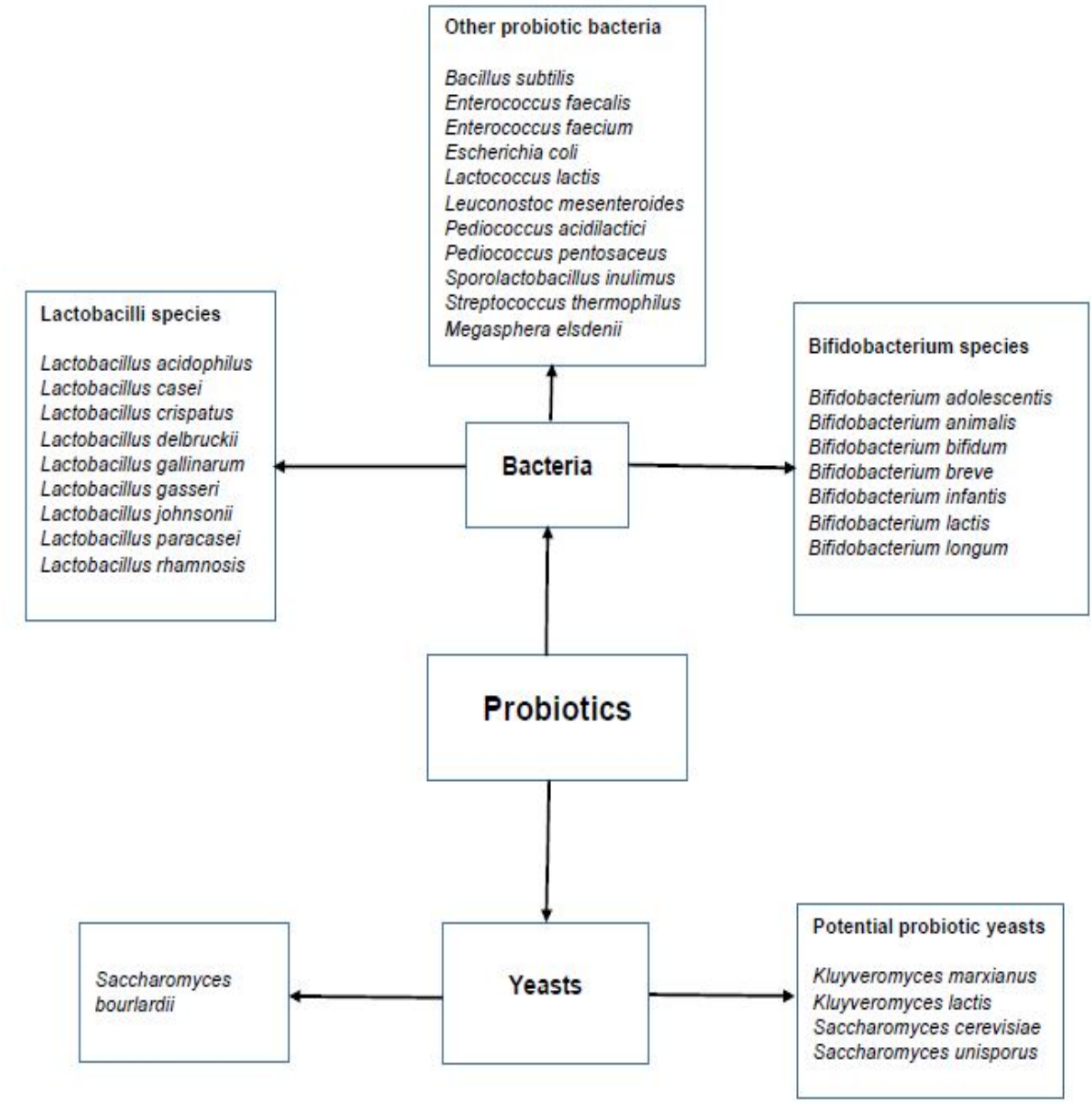

Figure 1. Examples of probiotics and potential probiotic microorganisms (Kesen & Aiyegoro, 2018).

2.2.2. Parabiotics

Parabiotics, also referred to as ghost or inactivated probiotics are defined as “non-viable microbial cells (either intact or broken) or crude cell extracts which when administered (either orally or topically) in adequate amounts, confer a benefit on the human

or animal consumer" (Nataraj, Ali, Behare, & Yadav, 2020). Paraprobiotic products may include intact non-viable microbial cells of probiotics or ruptured cell components such as teichoic acids, peptidoglycan-derived muropeptides, pili, fimbriae, flagella, polysaccharides, cell surface-associated proteins, cell wall-bound biosurfactants (Nataraj et al., 2020). Just like probiotics counterpart, consumption of parabiotics have been shown to confer desirable therapeutic and prophylactic properties include anti-inflammatory, gut barrier protection, anti-adhesion of pathogens onto human GIT, anti-biofilm formation, anti-viral, immunomodulatory, antihypertensive, hypocholesterolemic, anti-proliferative of cancerous cells, and antioxidant (Nataraj et al., 2020).

Parabiotics may have a critical application advantage over probiotic yeasts such as *S. bourladii* as viable yeast cells have been associated with 100 fungemia cases in immunocompromised people or those with gastrointestinal diseases (Kelesidis & Pothoulakis, 2012). Moreover, parabiotics have other advantages over probiotics including well-defined mode of action, availability in pure forms, availability of production processes for large industrial-scale-up, better accessibility of microbes associated molecular pattern (MAMP) during recognition and interaction with pattern recognition receptors (PRR), and easy storage (Nataraj et al., 2020).

2.2.3. Kefir as a probiotic product

The word kefir originated from the Turkish word *kef*, which means 'a pleasant taste' (Arslan, 2015). Other kefir synonyms include millet of the prophet, Mohomet grains, kefyr, kephir, kefer, kiaphur, knapon, kepi, and kippi. Its size ranges from 1-6 mm or sometimes up to 15 mm in diameter. It has a rough and convoluted surface and resembles a cauliflower floret (Garrote, Abraham, & De Antoni, 1997; Sarkar, 2008; Turkmen, 2017). Chemically, kefir grains are composed of 890-900 g/kg water, 2 g/kg fat, 30 g/kg protein, 60 g/kg sugars, and 7 g/kg ash, and these may vary depending on

the grain (Garrote et al., 1997). Microbiologically, kefir grain contains some of the well-known probiotics or potential probiotics including *Lactobacillus, Lactococcus, Leuconostoc*, *Streptococcus*, *Kluyveromyces, Candida, Saccharomyces and Pichia* (Plessas et al., 2016). It is widely consumed in the Caucasus Mountains, Europe, Asia, South and North America (Plessas et al., 2016). Consumption of kefir has been linked to its medicinal properties, which are attributed to its probiotics content and bioactive compounds produced in it. Its prophylactic and therapeutic properties such as anti-hypertension, anti-oxidation, anti-allergy, antitumor, anti-inflammation, and cholesterol lowering, and antimicrobial activities are being realized (Nejati, Junne, & Neubauer, 2020).

Three methods including traditional, Russian and large-scale industrial production are used in the production of kefir. The traditional method is a one-step fermentation, whereas the Russian method has two steps. The large-scale industrial production method includes the use of pure kefir cultures such as *Bifidobacterium* sp., *Lactobacillus* sp., and probiotic yeast (*S. boulardii*) rather than the naturally occurring kefir grain (Prado et al., 2015). Milk, rice, fruit juice or molasses are some of the matrix or beverages fermented with kefir grain or cultures to produce kefir drinks (Chunchom, Talubmook, & Deeseenthum, 2017; Kesenkaş et al., 2017; Liu, Wang, Lin, & Lin, 2002; Plessas et al., 2016).

2.2.4. Prebiotics

A prebiotic is “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health” (de Vrese & Schrezenmeir, 2008). Prebiotics include oligosaccharides, monosaccharides, polysaccharides, short-chain fatty acids, inulin, and fructans which are generally derived from plant roots, seeds, fruits, vegetables,

and marine herbs (Biswal, Pal, & Das, 2017). Ingestion of prebiotics has been associated with the potential to prevent or cure many diseases including disorders such as obesity, inflammatory bowel disease (IBD), diabetes, non-alcoholic fatty liver disease (NAFLD), antibiotic-associated diarrhea, colitis, constipation, cancer, and hepatic encephalopathy (Biswal et al., 2017). These health benefits attributed to prebiotics are due to their interaction with gut microbiota and subsequent fermentation in the GIT leading to increased production of short-chain fatty acids such as butyrate, propionate and acetate (Biswal et al., 2017). The interaction of short-chain fatty acids with metabolite-sensing G protein-coupled receptors (GPR41, GPR43 and GPR109A) in the GIT leads to short-chain fatty acid-mediated suppression of inflammation and carcinogenesis, not only in GIT but also in other organs (Sivaprakasam, Prasad, & Singh, 2016). However, these short-chain carbohydrates have been linked to GIT symptoms similar to those of irritable bowel syndrome. These symptoms are caused by their poor absorption, osmotic activity, and high fermentability (Killian et al., 2021).

2.2.5. Synbiotics

Synbiotic is defined as a mixture of prebiotics and probiotics that are aimed at conferring health benefits on the consumer by improving the survival and activity of beneficial microorganisms, the probiotic components (Gyawali et al., 2019). Many synbiotic products comprising of probiotic cocktails (such as Bifidobacterium spp. or *Lactobacillus* spp.) are used prophylactically and therapeutically. For example, synbiotic Synergy 1 and Synbiotic 2000 are some of the synbiotics in the market (Furrie et al., 2005; Rakel, 2017). The mixture in synbiotics has proven synergistic health effects on the hosts in that it enhances survival and establishment of probiotics in the GIT and selectively elicits the growth or activates the metabolism of health-promoting probiotics (Gyawali

et al., 2019). The health-promoting effect of the synbiotic mechanism is through modulation of the metabolic activity in the intestine and maintenance of the gut structure, flourishing of beneficial microbiota (with subsequent production of beneficial metabolites), and the inhibition of pathogens present in the gastrointestinal tract. Furthermore, the synergistic effect of synbiotics results in a reduction in undesirable metabolites as well as their inactivation (Gyawali et al., 2019).

2.2.6. Symbiosis

Naturally occurring fermented products such as kefir grains contain strains of lactobacilli*, Leuconostoc*, streptococci, lactococci; *Saccharomyces*, *Kluyveromyces*, *Candida* and *Pichia* among others, all bound together by kefiran (Plessas et al., 2017; Sarkar, 2008). Symbiosis is defined as a close and long-term biological interaction between two or more different organisms (Martín & Langella, 2019). The co-existence of microorganisms in products such as kefir grain is based on a symbiotic relationship. Three mechanisms have been hypothesized to be involved in nutrients (beneficial to bacteria in the symbiosis relationship) release by yeasts. Changes in yeast membrane permeability in the presence of glucose lead to a release of amino acids. Proteolytic and lipolytic enzymes produced by yeasts contribute to the breakdown of proteins and fats, leading to the release of amino acids/peptides, and fatty acids, respectively. Autolysis of yeast cells, which leads to the release of cell components including amino acids and vitamins (Lopitz-Otsoa, Rementeria, Elguezabal, & Garaizar, 2006; Ponomarova et al., 2017; Stadie, Gulitz, Ehrmann, & Vogel, 2013). The release of nutrients by yeast cells is an intrinsic characteristic of yeast species and not induced by bacteria (Ponomarova et al., 2017). Some yeast species including *Candida*, *Pichia* and *Kluyveromyces* can assimilate lactate causing the pH to rise, which favors bacterial growth. Yeast growth has been reported to be supported positively by bacteria through pH

reduction during lactic and acetic acid production (Stadie et al., 2013). The optimum growth pH for yeasts is about 4.5 -7. However, most species can grow at a pH of 2.0–2.5 (Fröhlich-Wyder, Arias-Roth, & Jakob, 2019).

2.3. Probiotics properties

A potential probiotic candidate should have more than one desirable attribute namely, prophylactic and therapeutic properties. Desirable probiotic properties including safety, survival in the GIT, antimicrobial activity, immunomodulation, antioxidant activity, and improvement of GIT health are briefly discussed below.

2.3.1. Probiotic safety

The safety of probiotics and probiotic products is an important aspect that needs consideration before they are approved for use. The starting point in the assessment of probiotics for safety is identification to strain level (Binda et al., 2020), followed by laboratory assessment for virulence factors and absence of acquired antimicrobial genes. Specific guidelines are issued by European Food Safety Authority including phenotypic cut-off for antimicrobial susceptibility and resistance values. Molecular methods such as whole-genome sequencing are also employed to detect the presence of antimicrobial resistance genes (Binda et al., 2020). For example, the presence of antibiotic-resistant genes has been detected in certain strains of *Enterococci* and *Lactobacillus lactis* (Czerucka, Piche, & Rampal, 2007) and there is a risk of passing these genes to enteric bacterial pathogens. Furthermore, genome sequencing can be used to detect the presence of genes encoding known virulence factors such as the production of bacterial toxins, invasion factors (for example hemolysis, phospholipases) (Binda et al., 2020). Alternatively, potential probiotics can be assessed phenotypically using agar or broth media for virulence factors (Binda et al., 2020).

2.3.2. Survival in GIT

Survival in the GIT is an important criterion for microorganisms to be classified as probiotic, which involves being able to resist harsh gastric juice contents such as pepsin, basic pancreatic enzymes lysozyme, and bile salts at physiological temperature (Binda et al., 2020). *Bifidobacterium bifidum, Lactobacillus acidophilus, Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Streptococcus thermophilus* survival was measured under physiological conditions *in vitro* and *in vivo* and were found to survive harsh GIT conditions (Marteau, Minekus, Havenaar, & Huis In't Veld, 1997) and their survival was due to maintenance of homeostasis through the PMF-dependent proton pump (Guan & Liu, 2020). *Saccharomyces cerevisiae* CIDCA8112 and *Kluyveromyces marxianus* were reported to exhibit immunomodulatory properties, which depended on the viability of the yeast species (Romanin et al., 2010). Furthermore, viability and growth of probiotics in the GIT are associated with several antagonistic properties towards enteropathogenic bacteria including competition for nutrients and binding sites, and the production of antimicrobial molecules (Revolledo, Ferreira, & Ferreira, 2009).

2.3.3. Antimicrobial properties

2.3.3.1. Organic acids

Organic acids (volatile and non-volatile) are among the main metabolites of probiotic microorganisms. Volatile organic acids include formic, acetic, propionic, and butyric whereas non-volatile organic acids include lactic, pyruvic, oxalic, and succinic (Iraporda et al., 2017; Puerari, Magalhães, & Schwan, 2012; Schwan, Magalhães-Guedes, & Dias, 2016). Organic acids contain one or more carboxylic acid groups, which are covalently linked in groups such as amides and esters. Catabolism of sugars under anaerobic conditions leads to the formation of organic acids as a means of gen-

erating nicotinamide adenine dinucleotide hydrogenase (NADH) and their accumulation closely parallels microbial growth (Papagianni, 2011). Tricarboxylic acid cycle (TCA) and glycolysis metabolic pathways result in the production of organic acids such as citric, lactic, itaconic, and malic. Organic acids including gluconic and acetic acids are produced by the two-step enzymatic metabolic pathway referred to as biotransformation (Papagianni, 2011). Figure 2 summarizes the production of organic acids by LAB during fermentation such as in kefir production.

The mode of action of organic acids on susceptible bacterial cells involves diffusion of non-dissociated acids through the cell membrane into the cytoplasm where protons are released causing acidification. The dissipation of potential protons from bacterial cell cytoplasm prevents energy generation (Diez-Gonzalez & Russell, 1997; Kundukad et al., 2020; Lambert & Stratford, 1999). The mechanisms on how organic acids kill or inhibit susceptible bacterial cell is diagrammatically shown in Figure 3. For example, a study on *S*. Typhimurium and *Escherichia coli* 0157: H7 showed that organic acids disrupted the outer cell membrane and thus enhanced the activity of other antimicrobial molecules (Alakomi et al., 2000; Kundukad et al., 2020).

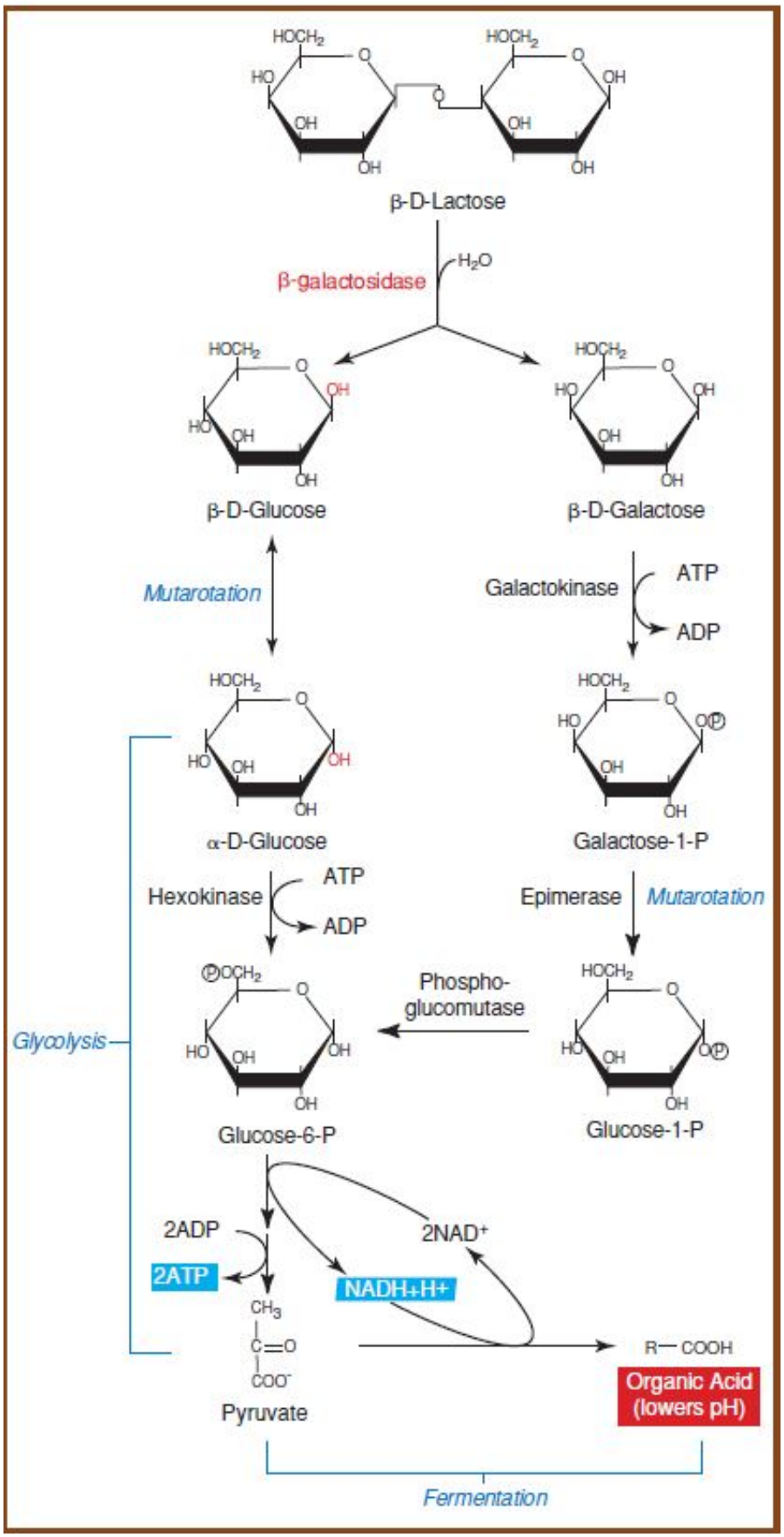

Figure 2. Example of production of organic acids by LAB (Leboffe & Pierce, 2012)

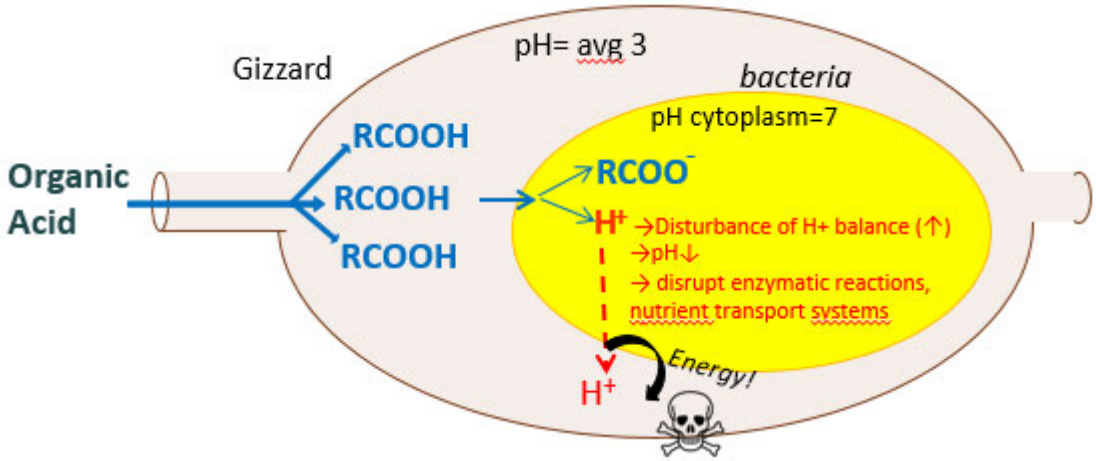

Figure 3. Example of how organic acids kill susceptible bacteria in poultry GIT (De, 2019)

2.3.3.2. Antimicrobial proteins

Probiotic microorganisms possess proteolytic enzymes. These enzymes are either proteinase or peptidases, which act in synergy with each other. Proteinases hydrolyze protein in the growth matrix and the peptidases break down the product of proteinase and other existing exogenous peptides into amino acids. Proteinase activity can result into release of protein molecules with antimicrobial activity such as the releasing of active bacteriocin from producing cells (Zhang & Gallo, 2016). Bacteriocins are proteins synthesised in ribosome by microorganisms of one strain and against another strains (Ahmad et al., 2017). *Lactococcus lactis* subsp. *Lactis* strains produce bacteriocins; nisin (Klewicka & Lipinska, 2016) and Lacticin 3147 (McAuliffe et al., 1998). The mechanisms of antibacterial peptides against susceptible microbial cells involve disruption of DNA, RNA, ATP synthesis, or protein synthesis inhibition, as well as disruption in the membrane and ionic potential (Biadała, Szablewski, Lasik-Kurdyś, & Cegielska-Radziejewska, 2020).

2.3.3.3. S-layer

Gram-positive bacteria including *Lactobacillus* species are covered by two-dimensional crystalline, glycoprotein cell surface (S-) layer lattice bound to the exterior cell

wall known as the S-layer protein. This protein is made up of two layers and performs 2 different functions including cell wall anchoring and mediation of protein self-assembly (Bönisch et al., 2018). This layer has antibacterial and antiviral properties (Fina Martin et al., 2019). *Lactobacillus* species S-layer proteins showed reduced infection of cell lines by *S*. Typhimurium due to the masking of *Salmonella* cell surface structures responsible for attachment onto GIT cells with *Lactobacillus* species S-layer proteins (Li, Yin, Yu, & Yang, 2011; Xue et al., 2015).

2.3.3.4. Kefirans

Kefiran, also referred to as exopolysaccharide, is chemically composed of glucose and galactose (Sarkar, 2008) and it constitutes 24 – 25% (w/w) of kefir grain (Plessas et al., 2017; Shen et al., 2018). Exopolysaccharides are biological polymers produced by microorganisms including probiotics to cope with adverse environmental conditions such as temperature, pH, antibiotics, and host immune defenses (Nguyen et al., 2020). Probiotics, especially LAB synthesize two types of exopolysaccharides including homopolysaccharides and heteropolysaccharides. Homopolysaccharide synthesis involves glucansucrase or fructansucrase and an extracellular sugar donor (sucrose) for the synthesis of glucans, but other fructose-containing oligosaccharides such as raffinose are utilized for the synthesis of fructans. Heteropolysaccharide synthesis is complex and involves several enzymes including glycosyltranferases, flippase, polymerase, glycosyltranferases and pyruvyl transferase (Nguyen et al., 2020).

The proposed mode of action of kefiran on susceptible microbial cells involves disruption of the cell membrane through pore formation and detergent-like effects (Barbosa, Santos, Lucho, & Schneedorf, 2011). This antimicrobial property was demonstrated by *Listeria monocytogenes* inhibition in an *in vitro* experiment (Jeong et al., 2017).

2.3.3.5. Adhesion

Cell adhesion is defined as a process whereby cells attach to each other or to a foreign surface with the aid of adhesins. Foreign surfaces may include other biotic or abiotic structures (Brückner & Mösch, 2012). Two mechanisms of adherence of bacteria onto probiotic yeast such as *S. boulardii* cells are proposed. Specific binding using type 1 fimbriae on bacteria such as *E. coli*, *E. aerogenes* and *Salmonella* cell with mannan oligosaccharides on yeast cells, and non-specific binding such as electrostatic and hydrophobic interaction (Adegbola & Old, 1985; Pérez-Sotelo et al., 2005; Tiago et al., 2012). *S. boulardii* cell wall contains a high level of mannose and hence the capacity to bind bacteria pathogens with mannose-binding fimbriae (Martins et al., 2010; Posadas et al., 2017). The adherence of enteric bacterial pathogen is postulated to be responsible for some probiotic effects such as inhibition of pathogenic bacteria signalling transduction pathway activation and subsequent translocation (Tiago et al., 2012). Hence their prophylactic and therapeutic application in human and animal husbandry to promote health possibly through the reduction of infection (Perez-Sotelo et al., 2005).

Adhesion through hydrophobic properties of probiotics is also important in competitive exclusion (outcompeting pathogens for binding sites). Probiotics with hydrophobic cell walls are able to adhere to GIT epithelial cells where they may provide prophylactic and therapeutic benefits (Fadda, Mossa, Deplano, Pisano, & Cosentino, 2017a). Hydrophobicity is species and strain-specific as demonstrated in many studies (Fadda, Mossa, Deplano, Pisano, & Cosentino, 2017b; Suvarna, Dsouza, Ragavan, & Das, 2018). Furthermore, the ability of probiotics to aggregate among themselves (auto-aggregation), with other probiotics, or with pathogens (co-aggregation) is another de-

sirable property. Auto-aggregation or co-aggregation leads to the formation of a sufficient population capable of conferring beneficial effects. For example, auto-aggregation by probiotic leads to the formation of a barrier that shields the host's GIT epithelium from colonization by pathogens (Popova et al., 2012), and co-aggregation with pathogens and this may allow the probiotics (especially yeasts) to trap pathogens and consequential shedding in feces.

2.3.3.6. Biofilm formation prevention

Biofilm is defined as a population of microbes attached to living and non-living surfaces (Brückner & Mösch, 2012). Biofilm formation on biotic and abiotic surfaces occurs in stages including reversible attachment of microbial cells using forces such as van der Waal forces. This is subsequently followed by hydrophilic or hydrophobic attachment using cell surface structures such as bacterial flagella, fimbriae, LPS, or adhesive proteins with the receptive surfaces leading to irreversible interaction. Production of extracellular polysaccharides and extracellular DNA proliferation occurs thereafter. The final step includes maturation and dispersal to establish at new sites (Sadekuzzaman, Yang, Mizan, & Ha, 2015). Biofilm is associated with chronic and asymptomatic carrier infection in infectious diseases such as typhoid fever. *Salmonella* Typhi forms a biofilm in the gallbladder, and it is reported that about 2–5% of *Salmonella* Typhi infected person developed persistence and asymptomatic carrier state as a result of the biofilm formation (Gunn et al., 2014). Biofilm formation in the GIT and other organs such as the liver is one of the virulence factors of bacterial pathogens. It is reported that biofilm is responsible for more than 60% of microbial infections in humans, and infections associated with biofilm are difficult to treat due to the antibiotic-resistant properties of microbes in biofilm (Sadekuzzaman et al., 2015). Slow growth and response to stress,

exclusion of antimicrobial agents by physical and/or chemical structure of exopolysaccharides are some of the mechanisms associated with antibiotic-resistant property of biofilm (Mah & O'Toole, 2001). Biomolecule such as alpha-amylase, an enzyme produced by potential probiotic yeasts was reported to prevent bacterial pathogen biofilm formation (Sadekuzzaman et al., 2015). Additionally, mechanisms such as competition between probiotics and pathogens for binding sites and nutrients also prevent biofilm formation in areas such as GIT (Revolledo et al., 2009).

2.3.3.7. Bacterial toxins deactivation

Enteric bacterial pathogens, including *Clostridium Perfringens, Staphylococcus aureus, Vibrio cholerae, Shigella dysenteriae, Clostridium difficile and Eshcerichia coli* (Shiga toxin-producing strains) and *Salmonella* species pathogenesis include production of toxins. *S. boulardii* produces serine protease that breaks down *Clostridium difficile* toxins (Czerucka et al., 2007). In addition, *S. boulardii* reduced the effect of toxin fluid secretion, decreased mucosal permeability, decreased mucosal damage, and release of inflammatory cytokines when administered to mice before being inoculated with *Vibrio cholerae* toxin (Czerucka et al., 2007).

2.3.3.8. Other probiotic metabolites with antimicrobial properties

Probiotic microorganisms metabolites such as sulfur dioxide, carbon dioxide and ethanol have antagonistic effects on bacterial pathogens. Sulfur dioxide produced by yeasts during fermentation produces sulfurous acid in an aqueous solution, which leads to an acidic solution, and therefore exerts its antimicrobial effect on acid-sensitive microorganisms. Additionally, sulfurous acid blocks microbial enzyme activity through reduction of disulfide linkage resulting in microbial cell death (Chichester & Tanner, 1972). The antimicrobial property of carbon dioxide produced by yeast during

fermentation is attributed to its dissolution in an aqueous solution which reduces pH (Erkmen, 2001; White & Zainasheff, 2010). Ethanol is a product of yeast metabolism and is found in fermented probiotic products such as kefir. It disrupts bacterial cell membranes through denaturation of proteins and dissolution of lipids, subsequently causing lysis of cells (McDonnell & Russell, 1999). Moreover, low ethanol concentration may not disrupt the cell membrane and has been linked to *Salmonella* growth inhibition due to ethanol-induced cell auto-aggregation causing physical hindrance to binary fission (Hassani et al., 2009).

2.3.4. Other health benefits of probiotics

2.3.4.1. Anti-carcinogenicity

The health and economic burden of cancer globally is immense. For example, in 2017, 24.5 million cancer incidents and 9.6 million cancer deaths were reported worldwide (Collaboration, 2019). The bioactive components of kefir (a probiotic product), such as peptides, polysaccharides, and sphingolipids have been reported to have anti-carcinogenic properties (Noğay, 2019). Several studies have indicated properties of kefir against several cancers including breast cancer, colorectal cancer, malignant T lymphocytes, chronic myelogenous leukemia, lung cancer, pancreatic cancer, prostate cancer and ovarian cancer (Chen, Chan, & Kubow, 2007; dos Reis et al., 2019; Rizk, Maalouf, & Baydoun, 2009; Sharifi et al., 2017). Clinical trials studies have reported both prophylactic and therapeutic potentials of probiotics in different types of cancers including colorectal, breast, liver, and bladder cancer (Śliżewska et al., 2020). Anti-carcinogenicity properties of kefir (conferred by probiotic components) is postulated to be due to mechanisms including regulation of inducible nitric oxide synthase *(iNOS)*, Nitric oxide synthase 2 (NOS-2) and nitric oxide synthase 3 (NOS3). Moreover, kefir

is reported to upregulate *BAX* and downregulate *BCL2* genes leading to apoptosis of cancer cells, promoting of anti-proliferative cytokines secretion especially IFN- *β* (Esener et al., 2018; Gao et al., 2013; Osada et al., 1993*)*.

2.3.4.2. Immunomodulation property

Immunomodulation is defined as “a process in which an immune response is altered to the desired level” (Encyclopedia, 2020). Immunomodulatory properties of probiotic products such as kefir are hypothesized to result from direct and indirect effects of microbial components and their metabolites such as organic compounds and bioactive peptides. Probiotics immunomodulatory properties are associated with their cell wall components, DNA, and metabolites, and therefore the ability of probiotics to elicit immunity may be independent of the viability of the probiotics such as yeast cells (Oelschlaeger, 2010). Bioactive peptides produced by probiotics in kefir are reported to activate macrophages, enhance the formation of nitric oxide and cytokines, and elicit the release of IgG and IgA by B lymphocytes in the GIT (Noğay, 2019). The role of IgG, IgA and B-lymphocytes as the first line of defense against infectious diseases in adaptive immunity is well established (Lindow, Fimlaid, Bunn, & Kirkpatrick, 2011; Nanton, Way, Shlomchik, & McSorley, 2012). Anti-inflammatory cytokines (IL-4, IL-6, IL-10) production as a result of kefiran (probiotics product) consumption by mice has been reported (Vinderola et al., 2005).

2.3.4.3. Improvement of GIT system

GIT is affected by many diseases such as irritable bowel syndrome, gluten intolerance, gastroenteritis, and lactose intolerance. Probiotic products such as kefir have shown efficacy in relieving symptoms such as abdominal pain and bloating when given to

patients who were suffering from GIT disorders (Yılmaz, Dolar, & Özpınar, 2019). *Lactobacillus* EV was used in inflammatory bowel disease-induced mice and was found to lessen the symptoms and improved body weight (Seo, Park, Ko, Choi, & Kim, 2018). LAB species in kefir produce beta-galactosidase, which breaks down lactose during milk kefir production and this, was demonstrated in a study in which lactose intolerant symptoms were reduced in the treatment group compared to the control (Hertzler & Clancy, 2003). This makes milk-based kefir fit for consumption by those with lactose intolerance (Hertzler et al., 2017; Noğay, 2019).

2.3.4.4. Other prophylactic and therapeutic properties of probiotics

Hypercholesterolemia is a major predisposing factor in cardiovascular disease and consumption of kefir has been reported to reduce the accumulation of serum triacylglycerol, total cholesterol, and low-density lipoprotein in mice (Choi et al., 2017). Reduction of cholesterol was due to kefir fatty acid oxidation increase through stimulation of phosphorylated AMP-activated protein kinase, peroxisome proliferator-activated receptor-α, and hepatic carnitine palmitoyltransferase-1 in mice livers (Farag, Jomaa, & El-Wahed, 2020).

Probiotic products such as kefir have been shown to have the potential to treat high blood pressure. Long-term feeding of mice for hypertension treatment led to lowering of abnormal heart enlargement, better cardiac contractility, and calcium-handling proteins, as well as reduction in the central nervous system regulation of the sympathetic activity (Silva-Cutini et al., 2019). The mechanism includes bioactive peptides with an angiotensin-converting enzyme (ACE)-inhibitory activity which lowered the blood pressure (Silva-Cutini et al., 2019). Furthermore, high blood pressure is associated with gut microbiota imbalance and subsequent dysregulation of the gut–brain axis. Probiotics in kefir and their metabolites improve GIT integrity and hence its function, and

protect against neuro-inflammation within cardio-regulatory nuclei (Silva-Cutini et al., 2019). In addition, kefir has been reported to protect GIT from toxins, allergens, and pathogens as well as promote cell proliferation, cell migration, resistance to apoptosis, synthesis of proteins and gene expression (de Almeida Silva, Mowry, Peaden, Andrade, & Biancardi, 2020).

Administration of goat and soya milk kefir to diabetic mice was reported to reduce blood glucose levels significantly and increase glutathione peroxidase (an antioxidant enzyme) activity resulting in improved pancreatic β-cells function (Nurliyani, 2015). Reactive oxygen and nitrogen species play important role in many diseases including cancer, autoimmune disorders, aging, cataract, rheumatoid arthritis, cardiovascular and neurodegenerative diseases (Pham-Huy, He, & Pham-Huy, 2008). Kefir has been shown to exhibit a strong anti-oxidant effect on 2, 2-diphenyl-1-picrylhydrazyl and 2, 2'-azino-di (3-ethylbenzthiazolin-sulfonate) (Farag et al., 2020), a free radical used to measure the radical scavenging activity of antioxidants.

Wound healing is another beneficial effect of probiotics in kefir. Topical application of kefir led to quicker healing compared to neomycin-clostebol in a study using mice due to its anti-biotic properties (Rodrigues, Caputo, Carvalho, Evangelista, & Schneedorf, 2005). Furthermore, the treatment of burn wounds with *Lactobacillus acidophilus* was efficacious in healing than Eucerin ointment ((Barzegari, Hashemzaei, Majdani, & Alihemmati, 2017)). The anti-inflammatory property of this *Lactobacillus* species was responsible for quick healing (Barzegari et al., 2017). Foot ulcers in diabetic patients were treated with kefir resulting in the proliferation and migration of human dermal fibroblast cells, reduced IL-1β and transforming growth factor-β1, and induction of basic fibroblast growth factor (Farag et al., 2020). Peptic ulcers are types of wound

that occurs in the GIT lining which compromises of tight junction making a person susceptible to infections (Qadir & Saba, 2019).

2.5. Conclusion

Probiotics include diverse microorganisms namely, *Lactobacillus*, *Bifidobacterium*, *Lactococcus* and *Streptococcus*. These are popular bacterial genera consumed in pure forms or used in fermentation of products such as yogurt, kefir or taken in lyophilized form. However, other microorganisms including *Escherichia coli*, *Bacillus, Enterococcus*, and some yeast, for example *S. boulardii* are proven probiotics. Probiotics confer their beneficial effects on the consumer via various mechanisms, including lowering intestinal pH, decreasing colonization and invasion by pathogenic organisms, and modifying the host immune response. Potential probiotics are screened for beneficial effects and virulence factors as well as antimicrobial susceptibility and anti-microbial resistance genes before they are accepted and approved for use.

2.6. References

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Chapter 3. *Salmonella* infection - prevention and treatment by antibiotics and probiotic yeasts: A review

This chapter provides a critical review of *Salmonella* infection and its current treatment. It seeks to understand the prophylactic and therapeutic potentials of probiotic microorganisms, their mechanisms of action in preventing and treating *Salmonella* and other enteric bacterial pathogens infection. In particular, the paper focuses on probiotic yeasts; however, probiotic bacteria are also briefly discussed. This chapter has been published as “Gut, Abraham Majak, Todor Vasiljevic, Thomas Yeager, and Osaana N. Donkor. *Salmonella* infection - prevention and treatment by antibiotics and probiotic yeasts: A review”. *Microbiology Society Journal* (2018). **https://doi.org/10.1099/mic.0.000709**

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Salmonella infection – prevention and treatment by antibiotics and probiotic yeasts: a review

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Abstract

Global *Salmonella* infection, especially in developing countries, is a health and economic burden. The use of antibiotic drugs in treating the infection is proving less effective due to the alarming rise of antibiotic-resistant strains of *Salmonella*, the effects of antibiotics on normal gut microflora and antibiotic-associated diarrhoea, all of which bring a growing need for alternative treatments, including the use of probiotic micro-organisms. However, there are issues with probiotics, including their potential to be opportunistic pathogens and antibiotic-resistant carriers, and their antibiotic susceptibility if used as complementary therapy. Clinical trials, animal trials and *in vitro* investigations into the prophylactic and therapeutic efficacies of probiotics have demonstrated antagonistic properties against *Salmonella* and other enteropathogenic bacteria. Nonetheless, there is a need for further studies into the potential mechanisms, efficacy and mode of delivery of yeast probiotics in *Salmonella* infections. This review discusses *Salmonella* infections and treatment using antibiotics and probiotics.

INTRODUCTION

The global burden of morbidity and mortality from human enteric pathogenic bacteria, including *Salmonella* species, is immense, despite the presence of antibiotic drugs [1–3]. Research has estimated that *Salmonella* infection causes 2.8 billion cases of diarrhoea annually worldwide. *Salmonella enterica* serovar Typhi (*S.* Typhi), the causative agent of typhoid fever, is reported to cause 16–33 million infectious cases, with an estimated 500 000 to 600 000 deaths, while non-typhoidal *Salmonella* (NTS) infections account for 90 million cases and 155 000 deaths worldwide annually [4]. The incidence of *Salmonella* infections has been exacerbated by the high prevalence of human immunodeficiency virus (HIV) infections in Africa, and it has been reported that there are 2000–7500 *Salmonella* infection cases per 100 000 HIV-infected adults [5]. In Australia, 127 195 cases of *Salmonella* infection were reported to the National Notifiable Diseases Surveillance System (NNDSS) from 2000 to 2013; however, the real cases of salmonellosis were underestimated, as it has been assumed that for every case of *Salmonella* infection reported, there are seven cases of salmonellosis in the community that have not been reported [6]. In 2010, Australia reported 40 000 salmonellosis cases, 2100 hospitalizations, 6750 complications and 15 deaths [6]. In the USA, *Salmonella*

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Keywords: *Salmonella* infection; probiotics; opportunistic pathogens; prophylactic; therapeutic.

Abbreviations: AAD, antibiotic associated diarrhoea; AGA1, A agglutinin anchorage subunit; AP, activator protein; B cells, lymphocytes from bone marrow; Caco 2, human colonic epithelial cell lines; cAMP, adenosine 3′, 5′ cyclic monophosphate; CD, cluster of differentiation; CED, cell death abnormality protein; CorA, magnesium transport protein CorA; CR3, complement receptor 3; DC, dendritic cell; EPS, extracellular polysaccharide; ERK 1/2, extracellular signal regulated kinases; FAO, Food and Agriculture Organization; FIG2, factor induced gene 2 protein; *fliC*, *Salmonella flagellin* gene; FLO, flocculation protein; G CSF, granulocyte colony stimulating factor; GIT, gastrointestinal tract; H antigen, flagella antigen; HIV, human immunodeficiency virus; IL, interleukin; IPEC, intestinal epithelial cell lines; JNK, c Jun N terminal kinases; kb, kilobase; Lg FLO1, gene encoding floc culin; Lpf, long polar fimbriae; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MEK, MAPK/ERK kinase; MEL, mannosylerthritol lip ids; MgtA, magnesium transporting ATPase, P type 1 for *S. typhimurium*; MgtB, magnesium transporting ATPase, P type 1 for *E. coli*; MSK1, mitogen and stress activated protein kinase 1; MUC1, mucin like protein; NF kB, nuclear factor kappa light chain enhancer of activated B cells; NNDSS, National Notifiable Diseases Surveillance System; NTS, non typhoidal *Salmonella*; O antigen, capsular antigen; pH, potential hydrogen; PqsA, *Pseudo monas quinolone* signal gene A; *S.* Dublin, *Salmonella enterica* serovar Dublin; *S.* Enteritidis, *Salmonella enterica* serovar Enteritidis; *S.* Heidelberg, *Sal monella enterica* serovar Heidelberg; *S.* Newport, *Salmonella enterica* serovar Newport; *S.* paratyphi, *Salmonella enterica* serovar Paratyphi; *S.* Typhi, *Salmonella enterica* serovar Typhi; *S.* Typhimuriym, *Salmonella enterica* serovar Typhimurium; SAIF, *S. boulardii* anti inflammatory factor; SAPK, stress activated protein kinase; SCARF1, scavenger receptor class F member 1; SPI, *Salmonella* pathogenicity island; SREC, scavenger receptor from endothelial cells; T cells, lymphocytes that mature in thymus; T3SS, type III secretion system; Tafi, thin aggregative fimbriae; TcdA, *C. difficile* toxin B; TcdB, *C. difficile* toxin B; TLR, Toll like receptor; TMP SMX, trimethoprim/sulfamethoxazole; TNF, tumour necrosis factor; Trk, potassium uptake pro tein; *tvi*A, virulence polysaccharide biosynthesis protein for *S. paratyphi*; *tvi*B, virulence polysaccharide biosynthesis protein for *S.* Typhi; Ty21a, atten uated live *S.* Typhi vaccine; Vi, capsular polysaccharide vaccine for typhoid fever; Vi rEPA, recombinant exoprotein A of *Pseudomonas aeruginosa* (Vi rEPA)/*S.* Typhi vaccine; ZnuABC, zinc import ATP binding protein.

is the leading cause of foodborne infections and associated medical costs amounted to $2.17 billion (for 1.4 million infections) in 2010 [7]. Bloodstream infections caused by *Salmonella enterica* in Asia accounted for 30 % of all community-acquired infections [8], while in Africa 29.1 % of community-acquired bloodstream infections were attributed to the same *Salmonella* species [9].

Antibiotics are becoming less effective against some bacterial pathogens, such as typhoidal *Salmonella* strains, and the rise of antibiotic-resistant bacteria means that there is a need for novel ways of preventing or treating infections caused by enteric pathogenic bacteria [10]. Studies on probiotics-based treatment/complementary treatment of *Helicobacter pylori* and *Clostridium difficile* have long been recognized as efficacious [11].

Probiotics are defined by the World Health Organization (WHO) and Food and Agriculture Organization (FAO) as 'live micro-organisms which when administered in adequate amounts confer a health benefit on the host' [12]. Species of *Lactobacillus* and *Bifidobacterium* are the most commonly used probiotics in the treatment of infectious diseases, including antibiotic-associated and travellers' diarrhoeas. Other micro-organisms, including *Saccharomyces boulardii*), *Streptococcus thermophilus, Enterococcus faecium*, *Leuconostoc* species, *Escherichia coli* Nissle 1917 strain and *Bacillus* species, are being researched *in vitro* or in animals and human trials, or are being used in humans for prophylaxis or therapeutic purposes [10, 13–15].

Specific criteria have been set for micro-organisms to qualify as effective probiotics. These include adherence to host cells in the gastrointestinal tract (GIT), ability to exclude or reduce the adherence of pathogens to the GIT, stimulation of immunity and the ability to persist and multiply in the GIT (resistance to acidic gastric juice, basic pancreatic juice, lysozyme and bile salts). Furthermore, other criteria include the ability to produce acids, hydrogen peroxide and bacteriocins that are antagonistic to the growth of pathogens and the ability to co-aggregate to form a normal sustaining flora. They must possess some of these properties to qualify as probiotics. Moreover, probiotic micro-organisms should be non-invasive, non-carcinogenic and non-pathogenic [12, 16, 17].

The objective of this paper is to provide a critical review of *Salmonella* infections and current treatment of salmonellosis, and to understand the prophylactic and therapeutic potential of probiotic micro-organisms and their mechanisms of action in preventing and treating *Salmonella* and other enteric pathogens infections. In particular, this paper focuses on probiotic yeasts, although probiotic bacteria are also briefly discussed.

SALMONELLA: THE BACKGROUND

Salmonella is a genus of the family *Enterobacteriaceae*. It is a Gram-negative, non-spore-forming, rod-shaped and facultative anaerobic bacterium. *Salmonella* cells move by means of a peritrichous flagellum. They are 2–5 μm long by 0.5–1.5 μm wide and, depending on the serotype, the *Salmonella* genome ranges from 4460 to 4857 kb. The bacterium was first identified in a veterinary laboratory in the 19th century in the USA. *Salmonella* is a lactose fermenter (some sub-species) and a hydrogen sulfite producer, and is oxidase-negative and catalase-positive. It hydrolyzes urea, utilizes citrate and decarboxylates lysine as its sole carbon source [5, 7].

The genus is classified into two species: *Salmonella enterica* and *Salmonella bongori*. Biochemical and genomic analysis of *Salmonella enterica* has led to further classification into subspecies, including *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica* [7, 18, 19]. The clinically important *Salmonella* species are classified under *Salmonella enterica*, which is further classified into more than 2,579 serovars on the basis of their antigenicity [7, 20].

Salmonella species are harboured in the intestinal tract of humans and farm animals. Reptiles and insects also act as *Salmonella* reservoirs. Moreover, eggs, poultry meat, pork, beef, dairy products, nuts, vegetables and water act as sources of *Salmonella*. The risk of infection is high in low- and middle-income countries or societies, with more than 100 infections per 100 000 people per year [6, 7, 21, 22]. Some *Salmonella* serotypes are host-specific, while others can infect more than one type of warm-blooded animal [5]. The *S.* Typhi and *Salmonella enterica* serovar Gallinarum serovars are restricted to human and poultry hosts, respectively, whereas *Salmonella enterica* serotype Dublin (*S.* Dublin) and *Salmonella enterica* serovar Choleraesus are adapted to cattle and pigs, respectively, but can infect other warm-blooded animals. However, other serovars, such as *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis), are generalists and are able to infect any warm-blooded animal [5].

The bacterium can be transmitted through faecal–oral routes, where susceptible hosts may acquire *Salmonella* through contaminated foods and water and therefore transmissions can be controlled through foods and water [6]. Moreover, infection with *Salmonella* from food or water can also be prevented with vaccination. *Salmonella* vaccines include killed whole-cell, Vi, live oral Ty2la and Vi-rEPA. The use of vaccine may reduce infections, but availability, efficacy, safety and cost are some of the issues that hamper its use and effectiveness [22, 23].

SALMONELLA PATHOGENESIS

After the ingestion of contaminated food or water, *Salmonella* colonizes the distal ileum and proximal colon [24, 25]. The infective dose for salmonellosis that is capable of establishing infection in the mucosa of the small intestine ranges from 10^5 to 10^6 cells [26]. *Salmonella* uses its flagella as a mode of movement as well as chemotaxis to target cells, the enterocytes. In humans, *Salmonella* cells use type I fimbriae, including long polar fimbriae (Lpf) and thin aggregative fimbriae (Tafi), to adhere to enterocytes. Type IV pili are

used by *S.* Typhi to attach to host cells [27]. Once *Salmonella* has adhered to the host cells on the apical side of M cells or enterocytes, it uses *Salmonella* pathogenicity islands (SPIs) – encoded type III secretion systems (T3SSs) – to be phagocytized into the receptive macrophages [27]. *Salmonella* cells can then be exocytosed into the interstitial spaces of the lamina propria, where they are randomly picked by macrophages, dendritic cells and polymorphonuclear cells and distributed to the host efferent lymph in the mesenteric lymph nodes before being transported to the spleen and liver via the bloodstream [28]. The attachment of *Salmonella* to the receptive epithelial cells and internalization into lamina propria causes inflammatory responses, including the release of pro-inflammatory cytokines. Pro-inflammatory cytokines cause acute inflammatory responses which lead to diarrhoea, ulceration and the destruction of the mucosa cells [29].

Apart from the invasiveness of *Salmonella cells*, enterotoxin and cytotoxin have been identified across all of the *Salmonella* sub-species. These toxins are reported to be similar to cholera toxins. Some of them have been found to be either heat-labile or heat-stable, and they have been reported to be associated with diarrhoea [30–32]. Enterotoxin was reported to induce the accumulation of fluid in the ligated murine ileal loop and was also found to have cytotoxic activity [33]. Cytotoxin inhibits protein synthesis, and it has been reported that it is responsible for intestinal mucosal surface damage, as well as enteric symptoms and inflammatory diarrhoea [25]. *S.* Typhi toxin is reported to be associated with persistent infection and the signs and symptoms of typhoid fever [34]. On the other hand, another study reported that there were no differences in virulence between mutant *Salmonella* without toxin phenotypes and wild-type with toxin phenotypes [35].

O antigen lipopolysaccharide (LPS) plays a role in the pathogenesis of *Salmonella* infections. All parts of LPS are important in the pathogenesis of *Salmonella,* but the length, structure, composition and surface roughness of O side-chains can influence the virulence. Failure to produce a full length of chain decreases virulence. The length of the chain influences resistance to the lytic action of the complement cascade. Furthermore, smooth surface strains are more resistant to the lytic action of the cascade than rough surface strains, and this has been postulated to be due to steric hindrance of complement cascade binding to the *Salmonella* cell [25].

Salmonella pathogenesis is also influenced by the virulence plasmids, which contain virulence genes. *S.* Typhimurium, *S.* Dublin and *S.* Enteritidis virulence plasmids have been reported to be responsible for systemic dissemination of infection in the mesenteric lymph nodes, spleen and liver. It has been reported that virulence plasmids are commonly found in *Salmonella* isolated from human or animal organs or blood, rather than in faeces, food, or environmental samples [25].

Salmonella also possesses other virulence factors (including flagella and flagellin), superoxide dismutase and ion acquisition systems [36]. Flagella increase invasiveness due to the motility of *Salmonella*, while flagellin has been reported to induce an inflammatory response. Bactericidal reactive oxygen species that have been produced against intracellular pathogens by the host can be inactivated by *Salmonella* superoxide dismutase. Moreover, *Salmonella* produces ion acquisition systems for the acquisition or transport of iron, magnesium, zinc and potassium, where their concentrations are low. *Salmonella* produces siderophores, including enterobactin and salmochelin. These siderophores are critical in accessing limited iron in the host. *Salmonella* also uses CorA, MgtA and MgtB systems to acquire limited magnesium. ZnuABC and Trk systems are used for zinc and potassium uptake, respectively. All of these ions are critical for the survival and pathogenesis of *Salmonella* [36].

DISEASES CAUSED BY *SALMONELLA* INFECTIONS

Infection of humans with *Salmonella* results in three main infectious diseases, namely typhoid fever, paratyphoid fever and NTS. Typhoid and paratyphoid fevers are caused by *S.* Typhi and *Salmonella enterica* serovar Paratyphi (*S.* Paratyphi), respectively, and are characterized by gastroenteritis and complications such as septicaemia, immunological symptoms, leukopenia and neurological sympotoms. These typhoidal and paratyphoidal complications account for deaths [7, 34]. On the other hand, *S.* Typhimurium, *S.* Enteritidis, *Salmonella enterica* serovar Newport (*S.* Newport) and *Salmonella enterica* serovar Heidelberg (*S. Heidelberg*) cause NTS infections, which are restricted to gastroenteritis (nausea, vomiting and diarrhoea) or occasional bacteraemia (dissemination of infection in the body), and are usually non-fatal [7].

LABORATORY DIAGNOSIS OF *SALMONELLA* INFECTION

Blood culture is the gold standard method for diagnosis of *S.* Typhi and *Salmonella* Paratyphi infections [37]. Blood volume, duration of illness, the presence of bacteraemia and antibiotic treatment commencement can impact on the reliability of the result obtained from blood culture [23].

Salmonella is serologically classified into six serotypes, which are detected on the basis of their antigenicity. The Widal test method, which detects the presence of *Salmonella* O and H antigens, is another method that can be used to diagnose *Salmonella* infections and is useful in areas where resources are limited. This method does not differentiate *Salmonella* species or serotypes and can cross-agglutinate with other non-*Salmonella Enterobacteriaceae* bacteria. False-negative Widal tests have been reported and false-positive results may also be expected in patients with malaria, dengue and disseminated tuberculosis [23]. The enzyme-linked immunosorbent assay (ELISA), which detects IgM and IgG antibodies against *Salmonella* surface molecules, is

another useful tool in the diagnosis of *Salmonella* infection. The Typhidot ELISA kit detects both IgG and IgM. Its sensitivity and specificity have been reported as >95 %, and 75 %, respectively. Typhidot-M, which only detects IgM, has a sensitivity of 90 % and a specificity of 93 % [23].

Validated molecular biology methods are also employed in the diagnosis of *Salmonella* infections from blood, faeces, foods and environmental samples [25]. The nested multiplex polymerase chain reaction method (PCR), which targets the *Salmonella* flagellin gene (*fliC*), polysaccharide capsule gene and virulence (*vi*) genes (*tviA* and t*viB*), is reported to offer better specificity, sensitivity and turnaround times compared to the other methods discussed [38].

TREATMENT OF *SALMONELLA* INFECTIONS BY ANTIBIOTIC DRUGS

Antibiotic drugs are critical in the treatment of infectious diseases and have considerably improved quality of life, in addition to reducing the mortality associated with bacterial infections. The selectivity of antibiotic drugs against invading bacteria ensures minimal harm to the patients and at the same time guarantees maximum eradication of the target bacteria [10].

NTS infections do not usually require treatment with antibiotic drugs, however complications such as meningitis and septicaemia do occur and require treatment with antibiotic drugs, including ciprofloxacin, ceftriaxone and ampicillin [22, 39]. Infections caused by *S.* Typhi and *S.* Paratyphi may involve serious complications and require treatment with antibiotics such as cefixime, chloramphenicol, amoxicillin, trimethoprim/sulfamethoxazole (TMP-SMX), azithromycin, aztreonam, cefotaxime or ceftriaxone to prevent death [23]. Dexamethasone is a corticosteroid drug and may be used when a complication such as delirium, obtundation, stupor, coma or shock occurs [23].

CURRENT ISSUES WITH THE USE OF ANTIBIOTIC DRUGS FOR TREATING *SALMONELLA* INFECTIONS

Bacterial infections have traditionally been treated with antibiotic drugs; however, certain bacterial species have developed resistance to current antibiotics. Bacteria with the ability to grow or survive in a concentration of antibiotic drug that is normally sufficient to be bactericidal or bacteriostatic are referred to as antibiotic drug-resistant bacteria, whereas antibiotic-susceptible bacteria are species that can be killed or have their growth inhibited by the recommended dose of antibiotic drug [40]. Resistance to an antibiotic drug may be innate or acquired through exposure of the bacteria to the antibiotic drug. Conjugation, transduction and transformation are the genetic mechanisms used by bacteria to acquire antibiotic-resistant genes. Conjugation involves the transfer of DNA on plasmids from one organism to another. In transformation, naked DNA is carried directly from one organism to another, while in transduction, the DNA is transferred by bacteriophage [40].

There is emerging resistance among *Salmonella* species to first-line antibiotic drugs, as well as to alternative medicines [21]. It was reported in Malawi in 2010 that 7 % of *S.* Typhi infection cases were multi-drug resistant, and in 2014 the figure increased to 97 % [41, 42]. In the USA, *S.* Enteritidis accounted for 50 % of ciprofloxacin-resistant infections, whereas *S.* Newport, *S.* Typhimurium and *S.* Heidelberg were reported to be responsible for 75 % of antibiotic-resistant infections, due to their resistance to ceftriaxone and ampicillin. The resistance of *Salmonella* species to antibiotic drugs has been shown to be serotype-specific according to metadata research [39].

The rise of antibiotic-resistance among pathogenic bacteria, including *Salmonella*, species is associated with a number of factors, including excessive use of antibiotic drugs as a result of easy access (over the counter and internet sales) in some countries [39]. The use of antibiotics for growth promotion in animal husbandry and for the protection of crops, together with poor hygiene practices, have also contributed to the overuse of antibiotic drugs, and hence resistance [10, 39, 40].

The inability to treat infectious bacterial diseases has resulted in high mortality and morbidity and substantial economic losses. It has been reported that in Europe, 25 000 people die and €1.5 billion is spent annually due to antibiotic-resistant infections, whereas in the USA, 23 000 deaths are reported and >$20 billion is spent on nosocomial antibiotic-resistant infections in hospitals in a year [40].

The effect of antibiotic drugs on the human microbiome is of great significance. Antibiotic drug use has been associated with interference with the normal flora, and as a consequence, disorders such as inflammatory bowel disease or allergies may happen due to the altered microbiome [10]. Furthermore, antibiotic-associated diarrhoea (AAD) is caused by changes to the microbiome resulting from the administration of antibiotics. This reduces carbohydrate digestion and short-chain fatty acid absorption and thus results in induced osmotic diarrhoea. Long hospital stay due to AAD contributes to the risk of nosocomial infections and is an increased economic cost [10].

PREVENTION AND ALTERNATIVE/ COMPLEMENTARY TREATMENTS OF *SALMONELLA* INFECTION BY PROBIOTICS

Probiotic micro-organisms exert their prophylactic and therapeutic properties against pathogenic micro-organisms in three main ways: they may modulate both innate and acquired immunity, act directly on the pathogens and produce antibiotic molecules [43]. These mechanisms of action are influenced by the probiotics metabolism, the cell surface molecules, the ability to secrete antibacterial molecules and the genetic makeup of the organisms [43].

Probiotic bacteria such as *Lactobacilli*, *Enterococci*, *Bifidobacteria*, *Pediococcus*, *E.coli, Streptococcus* and *Leuconostoc* species are normally found in the human GIT, where they form normal flora [44], and are commonly included in popular fermented functional foods to make their delivery easy [44–47]. Probiotic products can also be in the form of lyophilized capsules or powders or aqueous solutions [48]. Probiotic bacteria have been widely used in the treatment of infectious bacterial diseases and their efficacious application are summarized in Table 1. Apart from the treatment of infectious diseases briefly discussed below, these organisms confer other benefits, such as appropriate digestion, epithelial cell function, metabolism, enteric nerve function and angiogenesis to the host [10].

PROPHYLACTIC AND THERAPEUTIC EFFICACIES OF YEASTS

Yeasts are eukaryotes and are classified into two groups: ascomycetes and basidiomycetes [49, 50]. The ascomycetes division contains yeast species with probiotic potential, such as the genera *Saccharomyces*, *Schizosaccharomyces*, *Kluveromyces*, *Zygosaccharomyces* and *Devaryomyces* [49].

Studies have indicated that yeast can be used in the prevention and treatment of infectious bacterial diseases, including typhoid, paratyphoid and NTS. Currently, *S. boulardii* is the yeast strain being used as a probiotic [51–53], while other yeast species and strains have been proven to be efficacious in *in vitro* and animal trials [54]. In contrast to probiotic bacteria, which are affected by drugs that target enteric pathogenic bacteria, yeasts are not targeted when they are used as a complementary therapy [48]. Fig. 1 summarizes the antagonistic mechanisms of probiotics against bacterial pathogens. These mechanistic properties of probiotic yeasts against pathogens are discussed below and further studies are summarized in Table 2. Yeasts also have a wide range of other beneficial applications for humans, as illustrated in Fig. 2.

PROTECTION AND PRESERVATION OF TIGHT JUNCTIONS

Tight junctions are the apical epithelial layers that separate the interface lumen of the GIT and deep cell layers. It is composed of transmembrane proteins, cytoplasmic adaptors and the actin cytoskeleton. Tight junctions attach adjacent cells to each other and provide intercellular seals. They function as a physical barrier that prevents noxious objects, including pathogenic organisms, from entering into deeper layers within tissues. However, some micro-organisms, such as *Salmonella* species, have developed mechanisms to evade this barrier [55]. Probiotic micro-organisms, including yeast species, have been reported to not only maintain normal functions of the gut mucosa, but also protect it from toxins, allergens and pathogens. The protective effects of probiotics are attributed to cytoprotection, cell proliferation, cell migration, resistance to apoptosis, synthesis of proteins and gene expression [56]. *S. boulardii* is reported to inhibit pro-inflammatory cytokines such as IL-8 production by the host and prevent the activation of MAP kinases Erk1 /2 and JNK/ SAPK. *S. boulardii* anti-inflammatory factor (SAIF) was postulated to be responsible for tight junction protection and preservation. Furthermore, *S. boulardii* produce produces proteases that break down toxins produced by bacterial pathogens [57].

Inflammatory bowel diseases such as irritable bowel syndrome, gluten intolerance, gastroenteritis and *H. pylori* infections disrupt tight junctions and this can predispose the susceptible host to *Salmonella* and other enteric pathogen infections [56]. Mice with genetic and inducible colitis (hence disrupted tight junctions) were more prone to be colonized and infected by *S.* Typhimurium than mice without inflammatory diseases [58]. These inflammatory diseases are currently prevented and/or treated using *Saccharomyces* species [56] and this shows how yeasts may be used prophylactically in infection prevention with respect to enteropathogenic bacteria such as *Salmonella*. The infection rate was reduced in the yeast-treated group due to the protection of tight junctions through cytoprotection, cell proliferation, cell migration, resistance to apoptosis, synthesis of proteins and gene expression.

IMMUNOMODULATORY PROPERTIES

The immunomodulatory properties of probiotics are associated with their cell wall components, DNA and metabolites, and therefore their ability to elicit immunity may be independent of the viability of probiotics such as yeast cells [43]. The target host cells for immunomodulation by probiotics are enterocytes and gastrointestinal-associated immune cells. The sensitive cells can be stimulated due to the presence of β-glucan and mannose receptors for probiotic fragments or whole cells. The adhesion of probiotic organisms to sensitive cells or the production and release of soluble factors may modulate immunity or trigger signalling cascades in immune cells [43]. Yeast cell wall components, including mannoproteins and β-glucan, induce immunomodulatory responses when they interact with dentritic or other immune cells with receptors [59]. For example, the attachment of *S. boulardii* to dendritic cells (DCs) was reported to induce the secretion of immunoglobulins A and M and cytokines, including interleukin (IL)−1β, IL-12, IL-6, TNFα and IL-10. This immunomodulatory mechanisms was postulated to be due to tumour necrosis factor alpha (TNFα) and the transcriptional upregulation of C–C chemokine receptor type 7 mRNAs by yeast cells [60].

The cell wall components of *Saccharomyces cerevisiae*, including mannoprotein, act as nonspecific immune stimulators by interacting with macrophages through receptors. Yeast cell components, including β-glucan and mannoprotein, have adjuvant effects and can activate neutrophils, eosinophils, macrophages and complements [61].

The immunomodulatory properties of pathogenic fungal species are postulated to be due to the presence of β-glucan receptors on a susceptible host [62]. Beta-glucan is also

Table 1. Prophylactic and therapeutic properties of probiotic bacteria

Probiotic micro-organisms	Indicator enteric pathogens and/or animal models	Treatment mechanisms and outcomes	References
L.casei 11578, *Lactobacillus delbrueckii* ssp. *bulgaricus* 11 842 (*L. bulgaricus*), *Lactlbacillus fermentum* 1493 (*L. fermentum*) and the commercial probiotic product, PROB	Infection of neonatal broiler chicks with *S.* Enteritidis	Significant reduction of *S.* Enteritidis in the chick faeces in a time-dependent manner; feeding 24 h prior to infection was efficacious	[111]
L. casei, L. bulgaricus, Lactobacillus cellobiosus (L. cellobiosus), Lactobacillus helveticus (L. helvetticus) and *L. fermentum*	Infection of 1-day-old broiler chicks with *S. Enteritidis*	Reduced colonization of chicks' gastrointestinal tract	[112 114]
L. casei, L. bulgaricus, L. cellobiosus, L. helveticus and *L. fermentum*	Infection of neonatal broiler chicks with *S.* Enteritidis and *S.* Typhimurium	Caecal tonsils load of *S.* Enteritidis was reduced by 60 70 %, while the *S.* Typhimurium load in caecal tonsil was reduced by 89 95 % as a result of treatment with probiotic compared to control	[115]
Commercial probiotic floraMax	Infection of chicks and poults with *S.* Heidelberg	Reduced colonization and hence lower recovery of *S.* Heidelberg from caecal tonsil from both treated chicks and poults compared to control chicks and poults	[116]
Lactobacillus rhamnosus (*L. rhamnosus*) GG (ATCC 53103) and *B. longum* 46 (DSM14583)	*V. cholerae*	Removed 68 and 59 % enterotoxin in an aqueous solution, respectively	[117]
Bifidobacterium longum subsp. *longum/infantis*	*E.coli* 0157: H7	Prevented the production of toxin in the caecum and translocation of toxin from the GIT to the blood stream and hence reduced mortality	[118]
Lactobacilli, *Bifidobacterium bifidum* strains Bb12 and *Lactobacillus kefir*	*S.* Typhimurium	Secrete molecules that prevent invasion of epithelial cells	[43]
Lactobacillus acidophilus (L.acidophilus)	*In vitro* trial using human colonic adenocarcinoma cell line infected with *S.* Typhimurium	Attenuation of inflammatory response triggered by *S.* Typhimurium infection	[119]
E.coli Nissle 1917	Stimulation of intestinal epithelial cell line	Suppression of TNF-α induced IL-8 transcription and production Only viable *E.coli* Nissle 1917 showed immunomodulation	[120]
L. fermentum ME-3 and ofloxacin antibiotic, *L. plantarum* cell-free extract with co-trimoxazole	*S.* Typhimurium	Prevented invasion of organs and completely eradicated *S.* Typhimurium	[121, 122]
E.coli Nissle 1917 (EcN)	Infection of Caco-2 cells with *C. perfringens*	IL-1 β, IL-6, G-CSF and GM-CSF production was significantly increased in the absence of EcN, but decreased in the presence of EcN	[123]
Lactobacillus rhamnosus G and *Bifidobacterium lactis* Bb12.	*E. coli and S.* Typhimurium *in vitro* experiment	Significant co-aggregation of pathogens with probiotic bacteria	[124]
E.coli Nissle 1917 and L. acidophilus	*E.coli* 0157:H7 and cell lines	Suppressed production of pro-inflammatory cytokines and inhibited *E.coli* 0157:H7 virulence	[125]
E.coli Nissle 1917	*S.* Typhimurium, *Yersinia enterocolitica*, *Shigella flexneri*, *Legionella pneumophila* and *Listeria monocytogenes*	The ability of these probiotic bacteria to inhibit invasion is not dependent on direct contact with the pathogen; rather it is due to the production of not-yet-identified molecules	[126]
Bifidobacterium longum Bar33 and *B. lactis* Bar30	Infection of Caco-2 cells with *S.* Typhimurium and *E. coli H10407*	Displaced pathogenic bacteria from attachment site of CaCo-2	[127]
E.coli Nissle 1917	*C. difficile* and *C. perfringens*	Inhibited production of and deactivated toxins	[123]
L. plantarum 299 v, *L. rhamnosus* GG, *Bifidobacterium lactis* Bb12 and *L. rhamnosus* LGG	Infection of human mucusa cells with enteropathogenic *E. coli*, *S.* Typhimurium ATCC 12028 and *Clostridium histolyticum* DSM 627	Competition for the same receptor in the GIT and stimulation of mucin production by probiotic resulted in inhibition of pathogenic bacteria adhesion to the GIT; probiotics also degrade carbohydrate receptors for pathogens, exclude pathogens by establishing biofilms, produce receptor analogue for pathogens to bind to instead of binding to host cells and prevent binding of pathogens by producing surfactants	[43, 128]
Genetically engineered *L. lactis*	*C. difficile* and *H. pylori* in mice	Elicited immunity by expressing non-toxic fragments of TcdA and TcdB and produced *H. pylori* lipoprotein Lpp20, which elicited immunity *in vivo* and therefore prevented or treated *H. pylori* infections	[129]
Single-strain *Lactobacillus* species	*E. coli, Enterococci faecalis, Enterococcus faecium, Enterpbacter cloacae, Streptococcus salivarius, Listeria monocytogenes, S. aureus, Proteus mirabilis, P. aeruginosa* and *Bacteroides thetaiotaomicron* in *in*	Inhibited growth due to antibacterial metabolites other than hydrogen peroxide because of inhibition in anaerobic condition	[130]

Table 1. cont.

Probiotic micro-organisms	Indicator enteric pathogens and/or animal models	Treatment mechanisms and outcomes	References
	vitro experiment		
Bifidobacterium breve (*B. breve*) strain Yakult	*E. coli* (STEC) O157: H7 in mouse model	*B. breve* inhibited stx gene production by STEC cells	[131]
Clostridium butyricum strain MIYAIRI	Enteropathogenic *E.coli* (EHEC) 0157: H7 in mouse model	Inhibited toxin expression by producing butyric and lactic acid and reduced viability of EHEC *E.coli* 0157: H7 by producing butyric acid	[132]
Lactobacillus strains, three *Pediococcus* strains and four *Bifidobacterium* strains	*E.coli* (EHEC) 0157: H7 in *in vitro experiment*	All probiotics inhibit toxin production due to the production of organic acid, which resulted in low pH	[133]
Bifidobacterium thermophilum RBL67	Human colonic fermentation model using HT29-MTX cell lines; infection with *Salmonella* and *in vitro* trial	Probiotic prevented invasion and protected epithelial lining; probiotic also prevented expression of virulence factors by *Salmonella*	[134, 135]
Feed-grade lactobacilli (TGI)	Poultry (broiler) infection with *Salmonella*	Consumption of probiotic increased liveability in *Salmonella*-infected broilers compare to the control	[136]
L. plantarum MTCC5690	An animal trial using mice infected with *Salmonella*	Consumption of probiotic in fermented milk stimulated immunity and prevented GIT colonization by *Salmonella* and hence prevented infection	[137]
L. salivarius 59 and *Enterococci faecium* PXN33	An animal trial using poultry infected with *Salmonella*	Prevented colonization of Git by *S.* Enteritidis when used as multi-strain probiotic	[138]
L. rhamnosus GG (2×10^9 organisms per day)	A human trial involving 400 adult travellers	Reduced traveller's diarrhoea to 3.9 % in the treatment group compared to 7.4 % in the placebo group	[139]
Genetically engineered *E. coli* Nissle 1917	Animal trial using turkey infected with *Salmonella*	Ninety-seven per cent lower carriage of Salmonella in the GIT in the treated group compared to the control group, postulated to be due to the production of antimicrobial molecules by *E. coli* Nissle 1917	[140]
Genetically modified non-pathogenic *S.* Typhimurium	*S.* Typhimurium and murine model	Protected murine model due to competition for nutrients with pathogenic strains	[141]

found in probiotic yeast species such as *S. cerevisiae* (in the cell wall) and therefore a non-pathogenic yeast species may have the potential to modulate cell-mediated and humoral immunity in a host with its receptors [60].

Among the host receptors that recognize β-glucan are complement receptor 3 (CR3), dectin-1, scavenger receptor class F member 1 (SCARF1), cluster of differentiation 36 (CD36) and cell death abnormality protein 1 (CED1) [which is found in nematodes and is similar to human scavenger receptor from endothelial cells (SREC)] [62, 63]. CR3 is an integrin dimer and is expressed by immune cells such as monocytes, macrophages, DCs, neutrophils and natural killer cells. Dectin-1 is primarily expressed by macrophages, dendritic cells and neutrophils, while SCARF1 is expressed on macrophages. The binding of stimulators such as β-glucan to the above receptors on immune cells elicits immune responses. Some of these immune responses have been found to include phagocytosis, oxidative burst, neutrophil degranulation, fungal killing and the production of inflammatory lipid mediators, cytokines and chemokines. Chemokines recruit and coordinate the activation of other immune cells, including T cells, B cells and natural killer cells [60, 62]. CD36 is a sensor for β-amyloid, modified low-density lipoprotein, bacterial diacylated lipoproteins and lipoteichoic acid. These receptors mediate the binding of *Candida albicans* and *Cryptococcus neoformans* to mammalian cells via β-glucan and induce inflammatory cytokines and chemokines. However, collaboration with Toll-like receptor 2 (TLR2) is needed in order for these receptors to induce immune responses [62].

Mannose receptor is expressed by activated macrophages. Mannose is also recognized by langerin and dectin-2 and these also act as its receptors. Stimulation of mannose receptors can lead to pro-inflammatory or anti-inflammatory responses. Langerin (also known as cluster of differentiation 207) is a receptor on Langerhans cells, whereas dectin 2 is a receptor for mannan on a fungal cell wall. The type of response is dependent on the yeast cell wall components (the presence of β-glucan and mannoproteins) and the host cell receptors. Additionally, dectin 2 has an affinity for α-glucan, while langerin has an affinity for chitin [64].

The immunomodulatory properties of yeasts was demonstrated in *S. boulardii*, which has strong immunomodulatory properties; it induced the production of immunoglobulin A (IgA), tumour necrosis factor-alpha (TNF-α) and many ILs, including IL-1β, IL-5, IL-6, IL-10 and IL-12, as well as downregulating the production of IL-8 expression by acting on the NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway in uninfected enterocytes and on mitogen-activated protein kinases (MAPKs) and AP-1activator protein-1 (AP-1) in *S.* Typhimurium-infected enterocytes [54, 65]. *S. boulardii* was shown to reduce the production of pro-inflammatory

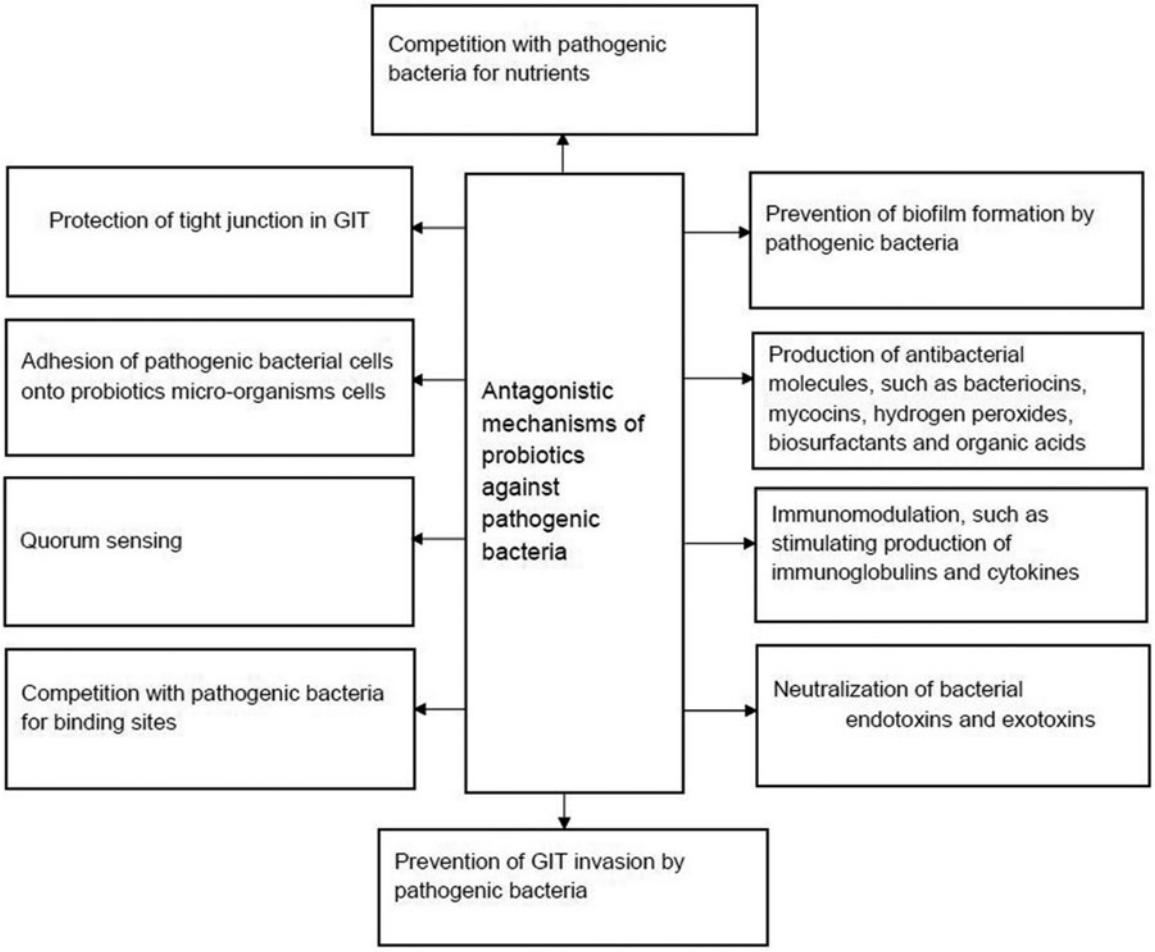

Fig. 1. Antagonistic mechanisms of probiotics against pathogenic bacteria [3, 52, 54, 70, 74, 86, 89, 92].

immune factors, including IL-6 and TNF-α, in a pathogenic *E. coli* infection colitis and it prevented *E. coli*-mediated apoptosis of T84 colonic cell lines [54]. In contrast to the above findings, the ability of *S. boulardii* to modulate immunity in healthy mucosa was reported to be minimal in research conducted on a murine model [59].

Yeast genera (including *Saccharomyces*, *Kluyveromyces* and *Issatchenkia*) isolated from kefir milk showed downward regulation of intestinal epithelial innate immune responses when cells were subjected to TLR ligands such as *Salmonella* flagellin and *E. coli* LPS. *Kluyveromyces marxianus* inhibited the expression of TNF-α and IL-1β cytokines by enterocytes when stimulated by LPS and flagellin. This yeast strain was also shown to block the NF-kB pathway and therefore inhibited pro-inflammatory cytokines, chemokines and the release of TNFα. The immunomodulatory ability of yeast species (especially *S. cerevisiae* CIDCA8112 and *Kluyveromyces marxianus*) isolated from kefir was shown to be dose-dependent. The viability of yeast cells was found to be a deciding factor in the downregulation of the innate response by human colonic epithelial cell lines (Caco-2). The inactivation of yeast strains by heat and UV irradiation completely destroyed the immunomodulatory effects [66, 67].

BINDING OF PATHOGENIC BACTERIA ONTO YEAST CELL WALLS

Cell adhesion is defined as a process whereby cells attach to each other or to a foreign surface with the aid of adhesins. In this context, foreign surfaces may include other biotic or abiotic structures [68]. In yeasts, adhesins are protein mosaics on the surface of cell walls which are involved in development, symbiosis and pathogenesis [69]. Currently, eight *S. cerevisiae* adhesins have been identified and these include FLO1, FLO5, FLO9, FLO10, FLO11 (or MUC1), FIG2, LgFLO1 and AGA1. The expression of these adhesins is determined by genetic factors, such as yeast species or environmental growth conditions, including growth medium, aeration or acidity [68, 69].

Table 2. Prophylactic and therapeutic properties of yeasts

Probiotic micro-organisms	Indicator enteric pathogens and/or animal models	Treatment mechanisms and outcomes	References
S. boulardii	Human trials	Improved tolerance to number of calories per day, reduced incidence of diarrhoea, reduced number of treatment days and reduced duration of diarrhoea	[74]
S. boulardii	*Salmonella* and *E.coli* in rat model	Neutralized LPS and therefore reduced its toxicity in the rat model; inflammatory lesions and necrotic bodies were seen in the control's liver and heart	[98]
S. cerevisiae UFMG A-905 from Brazilian distilled spirit cachaça, *S. cerevisiae* 982 from cheese and *S. boulardii* from chicken faeces	PBMCs (peripheral blood mononuclear cells) and mouse model	Reduction of inflammation and IL-6, TNF-α, interferon gamma (IFN-γ) and IL-10 by *S. cerevisiae* UFMG A-905 production, and stimulation of type 1 T helper (th1) response by *S. cerevisiae* 982 Induced TNF-α and IL-10 production Reduced the serum level of IL-6 in a mouse colitis model. Immunomodulatory properties through reduction of inflammation and IL-6, TNF-α, Interferon gamma (IFN- γ) and IL-10 production	[54]
S. cerevisiae	*Salmonella* species in *in vitro* experiment	Viable yeast bind better to *Salmonella* than non-viable yeast	[71]
Pichia kudriavzevii RY55	*E. coli*, *Enterococcus faecalis*, *Klebsiella* sp., *S. aureus*, *Pseudomonas aeruginosa* and *Pseudomonas alcaligenes* in *in vitro* experiment	Mycoccins inhibited the growth of pathogenic bacteria	[86]
Candida krusei isolated from fermented vegetables	*E. coli, S.* Typhimurium, *S. aureus* and *Bacillus cereus* in *in vitro* experiment	The killer toxin produced by yeast inhibited the growth of pathogenic bacteria	[88]
Yarrowia lipolytica	*Bacterial species* in *in vitro* experiment	Produces organic acids, including a-ketoglutaric, a pyruvic, citric and isocitric acid, which may have bactericidal or bacteriostatic effects on bacterial growth	[142]
S. cerevisiae	*In vitro* experiment on *Enterobacteriaceae* and lactic acid bacteria	Bactericidal or bacteriostatic effects due to production of carbon dioxide, sulfur dioxide, a high concentration of ethanol and secretion of organic acids which in turn reduce pH	[142, 143]
S. boulardii	*V. cholerae*, *C. difficile* and *C. perfringens* toxins in mouse model	Minimized the effects of toxin fluid secretion, and decreased mucosal permeability, mucosal damage and the release of inflammatory cytokines when administered to mice before they they were given the cholera toxin, and deactivated or inhibited production of toxins by *C. difficile* and *C. perfringens*	[74]
S. cerevisiae and *C. albicans*	*Pseudomonas*, *Staphylococcus epidermidis* and *Burkholderia pseudomallei* in *in vitro* experiment	Quorum-sensing molecules (farnesol) prevented biofilm formation by *Pseudomonas* and *Staphylococcus epidermidis* and enhanced the efficacy of B-lactams against *Burkholderia pseudomallei*	[3, 100]
S. boulardii	Human trial in children	Decreased severity and duration of infectious diarrhoea in children and shortened acute diarrhoea by almost a day in a clinical trial	[10]
S. cerevisiae IFST062013 isolated from fruit juice	*In vitro* experiment on Gram-negative and -positive bacteria	Significant antibacterial effects in gram-negative than gram-positive bacteria compared to antibiotic doxycycline, while cell lysate was more potent than whole cells or supernatants; induced pro- and anti-inflammatory mediators simultaneously and as a result enhanced the maintenance of balance between Th1- and Th2-type cytokines	[89]

Using *S. cerevisiae* as a model, yeast cells have been found to form six different communal structures. Sessile non-adhesive cells that do not produce adhesins on a solid surface exposed to air can form non-adhesive colonies, especially on laboratory agar media. However, in a liquid medium, non-adhesive yeast cells exist as individual cells in a planktonic form that makes the media look turbid. Yeast cells can produce self-adhesion genes and therefore auto-aggregate without aggregating to foreign biotic or abiotic surfaces. Alternatively, yeast cells can produce adhesins for self-aggregation as well as an adherent to foreign surfaces and thereby form a biofilm. Furthermore, adhesin-producing yeast cells in liquid media can form flocs on the bottom or flor on the surface. Lastly, yeast cells can develop filaments when they produce adhesins and adhere to the bottom in liquid substrates [68].

Intimate binding of *S.* Typhimurium pili to yeast *S. boulardii* has been demonstrated by transmission electron microscopy [70]. The underlying mechanism of binding is postulated to be due to the presence of mannose-specific

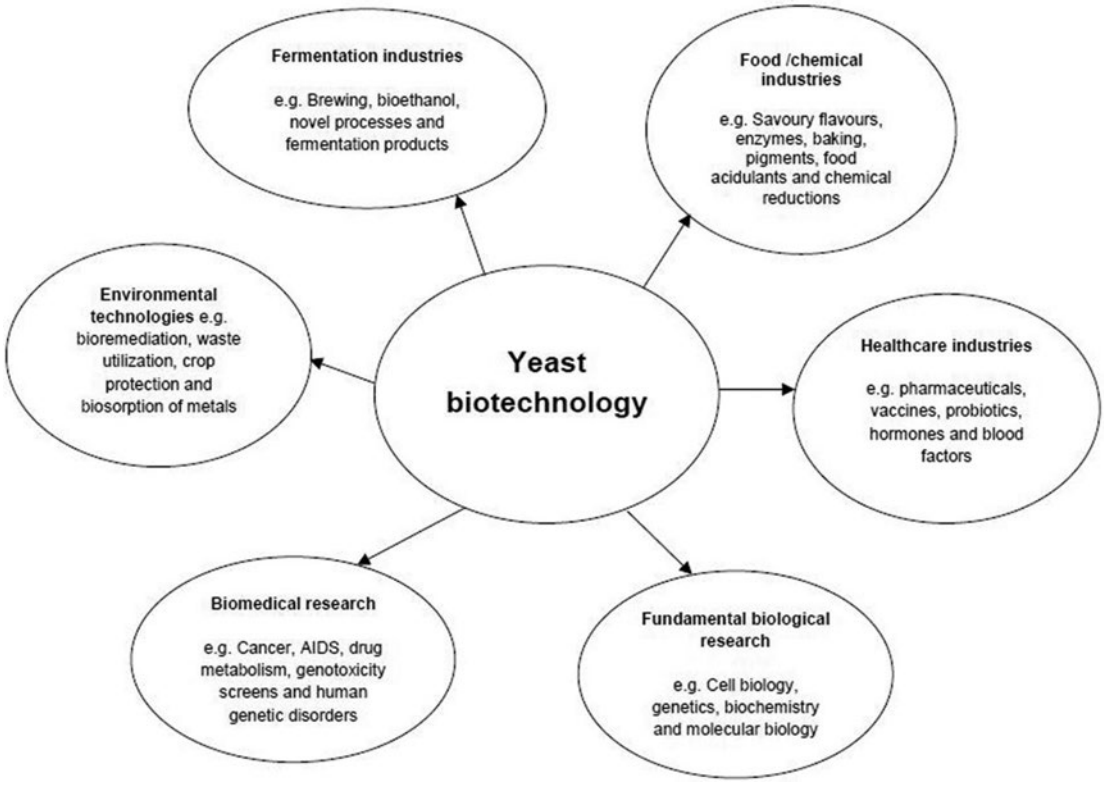

Fig. 2. Biotechnology applications of yeast [49].

adhesins/receptors such as fimbriae on bacteria cell walls that can bind to mannose on yeast cell walls. *S. boulardii* cell walls possess high mannose content and hence the capacity to bind bacteria pathogens with mannose-binding fimbriae [70, 71]. Bacterial pathogens, including *Salmonella* species, have been reported to bind better to probiotic yeasts than to parabiotic yeasts [71]. Moreover, adhesions of pathogenic bacterial cells onto yeast cell walls were found to be prominent when yeast growth was at the stationary phase compared to other growth phases [72]. The presence of sugars (including mannose, glucose and maltose media), and to some extent bile salts, in aqueous solutions was found to inhibit the binding of *S. boulardii* to pathogenic bacteria, including *Salmonella* species [70, 72]. Therefore, to improve the binding of *S. boulardii* to pathogenic bacteria, the consumption of foods or drinks rich in these sugars should be limited when yeast is used prophylactically or therapeutically.

Some bacteria, including *Salmonella* species, do show variation in the expression of fimbriae and therefore specific binding of yeast to the *Salmonella* may vary depending on the strains and/or genetic mutations [73]. Consequently, the efficacy of adhesion as a prophylaxis can be influenced by strains or genetic mutations that may occur over time.

Enteropathogenic bacteria, including *Salmonella* species and pathogenic *E.coli,* have been shown to preferentially and irreversibly bind to surfaces of *S. boulardii* [52, 71, 72]. The binding of pathogenic bacteria onto yeast cell walls limits their infectivity, since *S. boulardii* does not bind to the GIT; the bound bacterial cells pass transiently through the GIT and are excreted in the faeces [74].

The ability of *S. boulardii* to bind enteropathogenic bacteria is independent of viability; both probiotic and para-probiotic yeasts were shown to bind pathogenic bacterial cells [71, 75]. Interestingly, yeast species were reported not to bind to bacteria normally found in GIT, with the exception of the *S. cerevisiae* UFMG 905 strain, which bound *Bacteroides fragilis* [72]. *S. boulardii* has been reported to significantly reduce the internalization of *S.* Typhimurium in a human T84 cell monolayer when both yeast and the pathogen were applied together in an *in vitro* experiment [70]. Furthermore, *Pichia pastoris* X-33 and *S. boulardii* have been reported to reduce the binding of *S.* Typhimurium to human colorectal HCT-116 cells (by 47 and 37%, respectively) [76].

Mice infected with *S.* Typhimurium showed colonization along the GIT, but when the infected mice were administered with *S. boulardii*, the bacterial cells clustered around

the yeast cells, which was indicative of the adherence of *S.* Typhimurium onto *S. boulardii* cells [65].

GROWTH INHIBITION

The growth inhibitory properties of probiotics, especially yeasts, against bacteria have been proposed to include the production of a high concentration of ethanol, the synthesis of killer toxins, pH changes, organic acid production and competition for nutrients [77].

Competition for nutrients is considered to be the most important antagonistic property of yeast against other fungi in the context of postharvest fungal pathogens in fruits; yeast species have the capacity to quickly deplete glucose, fructose and sucrose, and therefore suppress the growth of other micro-organisms [77]. Moreover, some yeast species possess iron sequestering molecules that give them a competitive advantage to deplete iron, which is needed for growth and pathogenesis by many pathogens [77].

Killer toxins, also called mycocins, are extracellular proteins, glycoproteins or glycolipids that are produced by yeast species against other yeast species with receptors for the toxins. The toxins genes are carried on extra-chromosome elements, including double-stranded RNA virus and double-stranded linear DNA, or on a chromosome [77]. The toxins kill susceptible yeasts but do not affect the producer. The mechanism of action of killer toxins involves the inhibition of beta-glucan synthesis or the hydrolysis of beta-glucan in the cell wall of the target yeast, the inhibition of DNA synthesis in the target yeast, the cleavage of tRNA, the inhibition of calcium uptake and the leakage of ions due to the formation of channels on the cytoplasmic membrane [77]. Killer toxins are large glycoprotein molecules and consequently have the potential to induce unwanted immune responses in the host [78], and therefore further studies on molecular size and possible modification are needed with regard to antigenicity and toxicity before these toxins are used therapeutically[79]. Several yeast genera, including *Saccharomyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Kluyveromyces*, *Pichia*, *Torulopsis*, *Williopsis* and *Zygosaccharomyces*, can produce killer toxins [77].

Yeast metabolites such as sulfur dioxide, carbon dioxide and ethanol have been postulated to have antagonistic effects on enteropathogenic bacteria. Sulfur dioxide, which can be produced by yeasts during fermentation, when dissolved in aqueous medium, produces sulfuric acid, which lowers the pH and therefore exerts its bactericidal or bacteriostatic effect. Furthermore, sulfuric acid is postulated to block microbial enzyme activity through the reduction of disulfide linkage, resulting in an antagonistic property against micro-organisms [80]. Moreover, the antibacterial property of carbon dioxide produced by yeast during fermentation is attributed to its dissolution in aqueous solution, which lowers the pH [81, 82]. Ethanol, a product of yeast metabolism, disrupts bacterial cell membranes through the denaturation of proteins and the dissolution of lipids, subsequently causing the lysis of bacterial cells in an *in vitro* experiment [83]. Concentrations of carbon dioxide and ethanol that are bactericidal may also be harmful to host cells. Ethanol has been reported to affect red blood cells physically and biochemically. Ethanol-induced membrane fluidization, decreased haemogloblin content and concentration in the cytoplasm have been reported [84]. Furthermore, it has been reported that ethanol has negative effects on neurons, hepatocytes and enterocytes [85], and therefore further studies are needed before potential therapeutic application.

A study on *Pichia kudriavzevii* RY55 found that mycocins produced by this yeast species have growth inhibition effects on potential bacterial pathogens, including *E. coli*, *Enterococcus faecalis*, *Klebsiella spp.*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Pseudomonas alcaligenes.* However, the optimum temperature and pH for the toxins were lower and higher, respectively, than in the normal human gut environment. The maximum activity of the enzyme was observed at 30 °C and pH 5 [86]. Moreover, a killer toxin produced by *Candida krusei* that was isolated from fermented vegetables showed growth inhibition towards *E. coli*, *S.* Typhimurium, *S. aureus* and *Bacillus cereus* [87]. It has been reported that the killer toxin produced by *Williopsis Saturnus* shows a lack of bactericidal activity against *Streptococcus pneumoniae* [88].

S. cerevisiae IFST062013 isolated from fruit juice demonstrated moderate antibacterial activity compared to antibiotic doxycycline; the antagonistic effect was more pronounced against Gram-negative than Gram-positive bacteria. Moreover, a comparison of the effects of whole cells, cells lysates and supernatants indicated that cell lysates were more potent, which may be indicative of the antibacterial properties coming from the cell components rather than extracellular secretions. Nonetheless, the yeast species was reported to produce killer toxin and siderophore, and showed strong inhibition of bacterial biofilm formation [89].

PREVENTION OF INVASIVENESS AND SYSTEMIC INFECTION

The attachment of enteric bacterial pathogens, especially *Salmonella*, to receptive epithelial cells leads to internalization and hence infection, leading to symptoms and signs, including diarrhoea, ulceration and the destruction of the mucosa cells [29]. One of the mechanisms that has been proposed to explain how probiotics prevent invasion is competitive exclusion. This is defined as the ability of normal flora or probiotics, including yeast species, to limit the colonization of GIT, competing with invading pathogens by creating a restrictive physiological environment due to the production of antagonistic molecules and competition for binding sites and nutrients [90].

Lactobacillus kefiri CIDCA 8348, *L. plantarum* CIDCA 8327 and *Kluveromyces marxianus var. marxianus* CIDCA 8154 isolated from cheese whey fermented with kefir grain reduced the invasiveness of Caco-2/TC7 cells by *S.*

Enteritidis CIDCA 101. The precise mechanism and which of the probiotic micro-organisms (if it was not a synergistic effect) is responsible for the prevention of enterocyte invasion could not be explicitly identified in the research, as the three probiotic micro-organisms were used together [91]. *S. boulardii* prevented the invasiveness of *S.* Typhimurium and subsequent translocation to the spleen and liver in treated mice compared to untreated control mice, which had high bacterial counts in these organs [65].

BIOFILM FORMATION INHIBITION

Biofilms are defined as communities of micro-organisms attached to biotic or abiotic surfaces [68]. Bacterial biofilm formation occurs in stages, including the reversible attachment of bacterial cells on abiotic or biotic surfaces using forces such van der Waal forces. This is followed by hydrophilic/hydrophobic interactions between bacterial flagella, fimbriae, LPS or adhesive proteins with the receptive surfaces. When the bacteria have been irreversibly attached, the production of extracellular polysaccharide (EPS) and extracellular DNA proliferation occur. The final stage involves the maturation of the biofilm and subsequent dispersal for establishment at another site [92].

Biofilm formation in the GIT and other associated organs such as the liver is one of the virulence factors of bacterial pathogens, including enteropathogenic strains. It has been reported that biofilms account for more than 60 % of microbial infections in humans, and these infections are difficult to treat because of the antibiotic-resistant nature of micro-organisms in biofilms [92]. Typhoidal *Salmonella* infection, persistence and the asymptomatic carrier state are associated with biofilm formation in the gallbladder [93]. About 2–5 % of typhoid patients developed persistence and the asymptomatic carrier state as a result of biofilm formation [94].

Alpha-amylase, an enzyme produced by yeast cells, has been reported to prevent bacterial pathogen biofilm formation [92]. Moreover, other mechanisms, such as the creation of restrictive physiological environment by probiotics, result in competition for binding sites and nutrients, which also prevents biofilm formation [90].

It has been reported that at 10, 20 and 100 μg ml ^{1} doses of alpha-amylase decreased *S. aureus* biofilm formation by 72 %, 89 and 90% respectively, while it was able to reduce matrix formation by 82 % in an *in vitro* experiment [92]. *S. cerevisiae* and *Saccharomyces kluyveri* produce alpha-amylase [95], and so yeast probiotics may potentially be used to produce this enzyme to inhibit biofilm formation and thus prevent carrier stage development in patients infected with *S.* Typhi.

EFFECTS ON BACTERIAL TOXINS

Enteropathogenic bacteria, including *Clostridium perfringens*, *S. aureus*, *Vibrio cholerae*, *Shigella dysenteriae*, *C. difficile* and *E. coli* (Shiga toxin-producing), as well as *Salmonella* species, produce toxins in the gastrointestinal tract. The expression of the *Salmonella* enterotoxin (*stn*) gene, which encodes a 29 kDa protein, is a hallmark of *S.* Typhimurium virulence. The toxin is responsible for symptoms that include nausea, vomiting, abdominal pain, fever and diarrhoea [33, 96].

V. cholerae pathogenesis involves the activation of adenosine 3′, 5′-cyclic monophosphate (cAMP). Likewise, adenylate cyclase in the cytoplasmic membrane in enterocyte activation is mediated by *Salmonella* enterotoxins which lead to a high concentration of adenosine monophosphate [25]. This high concentration of adenosine monophosphate causes a loss of intestinal fluid. *S. boulardii* is reported to inhibit cholera toxin-stimulated chloride secretion through the reduction of cAMP [97], and therefore this ability of *S. boulardii* to inhibit chloride secretion and subsequent fluid loss due to *V. cholerae* toxin may well have similar effects on *Salmonella*-associated diarrhoea, since *Salmonella* toxin is genetically, immunologically and functionally similar to *V. cholerae* toxin [25, 97].

*S. boulardi*i has been reported to deactivate or inhibit the production of toxins by *C. difficile* and *C. perfringens*. *S. boulardii* produces serine protease with proteolytic activity against *C. difficile* toxins [74]. Furthermore, *S. boulardii* minimized the effects of toxin fluid secretion, decreased mucosal permeability, decreased mucosal damage and decreased the release of inflammatory cytokines when administered to mice prior to them being given the *V. cholerae* toxin [74].

The ability of yeast to bind or neutralize bacterial toxin is possibly probiotic strain-specific. *S. cerevisiae* LV02/CNCM I-3856 provided no protection when porcine IPEC-1 (intestinal epithelial cell lines 1) was infected with enterotoxigenic *E.coli*. The integrity of the IPEC-1 barrier was disrupted, which indicates that this strain does not act on the *E.coli* toxin [54].

LPS, an endotoxin of *Salmonella* and *E.coli*, is associated with sepsis, which can be life-threatening [96]. Alkaline phosphatases, an enzyme produced by *S. boulardii*, was shown to neutralize LPS and reduce its toxicity in a rat model, as well as reducing inflammatory lesions and necrotic bodies in the liver and heart of the treatment group compared to the control group [98].

EFFECTS OF QUORUM SENSING ON PATHOGENS

Micro-organisms produce extracellular compounds that measure microbial population density in the surrounding area and, as a result, regulate their population. This phenomenon is referred to as quorum sensing [99]. Quorum sensing in poly-microbial populations has both synergistic and antagonistic effects. When quorum sensing compounds such as farnesol, *N*-Acyl homoserine lactones, tyrosol and dodecanol are produced in sufficient quantities they cause the expression of

genes within the population. Genes expression results in microbial growth mode, virulence gene expression, biofilm formation or morphological changes [3].

The quorum-sensing molecules produced by micro-organisms not only affect poly-microbial communities, but also the hosts. The immunomodulatory properties of farnesol have been documented, including stimulation of the NF-κB pathway through MEK1/2-ERK1/2-MSK1-dependent phosphorylation of p65, which leads to the production of cytokines, namely IL-6 and IL-1α [3]. However, on a negative note, the alteration of monocytes to dendritic cells by farnesol has been reported. In brief, the effects of farnesol on immune cells lead to reduced ability to recruit and activate T cells and hence compromised immunity [3].

Farnesol, an alcohol derivative produced by *S. cerevisiae* or *C. albicans*, has been shown to prevent bacterial biofilm formation [3, 100]. Farnesol was reported to antagonize the production of quinolone signal via the inhibition of *Pseudomonas* quinolone signal gene A (PqsA). Furthermore, farnesol has the potential to be used as a complementary therapy for bacterial infections. It was shown to increase the susceptibility of *S. aureus* to antibiotics and had synergistic effects on the efficacy of nafcillin and vancomycin in the prevention of biofilm formation by *Staphylococcus epidermidis*. Additionally, farnesol enhanced the efficiency of B-lactams against *Burkholderia pseudomallei* [3].

An*in vitro* experiment in murine showed that macrophage cell line RAW264.7 acted in synergy with farnesol and yeast cell walls to increase the expression of pro-inflammatory cytokines [3].

ANTIBACTERIAL PROPERTIES OF YEASTS BIO-SURFACTANTS

Bio-surfactants, also referred to as glycolipids, are compounds made up of one or two sugar molecules, especially glucose or galactose residues in alpha or beta configuration on a lipid backbone. Bio-surfactants are found in bacteria, fungi, plants and animal cell membranes such as glycosylceramides, diacylglycerolglycosides and sterylglycosides [101]. Bio-surfactants are classified as rhamnolipids, sophorolipids, trehalolipids and man-nosylerythritol lipids. These bio-surfactants are produced by micro-organisms, some of which are probiotic bacteria or yeasts [102, 103]. These bio-surfactants have been reported to be functional in bioactive compounds such as glycosylceramides, sphingolipids, glycosphingolipids, sphingosines and ceramides. Their bioactivity has been associated with anti-proliferative responses, such as the inhibition of cell growth, proliferation, differentiation, interruption of the cell cycle, signal transduction, senescence transformation, inflammation and apoptosis [101].

Phytosphingosine, an endogenous bioactive molecule in fungi, plant and human skins, has been shown to inhibit Gram-positive bacteria growth and also has anti-inflammatory properties. Moreover, sphingolipids such as cerebrosides and gangliosides have antibiotic properties, in that they can bind pathogens or their toxins and remove them from the GIT [101].

Biosurfactants have been reported to prevent pathogenic bacteria adhesion from infection sites as well as biofilm formation. *Candida sphaerica* UCP 0995 biosurfactant, also known as lunasan, has anti-adhesive properties against some gram-positive bacteria, including *S. aureus* and *Streptococcus agalactiae*, while the polymeric biosurfactant produced by *Candida lipolytica* UCP 0988 has anti-adhesive properties against *S. aureus, Lactobacillus casei*, *Streptococcus mutan* and *E. coli* [101]. Mannosylerthritol lipids (MEL) and cellobiose lipids produced by fungi have antibacterial activities through the disruption of cell membranes, which leads to cell lysis. MEL types A and B produced by *Candida antarctica* and *Schizonella melanogramma* have antagonistic properties against gram-positive and gram-negative bacteria [101].

PROBLEMS ASSOCIATED WITH THE PROPHYLACTIC AND THERAPEUTIC USE OF PROBIOTICS

The safety of probiotic products is an important aspect that needs consideration before they are used. *S. boulardii* is generally safe when used in a healthy population; however, in 2012, 100 cases of fungaemia were reported worldwide in individuals with gastrointestinal track issues and those who were immunocompromised [51]. *Saccharomyces* fungaemia is critically severe in patients with gastrointestinal diseases [51]. Moreover, an allergic reaction from the administration of *S. boulardii* was been reported in an infant who had previously been diagnosed with food protein-induced enterocolitis syndrome [104].

Candida species have also been reported to possess virulence factors, including glycosidases, proteases, haemolysin, lipases and phospholipases [105]. The ability of yeast species to exist in a dimorphic form (e.g. through the formation of hyphae) has been reported to be one of their virulence factors, and both the *Saccharomyces* and *Candida* species have been shown to form hyphae [106]. The formation of hyphae was found to be triggered by nutrient deficiency, as well as the presence of 0.5 % isoamyl alcohol [107]. This is of great significance when kefir is used as a probiotic. Kefir is a probiotic low-content alcoholic drink [46], and therefore the potential for yeast species to develop hyphae is a safety risk and needs further research. Moreover, yeasts also have negative impacts on humans, including being food spoilers [52].

Prophylactic and therapeutic use of probiotic bacteria in infectious diseases caused by pathogens such as *Salmonella* has some drawbacks due to the risk of multi-drug resistance gene acquisition [45]. Antibiotic-resistant genes have been detected in *Enterococci* and *Lactobacillus lactis* [74]. Both *Bacillus subtilis* and *E.coli* Nissle 1917 are known to be susceptible to most antibiotic drugs and therefore pose no risk of antibiotic resistance, and so are safe to use as probiotics in prophylaxis, however their susceptibility to antibiotics

makes these bacteria unsuitable for complementary therapy in infectious bacteria treatment [108, 109]. Furthermore, probiotic bacteria have been implicated in sepsis and endocarditis in patients who are immunosuppressed or predisposed to translocation and systemic dissemination of bacteria [110]. These issues associated with probiotic bacteria make their use less attractive in infectious bacterial diseases and hence there is a need for alternative probiotic micro-organisms.

EFFECTS OF PROBIOTIC PRODUCT FORMULATION ON EFFICACY

Probiotics are commonly included in popular fermented functional foods, such as yoghurt, milk, cheese, soybean, fruits, sourdough, kefir and vegetable products, making their consumption easier and more enjoyable, while at the same time providing prophylactic and therapeutic benefits to consumers [44–46]. Probiotic products can also be in the form of lyophilized capsules or aqueous solutions. The survival of probiotics in lyophilized form during delivery in *in vivo* experiments has been reported to be higher than that observed in the aqueous suspension form [48]. However, 7–16 days at the optimum temperature (between 15–25 °C) is needed to resuscitate lyophilized yeasts cells. These requirements do not fit the temperature in the human GIT or the time period that substances stay in there, which may make the lyophilized yeast probiotic products less effective [48].

Furthermore, studies have shown that *S. boulardii* exhibited different revival rates in lyophilized form (between 50 and 60 %), whereas *S. cerevisiae* was found to have an even lower revival rate of about 20 % in aqueous solutions. These differences in the revival rates could be due to the different freeze-drying methods used by different manufacturers. Previous studies on *S. boulardii* and other *Saccharomyces* species that examined survival and recovery from different preserved forms showed diverse kinetics, such as viability for long storage times, revival and survival in the GIT. Despite this variability, lyophilization is the preferred method of preservation [48].

CONCLUSION AND FUTURE PERSPECTIVES

Probiotic bacteria and yeasts are currently used for prophylaxis and complementary therapy against infectious and non-infectious diseases. The rise of antibiotic resistance and the potential of probiotic bacteria to carry antibiotic-resistant genes, coupled with opportunistic pathogens, has increased the need for alternative biotherapeutic drugs. Yeast species isolated from various sources have antagonistic properties against enteric bacterial pathogens. The antagonistic mechanisms have been reported in many *in vitro* experiments and a few animal trials. The use of yeasts in humans as s probiotic is very limited. Currently, *S. boulardii* is the only probiotic yeast used for prophylaxis and therapies in various ailments, but it has been implicated in fungaemia and allergic reactions. Other yeast species with prophylactic and therapeutic potential with respect to infectious diseases such as *Salmonella* need further research.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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Chapter 4. Kefir characteristics and antibacterial properties-potential applications in the control of enteric bacterial infection

This chapter focuses on the prophylactic and therapeutic potential of kefir. Antibacterial properties of kefir on enteric bacterial pathogens are comprehensively discussed while other health benefits of kefir consumption especially immunomodulation and gastrointestinal improvement are briefly highlighted.

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Review

Kefir characteristics and antibacterial properties - Potential applications in control of enteric bacterial infection

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ABSTRACT

Kefir is an acidic and low alcoholic beverage produced by fermentation of milk, fruit juice or sugary water with kefir grains and its consumption is associated with prophylactic and therapeutic properties including its antagonistic effect on enteric pathogenic bacteria. Kefir grains have several bacteria and yeast species encased in an extracellular polysaccharide matrix. The beverage is consumed due to its attributed health benefits conferred by probiotics. Kefir drink has bactericidal and bacteriostatic effects on enteric bacterial pathogens. The mechanisms of kefir on bacterial pathogens involve destabilisation of the cell membrane, cell lysis, degradation of nucleic acid, inhibition of protein synthesis and binding onto yeasts. Exopolysaccharides, organic acids, peptides, S layer proteins, and others are responsible for these antagonistic mechanisms. Other prophylactic and therapeutic properties of kefir include anti inflammatory, constipation alleviating, reversal of lactose intolerance and general gastrointestinal tract improvement. This can lead to better health, and consequently resistance to infection.

Contents

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1. Introduction

The term kefir originates from kef, a Turkish word, which means 'a pleasant taste' (Arslan, 2015). Millet of the prophet, Mohomet grains, kefyr, kephir, kefer, kiaphur, knapon, kepi, and kippi are some other names used to refer to a kefir grain. It is a gelatinous granule measuring about 1–6 mm or sometimes up to 15 mm in diameter with irregular, rough and convoluted surface, which resembles a cauliflower floret (Garrote, Abraham, & De Antoni, 1997; Sarkar, 2008; Turkmen, 2017). The grain is made of a consortium of exopolysaccharides and many different types of microorganisms, mainly bacteria and yeasts (Plessas et al., 2016; Prado et al., 2015). The exopolysaccharide, also known as kefiran, is chemically composed of glucose and galactose (Sarkar, 2008). Kefir grains are used for production of kefir, a fermented acidic beverage with low alcohol content.

This ancient fermented milk drink originates from the Caucasus region (Nejati, Junne, & Neubauer, 2020). The consumption of kefir is widespread and popular in many countries including Caucasus Mountains of Russia, Europe, Asia, South and North America among others for its health benefits, which have been postulated to be conferred by microorganisms and their metabolites (Plessas et al., 2016). These microorganisms, especially lactic acid bacteria, have been reported to possess probiotic properties (Farag, Jomaa, & El Wahed, 2020; Gamba et al., 2020). Probiotics are defined by the World Health Organisation (WHO) and Food and Agriculture Organisation (FAO) as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). Consumption of kefir for many centuries has been linked to its medicinal properties, which are attributed to microorganisms and bioactive compounds produced in it, including antihypertensive, anti oxidative, antiallergenic, antitumor, anti inflammatory and cholesterol lowering functions; and antimicrobial activities that are increasingly being appreciated (Nejati et al., 2020).

There has been a significant rise in the global burden of morbidity and mortality associated with enteric bacteria because of resistance to current antibiotic drugs. For instance, resistance to multi drug of *Salmonella enterica* serovar Typhi (*Sal*. Typhi) increased from 7% in 2010 to 97% in 2014 in Malawi (Feasey et al., 2015). This multi drug resistance has huge health and socio economic effects, and has necessitated the search for alternative prophylactic and therapeutic natural products such as kefir (Gut, Vasiljevic, Yeager, & Donkor, 2018). Antagonistic properties of kefir against *Escherichia coli* (*E. coli*), *Listeria monocytogenes, S. enterica* serovar Typhimurium (*Sal*. Typhimurium), *S. enterica* serovar Enteritidis (*Sal*. Enteritidis), *Shigella flexneri, Yersinia enterocolitica, Candida albicans, Sal.* Typhi, *Shigella sonnei, Staphylococcus aureus* (*S. aureus*), *Bacillus subtilis, Enterococcus faecalis* have been reported (Leite et al., 2013). Antagonistic properties of kefir against enteric bacteria have been postulated to be due to its probiotic components and their bioactive metabolites including organic acids, bacteriocins, carbon dioxide, hydrogen peroxide, ethanol and diacetyl (Leite et al., 2013).

This review focuses on the prophylactic and therapeutic potential of kefir. Antibacterial properties of kefir on enteric bacterial pathogens are comprehensively discussed while other health benefits of kefir consumption on immunity and gastrointestinal improvement are briefly highlighted.

2. Production of kefir

Three methods are used to produce kefir, namely, traditional, Russian and a large scale industrial production. Traditional method involves a one step fermentation, whereas the commercial method, also known as the Russian method, involves a two step fermentation. The large scale industrial production involves the use of pure kefir cultures rather than the kefir grain. Kefir may be produced with kefir grains through fermentation with either milk, rice, fruit juice or molasses (Chunchom, Talubmook, & Deeseenthum, 2017; Kesenkaş, Gürsoy, & Ozbaş, 2017; Liu, Wang, Lin, & Lin, 2002; Plessas et al., 2016). Different types of milk may be used including full cream, part skim or skim milk from cow, ewe, goat, donkey, mare and camel (Apar, Demirhan, Ozel, & Ozbek, 2017; Esener et al., 2018; Sarkar, 2008), although goat milk is not recommended for kefir production due to its low viscosity and sensory properties (Tratnik, Božanić, Herceg, & Drgalić, 2006).

Generally as a requirement for the quality of kefir, milk used for production should be free from high microbial counts, pathogens and inhibitory substances such as antibiotics and sanitiser residues (Sarkar, 2008). Heat treatment of milk intended for kefir production is recommended and serves two purposes: firstly, to reduce microbial load of native milk that may interfere with fermentation, and secondly to release amino acids, decrease redox potential, remove inhibitory constituents, prevent syneresis and hydrolytic rancidity through denaturation of lipase (Sarkar, 2008). Different temperatures and time combinations (92 °C/20 min, 95 °C/15 min, 90–95 °C/2–3 min or 95 °C/10–15 min) have been suggested for heat treatment of milk; however, 95 °C/15 min is commonly used as shown in Fig. 1 (Sarkar, 2008). Other factors that affect kefir quality include kefir flora, grain to milk ratio, cultures used for kefir production, time and temperature of incubation, degree of agitation; as well as packaging type and storage conditions in industrially produced kefir (Sarkar, 2008). Codex Standard recommends that kefir should contain <10.0% milk fat, a minimum of 2.80% milk protein, 0.6% lactic acid titratable acidity, 10^7 cfu g^{-1} bacteria and a minimum of 10^4 cfu g^{-1} yeasts total counts (Codex, 2001).

2.1. Traditional method production

The traditional kefir production was originally based on the goat milk inoculated with kefir grains in a goat skin bag by hanging in the house during winter and outside during summer for a period of 20–48 h (Sarkar, 2008). Such an approach was based on a temperature requirement of 20–27 °C, which is optimum for microbial growth and fermentative activity (Sarkar, 2008). Fig. 1 shows a schematic representation of traditional kefir production. Sieving is performed at the end of fermentation to separate kefir grains from kefir. The kefir can be consumed immediately or stored at 4 °C. The

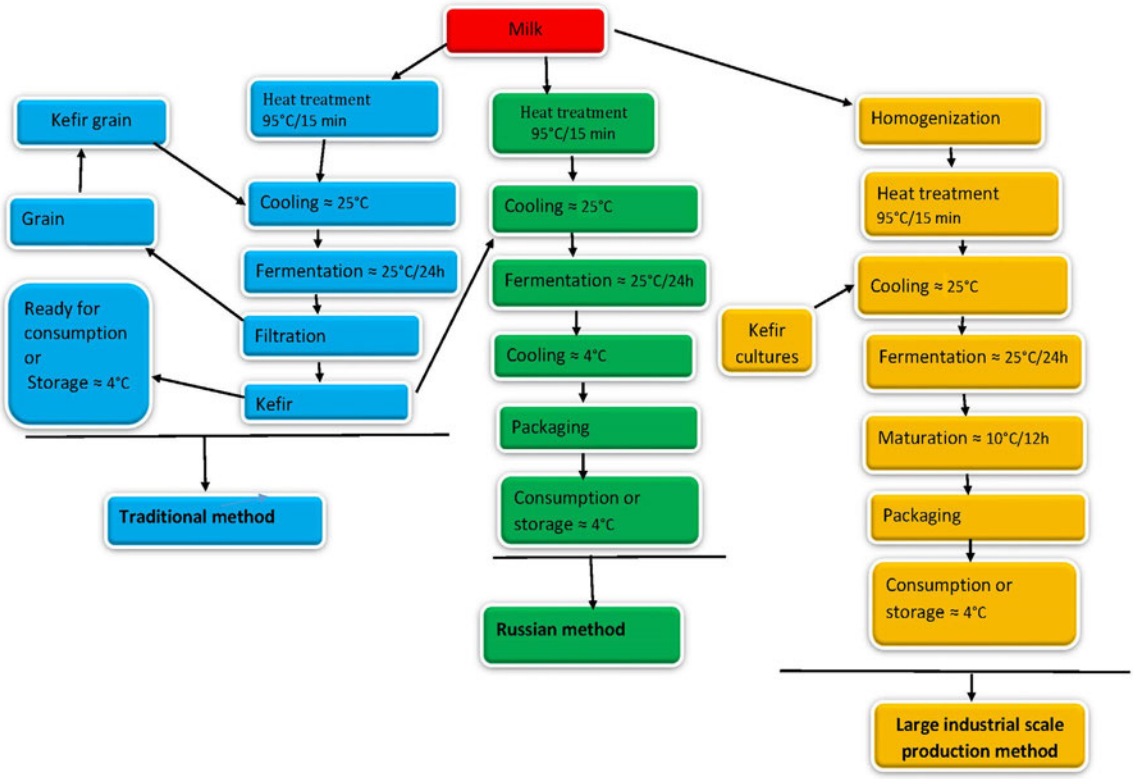

Fig. 1. A general outline of kefir production methods.

grain can then be used for a new fermentation or stored in milk for up to 7 days in the refrigerator for later use (Leite et al., 2013). Ratio of milk to kefir grain (commonly 1:10 or 1:50) is critical in the traditional method of kefir production as it affects pH, viscosity, lactose content and microbiological profile (Rattray & O'Connell, 2011). Furthermore, microbial composition of kefir is influenced by agitation during fermentation, with homofermentative lacto cocci and yeast being favoured (Farnworth & Mainville, 2008; Rattray & O'Connell, 2011; Wszolek, Kupiec Teahan, Guldager, & Tamime, 2006). Kefir grains contain mesophilic and thermophilic microorganisms with varying optimum growth temperature re quirements. Mesophilic organisms grow well below 30 °C while thermophilic prefer temperatures above 30 °C. However, tradi tional kefir productions apply a temperature between 20 °C and 27 °C, hence the common use of ambient temperature or 25 °C (Rattray & O'Connell, 2011; Sarkar, 2008). There has been a concern with the traditional method of kefir production, due to poor handling, source and quality of raw material as well as lack of in spection by health professional, making this method a potential hazard to consumers (Dias et al., 2012).

2.2. Russian method

Production of kefir using the Russian method (Fig. 1) is achieved through a two step fermentation. The first step includes inocu lating the milk with kefir grains and incubating at 25 °C for 1 day, followed by filtration to remove the grains. The grain free filtrate is then used to inoculate pasteurised milk and incubate at 18–24 °C for 18 h to produce kefir ready for consumption or packaging. The resultant grains are used to inoculate fresh milk (Ching Yun & Chin Wen, 1999; Farnworth & Mainville, 2008; Kesenkaş et al., 2017; Rattray & O'Connell, 2011; Wszolek et al., 2006).

2.3. Large scale industrial method

In a large scale industrial production of kefir (Fig. 1), pure kefir cultures are added to heat treated milk and fermentation is allowed to occur at 18–25 °C for 18–24 h. The resultant kefir is allowed to mature at 8–10 °C for 12 h before packaging (Kesenkaş et al., 2017; Leite et al., 2013). The large scale production of kefir is limited due to the problems associated with obtaining starter cultures with stable characteristics required to maintain standard quality (Dias et al., 2012).

3. Composition of kefir

3.1. Microbial composition and symbiosis

Kefir grains contain strains of *Lactobacilli, Leuconostoc*, *Strepto cocci*, *Lactococci*; *Saccharomyces*, *Kluyveromyces*, *Candida* and *Pichia* among others, all bound together by kefiran (Plessas et al., 2016; Sarkar, 2008). Mould species including Mucor, Alternaria and *Aspergillus* have been reported (Dertli & Çon, 2017). The microbial composition of kefir may influence the production and or efficacy of the antimicrobial compounds (since composition is different in different kefirs) hence the need to list in Tables 1 and 2 common microorganisms in kefir, which are not yet characterised for anti microbial activities. The colony counts of bacteria and yeasts in kefir fermented using grains differ but are generally in the range of 6.4×10^4 to 8.5×10^8 and 1.5×10^5 to 3.7×10^8 cfu mL^{-1}, respectively (Gut, Vasiljevic, Yeager, & Donkor, 2019; Witthuhn, Schoeman, & Britz, 2004). Other studies have reported kefir fer mented using grain as having 10^8–10^9 cfu mL^{-1} lactic acid bacteria, 10^5–10^6 cfu mL^{-1} acetic acid bacteria and 10^5–10^6 cfu mL^{-1} yeasts (Garrote, Abraham, & De Antoni, 2001; Robinson, 1991).

Table 1
Bacterial species identified in kefir.

Microorganism	Source	Reference
Bifidobacterium animalis; Bifidobacterium longum; Lactobacillus acidophilus; Lactobacillus bulgaricus; Lactobacillus krusei; Lactobacillus johnsonii; Lactobacillus plantarum; Lactobacillus reuteri; Lactobacillus rhamnosus; Lactococcus lactis	Kefir fermented using traditional kefir grain	Demir (2020)
Lactobacillus acidophilus; *Lactobacillus bulgaricus*; *Lactobacillus plantarum*; *Streptococcus thermophilus*; *Lactobacillus reuteri*; *Lactococcus lactis*	Kefir fermented using commercial kefir cultures	Demir (2020)
Lactobacillus kefiranofaciens, Lactococcus lactis; Leuconostoc mesenteroides; Lactobacillus helveticus; Acetobacter okinawensis; Acetobacter orientalis; Enterobacter aerogenes	Kefir fermented using traditional kefir grain	Gao and Zhang (2019)
Lactobacillus kefiranofaciens; Lactobacillus apis; Lactobacillus ultunensis; Enterobacter amnigenus;	Kefir fermented using traditional kefir grain	Dertli and Çon (2017)
Enterobacter hormaechei; Acinetobacter rhizosphaerae; Acinetobacter calcoaceticus; Enterococcus lactis; Pseudomonas azotoformans; Pseudomonas aeruginosa; Pseudomonas otitidis; Propionibacterium acnes		
Acetobacter orientalis; Lactobacillus gallinarum; Lactobacillus nagelii; Lactobacillus plantarum; Lactobacillus pentosus; Lactobacillus plantarum/Lactobacillus pentosus	Kefir fermented using traditional kefir grain	Gamba et al. (2020)

Table 2
Some yeast species found in kefir.

Yeast	Source	Reference
Candida krusei	Kefir fermented using commercial kefir cultures	Demir (2020)
Kluyveromyces lactis; Saccharomyces unisporus	Kefir fermented using traditional kefir grain	Gut et al. (2019)
Kazachstania turicensis; Kluyveromyces marxianus; Dekkera anomala	Kefir fermented using traditional kefir grain	Gao and Zhang (2019)
Issatchenkia orientalis; Dipodascus geotrichum; Saccharomyces cerevisiae; Kazachstania unispora; Candida parapsilosis; Candida zeylanoides; Rhodotorula dairenensis; Rhodotorula mucilaginosa; Yarrowia lipolytica; Cryptococcus victoriae	Kefir fermented using traditional kefir grain	Dertli and Çon (2017)
Kazachstania unispora; Galactomyces candidum; Geotrichum bryndzae; Pichia kudriavzevii	Kefir fermented using traditional kefir grain	Gamba et al. (2020)

Spatially, lactic acid bacteria (bacilli) dominate the outer layer of kefir grains while yeasts occupy the epicentre. In between, there is a balance in the number of bacteria and yeasts with a gradual variation depending on the distance from the centre. However, there is controversy on this location distribution (Chin Wen, Hsiao Ling, & Liu, 1999; Leite et al., 2013; Rea et al., 1996; Sarkar, 2008). Other studies reported presence of cocci on yeasts surfaces while bacilli sit between yeast cells (Neve & Heller, 2002).

The co existence of bacteria and yeasts in kefir grain is based on a symbiotic relationship. A previous study showed enhanced growth of *Lactobacillus nagelii* when co cultured with *Zygotor ulaspora florentina* because of improved nutrient availability released by *Z. florentina* (Bechtner, Xu, Behr, Ludwig, & Vogel, 2019; Stadie, Gulitz, Ehrmann, & Vogel, 2013). Three mechanisms have been postulated to be involved in nutrients release by yeasts. The first mechanism involves changes in membrane permeability in the presence of glucose leading to release of amino acids. Some yeasts produce proteolytic and lipolytic enzymes that contribute to breakdown of proteins and fats leading to release of amino acids and fatty acids, respectively. The second mechanism involves autolysis of yeast cells, which leads to the release of cell compo nents including amino acids and vitamins (Lopitz Otsoa, Rementeria, Elguezabal, & Garaizar, 2006; Ponomarova et al., 2017; Stadie et al., 2013). Autolysis of yeast cells is thought to be triggered by proteins, peptides and amino acids resulting in an altered membrane permeability and subsequent cell break down (Babayan & Bezrukov, 1985). The release of nutrients by yeast cells is an intrinsic characteristic of yeast species and not induced by bacteria (Ponomarova et al., 2017). The third mechanism involves some yeast species such as *Candida*, *Pichia* and *Kluyveromyces*, which can assimilate lactate leading to rise in pH resulting in improved growth of acid sensitive bacteria (Wang, Hao, Ning, Zheng, & Xu, 2018). However, this mechanism has been disputed by another study, which showed that the effect of yeast on bacteria in kefir was not caused by pH increase but by potential presence of other antibacterial metabolites (Ponomarova et al., 2017). On the other hand, yeasts growth was found to be supported positively by bacteria through pH reduction during lactic and acetic acid pro duction (Stadie et al., 2013). Most yeast species can grow at a pH of 2.0–2.5, however the optimum growth pH is about 4.5–7.0 (Frohlich Wyder, Arias Roth, & Jakob, 2019), whereas another study reported the optimum growth pH for *Saccharomyces* species used for brewing as 3.8 (Stadie et al., 2013).

3.2. Nutritional properties

Kefir grains are generally composed of 890–900 g kg^{-1} of water, 2 g kg^{-1} of fat, 30 g kg^{-1} protein, 60 g kg^{-1} sugars and 7 g kg^{-1} ash, which may vary depending on the grain and type of milk. For example, cow milk is reported to be richer in protein and fat than camel milk resulting in compositional differences (Sarkar, 2008). Kefir is also reported to be rich in vitamins (A, E, B_1, B_2, B_3, B_5, B_6, B_{12} and C) and minerals such as sodium, potassium, calcium, magne sium, phosphorus, iron, zinc, selenium, copper and manganese (Satir & Guzel Seydim, 2016).

4. Prophylactic and therapeutic potential of kefir against enteric bacterial pathogens

4.1. Effect of kefir extracts and microorganisms on enteric bacterial pathogen

Antagonistic properties of kefir against enteric bacteria have been associated with many factors such as production of organic acids, hydrogen peroxide, acetaldehyde, carbon dioxide, kefiran, bacteriocins, S layer proteins, adhesion of pathogens on yeasts cell walls, competition for nutrients and space (Leite et al., 2013; Menezes et al., 2020; Mobili et al., 2009; Shen et al., 2018). Fig. 2

depicts prophylactic and therapeutic properties of kefir. The antagonistic mechanisms on targeted microbial cells include destabilisation of cell membrane, degradation of nucleic acid and inhibition of protein synthesis (Dias, Silva, & Timm, 2018; Li, Yin, Yu, & Yang, 2011).

Enteric bacterial infections such as non typhoidal salmonellosis complications which can include meningitis, and septicaemia caused by *Sal.* Typhimurium, *S. enterica, S.* Enteritidis, *S. enterica* serovar Newport (*Sal*. Newport), and *S. enterica* serovar Heidelberg (*Sal.* Heidelberg) have typically been treated with ciprofloxacin, ceftriaxone or ampicillin (Medalla et al., 2016; WHO, 2003). Furthermore, other pathogens such as *Sal*. Typhi and *S. enterica* serovar Paratyphi infections require treatment and antibiotic drugs including cefixime, chloramphenicol, amoxicillin, trimethoprim sulfamethoxazole (TMP SMX), azithromycin, aztreonam, cefotaxime or ceftriaxone are used (Kumar & Kumar, 2017) while delirium, obtundation, stupor, coma, or shock occurrence is treated with dexamethasone (Kumar & Kumar, 2017). However, alternative preventative and complementary therapies to these drugs are important as antibiotic resistance is emerging, for example, *Salmonella* resistance to the above antibiotics has been reported (Bakken, 2014; Crump, Sjolund Karlsson, Gordon, & Parry, 2015; Feasey et al., 2015; Wong et al., 2015) hence consumption of kefir is one of the natural food sources to address this problem. This section is presented in two axes. The kefir antimicrobial molecules axis covers organic acids, kefiran, bacteriocins, S layer proteins, and other antimicrobial compounds such as ethanol. The antimicrobial activity of the microorganisms in the kefir axis covers adhesion and competition for nutrients and space.

4.1.1. Kefir antimicrobial extracts

4.1.1.1. S layer proteins. Kefir bacterial isolates have been found to possess S layer proteins on the cell wall (Xue et al., 2015) and these have been reported to possess antibacterial and antiviral properties (Fina Martin et al., 2019). Gram positive bacteria including *Lactobacillus* species are covered by two dimensional crystalline, glycoproteinaceous cell surface (S) layer lattice bound to the exterior cell wall known as the S layer protein. This protein is made up of two layers and perform two distinct functions including cell wall anchoring and mediation of protein self assembly (Bonisch et al., 2018).

In vitro studies using human cell lines including HT 29 and Caco 2 treated with *Lactobacillus* species S layer proteins showed reduced adherence of *Sal.* Typhimurium to the cell lines, consequently reducing the infection by the pathogen, compared with the control (Li et al., 2011; Xue et al., 2015). The mechanism of action was thought to be due to masking of *Salmonella* cell surface structures responsible for attachment onto enterocytes with *Lactobacillus* species S layer proteins. Infection of human cells by *Salmonella* leads to apoptosis induced by caspase 3 activation. *Lactobacillus* species S layer proteins inhibited caspase 3 activation and thus stopped *S.* Typhimurium induced apoptosis, hence prevented erosion of gastrointestinal tract (GIT) lining normally caused by infection (Li et al., 2011; Xue et al., 2015).

4.1.1.2. Kefiran effect on enteric bacterial pathogens. As previously mentioned in the introduction, kefiran is a branched glucogalactan, which is produced by lactic acid bacteria in kefir and constitutes 24–25% (w/w) of kefir grain (Plessas et al., 2016; Shen et al., 2018). Kefiran produced by kefir bacterial isolates showed inhibition of *Sal*. Enteritidis in a dose dependent manner and complete inhibition was observed at 2.5% kefiran concentration. Furthermore, the growth of *L. monocytogenes*, a foodborne pathogenic bacteria, was inhibited in the study by the same concentration (Jeong et al., 2017). Another study on kefiran (50 μg mL^{-1}) separated from kefir containing *Leuconostoc* spp., *Lactobacillus lactis, Acetobacter* spp., *Saccharomyces cerevisae, Kluyveromyces marxianus* and *Kluyveromyces lactis* reported strong growth inhibition. Antibiotics including ampicillin, azithromycin, ceftriaxone and oxacillin were used as control. This study showed that the inhibition zone for *S.* Typhimurium ranged from 15.7 to 24.4 mm for antibiotics controls, 26.2 mm for kefirans and 25.6 mm for kefir (Rodrigues, Caputo, Carvalho, Evangelista, & Schneedorf, 2005). Similarly, growth inhibition of Staph. aureus, *Streptococcus salivarius*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *C. albicans*, *L. monocytogenes*, and *E. coli* by kefiran and kefir in the study was similar to the antibiotics tested (Rodrigues et al., 2005). Proposed mechanisms of kefiran targeting microbial cells involved disruption of the cell membrane through pore formation and detergent like effects (Barbosa, Santos, Lucho, & Schneedorf, 2011).

4.1.1.3. Organic acid effects on enteric bacterial pathogens. The major chemical compounds produced by microbial component in

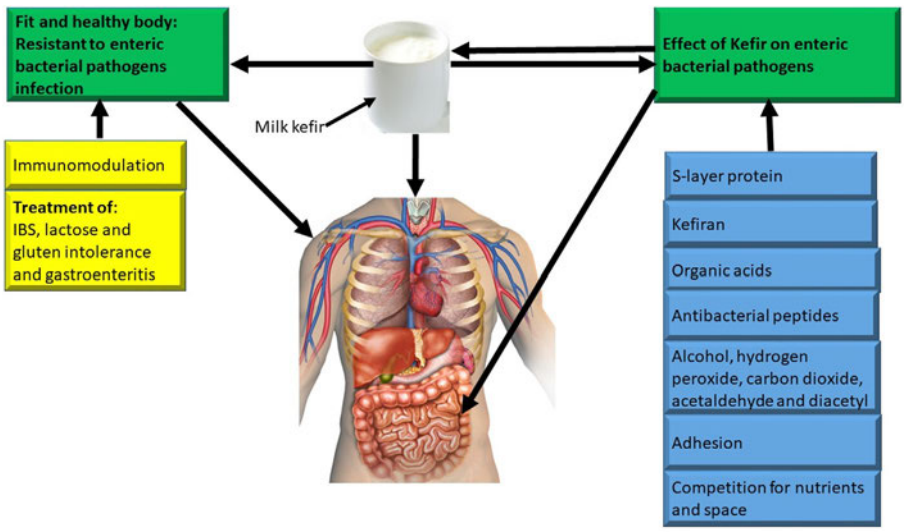

Fig. 2. Prophylactic and therapeutic properties of kefir against enteric bacterial infection.

kefir include carbonyl compounds (acetaldehyde, ethanol, diacetyl, acetoin, 2 butanone, and ethyl acetate), volatile organic acids and non volatile acids. Volatile organic acids include for mic, acetic, propionic, and butyric whereas non volatile organic acids include lactic, pyruvic, oxalic, and succinic (Iraporda et al., 2017; Puerari, Magalhaes, & Schwan, 2012; Schwan, Magalhaes Guedes, & Dias, 2016). Production of these compounds and their compositional ratios are influenced by a number of factors including microbial species, incubation temperature and the ratio of kefir grain/starter culture combination (Arslan, 2015; Sarkar, 2008). A study on the chemical composition of cow milk kefir reported a diverse composition of organic acids including lactic acid (0.73%), followed by acetic (0.65%) and malic acids (0.4%) (Arslan, 2015; Setyawardani, Rahardjo, Sulistyowati, & Wasito, 2014). Organic acids produced in kefir during fermentation are by products of carbohydrate catabolism, which results in the lowering of pH (Bosch et al., 2006). The action of organic acids on target bacteria involve diffusion of non dissociated acids through the cell membrane into the cytoplasm where protons are released and cause acidification. The dissipation of potential protons from bacterial cells, prevents energy generation (Diez Gonzalez & Russell, 1997).

A study on *Sal.* Typhimurium and *E. coli* 0157: H7 showed that lactic acid acts as a membrane permeabiliser through disruption of the outer cell membrane and thus enhances the activity of other antibacterial molecules (Alakomi et al., 2000). Lactic acid, one of the main organic acids in kefir (Arslan, 2015; Iraporda et al., 2017; Setyawardani et al., 2014), may aid activities of other antibacterial metabolites present in kefir to destroy target cells through permeability of cell membranes. Furthermore, lower pH enhances the attachment of proteinaceous antibacterial molecules to target bacterial cell surfaces and thus inhibit growth (Sadeghi, Raeisi, Ebrahimi, & Sadeghi, 2018).

Milk and milk products are commonly fermented with kefir; however, sugar solutions may also be used. Brown sugar (5%, v/v), high test molasses (6.5%, v/v) and purified molasses (6.5%, v/v) fermented with kefir grain (10%) completely inhibited *Sal.* Typhi murium. *E. coli* and *S. aureus* growth were also inhibited in this study (Gamba et al., 2019). The cause of growth inhibition was postulated to be due to presence of organic acids especially lactic and acetic acids at concentration greater than 70 and 40 mg 100 mL^{-1} respectively. Also, polyphenols or bacteriocins may have contributed in the growth inhibition (Gamba et al., 2019). *Sal.* Enteritidis, *Bacillus cereus* and *E. coli* growth was inhibited by non microbial fraction supernatant of kefir in a dose dependent manner in an in vitro study. However, neutralisation of acidity led to loss of growth inhibition signifying that low pH was responsible for the activity (Iraporda et al., 2017).

A study on the antibacterial properties of kefir against food borne pathogens and food spoilage organisms showed inhibitory effects. *Sal.* Enteritidis growth was totally inhibited at 36 h and 72 h; however, results showed that at 48 h, the growth was not inhibited. The cause of this discrepancy was likely due to the non synergistic effect of kefir metabolites, where some metab olites were found to have been produced at different times or degraded during incubation (Kim et al., 2016). Other pathogen growth, including that of *B. cereus, E. coli, Ps. aeruginosa, Cro nobacter sakazakii*, was inhibited at different levels (Kim et al., 2016). A similar study on survival of *Sal.* Enteritidis in kefir stored at 4 °C resulted in inactivation of the bacteria, which was proportionate to the titratable acidity (Chang et al., 2018). Similarly, the studies showed that other important foodborne pathogens (*B. cereus, E. coli*, *Enterobacter sakazakii*, *L. monocytogenes* and *S. aureus)* were inactivated (Chang et al., 2018; Kim et al., 2016).

4.1.1.4. The effect of antibacterial peptides on enteric bacterial path ogens. Bacteriocins are proteins produced by microorganism of one strain and active against those of a closely related strain but can also act against other unrelated species (Ahmad et al., 2017). Kefir microbial components including bacteria and yeasts have been reported to produce antibacterial molecules, which are proteina ceous in nature (Miao et al., 2016). These bacteriocin mechanisms are as discussed above and may be involved in DNA, RNA, ATP synthesis or protein synthesis inhibition, disruption in membrane and in ionic potentials (Biadała, Szablewski, Lasik Kurdyś, & Cegielska Radziejewska, 2020). Nisin, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis,* (common kefir bacterial isolates), is used in preservation of foods due to its antibacterial properties (Klewicka & Lipinska, 2016). Lacticin 3147, a broad spectrum bacteriocin also produced by Lc. lactis subsp. lactis (McAuliffe et al., 1998) is reported to inhibit *S. enterica* serovar Kentucky, and is found to be effective in the presence of organic acids (Scannell, Ross, Hill, & Arendt, 2000). Furthermore, antibacterial peptides produced by lactic acid bacteria inhibit S. Enteritidis growth (Leite et al., 2015). Supernatant from cheese whey fer mented with kefir isolates including Lactobacillus planatarium CIDCA 8327, *Lactobacillus kefiri* CIDCA 8348 and *K. marxianus* var. marxianus showed inhibitory effect on Sal. Enteritidis. The mech anism was thought to be due to substance active at low pH, possibly a synergistic effect of proteinaceous substance and the high acidity (Londero, Iraporda, Garrote, & Abraham, 2015). All these kefir iso lates have potential to produce antibacterial peptides in kefir and this has been shown in a study in which pathogenic bacteria growth was inhibited by proteinaceous antimicrobial molecules (Sindi, Badsha, Nielsen, & Ünlü, 2020).

4.1.1.5. Other metabolites as antibacterial compounds. Yeast species in kefir are responsible for production of some antimicrobial me tabolites such as carbon dioxide, tryptophol, tyrosol and 2 phenylethanol and ethanol (Nejati et al., 2020; Suharja, Henriksson, & Liu, 2014). Furthermore, headspace solid phase mi cro extraction (HS SPME) analysis of cow milk kefir found the organic compounds content to be as follows: ethanol (39.3%), 2 butanone (31.6%), ethyl acetate (8.9%), ethyl butyrate (5.5%), acetone (3.6%), 3 hydroxy 2 butanone (acetoin, 3.3%), 2,3 butanedione (diacetyl, 2.9%) and acetaldehyde (1.7%) (Aghlara, Mustafa, Manap, & Mohamad, 2009; Sarkar, 2008).

Carbon dioxide is one of the products produced during kefir fermentation. A previous study showed that kefir yeasts were able to produce high concentration of carbon dioxide, which depends on milk types, fermentation time and microbiological content of kefir (Tomar, Akarca, Ça lar, Bey kaya, & Gok, 2020). Antibacterial prop erties of carbon dioxide are thought to be due to its dissolution in an aqueous medium, which lowers pH making the medium bactericidal or bacteriostatic to pathogenic bacteria (Gut et al., 2018). Moreover, carbon dioxide under pressure was reported to pass through the cell membrane making the cells internal content acidic and hence inducing cell death (Erkmen, 2001).

Alcohol is one of the metabolites produced during kefir fermentation but at a very low concentration. However, alcoholic concentration varies and may reach 2.1% (Nunez, 2016). Alcohol effect on bacterial cells involves dissolution of cell membrane and results in uncontrolled transport of solutes. This decreases proton flux across the membrane and leakage of cofactors such as mag nesium. Furthermore, alcohol deactivates cytosolic enzymes including ATPase and glycolytic enzymes that are essential for survival and proliferation of bacterial cells (Huffer, Clark, Ning, Blanch, & Clark, 2011).

In addition to carbon dioxide and alcohol, kefir contains hydrogen peroxide, acetaldehyde and diacetyl in low

Table 3
Antibacterial properties of kefir fermented using kefir grain or pure kefir culture isolates.

Kefir fermentation	Indicator enteric pathogens	Mechanisms of action	Reference
Kefir fermented using traditional kefir grain	*Sal.* Typhimurium, *Sal.* Enteritidis, *E. coli* 0157: H7, *Staph. aureus*, *Listeria monocytogenes*	Growth of pathogenic bacteria inhibited caused by production of antibacterial metabolites	Aksu, Muratoglu, and Altunatmaz (2016)
	Sal. Typhimurium, *Staph. aureus, E.* coli	Significant suppression of Gram-negative bacteria caused by production of antibacterial metabolites	Gamba et al. (2020)
	Sal. Typhimurium, *Proteus mirabilis*, *E. coli*, *Shigella sonnei*	Suppression of Gram-negative bacteria caused by production of antibacterial metabolites.	Kontareva and Kryuchkova (2017)
	Sal. Typhimurium, *E.* coli	Inhibition of enteric bacterial growth caused by production of antibacterial metabolites.	Sulmiyati, Said, Fahrodi, Malaka, and Maruddin (2019)
Lactobacillus plantarum CIDCA 83114, *Lactobacillus kefir* CIDCA 8321, *Lactobacillus kefir* CIDCA 8348	*Sal.* Enteritidis	Prevented infection through co-aggregation; S-layer protein.	Golowczyc, Silva, Teixeira, De Antoni, and Abraham (2011)
S. boulardii, *Kazachstania unispora*, *Saccharomyces cerevisiae*, *Kodamaea ohmeri*	*Sal.* Typhimurium, *Staph. aureus, E.* coli	Significant suppression of Gram-negative bacteria through production of antibacterial metabolites.	Mohd Akmal and Mimi Sakinah Abdul (2019)
Lactobacillus kefir strains isolated from kefir	*Sal.* Enteritidis	Reduced invasion of cell lines due to the effect of S-layer protein.	Golowczyc, Mobili, Garrote, Abraham, and De Antoni (2007)
Lactobacillus diolivorans isolated from Brazilian kefir	*Sal.* Typhimurium	Nutrient competition and production of inhibitory compounds and reduced infection and growth inhibition.	Abatemarco Júnior et al. (2018)
Lactobacillus strains 8321, 83113 and 83114 isolated from kefir	*Sal.* Enteritidis, *Sal.* Typhimurium, *Salmonella* Gallinarum	Inhibited biofilm formation in vitro experiment through *Lactobacillus* strains surface protein.	Merino, Trejo, De Antoni, and Golowczyc (2019)
Lactobacillus spp. isolated from kefir	*Sal.* Typhimurium	Growth inhibition due to antibacterial metabolites.	Santos et al. (2003)
Lactobacillus kefiri	*Staph. aureus*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Sal.* Enteritidis	Growth inhibition due to antibacterial metabolites.	Carasi et al. (2014)

concentrations (Leite et al., 2013). These volatile compounds have been proven to have antibacterial properties but their low concentration in kefir (Aghlara et al., 2009; Bersezio et al., 2019; Daeschel & Penner, 2019) may reduce their antagonistic efficacy. However, they may work synergistically with other kefir antimicrobial metabolites to elicit desired antibacterial effects.

4.1.1.6. Kefir versus antibiotics. A comparative in vitro study demonstrated the antibacterial efficacy of undiluted kefir over ampicillin (10 mg mL^{-1}) and showed kefir's superior activity against *Sal.* Enteritidis, *S. aureus* and *E. coli* (AbdEl Mogheith, El Gendy, Sultan, & El Nesr, 2017). Antibacterial mechanisms were thought to be due to synergistic effects from organic acids, carbon dioxide, hydrogen peroxide, ethanol, diacetyl and peptides (AbdEl Mogheith et al., 2017). Another in vitro study reported synergistic action of bacteriocins produced by kefir bacteria with ceftriaxone, cefotaxime, ampicillin and EDTA against *Sal.* Typhimurium (Ahmad et al., 2017). A randomised clinical trial (Bekar, Yilmaz, & Gulten, 2011) showed that patients who consumed 250 mL of kefir daily while on a 14 day combined course of lansoprazole (30 mg), amoxicillin (1000 mg), and clarithromycin (500 mg) to treat *Helicobacter pylori* infections had 78.2% eradication rate compared with 50% eradication rate for the control group. In addition, this study reported less severe side effects in the treated group compared with the control group (Bekar et al., 2011). These results showed the potential of kefir to be used independently in a treatment or control of enteric pathogenic bacteria infection or as a complementary treatment with antibiotics. Table 3 summarises more research findings on antibacterial properties of kefir against pathogenic bacteria.

4.1.2. Antimicrobial activity of kefir microorganisms

4.1.2.1. Adhesion of enteric bacterial pathogens onto yeasts. Cell adhesion is defined as a process whereby cells attach to each other or to a foreign surface with the aid of adhesins (Gut et al., 2018). Yeast isolates from Brazilian fermented products including kefir co aggregated with *S.* Enteritidis through attachment of bacterial cells onto yeast cells (Menezes et al., 2020). Adherence of pathogenic bacteria on yeast cells is a potential way of controlling infections since bound pathogenic bacteria on yeast cell wall may limit infectivity and thus excreted via faeces. For example, non pathogenic yeasts such as Sac. cerevisae var boulardii do not bind to the epithelium of the GIT but pass transiently and are excreted in the faeces together with bound bacterial cells (Czerucka, Piche, & Rampal, 2007).

4.1.2.2. Competition for nutrients and space. Competition for nutrients is considered the most important antagonistic property of yeast species. Yeast species are reported to have the capacity to quickly deplete the nutrients such as glucose, fructose, and sucrose, and therefore may suppress the growth of other microorganisms including enteropathogenic bacteria (Muccilli & Restuccia, 2015). Additionally, some yeast species possess iron sequestering molecules, which gives them a competitive advantage to deplete iron, which is needed for growth and pathogenesis by many pathogens (Muccilli & Restuccia, 2015). Many yeast species have been isolated from kefir as shown in Table 2. These yeast species may not inhibit pathogens growth through antimicrobial molecules production or adhesion mechanism but may suppress their growth through nutrients depletion. In an in vitro study, *E.coli* growth was inhibited and one of the mechanisms suggested to be responsible for this growth inhibition was thought to be competition for nutrients between kefir microorganisms and *E. coli* (Garrote, Abraham, & De Antoni, 2000; Kim et al., 2018).

One of the mechanisms postulated on how probiotic microorganisms prevent invasion is by competitive exclusion. This is defined as the ability of normal flora or probiotics including yeast species to limit colonisation of the GIT, competing with invading

pathogens by creating restrictive physiological environment (Revolledo, Ferreira, & Ferreira, 2009). *S.* Typhimurium infection of human enterocyte like Caco 2 cells was prevented due to competitive exclusion by *Lactobacillus* spp. isolated from kefir grains (Santos, San Mauro, Sanchez, Torres, & Marquina, 2003). Furthermore, *Clostridium perfringens* colonisation of mice fed with milk kefir and soymilk kefir was statistically reduced compared with the control group, and the mechanisms was postulated to be competitive exclusion by kefir microbial components (Liu, Wang, Chen, Yueh, & Lin, 2006).

4.2. Other effects of kefir on enteric pathogenic bacterial infections

Kefir consumption is linked to GIT health due to prophylactic and/or therapeutic properties on diseases such as irritable bowel syndrome, lactose intolerance, ulcers and gastroenteritis caused by many ailments (Leite et al., 2013). Healthy GIT ensures intact tight junctions. Intact junctions function as a physical barrier that prevents noxious objects including pathogenic enteric bacteria from entering into deeper layers within tissues where they cause infection (Gut et al., 2018). Furthermore, consumption of kefir has been associated with health benefits such as anti obesity, anti hepatic steatosis, anti oxidative, anti allergenic, antitumor, anti inflammatory, cholesterol lowering (Rattray & O'Connell, 2011) and in this way make the person resistant to infection due to strong immunity and intact and healthy GIT lining. Fig. 2 depicts indirect ways kefir can help in infection prevention.

4.2.1. Impact on enteric bacteria through immunomodulation properties of kefir

Immunomodulatory properties of kefir are postulated to result from direct and indirect effects of microbiological components (lactic acid bacteria and yeasts) and metabolites (organic compounds and bioactive peptides) on the immune system (Gut et al., 2018; Noğay, 2019).

Kefir bioactive peptides are reported to activate macrophages, enhance formation of nitric oxide and cytokines, and elicit the release of IgG and IgA by B lymphocytes in the GIT (Noğay, 2019). The role of IgG, IgA and B lymphocytes as the first line of defence against infection in adaptive immunity is well documented (Lindow, Fimlaid, Bunn, & Kirkpatrick, 2011; Nanton, Way, Shlomchik, & McSorley, 2012). Another study reported anti inflammatory cytokine (IL 4, IL 6, IL 10) production as a result of kefiran consumption by mice (Vinderola et al., 2005). Anti inflammatory cytokines reduce inflammation response associated with pathogenic infection. For example, *Salmonella* pathogenesis involves stimulation of inflammatory responses including pro inflammatory cytokines release in the GIT. These cytokines cause acute inflammation which may lead to diarrhoea, ulceration, and destruction of the mucosa cells in the GIT (Gut et al., 2018). Therefore, the induction of anti inflammatory cytokines by kefir (Noğay, 2019) may make it a suitable candidate for treatment of *Salmonella* and other enteric bacterial infections independently or as a complementary treatment with antibiotic drugs.

4.2.2. Improvement of GIT system

GIT is affected by many disorders such as irritable bowel syndrome, gluten intolerance, gastroenteritis, and lactose intolerance. Some of these conditions can disrupt tight junctions between the GIT cells and as a consequence renders them susceptible to Salmonella and other enteric pathogenic infections (Rao & Samak, 2013). A trial using kefir in patients with GIT disorders reported significant relief from symptoms such as abdominal pain and bloating, resulting in improved quality of life (Yılmaz, Dolar, & Ozpınar, 2019). Kefir derived *Lactobacillus* extracellular vesicles was used in inflammatory bowel disease induced mice and was found to significantly lessen the symptoms associated with the disorder and improve body weight (Seo, Park, Ko, Choi, & Kim, 2018). Lactic acid bacterial species in kefir produce beta galactosidase, an enzyme that hydrolyses lactose during production and this was demonstrated in a previous study in which symptoms of lactose intolerance were reduced in the treatment group compared with the control (Hertzler & Clancy, 2003). This may make kefir suitable for consumption by those with lactose intolerance, while still benefiting nutritionally from this dairy product (Hertzler et al., 2017; Noğay, 2019).

Several studies have indicated a potential anti carcinogenicity property of kefir against colorectal, malignant T lymphocytes and chronic myelogenous leukaemia cancers (Chen, Chan, & Kubow, 2007; dos Reis et al., 2019; Rizk, Maalouf, & Baydoun, 2009; Sharifi et al., 2017). This anti carcinogenicity property of kefir is postulated to be due to mechanisms including regulation of inducible nitric oxide synthase (iNOS), Nitric oxide synthase 2 (NOS 2), and nitric oxide synthase 3 (NOS3) (Esener et al., 2018). Malignant processes, as well as chemotherapies, lead to immunodeficiency especially neutropenia further making patients more susceptible to infections (Bodey, 1986). Neutropenic mice were significantly more susceptible to *Sal.* Typhimurium than immunocompetent mice in a previous study (Dejager, Pinheiro, Bogaert, Huys, & Libert, 2010). Therefore, cancers such as colorectal cancer prevention and treatment using kefir may minimise susceptibility to enteric bacterial infection.

5. Conclusion

Consumption of kefir has many benefits including antibacterial, anti inflammatory, reversal of lactose intolerance and general GIT improvement as well as immunomodulatory properties. Studies have indicated potential application of kefir and its components in prevention and treatment of infectious enteric bacteria. Production of antibacterial peptides, organic acids, hydrogen peroxide, acetaldehyde, carbon dioxide, bacteriocins, and kefiran by microbial components of kefir are postulated to be responsible for antagonistic effects. The effect of kefir or its components on target microbial cells include destabilisation of cell membrane, degradation of nucleic acid and inhibition of protein synthesis. Microorganisms isolated from kefir have been shown to have antagonistic properties against pathogens including enteropathogenic bacteria by adhesion and competition for nutrients and space mechanisms. Further studies (including animal and clinical trials) are needed to fully understand and confirm the prophylactic and therapeutic roles of kefir in infectious enteric bacterial infection control.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Chapter 5. Antimicrobial properties of traditional kefir: an in vitro screening for antagonistic effect on *Salmonella* Typhimurium and *Salmonella* Arizonae

This chapter focuses on the analysis of two different traditional kefir drinks made from uncharacterized kefir grains. Antimicrobial effect of these drinks on *Salmonella* enterica serovar Arizonae and *Salmonella* enterica serovar Typhimurium was investigated. In addition,metabolites responsible for the observed effect were determined. This chaptered has been published as “Gut, Abraham Majak, Todor Vasiljevic, Thomas Yeager, and Osaana N. Donkor. Antimicrobial properties of traditional kefir: an in vitro screening for antagonistic effect on *Salmonella* Typhimurium and *Salmonella* Arizo-nae”. *International Dairy Journal* (2021). https://doi.org/10.1016/j.idairyj.2021.105180 .

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Antimicrobial properties of traditional kefir: An in vitro screening for antagonistic effect on *Salmonella* Typhimurium and *Salmonella* Arizonae

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ABSTRACT

The rise of antibiotic resistance in *Salmonella* has necessitated the need for alternative ways of preventing and controlling infections. Fermented products have been recognised to have prophylactic and therapeutic properties against diseases. This study focused on the analysis of antagonistic effect of two different traditional kefir grains on *Salmonella* Arizonae and *Salmonella* Typhimurium after 24 and 48 h fermentation. Kefir supernatants were analysed for ethanol, organic acid and protein composition using gas chromatography, high performance liquid chromatography and shotgun proteomics, respectively. Salmonellae were rapidly eradicated in kefir possibly due to action of lactic acid as kefir cell free supernatant contained high concentrations of lactic acid ranging from 83.59 to 229.92 mM. Other molecules with recognised antibacterial activities including carbonyl compounds, histone and cathelicidin were detected in the soluble phase that could have provided synergistic effect with the organic acids.

1. Introduction

The global morbidity and mortality caused by enteric bacterial pathogens are huge and increasing despite the availability and use of antibiotic drugs (Dixon & Hall, 2015; Kirk et al., 2015; Petri et al., 2008). Globally, *Salmonella* infections are estimated at 2.8 billion cases of diarrhoea per year. *Salmonella enterica* serovar Typhi (*S.* Typhi) causes 16–33 million infections and 500,000–600,000 deaths, with non typhoidal *Salmonella* infections accounting for 90 million cases and 155,000 deaths worldwide annually (Bula Rudas, Rathore, & Maraqa, 2015).

Traditionally, *Salmonella* infections are successfully treated with antibiotics but bacterial resistance to this treatment is emerging at an alarming rate (Crump, Sjolund Karlsson, Gordon, & Parry, 2015). For example, Malawi reported 97 % *Salmonella* multi drug resistant in 2014 compared with 7 % in 2010 (Feasey et al., 2015; Wong, Baker, Pickard, & Parkhill, 2015). Even in developed countries such as the USA, non typhoidal *Salmonella* infections caused by *Salmonella enterica* serovar Enteritidis, *Salmonella enterica* serovar Newport, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *Salmonella enterica* serovar Heidelberg have been reported to be resistant to many antibiotic drugs with varying magnitudes (Medalla et al., 2016).

The rise in resistance has necessitated the need for novel approaches of controlling and treating *Salmonella* infections including the use of probiotics. Probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). One possible source of probiotics is the kefir grains, which usually contain opportunistic probiotic microorganisms and exopolysaccharides (Plessas et al., 2016; Prado et al., 2015). *Lactobacillus, Lactococcus, Leuconostoc, Streptococcus, Kluyveromyces, Candida, Saccharomyces* and *Pichia* genera have been isolated from kefir grains (Plessas et al., 2016), and have been reported to have potential probiotic properties (Gut, Vasiljevic, Yeager, & Donkor, 2021). Consumption of kefir is widespread and is popular in areas such as the Caucasus Mountains of Russia, Europe, Asia, South, and North America due to the potential health benefits conferred by probiotic microorganisms and/or their metabolites (Plessas et al., 2016).

Antimicrobial properties of kefir against enteric bacterial pathogens including *Escherichia coli, Listeria monocytogenes, S.* Typhimurium, *Salmonella enterica* serovar Enteritidis, *Shigella flexneri,*

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Yersinia enterocolitica, Candida albicans, S. Typhi, *Shigella sonnei, Staphylococcus aureus, Bacillus subtilis*, and *Enterococcus faecalis* have been reported in the literature (Leite et al., 2013a; Zavala et al., 2016). Mechanisms of antibacterial activities are postulated to be due to bioactive molecules such as organic acids, bacteriocins, kefiran, carbon dioxide, hydrogen peroxide, ethanol, and diacetyl (Leite et al., 2013b; Rodrigues, Caputo, Carvalho, Evangelista, & Schneedorf, 2005). S layer proteins and adhesion of pathogens on yeast cell walls are other reported properties of kefir components that have effect on enteropathogenic bacterial infection (Leite et al., 2013a; Menezes et al., 2020; Mobili et al., 2009; Shen et al., 2018). Competition for nutrients (Garrote, Abraham, & De antoni, 2000; Kim et al., 2018; Muccilli & Restuccia, 2015), and attachment sites (Liu, Wang, Chen, Yueh, & Lin, 2006; Revolledo, Ferreira, & Ferreira, 2009; Santos, San Mauro, Sanchez, Torres, & Marquina, 2003) be tween kefir microbial cultures and pathogenic bacteria are other mechanisms reported to reduce colonisation (hence infection) in cell lines and animal studies.

The effect of kefir or its components on pathogenic bacterial cells involves destabilisation of the cell membrane, degradation of nucleic acid and inhibition of protein synthesis (Dias, Silva, & Timm, 2018; Li, Yin, Yu, & Yang, 2011). Therefore the aim of this study was to investigate the antimicrobial effect of two uncharacterised traditional kefir drinks on *S.* Typhimurium and *Salmonella* enterica serovar Arizonae (*S.* Arizonae), and to identify antimicrobial mol ecules in kefir responsible for the effect.

2. Materials and methods

2.1. Materials

Kefir grains were sourced from the Werribee starter culture collection (Victoria University, Melbourne, Australia), with two origins, namely: the Russian coded TVR and Kazakhstani coded HSK. *S.* Arizonae (ATCC 13314) and *S.* Typhimurium (ATCC 14028) were purchased from In vitro Technologies (Melbourne, Australia). Ultra high temperature (UHT) milk was purchased from Murray Goulburn, (Melbourne, Australia). Nutrient agar, nutrient broth, xylose lysine deoxycholate agar (XLD), Chloramphenicol (100 mg L^{-1}), and Muller Hinton agar, De Man, Rogosa and Sharpe (MRS) agar, and bacteriological peptone were purchased from Oxoid (Basingstoke, UK) while acetic acid was bought from Merck (Darmstadt, Germany). Sulphuric acid and absolute ethanol were supplied by Thermo Fisher Scientific (Melbourne, Victoria, Australia) and Rowe Scientific (Deveton, Victoria, Australia), respectively. Propionic, formic acids, 0.1 % cycloheximide, and 0.22 μm pore sterile filters were purchased from Sigma Aldrich (St. Louis, MO, USA). Acros Organic (Newark, NJ, USA) supplied butyric acid while AnalaR (BDH, Australia) supplied nitric and lactic acids.

2.2. Salmonella culture preparation

Well isolated *S.* Arizonae and *S.* Typhimurium colonies initially grown on nutrient agar and incubated at 37 °C for 24 h were inoculated into 10 mL nutrient broth and incubated at 37 °C for 24 h. Serial dilution was performed using peptone water to obtain *Salmonella* culture broth containing approximately 10^3 cfu mL^{-1}.

2.3. Effect of kefir and kefir supernatant on Salmonella

Kefir was prepared as described in literature (Kesenkaş, Gürsoy, & Ozbaş, 2017) with some modifications. Briefly, kefir grains were initially cultivated in milk to initiate proliferation at 25 °C for 24 h. The grains were removed from the fermented milk and washed with sterile water. Twenty grams of grain samples were inoculated into 200 mL UHT milk and incubated at 25 °C for up to 48 h. Ten millilitres of kefir were removed at 24 and 48 h and set aside for the prepa ration of kefir cell free supernatant (KCFS). In addition, 10 mL of kefir were taken at 24 and 48 h and inoculated with 1 mL of each *Sal monella* serovar culture separately. The *Salmonella* kefir mixture was vortexed for 10 s and incubated at 37 °C. Ten milliliters of UHT milk was also inoculated with 1 mL of each *Salmonella* serovar culture and used as the control. The kefir and UHT milk seeded with *Salmonella* were sampled at 0, 2, 4, 24 or 48 h by plating 0.1 mL onto XLD as spread plate at appropriate dilutions. *Salmonella* exposure to kefir for 2 and 4 h was to mimic time kefir spend in the gastrointestinal track where it is likely to exert prophylactic and therapeutic effect. The XLD plates were incubated aerobically at 37 °C for 24 h. Pink colonies with or without black centre were considered as *Salmonella* as pre viously described (Park, Ryu, & Kang, 2012) and counted.

Ten millilitres of KCFS were prepared as previously described (Kim et al., 2016) by centrifugation using a centrifuge (Eppendorf 5810 R, Hamburg, Germany) at 4000×*g* for 30 min at 4 °C. The resultant supernatants were sterilised by filtration using 0.22 μm pore sterile filters. For the control, UHT milk was inoculated with nitric acid to precipitate protein, centrifuged as described above and the pH adjusted to 6.5 using sodium hydroxide. Two millilitres of KCFS and control were each inoculated with 0.2 mL *Salmonella* culture and the mixtures were incubated at 37 °C and sampled at 0, 2, 4, or 8 h by removing 0.1 mL and plating onto the XLD plates. The mixtures were however gently vortexed before sampling. The XLD plates were incubated at 37 °C for 24 h and counting was performed as described above.

2.4. Salmonella survival in co culture with kefir grains

This experiment was performed to investigate the effect of gradual increase of potential antimicrobial compounds in kefir as opposed to the drastic exposure of *Salmonella* to final kefir (section 2.3) which may contain antimicrobial metabolites at high concen trations. Ten grams of traditional kefir grains separated from kefir (and washed with sterile water) was inoculated into 90 mL of UHT milk and seeded with 10 mL of *Salmonella* cultures (10^3 cfu mL^{-1}). Ninety millilitres UHT milk was also seeded with 10 mL of *Salmo nella* cultures and used as the control. Fermentation was carried out at 25 °C to simulate the traditional kefir fermentation and *Salmo nella* count was performed at 0, 8, 24, or 48 h by plating 0.1 mL (neat to 10^{-6} dilutions) onto XLD plates. Incubation and counting was performed as described above in section 2.3.

2.5. Determination of organic acids in kefir

Organic acids in kefir were analysed quantitatively using a high performance liquid chromatography (HPLC) as previously described (Ustunol & Gandhi, 2001) with some modifications. Three millilitre aliquots of kefir (prepared as in section 2.3) and UHT milk (control) were mixed with 50 μL of 15.5 mol L^{-1} nitric acid and then diluted with 1 mL of 0.01 mol L^{-1} sulphuric acid. The resulting mixture was centrifuged for 30 min at 4000×*g* at 4 °C using a centrifuge (Eppendorf). The supernatant was filtered into an HPLC vial for the quantitative analysis of organic acids. Analysis was performed using an HPLC system (Shimazdzu, Kyoto, Japan) fitted with an Aminex HPX 87H, 300 × 7.8 mm ion exchange column (Biorad Life Science Group, Hercules, CA, USA) and a guard column maintained at 65 °C. Sulphuric acid (0.01 mol L^{-1}) was used as a mobile phase. The flow rate was set at 0.6 mL min^{-1}. A UV/visible detector was used at 220 nm. Sample injection volume was set as 25 μL. Organic acids concentrations were calculated using 6 points standards (0, 125, 250, 500, 750, and 1000 mM; standard curve $R^2 > 0.99$). Acetic, butyric, formic, and lactic and propionic acid

were used as the standards. Analysis of the results was performed using a Lab Solution software (Shimazdzu, Kyoto, Japan).

2.6. Effect of lactic acid on Salmonella serovars

Bactericidal effect of lactic acid was determined by preparing five point concentrations (0, 57.5, 115, 175.5 and 230 mM) to cover the lowest and highest concentration detected in the kefir using analytical grade lactic acid and distilled sterile water. Two milli litres of each control was inoculated with 0.2 mL of *Salmonella* cultures (approximately 10^3 cfu mL^{-1}). Sampling was performed at <1, 30, 60, 90, 120 and 240 min by plating 0.1 mL onto XLD agar, incubated in 37 °C for 24 h before counting colonies. For control, water was inoculated with *Salmonella* and tested along with the samples.

2.7. Determination of alcohol content in kefir

Alcohol content of KCFS and control were analysed quantita tively by gas chromatography (Nikolaou et al., 2017) with some modifications. A gas chromatograph (Shimazdzu, Kyoto, Japan) with a SGE BP20 GC capillary column 12.0 m length, 0.22 mm inner diameter, 0.25 μm film thickness, (Fisher Scientific Hampton, USA) and flame ionisation detector at 200 °C were used. Samples were filter sterilised through a 0.22 μm membrane filter and 5 μL was injected into the column. The oven temperature was set as 35 °C for 5 min, and then increased to 200 °C at a rate of 10 °C min^{-1}, before rising to 200 °C at 10 °C min^{-1}. The injector tem perature was maintained at 200 °C, with a split ratio of 50:1 and the flow rate of 1.1 mL min^{-1}. Analysis of the results was performed using a Lab Solution software (Shimazdzu, Kyoto, Japan). Alcohol concentration was calculated using 6 point standards (0, 125, 250, 500, 750, and 1000 mM; standard curve $R^2 > 0.99$). Absolute ethanol was used as the standard.

2.8. Analysis of other organic compounds in kefir

Other organic compounds in KCFS were analysed using a gas chromatography mass spectrometer (GCMS) as previously described (Dursun, Güler, & Şekerli, 2017) with some modifications. A GCMS (QP2010 Plus, Shimadzu, Kyoto, Japan) was fitted with a ZB 5MS column (length 30 m, 0.25 mm, and 0.5 micron film thickness) and helium was used as the mobile phase. The starting temperature was set at 45 °C. The temperature rate was set at 25 °C min^{-1} and a final temperature at 300 °C, held for 3 min. The total run time was 13.7 min. Other parameters were set as follows: injection volume 1 μL; split ratio 100:1; ion source temperature 250 °C; interface temperature 301 °C; injector port temperature 250 °C; column flow rate 1.5 mL min^{-1}. The mass spectrometer was set as follows: acquisition mode as scan; start and end were set 40 and 1000 *m/z*, respectively. The solvent delay time was set at 0.5 min. Lab Solution database was used to identify compounds in KCFS.

2.9. Shotgun proteomics

KCFS samples were sent to the Monash Proteomics and Meta bolic Facility (Monash University, Melbourne Australia) for protein and peptide identification using shotgun proteomics. Samples were diluted to between 1 and 5 mg mL^{-1} total protein concentration, and approximately 10 μg^{-1} total protein of each was buffer exchanged into 50 mM ammonium bicarbonate and the protein was reduced in 2.5 mM DTT at 95 °C for 5 min followed by alkylation with 10 mM chloroacetamide for 30 min at ambient temperature. Trypsin was then added at the rate of 0.5 μg per 10 μg of protein and incubated at 37 °C overnight. All enzyme digests were analysed by LC MS/MS using the QExactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany) coupled online with a RSLC nano HPLC (Ultimate 3000, Thermo Scientific, Bremen, Germany). Two hundred nanograms of sample was injected and concentrated on a 100 μm, 2 cm nanoviper pepmap100 trap column with 97.5 % buffer A (0.1 % trifluoroacetic acid) at a flow rate of 15 min^{-1}. The peptides were then eluted and separated with a Thermo RSLC pepmap100, 75 μm × 50 cm, 100 Å pore size, reversed phase nano column with a 30 min gradient of 92.5 % buffer A (0.1 % formic acid) to 42.5 % B (80 % acetonitrile 0.1 % formic acid), at a flow rate of 250 nL min^{-1}. The eluent was nebulised and ionised using the Thermo nano electrospray source with a distal coated fused silica emitter (New Objective, Woburn, MA, USA) with a capillary voltage of 1900 V.

Peptides were selected for MS/MS analysis in full MS/dd MS2 (TopN) mode with the following parameter settings: TopN 10, resolution 70,000, MS/MS AGC target 5e5, 118 ms Max IT, NCE 27, 1.8 *m/z* isolation window, dynamic exclusion was set to 10 s. Results were analysed using MaxQuant to obtain protein identifications and their respective label free quantification values using in house standard parameters. Data were normalised based on the assumption that the majority of proteins do not change between the different conditions. Protein identification numbers were also used to verify protein names using Uniprot database.

2.10. Isolation and identification of lactic acid bacteria components of traditional kefir grains

Saccharomyces unisporus ATCC 10612 and *Kluyveromyces lactis* var. *lactis* ATCC 56498 were isolated from HSK and TVR respectively, identified using16S rRNA gene sequencing and characterized for probiotic properties in our previous study (Gut, Vasiljevic, Yeager, & Donkor, 2019). Therefore, this section of the current study was aimed at determining bacterial components in the two traditional kefirs that may be responsible for high lactic acid production.

Kefir drinks and grains were prepared as described under sec tion 2.3. Bacterial isolation and enumeration experiments were performed as described previously (Talib et al., 2019) with some modifications. Ten grams of TVR and HSK grains were diluted in 90 mL 0.1 % peptone water and each mixture was thoroughly ho mogenized using a BagMixer (Interscience, Saint Nom, France) for 2 min. Ten grams of each kefir (devoid of grains) were also diluted in 90 mL 0.1 % peptone water and homogenized. Both kefir and grain homogenates were serially diluted from 10^{-1} to 10^{-6} and 0.1 mL was inoculated onto MRS agar initially supplemented with 0.1 % cycloheximide to suppress yeast growth. MRS agar is a se lective medium commonly used in isolation of bacterial component in kefir (Chen, Tang, & Chiang, 2017).

MRSA plates were incubated at 25 °C since it was used in fermentation/production of traditional kefir and it was at this temperature LAB proliferate and produce high concentration of lactic acid. Incubation was extended to 7 days to ensure slow growing microbiota were not missed; however, colonies were counted and picked for identification on day three. Bacterial col onies were counted and grouped based on colour, size, shape, elevation, surface texture, edge, gram stain, and catalase reaction. Six colonies from each group were identified using Shimazdu Vitek MS (Kyoto, Japan) matrix assisted laser desorption/ionisation time of flight (MALDI TOF) (Viana, Magalhaes Guedes, Dias, & Schwan, 2019). MALDI TOF is emerging as method of choice for bacterial identification due to its simple protocol, quick turnaround time, sensitivity, specificity and cost saving and it has been used in identification of lactic acid bacteria (LAB) isolated from fermented product (Nacef, Chevalier, Chollet, Drider, & Flahaut, 2017), hence it use in this study. Briefly, well isolated colonies were picked and smeared onto a MALDI TOF specific slide coated with a solution of

energy absorbent, an organic compound commonly called matrix. The slide with the sample was loaded into the machine where desorption and ionization occurred leading to a generation of singly protonated ions from analytes in the sample. The protonated ions were then accelerated at a fixed potential, where they were sepa rated from each other based on their mass to charge ratio (m/z). The charged analytes were then detected and measured using the mass time of flight (TOF) (Singhal, Kumar, Kanaujia, & Virdi, 2015). The identification of the bacteria was automatically performed using the Spectral Archive and Microbial Identification System (SARAMIS database).

2.11. Statistical analysis

Experiments were performed in triplicates on two different occasions. Results were expressed as the mean ± standard devia tion (SD). Means differences between *Salmonella* treated with kefir or KCFS and controls were statistically analysed using the Student's t test. Mean differences were considered significant at $p < 0.05$. Statistical analysis was performed using SPSS version 26 Statistical software (IBM, New York, USA). The means for all the experiments followed a normal distribution.

For shotgun proteomics, statistical analysis was performed us ing Perseus. The LFQ data was converted to $\log_2$ scale, samples were grouped by conditions and missing values were imputed based on normal distributions after all proteins were eliminated that had 2 or less valid values. Protein fold changes were calculated and their significance was determined using a two sided T test with error corrected P values.

3. Results and discussion

3.1. Effect of kefir on Salmonella

S. Arizonae colony counts were reduced by almost 1.5 $\log_{10}$ ($p < 0.05$) during exposure to kefir within the first 2 h as shown in Fig. 1A. Extending this exposure (TVR and HSK) to 4 h led to com plete eradication (Fig. 1B); no surviving *Salmonella* cells were recovered on the XLD plates. *S.* Typhimurium exposure to kefir led to eradication within 2 h. This was in complete contrast to the control which experienced substantial and significant *Salmonella* growth over the experimental period (Fig. 1). Such a reduction or complete eradication with longer exposure time has been noted previously and attributed to presence of antimicrobial metabolites produced during fermentation (Alakomi et al., 2000; Fina Martin et al., 2019; Jeong et al., 2017). These results are consistent with previous studies in which *Salmonella* species were decimated by kefir (Aksu, Muratoglu, & Altunatmaz, 2016; Chang et al., 2018). *Salmonella* infection occurs after ingestion of contaminated food or drink, upon which *Salmonella* colonises the distal ileum and prox imal colon regions of the gastrointestinal tract (Hocking, 2012; Lonnermark et al., 2015). Kefir drinks made from these traditional grains and taken every day, may prevent *Salmonella* infection by exerting bactericidal effect in the gastrointestinal tract. It has been demonstrated in a clinical trial involving enteric bacterial pathogen and found effective when used as a complementary therapy in a previous study. Patients who consumed 250 mL of kefir daily while on a 14 day combined course of lansoprazole (30 mg), amoxicillin (1000 mg), and clarithromycin (500 mg) to treat *Helicobacter pylori* infections had 78.2% clearance rate in contrast to the control group, which had a 50 % eradication rate (Bekar, Yilmaz, & Gulten, 2011).

Similar to the whole kefir drinks, exposure of *S.* Arizonae and *S.* Typhimurium to the KCFS for 2, 4 or 8 h led to complete eradication of *Salmonella* but no observed effect from the control (Fig. 2). The bactericidal effect of KCFS on *Salmonella* is in agreement with previous studies (Iraporda et al., 2017; Londero, Iraporda, Garrote, & Abraham, 2015) which showed growth inhibition of enteric pathogenic bacteria including *Salmonella* by kefir supernatants. The effect was attributed to kefir metabolites such as such as lactic acid, ethanol, kefirans, and antibacterial peptides (Alakomi et al., 2000; Fina Martin et al., 2019; Jeong et al., 2017).

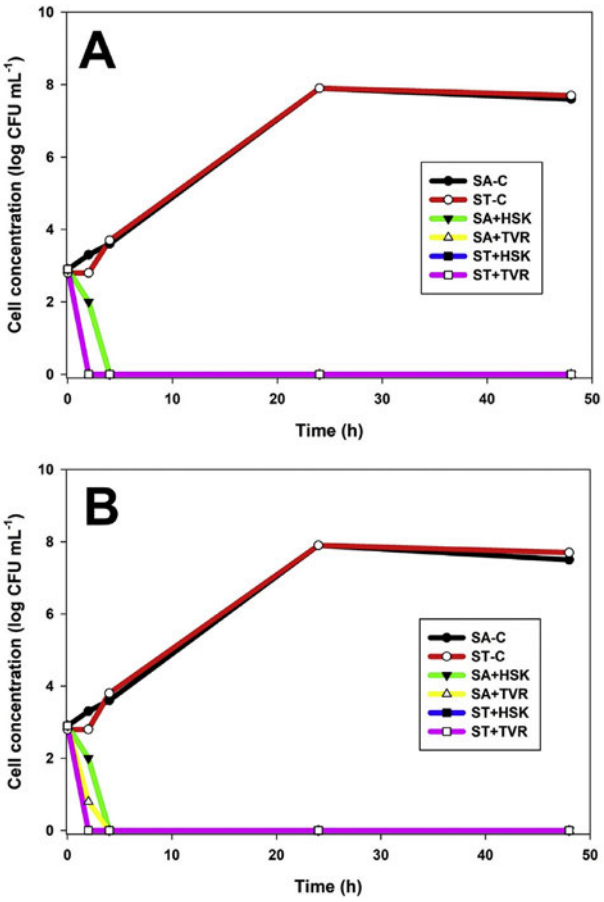

Fig. 1. Bactericidal effect of kefir fermented for 24 and 48 h on *Salmonella*. SA, S. Arizonae; ST, S. Typhimurium; the samples were prepared using HSK or TVR kefir grain originally sourced from Kazakhstan and Russia, respectively.

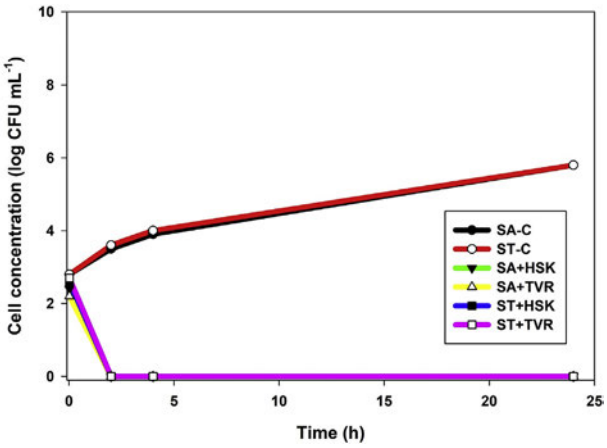

Fig. 2. Bactericidal effect of kefir supernatants on *Salmonella*. SA, S. Arizonae; ST, S. Typhimurium obtained by centrifugation of the samples prepared using HSK or TVR kefir grain originally sourced from Kazakhstan and Russia, respectively.

3.2. Salmonella survival and proliferation in co culture with kefir grains

Milk is the most commonly used liquid food in kefir preparation (Sarkar, 2008). Milk contamination with *Salmonella* has been associated with its transmission and subsequent infection of the consumers (Cummings, Virkler, Wagner, Lussier, & Thompson, 2018; Ford et al., 2016). Concurrent inoculation of UHT milk with *Salmonella* serovars and traditional kefir grains showed statistically significant decline of the *Salmonella* serovars counts after 8 h ($p < 0.05$; Fig. 3) for all kefir grain types. *Salmonella* serovars were not detectable after 24 h of co culturing with kefir.

After 8 h of fermentation, the pH of the fermenting kefir drinks was recorded as 6.6 and 6.8 for HSK and TVR respectively. At 24 h, the pH for the two kefir samples was at 4.9 and 5.2 for HSK and TVR, respectively. These low pH values were indicative of increased organic acid (lactic and acetic) concentration, which possibly contributed to the bactericidal effect, potentially in syn ergy with other antibacterial molecules or kefir microflora. Extending kefir fermentation for additional 24 h resulted in similar outcomes in *Salmonella* killing but lower kefir pH (pH 4.4 and 4.8 for HSK and TVR, respectively). Gradual exposure of *Sal monella* to antimicrobial agents is reported to increase *Salmonella* survival due to stress response. For example, exposure of *Salmo nella* to mild pH (pH 5.8) was reported to increase resistance to lower pH, heat, NaCl (2.5 M), crystal violet, and polymyxin B due to the development of cross tolerance induced by response to stress factors. In addition, subjecting *Salmonella* enterica cells to an initial acid shock or pH 5.8 or 4.5 before exposing it to pH 4.2–4.5 increased its survival rate (Keerthirathne, Ross, Fallowfield, & Whiley, 2016). However, in our study, gradual exposure of *Sal monella* to increasing antimicrobial molecules during kefir fermentation including decreasing pH did not enhance its survival. This study indicated that kefir may control *Salmonella* trans mission even if contaminated milk is used as the initial matrix for kefir fermentation.

3.3. Assessment of organic acids in kefir

Many organic acids such as lactic, acetic, butyric propionic, formic are found in kefir (Iraporda et al., 2017; Puerari, Magalhaes, & Schwan, 2012; Schwan, Magalhaes Guedes, & Dias, 2016) and were quantified in this study. As shown in Table 1, the concentra tion of lactic, acetic, propionic and butyric acids are significantly

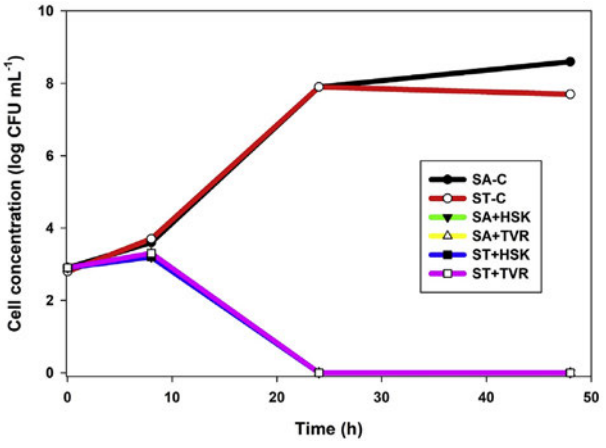

Fig. 3. *Salmonella* survival when co-cultured with kefir grains. SA, S. Arizonae; ST, S. Typhimurium; HSK, kefir from kefir grain originally from Kazakhstan; TVR, kefir from kefir grain originally from Russia.

higher ($p < 0.05$) than the control for all fermentation time (24 and 48 h). Extended fermentation time (48 h) did not lead to further significant production of organic acids, which could indicate depletion of precursor molecules for organic acids production, and/ or reduction of microbial activity as a result of increased acidity or presence of other metabolites (Shi, Chen, Li, Huang, & He, 2018). These results are in agreement with previous studies in which lactic and acetic acid reach the highest concentrations compared to other organic acids in kefir (Arslan, 2015; Setyawardani, Rahardjo, Sulistyowati, & Wasito, 2014). Furthermore, lactic and acetic acids are the principle organic acids with reported strong antibacterial properties partly because they are produced in substantial con centrations in kefir compared to other acids (Gamba et al., 2019, 2020). Antagonistic properties of lactic and acetic acids against pathogenic bacteria such as *Salmonella* is well documented even at pH levels above 5 (Hyunsook, 2018). A previous study on *S.* Typhimurium and *E. coli* 0157: H7 reported the bactericidal effect of lactic acid and the mechanism was postulated to be associated with disruption of the outer cell membrane making it permeable and thus enhanced the activity of other antibacterial molecules (Alakomi et al., 2000).

To confirm this important property of lactic acid, it was assessed separately for its impact on *Salmonella* in a model sys tem. Exposure of *Salmonella* to all the concentrations (57.5, 115, 175.5 or 230 mM) for <1, 30, 60, 90, 120 and 240 min resulted in complete eradication (data not shown). These results, especially the one observed in the initial stage of exposure, indicate that lactic acid rapidly kills *Salmonella* cells upon contact and there fore, the bactericidal effect observed in this study was most likely caused by lactic acid, possibly in synergy with other organic acids and proteinaceous antibacterial molecules. Furthermore, it ap pears that the effect may depend on the kefir matrix, which may provide some protection to *Salmonella* cells shielding them from the lethal effect of lactic acid. This is demonstrated by results in Figs. 1 and 2, which showed that KCFS eradicated *Salmonella* within 2 h while some *Salmonella* cells were able to survive beyond 2 h in unfiltered whole traditional kefir.

3.4. Assessment of alcohol content in kefir

Kefir is a fermented drink typically containing low levels of ethanol with the highest reported concentration of 2.1 % (Nunez, 2016). Yeast components of kefir grains such as *Kluyveromyces* and *Saccharomyces* species are responsible for the production of alcohol (Ho et al., 2012; Magalhaes et al., 2010). Table 1 shows the concentration of ethanol in two kefir drinks fermented for 24 or 48 h. Lower concentration of ethanol after 48 h of fermentation in HSK may be due to cessation of production as kefir microbial components reach stationary phase, coupled with possible loss through evaporation during fermentation. Ethanol has been known to be antiseptic which has a broad spectrum against bacteria. The lower content of ethanol may have contributed to antibacterial activity as its non lethal concentration has been linked to *Salmo nella* growth inhibition due to ethanol induced cell auto aggregation causing physical hindrance to binary fission (Hassani et al., 2009).

3.5. Other organic compounds with antimicrobial potential in kefir

Kefir contains many organic antimicrobial compounds such as acetaldehyde, diacetyl, acetoin, 2 butanone, and ethyl acetate, carbon dioxide, organic acids pyruvic, oxalic and succinic in addi tion to the five organic acids analysed above (Iraporda et al., 2017; Puerari et al., 2012; Schwan et al., 2016). Data from separation of these organic compounds in KCFS of HSK and TVR are presented in

Table 1
Determination of organic acids and ethanol content in kefir using HPLC and GC, respectively.[a]

Compound	UHT milk control	TVR		HSK	
		24 h	48 h	24 h	48 h
Lactic acid	9.69 ± 0.30[a]	83.59 ± 0.44[b]	110.11 ± 15.87[b]	227.89 ± 0.58[b]	229.92 ± 1.42[b]
Formic acid	33.84 ± 1.59[a]	29.46 ± 0.51[b]	29.82 ± 1.17[b]	29.20 ± 0.09[b]	29.24 ± 0.29[b]
Acetic acid	0.07 ± 0.05[a]	48.43 ± 0.66[b]	55.28 ± 0.97[b]	16.91 ± 0.16[b]	18.20 ± 0.05[b]
Propionic acid	0.14 ± 0.08[a]	1.87 ± 0.11[b]	2.82 ± 0.45[b]	4.18 ± 0.17[b]	4.58 ± 0.12[b]
Butyric acid	0.07 ± 0.04[a]	12.81 ± 0.07[b]	19.39 ± 2.60[b]	38.01 ± 0.10[b]	39.70 ± 0.18[b]
Ethanol	11.2 ± 1.2[a]	12.60 ± 0.14[a]	12.65 ± 0.070[a]	34.60 ± 3.11[b]	29.45 ± 5.56[b]

[a] Values (in mM) are means; means with different superscript letters are significantly different ($p < 0.05$).

Table 2. Traditional kefir is consumed after about 24 h fermentation and this study found that there was no difference between 24 and 48 h fermentation with respect to antibacterial activity. Therefore, kefir fermented for 24 h was analysed and compounds with known antibacterial properties including cyclohexanone (Nguyen et al., 2019) and carbon dioxide (Gut, Vasiljevic, Yeager, & Donkor, 2018) were detected in these drinks. These compounds may have contributed in bactericidal effect.

3.6. Assessment of antimicrobial proteins in kefir

Kefir microbial metabolites analysed in KCFS include those that are proteinaceous in nature of which some have antimicrobial properties (Miao et al., 2016). A total of 135 proteinaceous molecules were identified in both TVR and HSK kefir. However, only 34 and 26 were of interest in TVR and HSK, respectively (Table 2), and were produced in kefir possibly by proteolytic activity of kefir

Table 2
Organic compounds and proteins in kefir identified by GCMS and shotgun proteomics, respectively.[a]

Compound	GCMS analysis				Shotgun proteomics			
	HSK	TVR	Molecular weight	Similarity (%)	Protein name	Database identification number	HSK	TVR
1-Hydroxy-2 propanone/acetol	✓	✓	74	94	60S ribosomal protein L35a	Q56JY1; E1BHM9; E1BMJ8	✓	✓
1-Monoacetine/glycerol monoacetate	✓	×	134	93	14-3-3 protein zeta/delta,	P63103	×	✓
2,3 Butanediol/	✓	×	90	97	40S ribosomal protein S24	Q56JU9	✓	×
2-Bromo-hexane	✓	×	164	90	40S ribosomal protein S29	P62276; E1BNY4	×	✓
3 Methyl 2-hexene	✓	×	98	85	60S ribosomal protein L13	Q56JZ1; F1MK30	✓	×
3 Methyl 2-hexene	×	✓	98	85	60S ribosomal protein L20-B; 60S ribosomal protein L20-A	P0CX24; P0CX23	✓	✓
3-Deoxy-D-mannoic lactone	✓	✓	162	92	60S ribosomal protein L21	F1N7U3; F1MUN0; Q861S4	×	×
3-Methyl butanoic methyl	✓	✓	170	83	60S ribosomal protein L34	F1ML72; P87262; P40525	✓	×
4-Hydroxydihydro-2furanone	✓	✓	102	93	60S ribosomal protein L35a	Q56JY1; E1BHM9; E1BMJ8	×	✓
5 Hydroxymethylfurfural	×	✓	126	87	60S ribosomal protein L36	Q3T171; G3N2X2	✓	×
5-Hydroxymethyl-furanone	✓	✓	114	80	60S ribosomal protein L37a	Q3MIC0	✓	×
Acetic acid	✓	✓	60	94	Actin, cytoplasmic 2	P63258	✓	×
Butanamide	✓	✓	119	86	Acyl-CoA-binding protein	P07107	×	✓
Butyric acid compound	✓	✓	118	87	Cathelicidin-2, Cathelicidin-6, Cathelicidin-7	P19660; P54228; P56425	×	✓
Carbon dioxide	✓	✓	44	97	Cystatin-B	F6QEL0	×	✓
Cyclohexanone	✓	✓	98	85	Enolase 1	Q861S4	✓	×
Diaglyceraldehyde dimer	✓	✓	180	84	Eukaryotic translation initiation factor 2 subunit gamma	P32481	✓	✓
Diaglycerol	✓	×	166	83	Fatty acid-binding protein, heart	P10790	×	✓
Ethyl ethanol	✓	✓	46	90	Heterogeneous nuclear ribonucleoproteins A2/B1	Q2HJ60	✓	×
Ethyl vinyl ketone	✓	✓	84	92	Histone H2B	F2Z4F9	×	×
Formic acid	✓	✓	46	96	Histone H4	P62803	✓	✓
Furfuryl alcohol	✓	✓	98	97	Na (+)-dependent phosphate cotransporter 2B	P26201	×	✓
Glyceraldehyde	✓	✓	180	190	NADP-specific glutamate dehydrogenase 1	P07262	✓	×
Lactic acid	✓	✓	90	97	Nucleobindin 2	Q0IIH5	×	✓
Propanal	✓	×	90	80	Peptidyl-prolyl cis trans isomerase	P14832	✓	×
Propanoic acid	×	✓	74	97	Peptidyl-prolyl cis trans isomerase A	P62935; G3X8B1; G3MZS9; A4FV72	✓	×
Propyl-2-4- imidazolidinedione	✓	×	142	84	Prostaglandin-H2 D-isomerase	O02853	✓	×
Pyran dione compound	✓	×	112	77	Protein BMH1; Protein BMH2	P34730; P29311	✓	×
Tetrahydrofuran compound	✓	✓	130	90	Ran-specific GTPase-activating protein 1	P41920	✓	×
1-Hydroxy-2 propanone/acetol	✓	✓	74	94	Ribonuclease pancreatic	P61823; P00669; P39873	×	✓
1-Monoacetine/glycerol monoacetate	✓	✓	134	93	Sodium-dependent phosphate transport protein 2B	Q27960; F1N6D4	✓	✓
2,3 Butanediol/	✓	×	90	97	Tubulin beta-5 chain, Tubulin beta-2B chain, Tubulin beta-3 chain	G3X7R7; G3N1W7; Q2T9S0	✓	×
2-Bromo-hexane	✓	×	164	90	Vacuolar protein sorting-associated protein 53 homolog	E1BJW7	×	✓
3 Methyl 2-hexene	✓	×	98	85				

[a] ✓ detected in kefir; × not detected in kefir; where there was difference in percentage of similarity for the same compound in the two traditional kefirs, the lower percentage was recorded.

microbiota or as products of trypsin action used during shotgun proteomics. Among these proteins of interest, histone (in HSK and TVR) and cathelicidin (in TVR) are known to have antimicrobial properties (Hoeksema, van Eijk, Haagsman, & Hartshorn, 2016; Xia, Zhang, & Wang, 2015). A previous study showed that cathelicidin attenuated clinical symptoms of *Salmonella* infection and reduced its internalisation in a mice study (Xia et al., 2015) while histone protect epithelial cells against *S.* Typhimurium infection (Hoeksema et al., 2016). Furthermore, many studies have reported antibacterial properties of peptides derived from fermented dairy based products (Sah, Vasiljevic, McKechnie, & Donkor, 2018).

The mechanisms of antibacterial peptides against susceptible bacteria involve DNA, RNA, ATP synthesis, or protein synthesis in hibition, as well as disruption in the membrane and ionic potential (Biadała, Szablewski, Lasik Kurdyś, & Cegielska Radziejewska, 2020). A previous study on cheese whey kefir supernatant (fer mented with kefir isolates including *Lactobacillus planatarium CIDCA 8327, Lactobacillus kefiri* CIDCA 8348, and *Kluyveromyces marxianus* var. marxianus) showed growth inhibitory effect on S. Enteritidis. The molecule responsible for the antibacterial effect was found to be a peptide molecule that is active at a low pH (Londero et al., 2015). Stability of histone and cathelicidin in presence of trypsin or their release by this enzyme action in this study, indicates that a similar release of these peptides in vivo, in the GIT, may exert their potential prophylactic or therapeutic role (Baird & Craik, 2013).

3.7. Assessment of bacterial composition of traditional kefir grains

Observed antibacterial properties of kefir in this study have been associated with its microbial flora metabolites especially the lactic acid, hence the need to isolate, enumerate and identify LAB which may be responsible for its production as the yeast compo sition of the kefir were characterized in our previous study (Gut et al., 2019). *Lactobacillus* species are reported to be the main pro ducers of lactic acid (Abedi & Hashemi, 2020) and are routinely cultured on MRS agar. Furthermore, other LAB species such as *Lactococcus lactis* strains are known producers of lactic acid and can be grown on MRS agar (Cock & de Stouvenel, 2006), hence the use of MRS agar. The production of formic, acetic, propionic, and butyric acids have been shown to be a characteristic of LAB fermentation in milk (Leite et al., 2013a; Ozcelik, Kuley, & Ozogul, 2016). Since these organic acids were detected in kefir including high concentration of lactic acid, LAB were the likely source of production.

Bacterial colonies from TVR grain and kefir were medium in size, entire, convex, and white in colour, catalase negative and Gram positive rods. The lactic acid bacteria count in TVR were 5.60 ± 0.17 and 8.66 ± 0.08 log10 cfu mL^{-1} in grain and kefir respectively. Isolated colonies from HSK grain and kefir were small, entire, translucent, and were catalase negative Gram positive cocci with a count of 6.46 ± 0.09 and 8.69 ± 0.15 log10 cfu mL^{-1} in grain and kefir respectively. Colony morphology, catalase reaction and Gram stain of the bacterial isolates from these traditional kefir grains are consistent with previous studies (Ismail, Yulvizar, & Mazhitov, 2018).

The population of LAB isolated from two types of traditional kefir grains and drinks were also consistent with previous studies (Garrote, Abraham, & De Antoni, 2001; Robinson, 1991). Identifi cation of the isolates was deemed necessary since some bacterial strains found in kefir are known to produce high concentration of lactic acid compared to other strains (Garrote et al., 2001; Londero et al., 2015; Puerari et al., 2012). Isolates from TVR and HSK were identified as *L. kefiri* and *L. lactis*, respectively, by MALDI TOF. The principle of MALDI TOF identification is based on ribosomal protein mass fingerprint. Ribosomal protein mass spectrum of the un known isolate is compared with that of spectra of known micro organisms in the database (Singhal et al., 2015). Isolation of *L. kefiri* and *L. lactis* from the two traditional kefir grains is consistent with previous studies (Demir, 2020; Dertli & Çon, 2017).

Kefir grain contains many bacterial species, however, and since the focus was on lactic acid bacteria in this study, MRSA and the experimental conditions only selected for LAB. Further future studies using selective growth media and conditions may be needed to screen for the different bacterial species found in kefir. Kefir containing *L. kefiri* showed antagonistic effect on *Salmonella enterica* serovar Enteritidis in a previous study (Londero et al., 2015) while *L. lactis* strains are reported to produce bacteriocins including nisin and lacticin (Gut et al., 2021). However, antimicrobial prop erties of probiotics are strain dependent (Campana, van Hemert, & Baffone, 2017), hence the need to investigate the anti salmonella effect of these bacteria isolated from traditional kefir.

4. Conclusion

The aim of the study was to assess uncharacterised traditional kefir grains potential as alternative or complementary therapies, and control measure for *S.* Typhimurium and *S.* Arizonae, and this study found that the two traditional kefir drinks were very effi cient in eradicating these *Salmonella* serovars upon exposure. Hence, these traditional grains kefir have potential to be used prophylactically or therapeutically in *Salmonella* infections. The fact that the kefir drinks contain high concentrations of organic acids, especially lactic acid, were likely the reasons for bactericidal effect observed. This effect was likely augmented by the presence of other metabolites with known antibacterial properties such as aldehyde, carbon dioxide, histone and cathelicidin, detected in the serum phase of kefir.

To the best of our knowledge, we are the first to show that the antagonistic effect of traditional kefir is bactericidal and is caused by lactic acid. Furthermore, this is the first study to use shotgun proteomics to show the presence antimicrobial peptides (or their precursor proteins); histone and cathelicidin in traditional kefir. Further studies are required to determine the bactericidal effect of the two traditional kefir drinks on other pathogens. Additional comprehensive studies are needed to identify and characterise microbiota in the two traditional kefir gains using various growth media and conditions, and for probiotic properties.

Authors' contributions

Planning and designing experiment: Abraham Majak Gut, Osaana Donkor, Thomas Yeager, Todor Vasiljevic.

Performing experiments: Abraham Majak Gut.

Supervision: Osaana Donkor, Thomas Yeager, Vasiljevic.

Results analysis: Abraham Majak Gut, Osaana Donkor, Thomas Yeager, Todor Vasiljevic.

Writing - original draft: Abraham Majak Gut.

Writing – reviewing and editing: Abraham Majak Gut, Osaana Donkor, Thomas Yeager, Todor Vasiljevic.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Chapter 6. Characterization of yeasts isolated from traditional kefir grains for potential probiotic properties

This chapter focuses on isolating, identifying, and characterizing yeasts from two traditional kefir grains for basic probiotic properties including survival in GIT simulated conditions, production of hydrolytic enzymes, hydrophobicity, and auto-aggregation. This chaptered has been published as “Gut, Abraham Majak, Todor Vasiljevic, Thomas Yeager, and Osaana N. Donkor. Characterization of yeasts isolated from traditional kefir grains for potential probiotic properties”. Journal of Functional Foods (2019). https://doi.org/10.1016/j.jff.2019.04.046.

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Characterization of yeasts isolated from traditional kefir grains for potential probiotic properties

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ABSTRACT

Kefir is a mixed fermented product with numerous attributed health benefits due to presence of a complex culture composed of bacteria and yeasts in an exopolysaccharide matrix. This work aimed at isolating and identifying yeast species from two types of traditional kefir grains and establishing some potential probiotic properties including survival in the gastrointestinal tract, auto-aggregation, hydrophobicity and hydrolytic enzymes production. All the isolates showed good survival rates in simulated gastrointestinal tract solution, with < 0.5 $\log_{10}$ reduction. *Kluyveromyces lactis* was characterized with a high level of hydrophobicity (88.75%) but moderate auto-aggregation whereas *S. unisporus* showed moderate hydrophobicity and auto-aggregation. Indicator enteric bacteria adhered onto both viable and non-viable yeast isolates and controls. In comparison to *Saccharomyces boulardii* strains used as controls, both kefir yeast strains showed low alpha hemolytic and proteolytic activities, but exhibited no phospholipase activity. *Kluyveromyces lactis* and *Saccharomyces unisporus* isolated, were identified on the basis of 26s rDNA and ITS region sequencing. Overall, the yeast isolates showed some potential probiotic properties.

1. Introduction

Kefir is an acidic and low alcoholic probiotic product made from kefir grain, which is a consortium of exopolysaccharides and many microorganisms (Plessas et al., 2016; Prado et al., 2015). The term kefir is derived from *kef*, a Turkish word which is translated as 'pleasant taste' (Arslan, 2015). Chemically, kefir grains are generally composed of 890–900 g/kg water, 2 g/kg fat, 30 g/kg protein, 60 g/kg sugars and 7 g/kg ash, and these may vary depending on the grain. Physically, grains appear as cauliflower florets with size ranging from 0.3 to 3.5 cm in diameter (Garrote, Abraham, & De Antoni, 1997). Bacterial components of kefir grains include *Lactobacillus, Lactococcus, Leuconostoc* and *Streptococcus* genera while yeast genera include *Kluyveromyces, Candida, Saccharomyces and Pichia* (Plessas et al., 2016). Spatial distribution of microorganisms in kefir is still controversial, however, it has been generally reported that yeasts are located in the inner and intermediate inner section of the grains while bacteria exist on the surface areas of grains (de Oliveira Leite et al., 2013). Kefir can be produced commercially using two-step fermentation process (Russian method) or traditional one-step fermentation method by inoculating milk, fruit juices or molasses with kefir grains (Plessas et al., 2016).

Kefir is widely consumed in Caucasus Mountains of Russia, Europe, Asia, South and North America for health benefits conferred by probiotic microorganisms (Plessas et al., 2016). Probiotics are defined by the World Health Organization (WHO) and Food and Agriculture Organization (FAO) as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). Kefir consumption has been associated with benefits in management and treatment of gastrointestinal problems, hypertension, allergies, cancers, and ischemic heart disease. Furthermore, antibacterial properties against pathogenic bacteria such as *Salmonella* have been reported (Zavala et al., 2016). These prophylactic and therapeutic properties are associated with probiotic microorganisms' interactions with the hosts. Moreover, probiotics prophylactic and therapeutic properties are also attributed to their bioactive metabolites including organic acids, bacteriocins, carbon dioxide, hydrogen peroxide, ethanol and diacetyl (de Oliveira Leite et al., 2013). Probiotics are expected to meet certain criteria including; the ability to persist and multiply in the gastrointestinal tract (GIT) (resistance to acidic gastric juice, basic pancreatic juice, lysozyme, and bile salts), ability to auto-aggregate and

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to form normal sustaining flora, and should be non-pathogenic (Gut, Vasiljevic, Yeager, & Donkor, 2018). Proteinases, phospholipases and hemolysins are some of the key hydrolytic enzymes that may contribute to invasiveness, persistent infections, host immune evasion, proliferation and colonization, as well as provision of nutrients to pathogenic yeasts such as *C. albicans* (Anoop, Rotaru, Shwed, Tayabali, & Arvanitakis, 2015; Ramesh et al., 2011). However, some of these hydrolytic enzymes may be beneficial in treatment of proteinous toxins producing bacteria (Gut et al., 2018).

This work thus was focused on isolating, identifying and characterizing the cultural kefir yeasts from two traditional kefir grains. The isolates were examined for GIT survival, auto-aggregation, growth at 37 °C, hydrophobicity, antibacterial properties as well as screened for hydrolytic enzymes including proteolysins, phospholipases and hemolysins.

2. Materials and methods

2.1. Isolation and enumeration of yeast isolates

Kefir grains were obtained from the Werribee starter culture collection (Victoria University, Melbourne, Australia). They had two origins - kefir grain coded TVR was originally from Russia while the other grain coded HSK originated from Kazakhstan. Yeast species were isolated as previously reported with some modifications (Garofalo et al., 2015). Briefly, samples of kefir grains were initially cultivated in pasteurized milk to initiate their growth proliferation. They were subsequently removed from the fermented milk and washed with sterile water. The grain samples were inoculated into 200 mL ultra-high temperature (UHT) milk (Devondale, Murray Goulburn, Melbourne, Australia), incubated at 30 °C for 24 h, and then moved to 25 °C incubator (Thermoline, Wetherill park, Australia) for further 24 h. Ten grams of the freshly cultured (TVR and HSK) grains were diluted in 90 mL 0.1% peptone water (Oxoid, Basingstoke, United Kingdom) and each mixture was thoroughly homogenized using a BagMixer (Interscience, Saint Nom, France) for 2 min. Ten grams each of remaining kefir (devoid of grains) were also diluted in 90 mL of 0.1% peptone water. Serial dilutions from 10^{-1} to 10^{-5} were performed and 100 µL were inoculated onto Rose-Bengal chloramphenicol agar (RBCA, Oxoid, Basingstoke, United Kingdom). Chloramphenicol was prepared by following manufacturer's protocols and 3 mL of sterile deionized water was added to vial and mixed thoroughly. The vial content was then added to 500 mL RBCA agar base. Chloramphenicol was added to RBCA in order to inhibit growth of bacteria component of kefir. Plates were incubated aerobically at 25 °C for 5 days. Yeasts growth on agar plates were counted and morphologically grouped as reported in a previous study (Garrote et al., 1997). Briefly, colonies were grouped based on color, size, form, elevation and margin, and also on the basis of cell morphology using optical microscopy (Olympus Optical, Tokyo, Japan). Colonies from HSK and TVR were coded as HSK18099-11 and TVR18099-12 respectively.

2.2. Probiotics potential evaluations

2.2.1. Growth at human body temperature

Well isolated colonies of the yeast isolates were streaked onto yeast YEPD agar and incubated at 37 °C aerobically for 10 days.

2.2.2. Survival in simulated gastrointestinal tract conditions

Survival of kefir yeast isolates in simulated GIT was performed as reported (Minekus et al., 2014) with some modifications. Briefly, 10 mL of kefir yeast isolates and controls (*S. boulardii* SB48 MYA-796, and *S. boulardii* SB49 MYA-79) in YEPD broth initially incubated at 25 °C for 24 h in a shaking incubator at 100 horizontal strokes/min (Innova 4230 New Brunswick Scientific, Edison, NJ, USA) was added to 10 mL sterile 0.1% peptone water. The yeast mixture was serially diluted up to 10^{-7} using 0.1% peptone water, 100 µL of each dilution was plated onto YEPD agar, and incubated at 25 °C aerobically for 5 days. This was used as a control.

Another 10 mL aliquot of the yeast cultures mentioned above were mixed with 7.5 mL of simulated gastric fluid (SGF) containing 6.9 mL KCl (0.5 M), 0.9 mL KH_2PO_4 (0.5 M), 12.5 mL $NaHCO_3$ (1 M), 11.8 mL NaCl (2 M), 0.4 mL $MgCl_2{\cdot}6H_2O$ (0.15 M) and 0.5 mL $(NH_4)_2CO_3$ (0.5 M). Two milliliters of 20,000 U/mL (3.7 g 543 unit/g in 100 mL sterile milli-Q water) porcine pepsin solution was added. Furthermore, 5 µL 0.3 M $CaCl_2$ solution and 0.295 mL of water were added. Final pH was adjusted to 3.0. Twenty milliliters of the mixtures containing yeasts were incubated at 37 °C aerobically for 2 h in a shaking incubator as above. The simulated gastric chymes containing yeast were further mixed with 11 mL of simulated intestinal fluid (SIF). The SIF solution contained 6.8 mL KCl (0.5 M), 0.8 mL KH_2PO_4 (0.5 M), 42.5 mL $NaHCO_3$ (1 M), 9.6 mL NaCl (2 M), 1.1 mL $MgCl_2{\cdot}6H_2O$ (0.15 M), 5 mL pancreatin stock solution (800 U/mL in simulated intestinal fluid electrolyte), 2.5 mL bile salt (160 mM), 40 µL $CaCl_2$ (0.3 M) and 1.31 mL sterile water. Final pH was adjusted to 7.0, and the solutions were incubated at 37 °C aerobically for 2 h in a shaking incubator at 100 horizontal strokes/min. Serial dilutions from the simulated GIT mixture were performed as above and YEPD agar was inoculated with 100 µL of each dilution and incubated at 25 °C aerobically for 5 days.

2.2.3. Hydrophobicity

Hydrophobicity experiment was carried out as previously described (Fadda, Mossa et al., 2017a) with some modifications. Yeast cultures in YEPD broth incubated at 25 °C for 24 h were centrifuged (Eppendorf AG, Hamburg, Germany) at 4000*g* for 15 min at 4 °C and washed twice with 1X PBS. The yeast pellets were re-suspended in 1X PBS and optical density at 560 nm (OD_{560nm}) was measured using spectrophotometer (Shimadzu, Kyoto, Japan). The yeast cultures in 1X PBS optical densities were adjusted to range between 0.4 and 1 using the same buffer. Three milliliters of the yeasts suspension and 0.6 mL *n*-hexadecane (Sigma, St. Louis, USA) were mixed and vortexed for 2 min before incubation at 37 °C aerobically for 1 h to separate *n*-hexadecane and aqueous phases. The aqueous phase optical density of the solution was measured using spectrophotometer (Shimadzu, Kyoto, Japan) at 560 nm. Percentage hydrophobicity was calculated as a reduction in OD_{560nm} using the formula:

$$\%\ \text{hydrophobicity} = \frac{OD0 - OD}{OD} \times 100$$

where OD0 and OD are OD_{560nm} before and after extraction with *n*-hexadecane respectively.

2.2.4. Auto-aggregation

Auto-aggregation experiment was performed as reported in the literature (Fadda, Mossa et al., 2017a) with some modifications. Yeast cultures incubated in YEPD broth at 25 °C aerobically for 24 h were centrifuged at 4000*g* for 15 min at 4 °C and washed twice in 3 mL 1X PBS per wash after each centrifugation. The yeast cultures in 1X PBS optical densities were adjusted to range between 0.4 and 1 using the same buffer. OD_{560nm} of the suspensions were measured using spectrophotometer (Shimadzu, Kyoto, Japan) before incubation at 37 °C for 2 h. The top phase of the solution was carefully removed after incubation and OD measured again at 560_{nm}. Percentage auto-aggregation was calculated as follows:

$$\%\ \text{auto} - \text{aggregation} = \left[1 - \left(\frac{ODt}{D0}\right)\right] \times 100$$

where OD is OD_{560nm} before incubation and OD_t is OD_{560nm} after incubation.

2.3. Safety screening

2.3.1. Phospholipase production

Phospholipase production screening was performed as described in

literature (Deorukhkar, Saini, & Mathew, 2014) with some modifications. Ten microliters of yeast suspension from YEPD broth was spotted onto Sabouraud dextrose agar (SDA) plus egg yolk (16.25 g SDA, Oxoid, Basingstoke, United Kingdom; 20 mL egg yolk emulsion, Sigma-Aldrich, Castle Hill, Australia, 230 mL sterile distilled water), with a final pH 7. Plates were incubated at 30 °C and 37 °C aerobically for up to 5 days. Phospholipase activity was expressed as ratio of diameter of colony to that of colony plus translucent zone around the colony. Diameter was measured using digital caliper (Instrument Choice, Dry Creek, Australia). *C. albicans* (ATCC 10231) was used as a positive control.

2.3.2. Hemolytic activity

Hemolysin production was screened as described in literature (Deorukhkar et al., 2014; Luo, Samaranayake, & Yau, 2001) with some modifications. Briefly, spot inoculation with 10 µL yeasts suspension in YEPD onto SDA enriched with 7% (of final volume) sheep defibrinated blood (Oxoid, Basingstoke, United Kingdom) was performed. Final medium pH was adjusted to 5.6. Plates were incubated at 30 °C and 37 °C for up to 5 days. Hemolytic activity was expressed as ratio of diameter of colony to that of colony plus translucent or clear zone around the colony. Diameter was measured using digital caliper (Instrument Choice, Dry Creek, Australia). *C. albicans* was used as a positive control.

2.3.3. Proteolytic activity

Screening for proteolytic enzymes production was performed as previously described (AlGburi et al., 2016; Deorukhkar et al., 2014) with some modifications by spot inoculating 10 µL of yeast suspensions in YEPD broth onto SD plus milk agar (100 mL UHT skim milk, 16.25 g SDA, and 100 mL deionized sterile water), with a final medium pH of 7.3. Plates were incubated at 30 °C and 37 °C for up to 5 days. Proteolytic activity was expressed as ratio of diameter of colony to that of colony plus clear zone around the colony. Diameter was measured using digital caliper (Instrument Choice, Dry Creek, Australia). *C. albicans* was used as a positive control.

2.4. Identification of yeast isolates

Different colony types from RBCA were picked and streaked onto yeast extract peptone dextrose (YEPD) agar (Oxoid, Basingstoke, United Kingdom) and incubated at 30 °C for 3 days. These isolates on YEPD agar were sent to Microgenetix, a National Association of Testing Authorities (NATA) accredited laboratory for identification using 26s fungal ribosomal DNA. MicroSEQ® D2 LSU rDNA Fungal Identification Kit was used as per manufacturer's protocols (Scientific, 2015). Further identification work was performed on TVR18099-12 colonies using internal transcribed spacer (ITS) region sequencing and Accugenix ITS database (AccuBLAST) by the same laboratory as per literature (Schoch et al., 2012). 26s rDNA was previously used in identification of kefir grains yeast isolates including Saccharomyces cerevisiae, Saccharomyces unisporus, Issatchenkia occidentalis and Kluyveromyces marxianus. (Diosma, Romanin, Rey-Burusco, Londero, & Garrote, 2014).

2.5. Antibacterial properties

2.5.1. Growth inhibition

Bacteriostatic and bactericidal analysis was performed as described in the literature (Rajkowska & Kunicka-Styczyńska, 2012) with some modification. Slabs of yeasts isolates previously grown on YEPD agar at 30 °C for 24 h were placed on Muller-Hinton agar (Oxoid, Basingstoke, United Kingdom) previously inoculated with 10^4 cfu/mL *Escherichia coli* ATCC 43895 (*E. coli*) and *Enterobacter aerogenes* VUN 00025 (*E. aerogenes*) as spread plate and incubated at 37 °C for 24 h.

Production of antibacterial molecules or pH effects analysis was carried out as reported (Bajaj, Raina, & Singh, 2013) with modification. Fermentation was performed by growing yeast isolates and controls in killer toxin medium (KTM) consisting of YEPD plus glycerol (50 g/L, Sigma, St. Louis, USA), buffered at pH 5 using 50 mM citrate-phosphate buffer, and fermented at 30 °C under shaking (180 rpm) for 24–72 h. Fermented broth was centrifuged (Eppendorf AG, Hamburg, Germany) at 4000*g* for 30 min at 4 °C. Supernatant from KTM was used for well diffusion assay as described previously (Bajaj et al., 2013).

2.5.2. Sedimentation and adhesion assay

Adhesion of bacteria onto yeast cells was performed as previously described (Tiago et al., 2012) with some modification. Briefly, 1 mL (approximately 10^8 cfu/mL) yeast in YEPD broth initially incubated at 25 °C for 24 h in a shaking incubator at 100 horizontal strokes/min (Innova 4230 New Brunswick Scientific, Edison, NJ, USA) was added to a 15 mL centrifuge tube with 0.5 mL of *E. coli* and *E. aerogenes* (approximately 10^9 cfu/mL). The bacteria-yeast mixture was vortexed for 1 min and incubated at 37 °C for 4 h. Slide agglutination was also performed and macroscopically observed. One hundred microliter aliquot of supernatant was serially diluted and plated onto Nutrient agar (Oxoid, Basingstoke, United Kingdom) supplemented with 0.1% cycloheximide (Sigma, St. Louis, USA) to suppress yeasts growth. Plates were incubated at 37 °C for 24 h. Indicator bacterial colonies were counted and expressed as $\log_{10}$ cfu/mL. For the controls, 1 mL of sterile YEPD broth was added to 0.5 mL of indicator bacteria and treated as above. The procedure was repeated for non-viable yeasts sedimentation and adhesion assay after the yeasts initially grown in YEPD broth were inactivated by autoclaving at 121 °C for 15 min.

To visualize the adherence of bacteria onto yeast cells, 10 µL of the sediments were smeared onto microscopic slides. Gram stain was performed as described (Claus, 1992) and analyzed under optical Motic microscope (Motic, Melbourne Australia).

2.6. Statistical analysis

Experiments were replicated at least twice with subsequent three subsampling. The data was analyzed with a randomized split plot block design, using replications as the block. The isolates at two levels were the main plot. All data were expressed as mean and with standard error of the mean. Statistical analysis was performed using SPSS Statistical software (IBM, New York, USA).

3. Results and discussions

3.1. Isolation and enumeration of yeast isolates

Table 1 summarizes yeast species isolated from traditional kefir product. Two distinct colony morphologies were isolated on RBCA shown in Fig. 1. HSK18099-11 colonies morphology from both grain

Table 1
Traditional kefir grains morphological and numerical characterization.

Yeast strains	TVR18099-12	HSK18099-11
Colony morphology	White centre with pink edge, convex and round	Round smooth, shiny pink and convex colonies
Cellular morphology	Globose to ellipsoidal unicellular and some budding	Globose to ellipsoidal, unicellular and budding
Count in grain $\log_{10}$ cfu/g	5.4	6.28
Count in Kefir $\log_{10}$ cfu/g	6.05	5.81

Mean SD (SE) = 0.05.

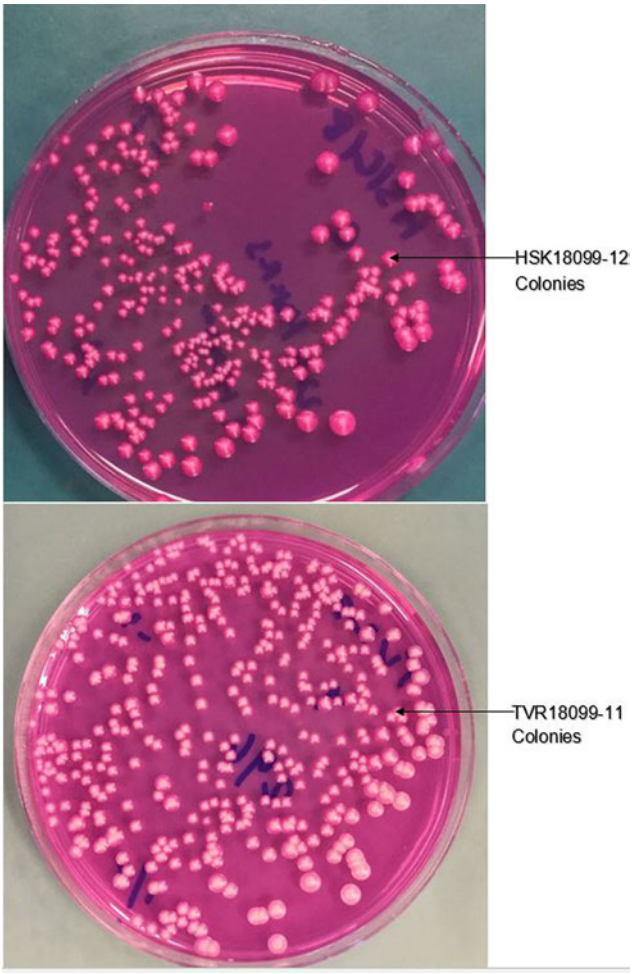

Fig. 1. HSK18099-11 and TVR18099-12 colonies morphology appearance on Rose Bengal Chloramphenicol Agar incubated at 25 °C for 5 days.

and kefir appeared similar, likewise, the morphology appearance of colonies for TVR. Both colony and cellular morphologies of these kefir isolates were consistent with previous study in terms of colonies and cell appearance (Garrote et al., 1997). Fig. 2 shows traditional kefir grains used in this study after separation from kefir, and washed with sterile water. The appearances of the grains were consistent with the description of kefir grains in literature (Garrote et al., 1997). It appeared TVR18099-11 was readily released into kefir, hence the high cell count in kefir compared to the grain. On the other hand, HSK18099-12 was retained in the grain resulting in low cell count (Table 1). The numbers of yeasts in these traditional kefirs were similar to a previous study (Silva, Santos, Santana, Silva, & Coaceicao, 2018) which recorded 5.6 cfu/g ($\log_{10}$) yeasts cells in kefir drinks.

3.2. Survival in simulated GIT

Kefir yeast isolates and 2 strains of *S. boulardii* used as controls decreased by < 0.5 $\log_{10}$ under GIT simulated conditions for 4 h (Table 2). All the kefir yeasts isolates showed high survival rates in simulated GIT conditions comparable to controls strains (SB48/MYA 796™ and SB49/MYA 797™) currently used as prophylactic and therapeutic strains in some human ailments (Czerucka, Piche, & Rampal, 2007; Hudson et al., 2016; Palma et al., 2015). MYA 796 was the least affected yeast strain by GIT conditions, and the variation of $\log_{10}$ reduction may be due to strain differences. The ability of these kefir yeast isolates to survive in the GIT may be due to the fact that kefir is an acidic and low alcohol beverage (Prado et al., 2015) and therefore have likely developed resistance to harsh conditions. HSK and TVR kefir pH were 4.7 and 4.5 respectively after 48 h of incubation as described above. Resistance of *S. boulardii* MYA 797 to GIT conditions has been postulated to be due to thicker cell walls compared to other *Saccharomyces cerevisiae* strains including W303 and BY4741 (Hudson et al., 2016). However, exposure of *S. boulardii* MYA 797 to Caspofungin, an antifungal drug that interferes with synthesis of cell wall, was found to significantly reduce resistance of these strains to GIT simulated environment (Hudson et al., 2016). Role of cell wall thickness of kefir yeast isolates in GIT survival in this study needs further investigation. Survival in the GI tract is an important criteria for microorganisms to be classified as probiotic, and involves being able to resist acidic gastric juice such as pepsin, basic pancreatic enzymes lysozyme, and bile salts at physiological temperature (Gut et al., 2018). Survival of these yeast strains in simulated GIT showed their resistance to digestive enzymes including pepsin, pancreatins as well as bile salts and low pH. *S. cerevisiae* CIDCA8112 and *Kluyveromyces marxianus* were reported to exhibit immunomodulatory properties which depended on viability of the yeast species (Romanin et al., 2010). Furthermore, viability of yeast probiotics is associated with several antagonistic properties towards enteropathogenic bacteria including competition for nutrients, binding sites and production of antibacterial molecules (Revolledo, Ferreira, & Ferreira, 2009).

3.3. Growth at human body temperature

Growth for both isolates and controls is shown in Table 2. Both the controls and TVR18099-12 were able to grow at 37 °C whereas HSK18099-11 did not grow at 37° C (Table 2). This was in agreement with previous studies where some yeast species grew at 30 °C but no at 37 °C (Lodder & Kreger-Van, 1952). Failure of HSK18099-11 to grow at this human body temperature may not disqualify it as a potential probiotic since prophylactic and therapeutic potentials of yeasts are not limited to viable and proliferating cells only. Prophylactic and therapeutic efficacy of non-viable yeast cells have been reported (Gut et al., 2018).

3.4. Auto-aggregation

Auto-aggregation of kefir isolates and controls are shown in Table 2. It is defined as aggregation among yeast cells to form flocs or flor which provides competitive advantage over other microorganisms including enteric bacterial pathogens in a harsh environment such as human GIT (Brückner & Mösch, 2012). Auto-aggregations of kefir isolates were slightly comparable to that of *S. boulardii* strains used as controls. The percentage auto-aggregations of isolates and controls were consistent with previous studies (Fadda, Mossa et al., 2017a; Gil-Rodríguez, Carrascosa, & Requena, 2015a, 2015b). Formation of cell aggregates provides shielding to cells in the center against harmful environmental conditions (Suvarna, Dsouza, Ragavan, & Das, 2018). *In vitro* auto-aggregation can be influenced by duration of incubation used during analysis to separate aqueous and *n*-hexadecane phase (Garrote et al., 2015). On the other hand, yeasts auto-aggregation has been reported to be strain-specific (Garrote et al., 2015; Suvarna et al., 2018). Therefore, variations of auto-aggregation in the current study was likely due to these factors which have similarly been reported by Fadda, Mossa et al. (2017a, 2017b) using similar strains.

3.5. Hydrophobicity

Kefir yeast isolates were analyzed for hydrophobicity (Table 2). Hydrophobicity is defined as a non-specific interaction between microbial and host cells. This interaction is mediated by cell-surface proteins and lipoteichoic acids (Todorov et al., 2008). In this study, TVR18099-12 showed significantly higher hydrophobicity and therefore was capable of interacting with other cell bodies compared to HSK18099-11 and controls. Similar findings showed significantly lower hydrophobicity for control strains compared to other strains (Fadda, Mossa et al., 2017a). Hydrophobicity is crucial in adhesion of probiotic microorganisms onto GIT epithelial cells where they may provide

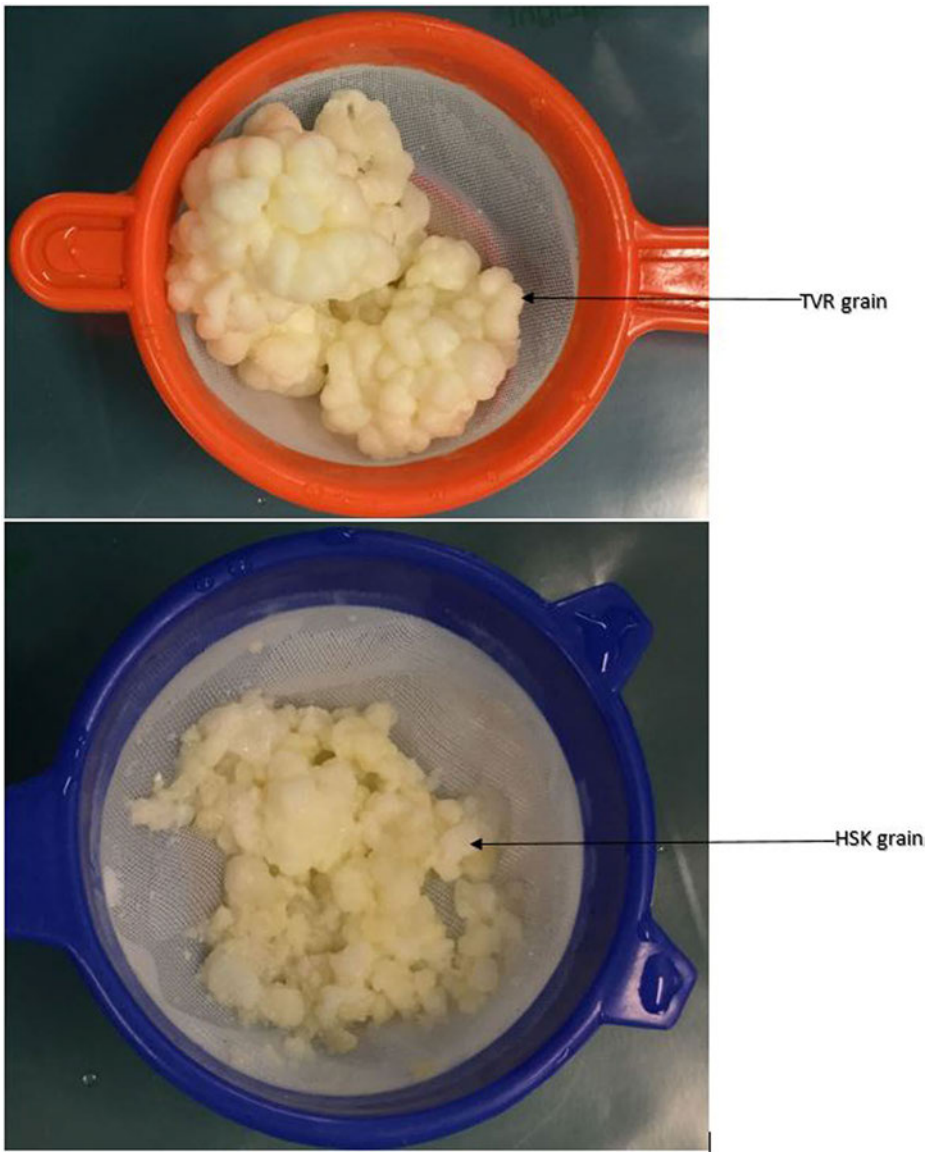

Fig. 2. Traditional kefir grains after incubation at 30 °C for 24 hrs and moved to 25 °C in Devon dale UHT full cream milk and washed with sterile water.

prophylactic and therapeutic benefits (Fadda, Mossa et al., 2017a). Hydrophobicity is species and strain specific as demonstrated in this study (Fadda, Mossa et al., 2017b; Suvarna et al., 2018). Furthermore, similar to a previous study, there was no correlation between auto-aggregation and hydrophobicity (Fadda, Mossa et al., 2017a).

3.6. Hydrolytic enzymes screening

3.6.1. Phospholipase activity

The kefir yeast isolates and probiotic controls did not produce these enzymes (Fig. 3) and as shown in Table 3. Only positive control (C. *albicans*) produced phospholipase hence the zone of precipitation

Table 2
Yeasts isolates probiotics properties.

Yeast strains	Initial mean count (Log_{10} cfu/mL) - T0[a]	GIT survival (Log_{10} cfu/mL) - T1[a]	Decrease in viability (Log_{10} cfu /mL) - T2[a]	Growth at 37 °C	Hydrophobicity (% index)[b]	Auto-aggregation (% index)[c]
TVR18099-12	7.06	6.82	0.24	+ + +	88.75	35.48
HSK18099-11	6.80	6.54	0.26	–	30.00	43.33
MYA 796™	7.04	6.95	0.09	+ + +	15.58	42.86
MYA 797™	7.07	6.66	0.41	+ + +	21.18	30.59

– = no growth; + + + = very good growth.
MYA 796™ and MYA 797™ are two strains of *S. boulardii* corresponding to SB48 and SB49 respectively. The difference between GIT treated and untreated counts for both isolates and controls are statistically significant.
[a] Standard error mean for GIT = 0.06.
[b] Standard error mean for hydrophobicity = 0.03.
[c] Standard error mean for auto-aggregation = 0.03.

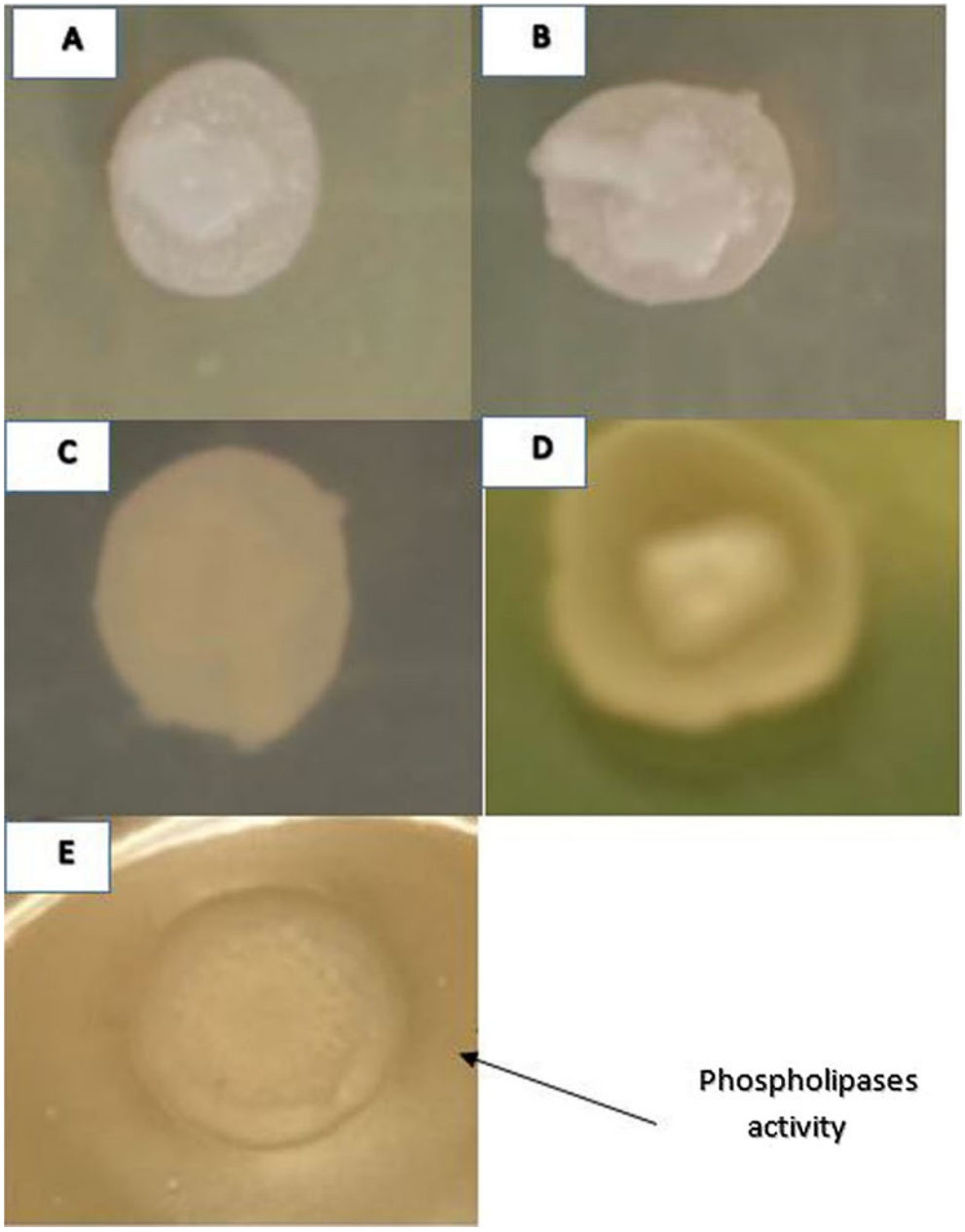

Fig. 3. C. albicans, phospholipase activity in SDA supplemented with 8% egg yolk and incubated at 30 °C for 5 days A = HSK18099-11, B = MYA 797™, C = MYA 796™, D = TVR18099-12, E = C. albicans.

Table 3
Hydrolytic enzymes activity of yeasts.

Yeast strains	Hemolytic activity[1]		Proteolytic activity[2]	Phospholipase production[3]
	α-Hemolysis	β-Hemolysis		
TVR18099-12	0.90	1	0.92	1
TVR18099-11	0.76	1	0.67	1
MYA 796™	0.77	1	0.78	1
MYA 797 ^{T}	0.84	1	0.82	1
Calb	0.78	0.94	0.76	0.72

Pz = diameter of colony to the colony plus clearing/precipitation diameter ratio; high, Pz < 0.40; medium, Pz = 0.41–0.60; low, Pz = 0.61–0.99; none, Pz = 1. Calb = *Candida albicans*.
MYA 796™ and MYA 797™ are two strains of *S. boulardii* corresponding to SB48 and SB49 respectively. Calb = *Candida albicans*.
[1] Hemolytic activity Mean SD (SE) = 0.01.
[2] Proteolytic activity Mean SD (SE) = 0.02.
[3] Phospholipases activity SEM = 0.02.

around the colony shown in Fig. 3 (Mayer, Wilson, & Hube, 2013; Park, Do, & Jung, 2013). This activity was in agreement with previous studies in which C. *albicans* produced this enzyme (Deorukhkar et al., 2014; Ramesh et al., 2011; Yang, 2003). Lack of phospholipase production by kefir grain yeasts isolates make them safe in respect to this enzyme as it is associated with yeast virulence (Ramesh et al., 2011).

3.6.2. Hemolytic activity

All the kefir yeast isolates and *S. boulardii* strains produced low levels of alpha hemolysins comparable to *C. albicans* as shown in Table 3 and Fig. 4. However, only *C. albicans* produced weak beta-hemolysis on further incubation up to 72 h, as illustrated by greenish-black halo around the colony (Fig. 5E). Similar findings have been reported previously in which *C. albicans* produced beta hemolysis after 48 h incubation (Luo et al., 2001). Mammalian systems lack free iron which is essential for microbial proliferation and pathogenesis. Some pathogenic microorganisms possess hemolysins which assist with breakdown of hemoglobin in order to access hemoglobin-bound iron (Luo et al., 2001). Hemolysins are either proteinaceous enzymes or non-proteinaceous toxins which cause cell lysing. The mechanism involve creating a pore in the cell membrane. Fungal hemolysins are reported to act slowly on cells resulting in cell death (Vesper & Jo Vesper, 2004). Alpha hemolysin in the blood may cause partial breakdown of red blood cells whereas beta hemolysin breakdown cells completely (Vesper & Jo Vesper, 2004). Histamine release induced by *E. coli* alpha hemolysin through immunomodulation was reported in rat model (Gross-Weege, König, Scheffer, & Nimmich, 1988; Scheffer, König, Braun, & Goebel, 1988). The production of alpha hemolysin by the isolates, is an important property which may be employed to fight infection by pathogenic bacteria.

3.6.3. Proteolytic activity

All kefir yeast isolates and controls showed very weak proteolytic activity shown in Table 3 and Fig. 5. Proteolytic enzymes are associated with active entry of pathogens into the host tissue (Mayer et al., 2013; Sacristan et al., 2011). However, production of proteolytic enzymes by yeasts may provide prophylactic and therapeutic benefits to the host as *S. boulardii* serine protease has been reported to break down *Clostridium difficile* and *Clostridiums perfringens* toxins (Czerucka et al., 2007; Hudson et al., 2016; Palma et al., 2015). Therefore, production of proteolytic enzymes may protect host against infections by toxins produced by enteropathogenic bacteria such as *Salmonella, Vibrio, Clostridiums, E. coli* and *Bacillus* species (Gut et al., 2018).

3.7. Identification of yeast isolates

The yeast isolates from two traditional kefir grains showed potential probiotic properties and after identification and characterization, two phylogenetic trees were obtained as shown in Fig. 6. A phylogenetic tree by definition shows evolutionary relationships among species (Mooers & Heard, 1997). Isolates HSK18099-11 and TVR18099-12 were identified as *Saccharomyces unisporus* ATCC 10612 (*S. unisporus*) and *Kluyveromyces lactis* var. *lactis* ATCC 56498 (*K. lactis*)/*Kluyveromyces marxinus ATCC16045* (*K. marxinus*) respectively. The first part of the figure shows a good separation of *S. unisporus* from its evolutionarily related species including *Kazachstania africana*, *Saccharomyces cerevisiae* and *Saccharomyces bayanus*. Therefore, further differentiation identification method was not required. However in a previous study *S. unisporus* isolated from Tibetan kefir grains could not be differentiated from Kazachstania unisporus and Kazachstania exigua using 26s rDNA (Zhou, Liu, Jiang, & Dong, 2009). On the other hand, the second part of the figure showed close similarity between *K. lactis* and *K. marxinus,* which were not effectively differentiated using 26s ribosomal DNA. This was in agreement with a previous study in which two *Kluyveromyces* species could not be differentiated on the basis of their amino acid sequence (Lertwattanasakul et al., 2015). However, in another study, it was reported that *K. lactis* and *K. marxinus* were clearly separated and identified from kefir grain using 26s rDNA (Zhou et al., 2009). The TVR18099-12 colonies labelled as *K. lactis/K. marxinus* by 26s rDNA (Fig. 6) were further analyzed using ITS sequencing, and identified as *K. lactis* ATCC 56498. ITS sequencing was used successfully to identify *Kluyveromyces* species and strain levels in previous study (Belloch, Barrio, García, & Querol, 1998).

The isolation and identification of cultural yeasts species in the two traditional grains were consistent with a FAO report which states that kefir grain contains *Saccharomyces unisporus* and Kluyveromyces *species,* Saccharomyces cerevisiae, Issatchenkia occidentalis (Diosma et al., 2014; Magalhães, Pereira, Campos, Dragone, & Schwan, 2011).

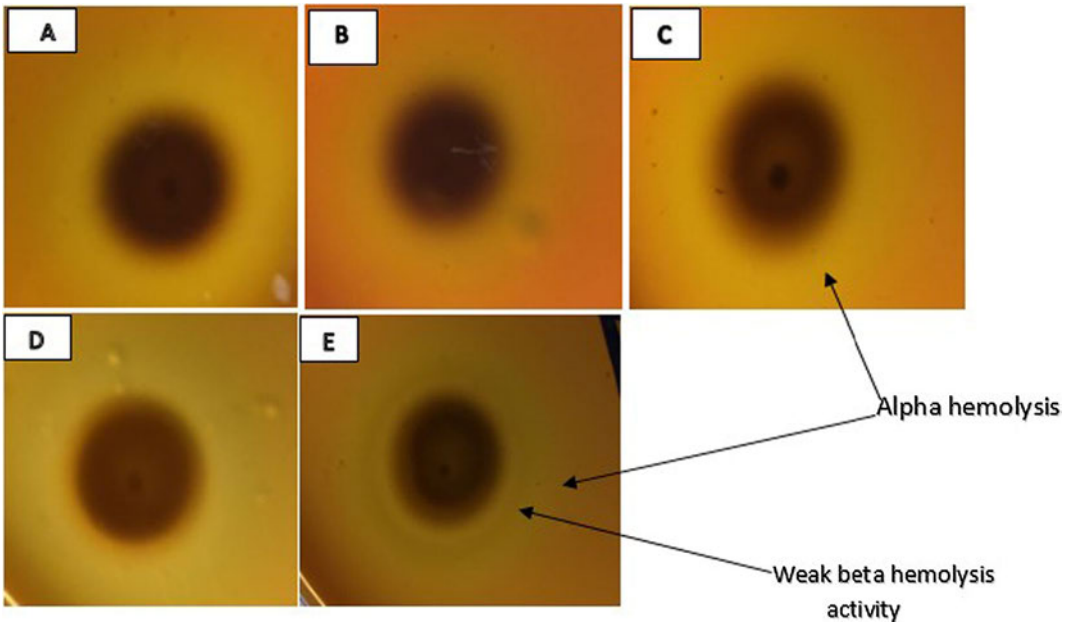

Fig. 4. Hemolytic activity in SDA supplemented with 7% defibrinated sheep blood and incubated at 30 °C for 5 days. A = HSK18099-11, B = MYA 797™, C = MYA 796™, D = TVR18099-12, E = C. albicans.

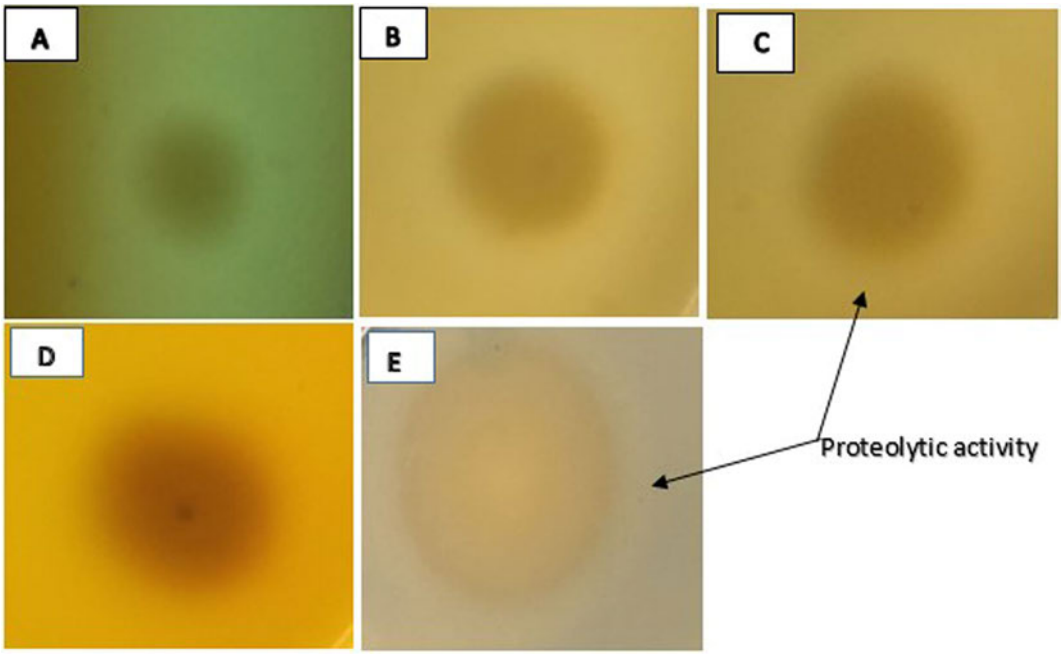

Fig. 5. Proteolytic activity in 16.25 g SDA, 100 mL UHT skim milk, and 100 mL deionised sterile water and incubated at 30 °C for 5 days. A = HSK18099-11, B = MYA 797™, C = MYA 796™, D = TVR18099-12, E = C. albicans.

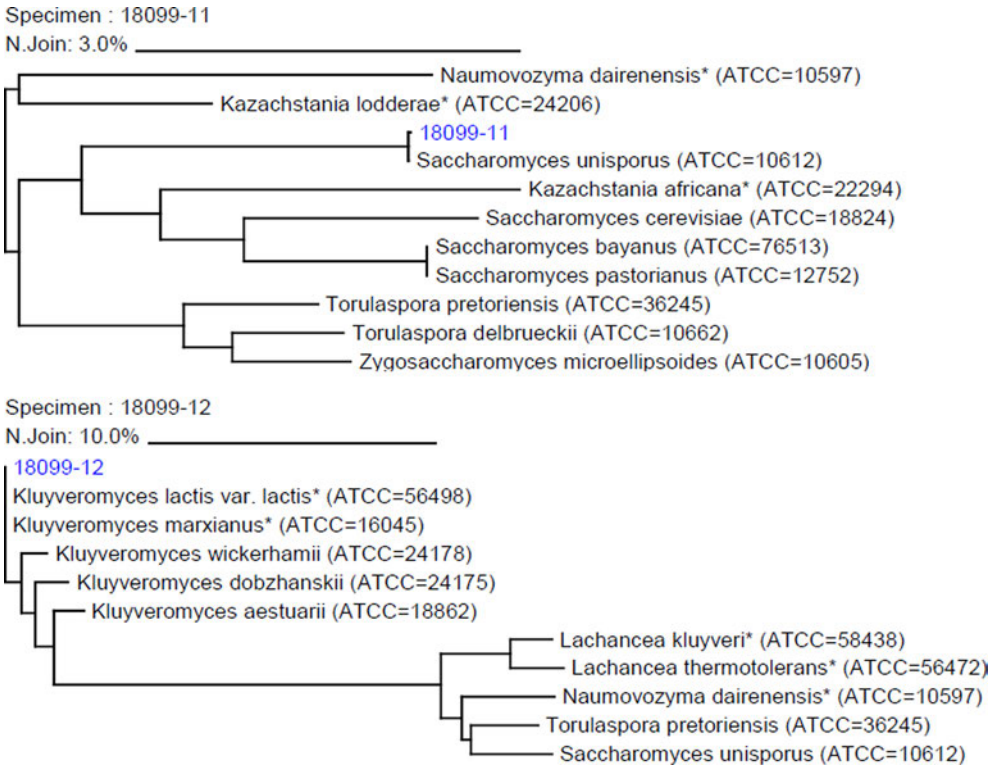

Fig. 6. Phylogenetic tree analysis of 26s rDNA K. lactis/K. marxinus and S. unisporus obtained by MicroSEQ® D2 LSU rDNA Fungal Identification Kit following manufacturers protocols. The tree shows evolutionary closeness of the traditional kefir grain yeasts isolates to the other yeast species.

3.8. Antibacterial properties

3.8.1. Bacterial growth inhibition

K. lactis and the controls slabs on the lawn and well diffusion assay showed no growth inhibition of indicator enteric bacteria, however *S. unisporus* showed weak growth inhibition of *E. coli* and *E. aerogenes* just under the slab (figure not shown). The controls results are consistent with previous study in which *S. boulardii* did not inhibit some gram negative enteropathogenic bacteria growth when tested using this method (Rajkowska, Kunicka-Styczyńska, & Rygala, 2012). Both control and kefir yeast isolates supernatants showed no growth inhibition effects (picture not shown). There are controversies on bacteriostatic and bactericidal effects of Saccharomyces species including the controls and *S. unisporus* (Rajkowska et al., 2012), and the fact that insufficient data on antibacterial properties of *K. lactis* is available, further in-depth research is needed.

Table 4
Adhesion and sedimentation assay.

	Viable yeasts					Non-viable				
Indicator bacteria	Control	*MYA 796™*	*MYA 797™*	KL	SU	Control	*MYA 796™*	*MYA 797™*	KL	SU
E. aerogenes	9.05	8.08	8.29	8.47	8.46	8.85	8.18	8.23	8.31	8.34
E. coli	8.89	8.29	8.52	8.50	8.69	8.73	8.38	8.42	8.39	8.48

The indicator bacterial count in supernatants of yeasts-bacteria mixture are statistically lower compare to the control (Paired T sample *t*-test, $p < 0.05$). Mean SD (SE), 0.03.

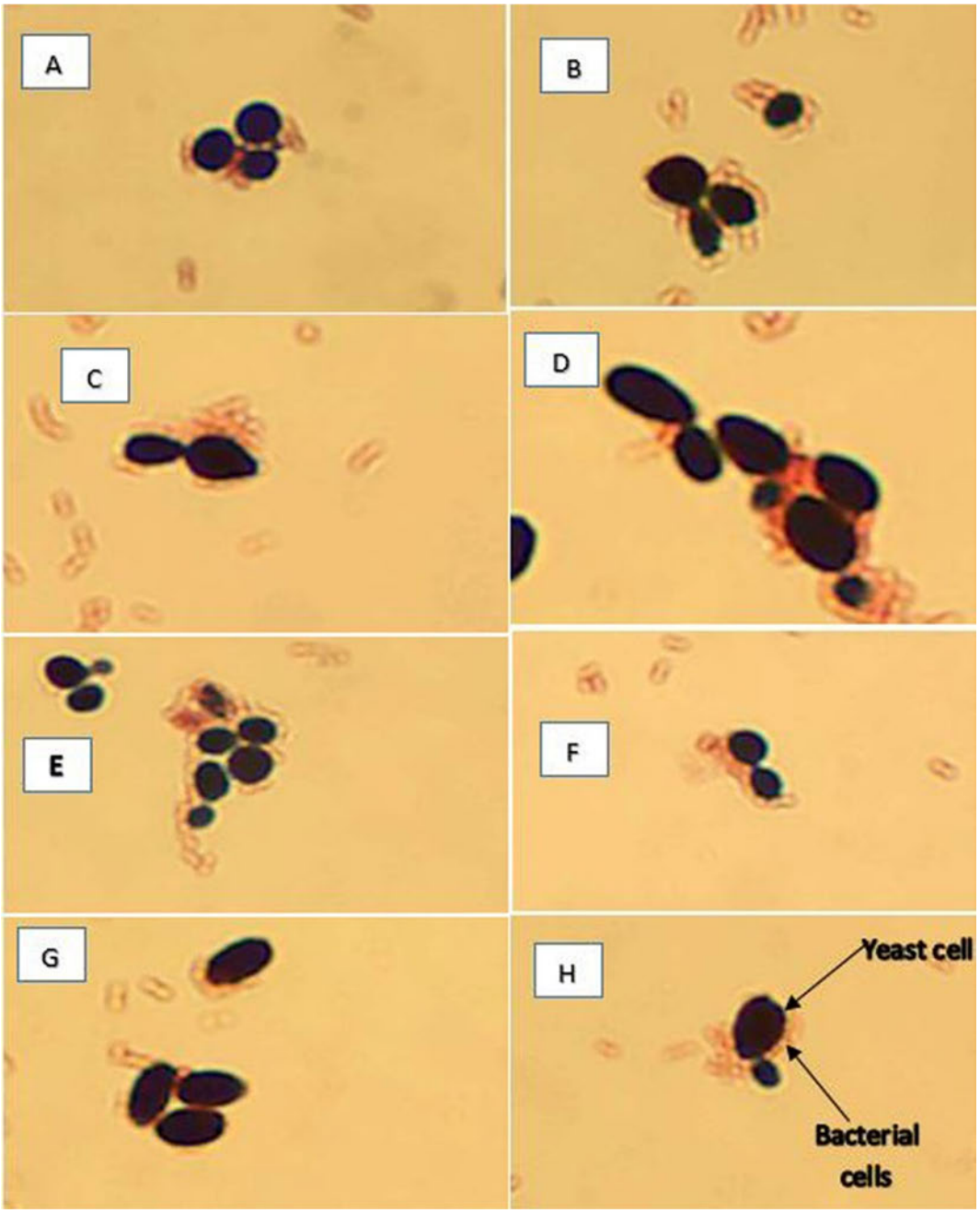

Fig. 7. Gram stain showing adherence of *E. coli* and *E. aerogenes* onto yeasts cell wall observed under optical Motic microscope at 100x magnification. A = *E. aerogenes* plus *K. lactis,* B = *E. aerogenes* plus *S. unisporus,* C = *E. aerogenes* plus MYA 796™, D = *E. aerogenes* plus MYA 797™, E = *E. coli* plus *K. lactis,* F = *E. coli* plus *S. unisporus,* G = *E. coli* plus MYA 796™ and H = *E. coli* plus MYA 797™.

3.8.2. Adherence of bacteria onto yeasts cells

Adherent of enteric bacteria indicators was analyzed both qualitatively and quantitatively. Table 4 shows both viable and non-viable kefir isolates and controls with statistical significant differences. The data above was also supported by slide agglutinations (figures not shown) and optical microscopic examination as shown in Fig. 7. Two

mechanisms of adherence of bacteria onto yeast cells are proposed. Specific binding using type 1 fimbriae on bacteria such as *E. coli*, *E. aerogenes* and *Salmonella* cell with mannan oligosaccharides on yeast cells, and non-specific binding such as electrostatic and hydrophobic (Adegbola & Old, 1985; Pérez-Sotelo et al., 2005; Tiago et al., 2012). These results are consistent with previous results where *E. coli* was reported to bind both viable and non-viable *S. boulardii* and *Saccharomyces cerevisiae* UFMG 905. Binding of enteric bacteria onto yeast cells is reported to be irreversible leading to transient passage of bacteria through GIT. *S. boulardii* does not bind to GIT (Gut et al., 2018). The adherence of enteric bacterial pathogen is postulated to be responsible for probiotic effects such as inhibition of signalling transduction pathway activation and subsequent translocation (Tiago et al., 2012) and hence their prophylactic and therapeutic application in human (Gut et al., 2018), and animal husbandry to promote health possibly through reduction of infection (Perez-Sotelo et al., 2005). Survival in GIT of these yeast isolates as shown in this study may lead to increased numbers, and hence increase capacities to scavenge (adhered bacterial cells) potential pathogenic gram negative enteric bacteria from the gut and subsequent flushing out in the feces. Moreover, since these yeasts survive in GIT and are not affected by antibiotics (for example, not affected by Chloramphenicol in this study (Fig. 1 and Table 1), data not shown) aimed at bacteria, their use as complementary therapy with antibiotics during enteric bacterial infection may also improve treatment through increased numbers and subsequent mopping out enteric bacteria from the GIT. Furthermore, the binding of opportunistic enteric bacteria onto non-viable yeasts is of great significance since consumption of viable yeast probiotics is associated with fungemia especially in immunocompromised individual or those with GIT issues (Gut et al., 2018).

4. Conclusion and future perspective

The two traditional kefir grains contained yeasts with potential probiotic properties. GIT survival, hydrophobicity, auto-aggregation and hydrolytic enzymes production of kefir yeast isolates was comparable to *S. boulardii* strains. Both kefir yeast isolates and *S. boulardii* produced weak alpha hemolytic and proteolytic activities, but none produce phospholipases at 30 °C. None of the yeasts produced hydrolytic enzymes at 37 °C. The 2 isolates showed adherence to enteric bacteria comparable to the controls. However, further in-depth studies are needed to establish their prophylactic and therapeutic properties. The isolates were identified as *S. unisporus* and *K. lactis*.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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Chapter 7. Anti-salmonella properties of kefir yeast isolates*:* an *in vitro* screening for potential infection control

This chapter focuses on analyzing kefir yeast isolates for potential application in *Salmonella* infection control and prevention. *Salmonella* adhesion onto kefir yeast isolates as well as growth inhibition due to antibacterial metabolites were analyzed in *in vitro* experiments in comparison to *Saccharomyces boulardii* strains. This chapter has been published as “Gut, Abraham Majak, Todor Vasiljevic, Thomas Yeager, and Osaana N. Donkor. Anti-salmonella properties of kefir yeast isolates*:* an *in vitro* screening for potential infection control”. *Saudi Journal of Biological Sciences* (2021). **https://doi.org/10.1016/j.sjbs.2021.09.025**

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This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

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Anti-salmonella properties of kefir yeast isolates: An in vitro screening for potential infection control

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ABSTRACT

The rise of antibiotic resistance has increased the need for alternative ways of preventing and treating enteropathogenic bacterial infection. Various probiotic bacteria have been used in animal and human. However, *Saccharomyces boulardii* is the only yeast currently used in humans as probiotic. There is scarce research conducted on yeast species commonly found in kefir despite its claimed potential preventative and curative effects. This work focused on adhesion properties, and antibacterial metabolites produced by *Kluyveromyces lactis* and *Saccharomyces unisporus* isolated from traditional kefir grains compared to *Saccharomyces boulardii* strains. Adhesion and sedimentation assay, slide agglutination, microscopy and turbidimetry assay were used to analyze adhesion of *Salmonella* Arizonae and *Salmonella* Typhimurium onto yeast cells. *Salmonella* growth inhibition due to the antimicrobial metabolites produced by yeasts in killer toxin medium was analyzed by slab on the lawn, turbidimetry, tube dilution and solid agar plat ing assays. Alcohol and antimicrobial proteins production by yeasts in killer toxin medium were analyzed using gas chromatography and shotgun proteomics, respectively. *Salmonella* adhered onto viable and non viable yeast isolates cell wall. Adhesion was visualized using scanning electron microscope. Yeasts fermented killer toxin medium showed *Salmonella* growth inhibition. The highest alcohol concen tration detected was 1.55%, and proteins with known antimicrobial properties including cathelicidin, xanthine dehydrogenase, mucin 1, lactadherin, lactoperoxidase, serum amyloid A protein and lactotrans ferrin were detected in yeasts fermented killer medium. These proteins are suggested to be responsible for the observed growth inhibition effect of yeasts fermented killer toxin medium. *Kluyveromyces lactis* and *Saccharomyces unisporus* have anti salmonella effect comparable to *Saccharomyces boulardii* strains, and therefore have potential to control *Salmonella* infection.

1. Introduction

Salmonella resistance to current antibiotic drugs is rising at an alarming rate worldwide especially in Africa and Asia. For example in Malawi, the percentage of multi drug resistant *Salmonella enter ica* serovar Typhi increased from 7% in 2010 to 97% in 2014 (Feasey et al., 2015). This has raised the need for alternative prophylaxis and therapies. One of the ways of preventing and treating infec tious diseases in human and animal is the use of probiotic microor ganisms (Feye et al., 2019; Gut et al., 2018; Kogan & Kocher, 2007). Probiotics are defined by World Health Organization (WHO) as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). *Lactobacillus, Bifidobacterium, Streptococcus thermophilus, Enterococcus, Leuconos toc* species, *Escherichia coli* Nissle 1917, *Bacillus* species and *Saccha*

Abbreviations: WHO, World Health Organization; FAO, Food Agriculture Organization; ATCC, American type Culture Collection; YEPDA, Yeast Extract Peptone Dextrose Agar; YEPDB, Yeast Extract Peptone Dextrose Broth; PBS, Phosphate buffered saline; CFU, Colony Forming Unit; DSR, Desk Sputter Coater; HCL, Hydrochloric Acid; NaOH, Sodium hydroxide; KTM, Killer Toxin Cedium; CFS, Cell Free Supernatant; DTT, Dithiothreitol; LC-MS/MS, Liquid Chromatography with tandem mass spectrometry/Liquid Chromatography with tandem mass spectrometry; HPLC, High-performance liquid chromatography; mL, Milliliter; Min, Minute; h, Hour; RSLC, Rapid Separation Liquid Chromatography; AGC, Automatic Gain Control; SPSS, Statistical Package for the Social Sciences; IBM, International Business Machines; SD, Standard Deviation; LFQ, Label Free Quantitation; GIT, The gastrointestinal tract; DNA, Deoxyribonucleic Acid; RNA, Ribonucleic Acid; ATP, Adenosine triphosphate.

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romyces boulardii are currently used as probiotics in humans against bacterial pathogens infection (Bakken, 2014; Bekar et al., 2011; Khodadad et al., 2013; Nami et al., 2015). Probiotics are com monly found in popular fermented functional foods such as yoghurt, milk, kefir, cheese, soybean, fruits, sourdough and veg etable products (Plessas et al., 2016; Prado et al., 2015; Priyodip et al., 2017; Saarela et al., 2000) or formulated into either lyophi lized forms as capsules, powders or in aqueous solutions (Martins et al., 2009). These microorganisms exert antagonism against susceptible bacteria by production of antibacterial mole cules, prevention of biofilm formation and pathogen invasiveness; adhesion of bacteria cells onto cell walls, and degradation of bacte rial toxins among others (Gut et al., 2018).

Kefir drink is a probiotic product made from a kefir grain, a con sortium of many microorganisms and exopolysaccharide, con sumed in many parts of the world including the Caucasus Mountains of Russia, Europe, Asia, South and North America due to its health benefits conferred by the probiotic components (Garrote et al., 1997). Bacterial cultures of kefir include *Lactobacil lus, Lactococcus, Leuconostoc* and *Streptococcus* genera whereas the yeast cultures include *Kluyveromyces, Candida, Saccharomyces* and *Pichia* (Plessas et al., 2016).

The aim of this study was to screen kefir yeast isolates for potential application in *Salmonella* infection control. The study hypothesized that yeasts isolated from kefir and *Saccharomyces boulardii* may show *Salmonella* binding capability, as well as growth inhibition due to production of antimicrobial metabolites. Specifically, *Salmonella enterica* serovar Arizonae (*S.* Arizonae) and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) adhesion onto *Saccharomyces unisporus* ATCC 10612 (*S. unisporus*) and *Kluyveromyces lactis* var. *lactis* ATCC 56498 *(K. lactis)* as well as growth inhibition due to antibacterial metabolites were ana lyzed in an *in vitro* experiments in comparison to *Saccharomyces boulardii* strains {*Saccharomyces* var *boulardii* MYA 796 (SB48) and *Saccharomyces* var *boulardii* MYA 797 (SB49)}.

2. Materials and methods

2.1. Materials

Microbiological cultures including kefir yeast isolates (*S. unisporus*d and *K. lactis*), *S. boulardii* strains (Victoria University culture collection). *S.* Arizonae ATCC 13314 and *S.* Typhimurium ATCC 14028 (In vitro Technologies, Melbourne, Australia). Microbi ological media including yeast extract peptone dextrose agar (YEPDA), YEPD broth (YEPDB), nutrient agar, chloramphenicol drug (100 mg/L), and nutrient broth (Basingstoke, United Kingdom). Phosphate buffered saline (PBS), cycloheximide (0.1%), bile extract porcine (containing glycine & taurine conjugate of hyodeoxycholic acid), glycerol, citrate phosphate buffer, 0.22 μm sterile filters (Sigma Aldrich, St. Louis, USA) and absolute ethanol (Rowe Scien tific, Melbourne, Australia).

2.2. Preparation of Salmonella and yeast cultures

Salmonella and yeast cultures were prepared as described in a previous study (Tiago et al., 2012) with some modifications. Well isolated *S.* Arizonae and *S.* Typhimurium colonies initially grown on nutrient agar and incubated at 37 °C for 24 h were inoc ulated into 10 mL nutrient broth and incubated at 37 °C for 24 h. One milliliter of each culture was serially diluted to approximately 10^3 CFU/mL. Another set of *Salmonella* cultures with approximately 10^9 CFU/mL (undiluted culture) was set aside. Well isolated yeast colonies on YEPDA were picked and inoculated into 10 mL YEPDB and incubated at 25 °C for 24 h in a shaking incubator at 100 hor izontal strokes/min (Innova 4230 New Brunswick Scientific, Edi son, NJ, USA). The yeast cultures with approximately 10^8 CFU/mL were labelled accordingly. All microbial cultures were kept in a refrigerator at 4 °C prior to use when necessary.

2.3. Adhesion of Salmonella onto yeast cell wall

2.3.1. Salmonella adhesion onto yeast cell wall

Adhesion of *Salmonella* onto yeast cell wall was performed as previously described (Tiago et al., 2012) with some modifications. One milliliter of viable yeast culture broth (approximately 10^8 CFU/mL) was added to a 15 mL centrifuge tube containing 0.5 mL of *Salmonella* culture (approximately 10^9 CFU/mL). The bacteria yeast mixture was vortexed for 30 s and incubated at 37 °C for 4 h. One hundred microliter was removed from the top and was serially diluted in 0.1% peptone water to 10^{-7}. One milli liter of each dilution was added to a Petri dish and then 20 mL mol ten nutrient agar at 45 °C (supplemented with 0.1% cycloheximide to suppress yeasts growth) was added and mixed gently. Plates were incubated at 37 °C for 24 h. *S.* Arizonae and *S.* Typhimurium colonies were counted and expressed as $\log_{10}$ CFU/mL. For the con trols, 1 mL sterile YEPDB (containing no yeast cells) was added to 0.5 mL of each *Salmonella* serovars and treated as above.

To assess adhesion of *Salmonella* onto non viable yeast cells, 10 mL of yeast cultures were heated at 100 °C for 5 min to obtain inactivated cells. Adhesion and sedimentation were performed as per the protocol for viable yeast cells described above.

Slide agglutination was performed as described in the literature (Perez Sotelo et al., 2005) by inoculating 0.02 mL viable and non viable yeast suspension with 0.01 mL *Salmonella* on microscopic slides. The two cultures were mixed using sterile inoculating loop before gentle rocking the slide. Agglutination was observed under illumination (Perez Sotelo et al., 2005).

Table 1
Adhesion of *Salmonella* onto yeast cell wall: quantitative and qualitative analysis results.

	Yeast strains/ control	*S.* Arizonae Count (log10 CFU/ mL)	*S.* Typhimurium Count (log10 CFU/ mL)
Live yeast	Control (YEPD)	9.14 ± 0.05[b]	9.20 ± 0.05[b]
	SB48	8.69 ± 0.40 ++[a]	8.66 ± 0.14 +++[a]
	SB49	8.63 ± 0.35++[a]	8.82 ± 0.18 +++[a]
	KL	8.88 ± 0.10 ++[a]	8.81 ± 0.11 +++[a]
	SU	8.97 ± 0.07 ++[a]	8.84 ± 0.20 +++[a]
Heat-killed yeast	Control (YEPD)	8.92 ± 0.04[b]	8.93 ± 0.06[b]
	SB48	8.67 ± 0.05 ++ [a]	8.32 ± 0.17 ++ [a]
	SB49	8.68 ± 0.07++ [a]	8.28 ± 0.19 ++ [a]
	KL	8.68 ± 0.08++ [a]	8.69 ± 0.11 ++ [a]
	SU	8.69 ± 0.09 ++ [a]	8.67 ± 0.09 ++ [a]
Bile	Control (bilee)	9.04 ± 0.05[b]	8.96 ± 0.07[b]
	SB48	8.74 ± 0.10 ++[a]	8.68 ± 0.09 ++[a]
	SB49	8.72 ± 0.13 ++[a]	8.86 ± 0.03 ++[a]
	KL	7.72 ± 0.07 ++[a]	8.88 ± 0.02++[a]
	SU	8.78 ± 0.06 ++[a]	8.67 ± 0.13 ++[a]
pH 2.0, 4.0, 5.0 and 7.0, and 8.0 (live yeast)	SB48	++	+++
	SB49	++	+++
	KL	++	+++
	SU	++	+++

The experiments was performed twice and in triplicate and the values are reported as the mean plus standard deviation; + = very weak agglutination seen after 5 s of gentle rocking of slide; ++ medium level agglutination seen after 5 s of gentle rocking of slide; +++ = very strong instant agglutination seen after rocking of slide. Means with different superscript are significantly different ($p < 0.05$

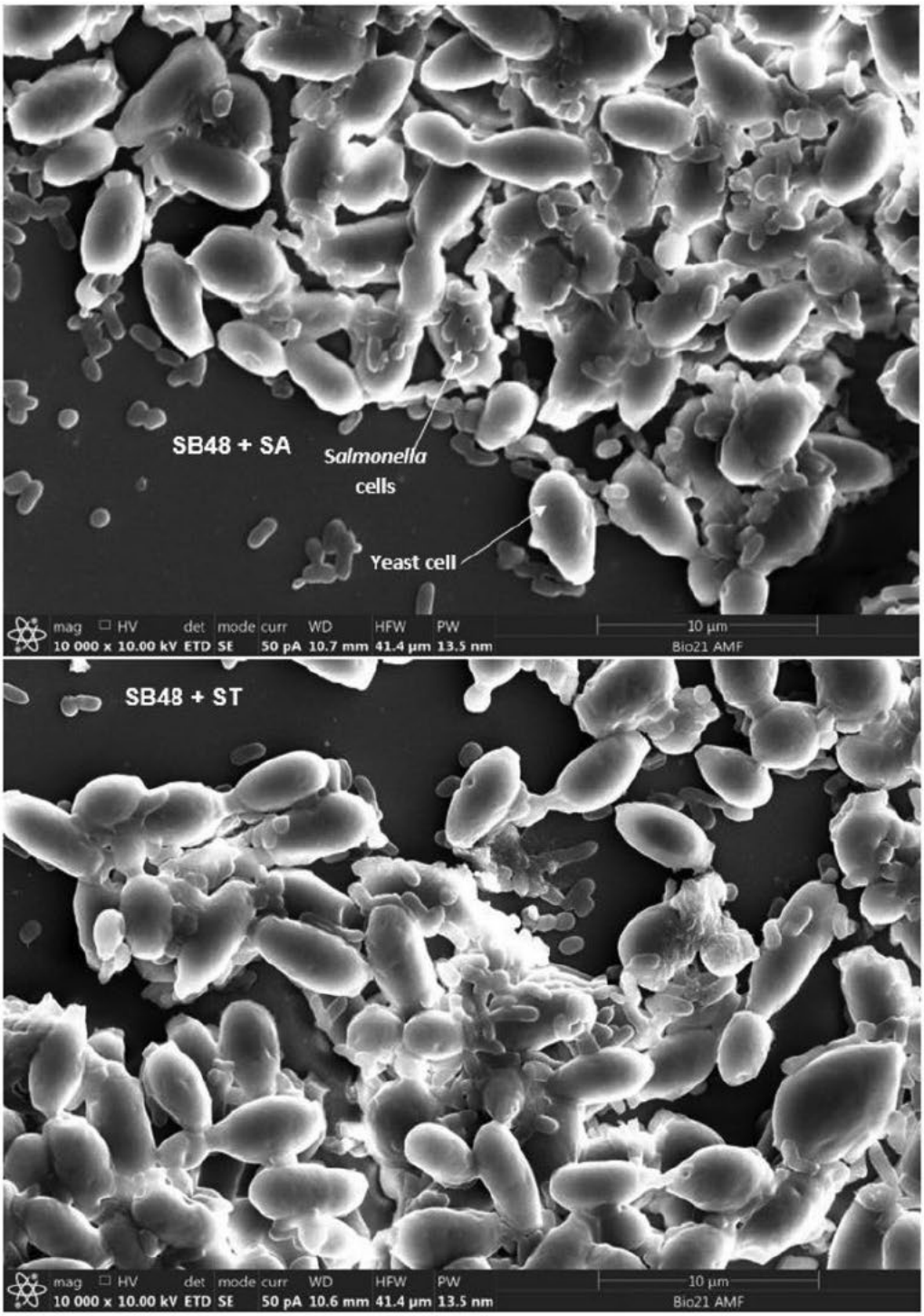

Fig. 1A. Adhesion of *Salmonella* servers onto SB48 cell walls observed using scanning electron microscope. SA = *S.* Arizonae; ST = *S.* Typhimurium.

Salmonella (0.01 mL, 10^9 CFU/mL) and yeast (10^8 CFU/mL 0.02 mL) were added to a clean microscope slide and mixed using sterile inoculating loop. The smear was allowed to air dry before fixing with 0.1% glutaraldehyde, washed three times with 1X PBS and allowed to air dry again. Smear on slides were dehydrated using classical dehydration process (Kiekens et al., 2019; Piroeva et al., 2013) with modification. Briefly, slides were soaked stepwise in 50%, 75% and absolute ethanol consequentially for 30 min at room temperature before drying at 37 °C for 1 h. The slides were then glued onto scanning electron microscope stubs and gold coated by sputtering for 5 min using a desk sputter coater DSR1 (Markham, Canada). The adhesion was qualitatively observed using a Teneo scanning electron microscope (Thermo Fisher Scien tific, Hillsboro, USA). Adhesion of *Salmonella* onto both viable and heat inactivated yeast cell wall was analyzed at magnification of 10,000×.

2.3.2. Salmonella growth in presence of inactivated yeast cells

Growth behaviour of *Salmonella* in presence of inactivated yeast cells was analyzed using turbidimetry assay. Six milliliters of yeast

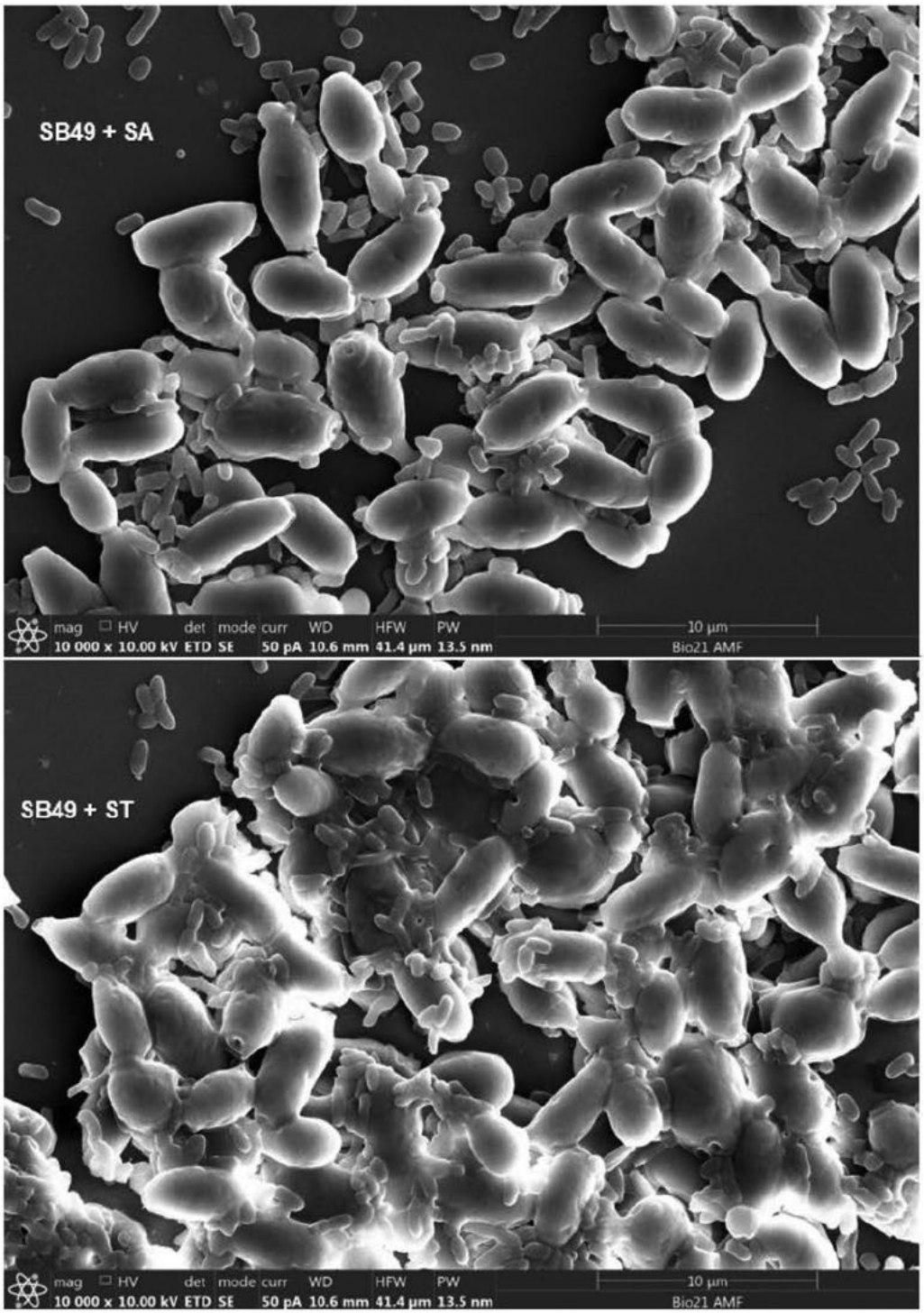

Fig. 1B. Adhesion of *Salmonella* servers onto SB49 cell walls observed using scanning electron microscope. SA = *S.* Arizonae; ST = *S.* Typhimurium.

(10^9 CFU/mL) and 3 mL of *Salmonella* (10^9 CFU/mL) cultures were centrifuged (Eppendorf 5810 R, Hamburg, Germany) at 4000g for 15 min at 4 °C and washed twice with 1X PBS respectively. The yeast pellets were re suspended in 6 mL of 1X PBS whereas the *Salmonella* pellets were re suspended in 3 mL 1X PBS and 3 mL nutrient broth (as source of nutrients for growth). One milliliter of the yeast suspension (10^2 CFU/mL) and 0.5 mL of the *Salmonella* suspension (10^3 CFU/mL) were added to a 15 mL centrifuge tube and vortexed for 20 s to mix. *Salmonella* cultures in 1X PBS and nutrient broth were used as control. All the tubes containing cultures including controls were supplemented with 0.1% cyclo heximide (4 mL/L) to inactivate yeasts. Prepared *Salmonella* yeasts mixtures (0.2 mL) and controls were dispensed into flat bottom 96 well microtiter plate (Biorad, California, USA). The optical densities were read at 0, 2, 4, 6, 12 and 24 h using micro plate reader at 595 nm (Biorad, California, USA).

2.3.3. Factors affecting Salmonella adhesion onto yeast cell wall

The pH of yeast and *Salmonella* culture broths were adjusted separately to 2.0, 4.0, 5.0, 7.0, and 8.0 with either 1 M HCl or 1 M

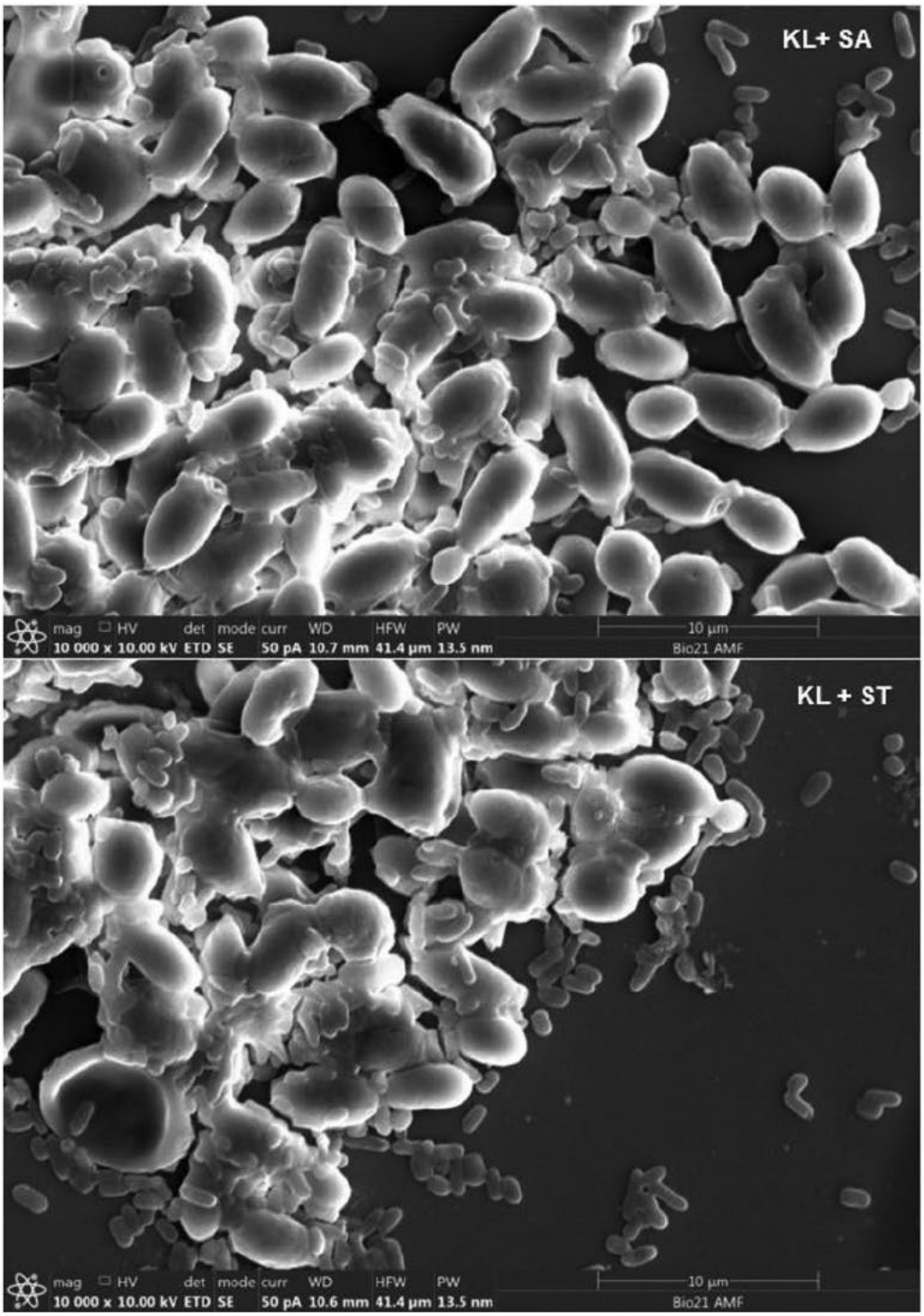

Fig. 1C. Adhesion of *Salmonella* servers onto KL cell walls observed using scanning electron microscope. SA = *S.* Arizonae; ST = *S.* Typhimurium, *KL K. lactis.*

NaOH. *Salmonella* (20 µL, 10^9 CFU/mL) and yeast (10 µL, 10^8 CFU/mL) of same pH were added onto a clean microscope slide, mixed using a sterile inoculating loop, rocked gently and observed under illumination for agglutination as described in the literature (Perez Sotelo et al., 2005). Both viable and non viable yeasts were tested.

Effect of bile salt on *Salmonella* adhesion onto yeast cell wall was performed as described in the literature (Tiago et al., 2012) with some modifications. Briefly, 6 mL each of yeast (10^8 CFU/mL) and *Salmonella* (10^9 CFU/mL) broths were cen trifuged (Eppendorf 5810 R, Hamburg, Germany) separately at 4000*g* for 15 min at 4 °C and washed twice with 1X PBS. The pellets were each re suspended in 6 mL 1X PBS containing 3 g/L bile (sup plemented with 0.1% cycloheximide 4 mL/L). For the control, *S.* Ari zonae and *S.* Typhimurium prepared separately without yeast cells, were similarly treated as above. Qualitative and quantitative anal ysis were performed as described under adhesion and sedimenta tion assays. However, experimental conditions including time, temperature, initial culturing of yeast and *Salmonella* in bile free

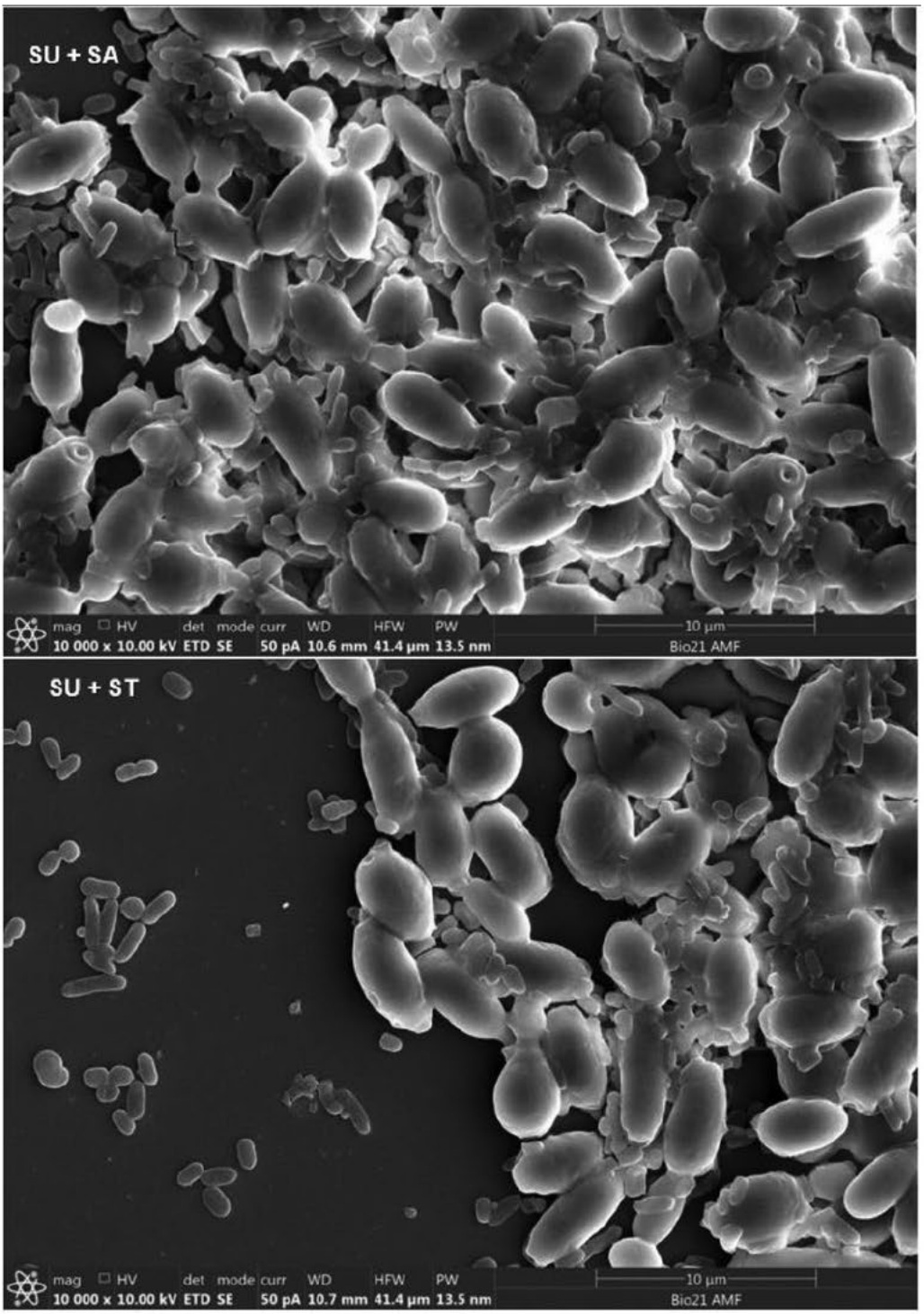

Fig. 1D. Adhesion of *Salmonella* servers onto SU cell walls observed using scanning electron microscope. SA = *S.* Arizonae; ST = *S.* Typhimurium, SU *S. unisporus.*

media were maintained to reflect standardized simulated intesti nal digestive system (Minekus et al., 2014).

2.4. Salmonella growth inhibition by yeast metabolites

2.4.1. Salmonella growth inhibition assay

Salmonella growth inhibition by yeast colonies was performed as described in the literature (Gut et al, 2019). Yeast colonies on YEPDA grown for 72 h at 25 °C were cut and placed onto Muller Hinton agar (supplemented with 0.1% cycloheximide) initially inoculated with 10^4 CFU/mL of *S.* Arizonae and *S.* Typhimurium using spread plate technique. The Muller Hinton agar plates were incubated at 37 °C for 24 h. Plates were checked for growth inhibi tion indicated by complete or partial clearing around the yeast slabs.

Further experimentation to investigate *Salmonella* growth inhi bition (Bajaj et al., 2013) was carried out with some modifications. Yeasts were grown in a killer toxin medium (KTM) composed of YEPDB plus glycerol (50 g/L), buffered at pH 5 using 50 mM citrate phosphate buffer, and incubated at 25 °C under shaking

(180 rpm) for 24 48 h. KTM has been reported to enhance production of killer toxin by yeast species (Bajaj et al., 2013). Sampling for KTM cell free supernatant (KTM CFS) was performed at 24 and 48 h. The yeast fermented KTM was centrifuged (Eppendorf 5810 R, Hamburg, Germany) at 4000*g* for 30 min at 4 °C and filter sterilized using 0.22 μm membrane filter.

KTM CFS was analyzed for antibacterial potential using established turbidimetry assay (broth dilution) with some modifications (Balouiri et al., 2016). Briefly, 0.1 mL of 10^4 CFU/mL of *Salmonella* culture in fresh nutrient broth was dispensed into flat bottom 96 well microtiter plate (Bio Rad, California, USA) containing 0.1 mL of KTM CFS. The optical density was read using microplate reader at 595 nm (Biorad, California, USA) at 0, 2, 4, 6, 12 and 24 h. Growth curves were constructed using a Microsoft Excel.

To determine if the effect of KTM CFS on *Salmonella* demonstrated by turbidimetry assay is bacteriostatic or bactericidal, 5 mL of the KTM CFS was inoculated with 0.5 mL of *Salmonella* cultures (approximately 10^3 CFU/mL). Sampling was performed at < 1, 30, 60, 90, 120 and 240 min by plating 0.1 mL onto nutrient agar, incubated in 37 °C for 24 h before counting colonies. For control, unfermented KTM was inoculated with *Salmonella* and tested along with the samples.

2.4.2. Determination of alcohol content in KTM and its effect on *Salmonella* species

Alcohol content of KTM CFS was analyzed quantitatively by gas chromatography as described in a previous study (Nikolaou et al., 2017) with some modifications. Gas chromatography (Shimazdzu, Kyoto, Japan) with SGE BP20 GC capillary column (12.0 m length, 0.22 mm inner diameter, 0.25 μm film thickness, Fisher Scientific, Hampton, USA) and flame ionization detector at 200 °C was used. Samples were filter sterilized through a 0.22 μm membrane filter (Sigma Aldrich, St. Louis, USA) and 5 μL was injected into the column. The oven temperature was set as 35 °C for 5 min, then increased to 200 °C at a rate of 10 °C/min. The injector temperature was maintained at 200 °C, with a split ratio of 50:1 and flow rate of 1.1/min. Analysis of the results were performed using a Lab Solution software (Shimazdzu, Kyoto, Japan). Alcohol concentration was calculated using 6 point standards (0, 125, 250, 500, 750 and 1000 mM/L; standard curve (R^2 > 0.99) created with absolute ethanol.

To determine if the concentration of ethanol in yeast fermented KTM is responsible for *Salmonella* growth inhibition demonstrated by the turbidimetry assay, ethanol control (1, 1.5 and 2% in sterile distilled water) effect on *Salmonella* was investigated. Five milliliters of each control concentration was inoculated with 0.5 mL of *Salmonella* cultures (approximately 10^3 CFU/mL). Sampling time, plating, incubation and colony counting were performed as described under 2.4.1.

To determine if the growth inhibition was due to other volatile metabolites other than ethanol, KTM CFS was heated in loosely capped centrifuge tubes at 60 °C for 10 min in a shaking incubator at 120 horizontal strokes per min. Five milliliters of the heat treated KTM CFS was inoculated with 0.5 mL of *Salmonella* cultures (approximately 10^3 CFU/mL) prepared above. Sampling time, plating, incubation and colony counting were performed as described under 2.4.1.

2.4.3. Shotgun proteomics

Yeast fermented KTM and unfermented KTM (control) samples were analyzed for proteins and peptides identification using shotgun proteomics. Samples were diluted to between 1 and 5 mg/mL total protein concentration, and approximately 10 μg/L total protein of each was buffer exchanged into 50 mM ammonium bicarbonate and the protein was reduced in 2.5 mM DTT at 95 °C for 5 min followed by alkylation with 10 mM chloroacetamide for 30 min at ambient temperature. Trypsin was then added at the rate of 0.5 μg per 10 μg of protein and incubated at 37 °C overnight. All enzyme digests were analyzed by LC MS/MS using the QExactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany) coupled online with a RSLC nano HPLC (Ultimate 3000, Thermo Scientific, Bremen, Germany). Two hundred nanograms of sample was injected and concentrated on a 100 μm, 2 cm nano viper pepmap100 trap column with 97.5% buffer A (0.1% Trifluoroacetic acid) at a flow rate of 15 min^{-1}. The peptides were then eluted and separated with a Thermo RSLC pepmap100, 75 μm × 50 cm, 100 Á pore size, reversed phase nano column with a 30 min gradient of 92.5% buffer A (0.1% formic acid) to 42.5% B (80% acetonitrile 0.1% formic acid), at a flow rate of 250 nL min^{-1}. The eluent was nebulised and ionised using the Thermo nano electrospray source with a distal coated fused silica emitter (New Objective, Woburn, MA, USA) with a capillary voltage of 1900 V. Peptides were selected for MS/MS analysis in full MS/dd MS2 (TopN) mode with the following parameter settings: TopN 10, resolution 70000, MS/MS AGC target 5e5, 118 ms Max IT, NCE 27, 1.8 *m/z* isolation window, dynamic exclusion was set to 10 s. Results were analyzed using MaxQuant to obtain protein identifications and their respective label free quantification values using in house standard parameters. Data were normalized based on the assumption that the majority of proteins do not change between the different conditions. Protein identification numbers were also used to verify protein names using Uniprot database.

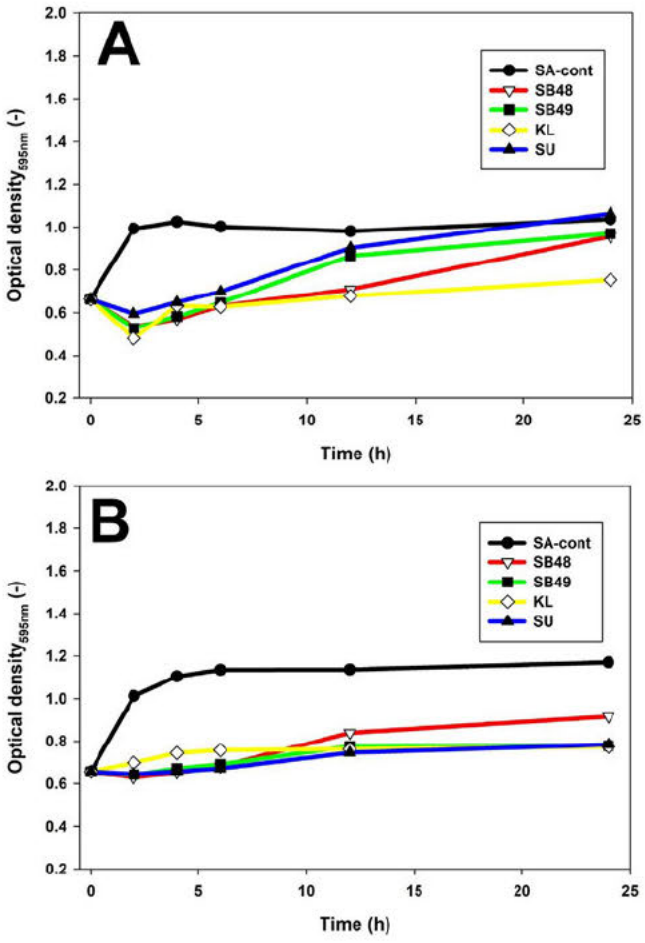

Fig. 2. *Salmonella* real time growth analysis in presence of cyloheximide inactivated yeast cells. A, *S.* Arizonae; B, *S.* Typhimurium.

2.5. Statistical analysis

Experiments were performed in triplicates and subsampled. Results were expressed as the mean ± standard deviation (SD). Means differences were statistically analyzed using Student's *t* test. Mean differences were considered significant at $p < 0.05$. Statistical analysis was performed using SPSS version 26 Statistical software (IBM, New York, USA). Growth curves were constructed using Microsoft Excel 2016 (Microsoft, Washington, United States). The means for all the experiment followed a normal distribution. For shotgun proteomics, statistical analysis was performed using Perseus. The LFQ data was converted to $\log_2$ scale, samples were grouped by conditions and missing values were imputed based on normal distributions after all proteins were eliminated that had 2 or less valid values. Protein fold changes were calculated and their significance was determined using a two sided *T* test with error corrected P values.

3. Results

3.1. Adhesion of Salmonella onto yeast cells

Adherence of *S.* Arizonae and *S.* Typhimurium onto viable and non viable yeast cells was qualitatively and quantitatively analyzed (Table 1). Colony count of the two *Salmonella* serovars after sedimentation with *K. lactis*, *S. unisporus* and *S. boulardii* strains were lower ($p < 0.05$) compared to the controls for both viable and non viable yeast cells. Agglutination on the slides which is an indication of aggregation (between *Salmonella* and yeast cells) was also observed in all cases (Table 1). Figs. 1A, 1B, 1C and 1D show clear adherence of *Salmonella* onto viable yeast strains observed using a scanning electron microscope. Observed adhesions of *Salmonella* onto non viable kefir yeast isolates and *S. boulardii* strain (data not shown) appeared similar to that onto viable yeasts. Scanning electron microscope results provide clear visual evidence that *Salmonella* count reduced after treatment with yeast, likely caused by attachment to yeast cells and subsequent settling at the bottom of the tubes.

The growth behavior of *Salmonella* in presence of inactivated yeast cells was determined at different time points using optical density (Fig. 2). Growth rate of *S.* Arizonae was lower in all yeast cells compared to the control ($p < 0.05$) until 12th h (Fig. 2). On the other hand, *S.* Typhimurium had its growth significantly suppressed by all yeasts compared to the control ($P < 0.05$) until the experiment was stopped at 24 h (Fig. 2).

3.2. Factors affecting Salmonella adhesion onto yeast cell walls

The effect of pH on adhesion was investigated qualitatively at pH 2, 4, 5, 7 and 8 (Table 1). The gastrointestinal tract (GIT) pH ranged from 3 to 7, with the stomach being more acidic (Minekus et al., 2014). There was no difference in observable agglutination with all the pH levels, which indicated that pH did not interfere with adhesion.

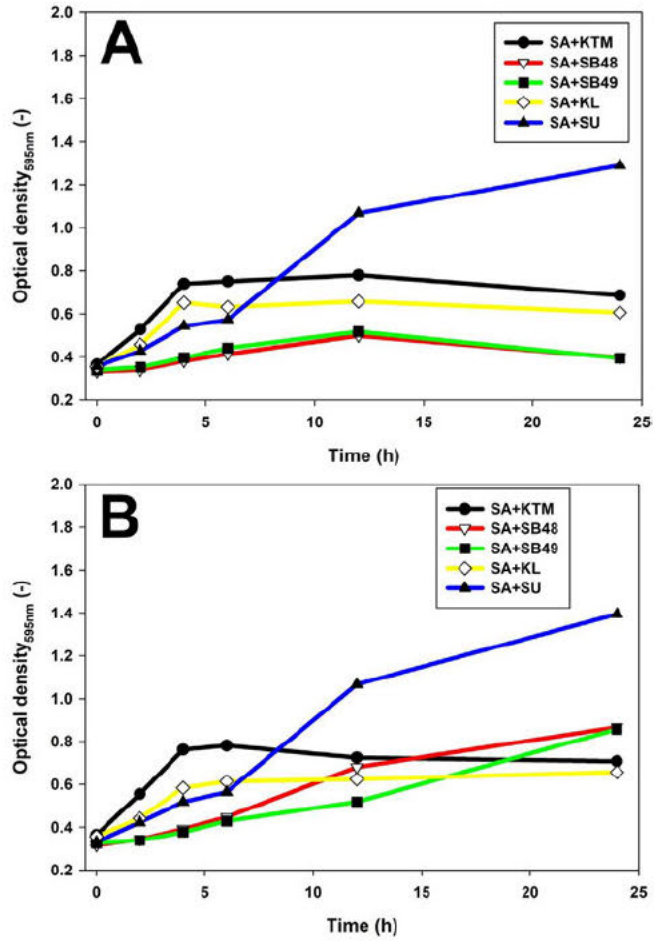

Fig. 3. *S.* Arizonae growth inhibition by yeast fermented KTM using optical turbidimetry assay. A, KTM fermented for 24 h; B, KTM fermented for 48 h.

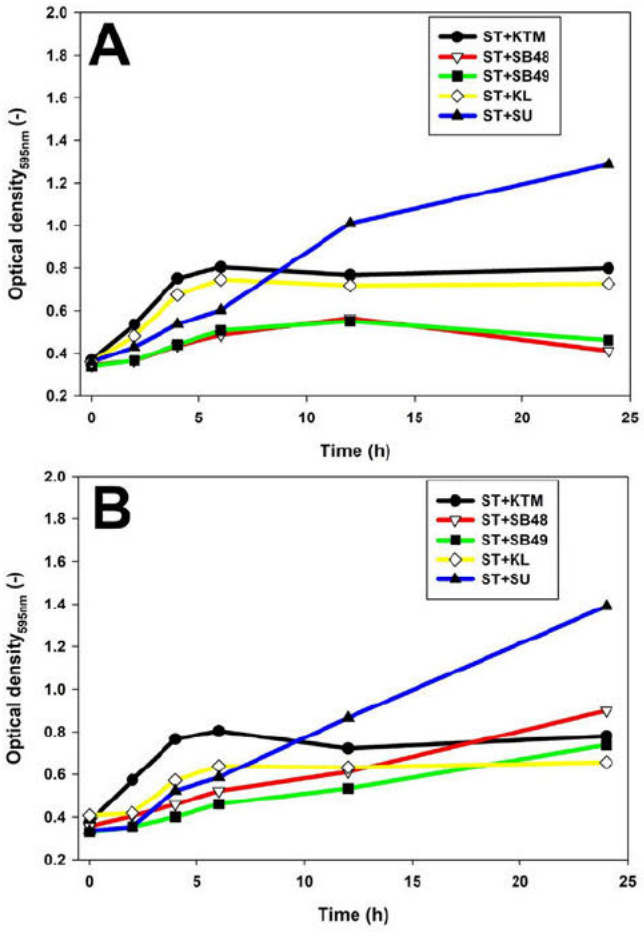

Fig. 4. *S.* Typhimurium growth inhibition by yeast fermented KTM using optical turbidimetry assay. A, KTM fermented for 24 h; B, KTM fermented for 48 h.

Bile salt (0.3%) had no effect on adhesion of *Salmonella* onto *K. lactis*, S. *unisporus*, and *S. boulardii* strains (Table 1). Slide agglutination in presence of bile did not interfere with adhesion of *Salmonella* onto yeast cell walls with exception of *K. lactis*, which showed weak clumping on slide agglutination.

3.3. Salmonella growth inhibition by yeast antimicrobial metabolites

3.3.1. Salmonella growth inhibition by yeasts metabolites

Potential presence of anti *Salmonella* molecules produced by yeast was investigated using slab on the lawn assay, and growth inhibition was not observed (data not shown). The effect of potentially antimicrobial metabolites in KTM CFS was assessed (Figs. 3 & 4) using a turbidimetry assay. *Salmonella* growth was suppressed by *S. unisporus* KTM CFS in the first 5 h, but started to grow rapidly thereafter, compared to the control. However, *K. lactis* exhibited growth rate suppression of *S.* Typhimurium at all time points analyzed. Forty eight hour fermentation of KTM with *K. lactis* showed stronger growth inhibition of *Salmonella* compared to 24 h (Figs. 3 & 4). *S. boulardii* strains showed significant growth rate reduction of *Salmonella* compared to kefir yeast isolates (Figs. 3 & 4). Fig. 5 showed that *Salmonella* count remained constant without significant decrease or increase in yeast fermented KTM, which indicated growth inhibition effect.

3.3.2. Assessment of alcohol content in KTM

To confirm the presence of alcohol in KTM CFS, GC analysis was performed with data shown in Table 2. Fermentation of KTM with *S. boulardii* showed significantly higher concentration of alcohol after 24 h compared to that at 48 h. The concentration of alcohol produced by kefir yeast isolates in KTM CFS from 48 h fermentation was also lower than that at 24 h (Table 2). Experimentation to determine if alcohol in yeasts fermented KTM is responsible for growth inhibition above showed no effect on *Salmonella*, possibly due to low concentration (data not shown). Heat treatment of the fermented KTM CFS to remove potential volatile antimicrobial compounds did not have effect on *Salmonella* growth for *K. lactis*, and *S. boulardii* strains. However, *S. unisporus* fermented KTM CFS growth enhancing effect on *S. arizonae* was lost (Fig. 6).

Table 2
Analysis of alcohol content in fermented KTM using GC.

Yeast strain	Alcohol concentration (% V/V)	
	24 h KTM fermentation	48 h KTM fermentation
SB48	1.55 ± 0.16[a]	0.64 ± 0.01[a]
SB49	1.42 ± 0.01[a]	0.43 ± 0.04[a]
KL	1.30 ± 0.06[a]	1.21 ± 0.17[a]
SU	1.46 ± 0.01[a]	1.32 ± 0.07[a]
KTM blank	0.08 ± 0.05[b]	0.08 ± 0.05[b]

Means with different superscript are significantly different ($p < 0.05$)

3.3.3. Assessment of antimicrobial proteins in KTM

Production of proteins with potential antimicrobial properties in yeasts fermented KTM was assessed using shotgun proteomics. Proteins shown in Table 3 were produced in substantial quantities

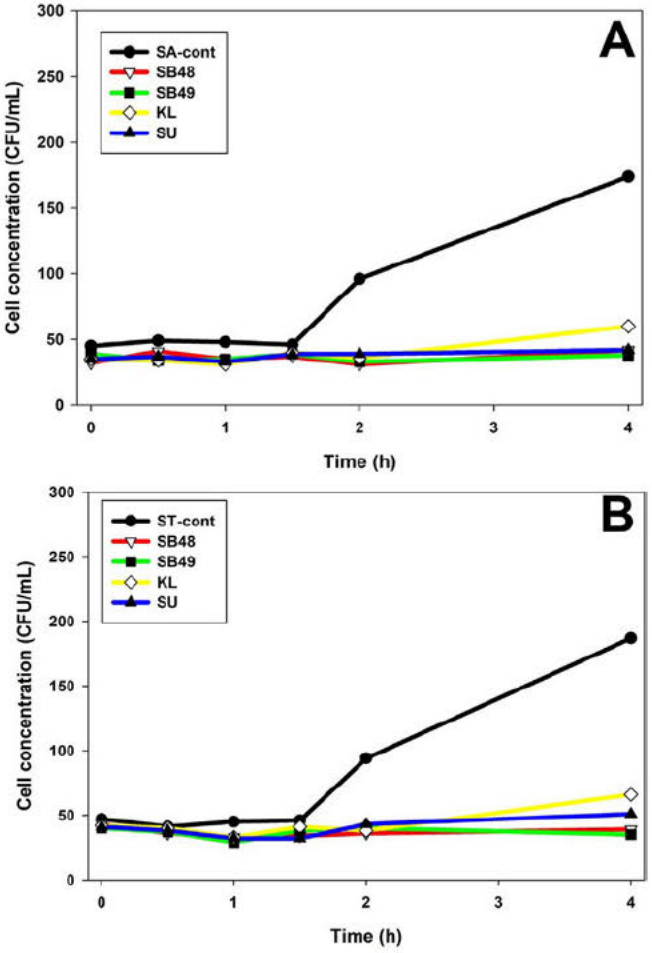

Fig. 5. Determination of bacteriostatic and bactericidal effect of KTM on *Salmonella* using colony forming unit counting essay. A; *S.* Arizonae; B *S.* Typhimurium.

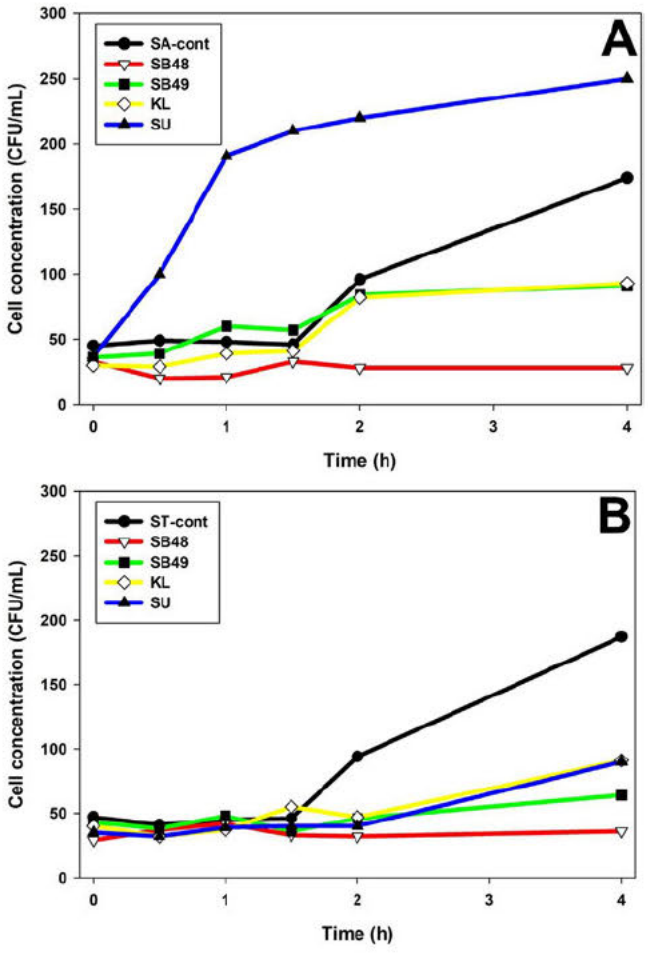

Fig. 6. Determination of bacteriostatic and bactericidal effect of heat treated KTM on *Salmonella* using colony forming unit counting essay. A; *S.* Arizonae; B *S.* Typhimurium.

Table 3
Shotgun proteomic analysis of yeast fermented KTM.

SB48	SB49	KL	SU
40S ribosomal protein S14-A	40S ribosomal protein S14-A	Fructose-bisphosphate	60 kDa chaperonin
40S ribosomal protein S14-B	40S ribosomal protein S14-B	aldolase	60S ribosomal protein L20-B
40S ribosomal protein S29	60S ribosomal protein L20-B	Lactadherin*	60S ribosomal protein L20-A
60 kDa chaperonin	60S ribosomal protein L20-A	40S ribosomal protein S14-A	60S ribosomal protein L21
60S ribosomal protein L13	60S ribosomal protein L36	40S ribosomal protein S14-B	60S ribosomal protein L28
60S ribosomal protein L20-B	Actin cytoplasmic 1	40S ribosomal protein S24	60S ribosomal protein L34
60S ribosomal protein L20-A	Adenosylhomocysteinase	40S ribosomal protein S29	60S ribosomal protein L36
60S ribosomal protein L28	Alcohol dehydrogenase 2	60 kDa chaperonin	60S ribosomal protein L8
60S ribosomal protein L34	Alcohol dehydrogenase 1	60S ribosomal protein L13	Actin, cytoplasmic 1
60S ribosomal protein L35a,	ATP-dependent molecular chaperone	60S ribosomal protein L14	Alcohol dehydrogenase 2
60S ribosomal protein L35a	HSC82;ATP-dependent molecular	60S ribosomal protein L20-B	Alcohol dehydrogenase 1
60S ribosomal protein L36	chaperone HSP82	60S ribosomal protein L20-A	ATP-dependent molecular chaperone HSC82; ATP-
60S ribosomal protein L37a	Cathelicidin-2*	60S ribosomal protein L21	dependent molecular chaperone HSP82
60S ribosomal protein L8	Cathelicidin-6*	60S ribosomal protein L28	Broad substrate specificity ATP-binding cassette
Actin, cytoplasmic 1	Cathelicidin-7*	60S ribosomal protein L34	transporter ABCG2 ATP-binding cassette sub-family
Acyl-CoA-binding protein	Cysteine-rich secretory protein 2	60S ribosomal protein L34-A	G member 2
Adenosylhomocysteinase	Elongation factor 2	60S ribosomal protein L34-B	Calmodulin
Alcohol dehydrogenase 2	Enolase 1	60S ribosomal protein L35a	Cathelicidin-2*
Alcohol dehydrogenase 1	Enolase 2	60S ribosomal protein L36	Cathelicidin-6*
ATP synthase subunit alpha	Fructose-bisphosphate aldolase	60S ribosomal protein L37a	Cathelicidin-7*
mitochondrial	Glucan 1,3-beta-glucosidase I/II	60S ribosomal protein L8	CD59 glycoprotein
ATP-dependent molecular	Glyceraldehyde-3-phosphate	Actin, cytoplasmic 1	Cystatin E/M
chaperone HSC82	dehydrogenase 2 Glyceraldehyde-3-	Acyl-CoA-binding protein	Cystatin-C
ATP-dependent molecular	phosphate dehydrogenase 3	Adenosylhomocysteinase	Cysteine-rich secretory protein 2
chaperone HSP82	Heat shock protein SSB2	Alcohol dehydrogenase 2	Endoplasmic reticulum chaperone BiP
Broad substrate specificity	Heat shock protein SSB1	Alcohol dehydrogenase 1	Enolase 1
ATP-binding cassette	Histatherin	ATP synthase subunit alpha	Enolase 2
transporter ABCG	Immunoglobulin J chain	mitochondrial	Eukaryotic initiation factor 4A-I
ATP-binding cassette sub-	Inositol polyphosphate-5-phosphatase E	ATP synthase subunit beta	Fatty acid-binding protein, heart
family G member 2	Keratin 24	mitochondrial	Folate receptor alpha,
Butyrophilin subfamily 1	NPC intracellular cholesterol transporter 2	ATP-dependent molecular	Folate receptor 2Fructose-bisphosphate aldolase
member A1	Nucleobindin 2	chaperone HSC82;	Glucan 1,3-beta-glucosidase I/II
Calmodulin	Peptidyl-prolyl cis-trans isomerase	ATP-dependent molecular	Glycoprotein 2
Cathelicidin-2*	Pyruvate kinase 1	chaperone HSP82	Heat shock protein SSB2
cathelicidin-4*	Secretoglobin family 1D member	Beta-1,4-galactosyltransferase	Heat shock protein SSB1
cathelicidin-6*	Selenoprotein M	1	Heterogeneous nuclear ribonucleoproteins A2/B1
cathelicidin-7*	Translationally-controlled tumor protein	Beta-2-microglobulin	Histatherin
CD59 glycoprotein	homolog	Broad substrate specificity	Immunoglobulin J chain
CD9 antigen	Transthyretin	ATP-binding cassette	Inositol polyphosphate-5-phosphatase E
Cystatin E/M	Triosephosphate isomerase	transporter ABCG2	Keratin 24
Cysteine-rich secretory	Ubiquitin-like protein SMT3	Butyrophilin subfamily 1	Lactadherin
protein 2	Uncharacterized protein	member A1	Lactoperoxidase
Elongation factor 2	Uncharacterized protein	Calmodulin	Lipoprotein lipase
Endoplasmic reticulum	Vacuolar protein sorting-associated	Cathelicidin-1*	Monocyte differentiation antigen CD14
chaperone BiP	protein 53 homolog	Cathelicidin-2*	Mucin-1*
Enolase 1		Cathelicidin-4*	NPC intracellular cholesterol transporter 2
Enolase 2		Cathelicidin-6*	Nucleobindin 1
Enoyl-CoA hydratase,		Cathelicidin-7*	Nucleobindin 2
mitochondrial		CD59 glycoprotein	Parathyroid hormone-related protein
Eukaryotic initiation factor		CD9 antigen	Peptidyl-prolyl cis-trans isomerase A
4A-I Eukaryotic initiation		Cellular repressor of E1A	Polymeric immunoglobulin receptor
factor 4A-II		stimulated genes 1	Prostaglandin-H2 D-isomerase
Eukaryotic translation		Collagen alpha-2(I) chain	Pyruvate kinase 1
initiation factor 2 subunit		Cystatin domain	Secretoglobin family 1D member
gamma		Cystatin E/M	Selenoprotein M
Fatty acid-binding protein		Cystatin-C	SET nuclear oncogene
heart		Cysteine-rich secretory protein	Solute carrier family 38 member 10
FGG protein		2	TGOLN2 protein
Fibrinogen gamma-B chain		Elongation factor 2	Translationally-controlled tumor protein homolog
Folate receptor alpha		Endoplasmic reticulum	Transthyretin
Fructose-bisphosphate		chaperone BiP	U6 snRNA-associated Sm-like protein LSm4
aldolase		Enolase 1	Ubiquitin-40S ribosomal protein S27a Ubiquitin-60S
Glucan 1,3-beta-glucosidase		Enolase 2	ribosomal protein L40 Polyubiquitin-B
I/II		Enoyl-CoA hydratase,	Polyubiquitin-C
Glyceraldehyde-3-		mitochondrial	Ubiquitin-like protein SMT3
phosphate dehydrogenase 2		Eukaryotic initiation factor 4A-	Uncharacterized protein
Glyceraldehyde-3-		I Eukaryotic initiation factor	Uncharacterized protein
phosphate dehydrogenase 3		4A-II	Uncharacterized protein
Glycoprotein 2		Eukaryotic translation	Vacuolar protein sorting-associated protein 53
Heat shock protein SSB2;		initiation factor 2 subunit	homolog
Heat shock protein SSB1		gamma	WAP four-disulfide core domain 2
Heterogeneous nuclear		Fatty acid-binding protein	Xanthine dehydrogenase/oxidase
ribonucleoproteins A2/B1		heart	

Table 3
Shotgun proteomic analysis of yeast fermented KTM.

SB48	SB49	KL	SU
Histatherin		FGG protein	
Ig-like domain-containing protein		Fibroblast growth factor-binding protein 1	
Immunoglobulin J chain		Folate receptor alpha	
Inositol polyphosphate-5-phosphatase E		Glucan 1,3-beta-glucosidase I/II	
Keratin 24		Glyceraldehyde-3-phosphate dehydrogenase	
Lactadherin*		Glyceraldehyde-3-phosphate dehydrogenase 2	
Lactoperoxidase*		Glyceraldehyde-3-phosphate	
Lactotransferrin*		dehydrogenase 3	
Lipoprotein lipase		Glycoprotein 2	
Monocyte differentiation antigen CD14		Glycosylation-dependent cell adhesion molecule 1	
Mucin-1*		Granulin precursor	
NPC intracellular cholesterol transporter 2		Heat shock 70 kDa protein 1A	
Nucleobindin 2		Heat shock protein SSB2	
Nucleobindin-1		Heat shock protein SSB1	
Parathyroid hormone-related protein		Helix-destabilizing protein	
Peptidyl-prolyl cis-trans isomerase		Heterogeneous nuclear ribonucleoprotein K	
Peptidyl-prolyl cis-trans isomerase A		Heterogeneous nuclear ribonucleoproteins A2/B1	
Perilipin-2		HHIP like 2	
Platelet glycoprotein 4		Histatherin	
Polymeric immunoglobulin receptor,		Ig-like domain-containing protein	
Prostaglandin-H2 D-isomerase		Immunoglobulin J chain	
Protein BMH1		Inorganic pyrophosphatase	
Protein BMH2		Inositol polyphosphate-5-phosphatase E	
Ribosomal protein L21e		Keratin 24	
60S ribosomal protein L21		Lactoperoxidase*	
Secretoglobin family 1D member		Lactotransferrin*	
Selenoprotein M		Lipoprotein lipase	
Serum amyloid A protein*		Monocyte differentiation antigen CD14	
SET nuclear oncogene		Mucin-1*	
snRNA-associated Sm-like protein LSm4		Myosin heavy chain 9	
Sodium-dependent phosphate transport protein 2B		NPC intracellular cholesterol transporter 2	
Solute carrier family 38 member 10		Nucleobindin 2	
Sulfhydryl oxidase		Parathyroid hormone-related protein	
TGOLN2 protein		Peptidyl-prolyl cis-trans isomerase	
Translationally-controlled tumor protein homolog		Peptidyl-prolyl cis-trans isomerase A	
Transthyretin		Perilipin-2	
Triosephosphate isomerase		Platelet glycoprotein 4	
Ubiquitin-40S ribosomal protein S27a		Polymeric immunoglobulin receptor	
Ubiquitin-60S ribosomal protein L40		Prostaglandin-H2 D-isomerase	
Polyubiquitin-B, Polyubiquitin-C		Pyruvate kinase 1	
Ubiquitin-like protein SMT3		Ribonuclease pancreatic	
Uncharacterized protein		Ribosomal protein L37	
Uncharacterized protein		Secretoglobin family 1D member	
Uncharacterized protein		Selenoprotein M	
Vacuolar protein sorting-associated protein 53 homolog		SET nuclear oncogene	
WAP four-disulfide core domain 2		Sodium-dependent phosphate transport protein 2B	
Xanthine dehydrogenase/oxidase*		Solute carrier family 38 member 10	
Zinc-alpha-2-glycoprotein		Sulfhydryl oxidase	
		TGOLN2 protein	
		Translationally-controlled tumor protein homolog	
		Transthyretin	
		Triosephosphate isomerase	
		U6 snRNA-associated Sm-like protein LSm4	
		Ubiquitin thioesterase	
		Ubiquitin-60S ribosomal protein L40, Polyubiquitin-C, Polyubiquitin-B	
		Ubiquitin-60S ribosomal protein L40 Ubiquitin-40S ribosomal protein S31	
		Polyubiquitin	
		Ubiquitin	
		Ubiquitin-like protein SMT3	
		Uncharacterized protein	
		Uncharacterized protein	
		Uncharacterized protein	
		Uncharacterized protein	
		Vacuolar protein sorting-associated protein 53 homolog	
		WAP four-disulfide core domain 2	
		Xanthine dehydrogenase/oxidase*	
		Zinc-alpha-2-glycoprotein	

Key: * = protein with known antimicrobial properties.

in yeasts fermented KTM, but present in trace amount or absent in KTM blank.

4. Discussion

Adhesion is defined as a process whereby cells attach to surfaces of each other with the aid of adhesins (Brückner & Mösch, 2012). The adherence of *Salmonella* and other enteric bacterial pathogens onto yeast cell wall is postulated to be due to specific binding of type 1 fimbriae on bacteria cell with mannan oligosaccharides on yeast cell wall (Gut et al., 2018). Moreover, non specific adhesion mechanisms including electrostatic and hydrophobic attachment between bacteria and yeasts have been reported (Adegbola & Old, 1985; Pérez Sotelo et al., 2005; Tiago et al., 2012).

Adhesion of the two *Salmonella* serovars onto kefir yeast isolates and *S. boulardii* strains (Table 1, Fig. 1A D) are consistent with a previous study in which Gram negative enteric bacteria (*Escherichia coli* and *Enterobacter aerogenes*) were bound to kefir yeast isolates and *S. boulardii* strains (Gut et al., 2019). Furthermore, similar results showed that enteropathogenic bacteria including *S.* Typhimurium (ATCC 14028) adhered onto viable and non viable yeast cells (França et al., 2015; Martins et al., 2010; Posadas et al., 2017). The growth trend of *Salmonella* in the presence of inactivated yeasts (Fig. 2) was likely due to *Salmonella* binding to yeast cells and subsequent sedimentation. Bacteria cells in tight auto aggregation have been reported to show no cell division (Hassani et al., 2009). This therefore resulted in reduced *Salmonella* population, as was indicated in a less turbid medium compared to the control. Both kefir yeast isolates and *S. boulardii* strains were found to survive well in simulated GIT conditions (Gut et al., 2019). Yeasts survival and proliferation may lead to higher numbers in GIT due to potential growth, increasing the capacity to bind *Salmonella* and subsequent shedding in feces. Furthermore, the advantage of yeasts is the fact that they are not affected by drugs targeting infectious bacteria such as *Salmonella,* making them suitable candidates for complementary therapy with antibiotics. For example chloramphenicol is an antibiotic typically used in *Salmonella* treatment (Gut et al., 2018) and works against bacteria by binding to ribosomes and blocking protein synthesis but does not affect yeasts (Das & Patra, 2017; Gut et al., 2019). The current study also established that non viable yeast cells were as effective in *Salmonella* attachment as live yeast cells. This may have critical therapeutic or prophylactic application advantage as viable yeast cells have been associated with at least 100 fungemia cases (Gut et al., 2018). For example, the use of viable yeast cells in immunocompromised people or those with gastrointestinal disease has been reported to pose serious threat of fungemia (Kelesidis & Pothoulakis, 2012). Use of non viable kefir yeast either prophylactically or as a complementary therapy for *Salmonella* infection may be a better choice for people with GIT diseases or compromised immunity.

Effect of pH on adhesion of *Salmonella* onto yeast cell walls (Table 1) was in line with a previous research (Tiago et al., 2012), which reported that pH between 4 and 8 had no effect on bacteria attachment onto yeast cell wall. Adhesion of *Salmonella* onto yeast cell wall at GIT pH is important because it is where invasion of pathogen and gastroenteritis occur. Therefore, if *Salmonella* could attach to yeast cells in the GIT under acidic condition, invasion may be prevented. Furthermore, bile salt has high surface activity (Attili et al., 1986) and reduces surface hydrophobicity (Tiago et al., 2012), therefore may prevent adhesion. However, 0.3% bile salt did not prevent adhesion in this study (Table 1) which correlated with previous reports (Gómez et al, 2002; Guglielmetti et al., 2009; Tiago et al., 2012). Adherence in presence of bile is important since it will not interfere with potential prophylactic or therapeutic application in GIT.

Growth inhibitory properties of yeast against bacteria have been previously proposed to include production of ethanol and other antibacterial metabolites such as killer toxins (Bajaj et al., 2013; Muccilli & Restuccia, 2015). Many mechanisms of probiotics including yeasts against susceptible microbial cells have been proposed and involve destabilization of the cell membrane, cell lysis, degradation of nucleic acid, inhibition of protein synthesis and binding onto yeasts (Gut et al., 2021).

Lack of *Salmonella* growth inhibition by yeast colonies correlated with a previous study on *S. boulardii* effect on enteric bacterial growth (Rajkowska et al., 2012). However, a study reported that killer toxin produced by bakery *Saccharomyces* showed growth inhibition of *Escherichia coli* and *S.* Typhimurium as a result of cell membrane destruction (Alsoufi & Aziz, 2017). Therefore further experimentation involving use of KTM was required. Stronger growth inhibition exhibited by KTM fermented with *K. lactis* for 48 h (Figs. 3 and 4) may be due to accumulation of antibacterial metabolites produced by *K. lactis* when fermentation time was prolonged, consistent with a previous report in which *K. lactis* and *Kluyveromyces marxinus* showed antagonistic effect on *Salmonella* Paratyphi B, *S.* Typhimurium ATCC 14028 and *Salmonella* Enteritidis when fermentation time was extended by downregulating chromosomal sopD gene (Ceugniez et al., 2017). *Salmonella* growth promotion by *S. unisporus* KTM CFS observed in this study after the initial 5 h (Figs. 3 and 4) was likely due to the loss of potential antibacterial molecules coupled with possible presence of growth factors such *as* amino acids and vitamins released by yeasts (Bechtner et al., 2019; Gut et al., 2021; Stadie et al., 2013). This growth promoting effect of *S. unisporus* on *S.* Arizonae was lost likely due to heat inactivation or evaporation of some volatiles (Fig. 6). However, *Salmonella* population generally remained stable in the presence of yeast fermented KTM due to its bacteriostatic effect compared to the control which showed significant increase (Fig. 5).

Studies have confirmed production of alcohol by *Kluyveromyces* and *Saccharomyces* species commonly isolated from kefir (Ho et al., 2012; Magalhães et al., 2010). Concentration of alcohol produced by kefir yeast isolates in Table 2, was consistent with reported alcohol concentrations in the literature (Magalhães et al., 2010; Nuñez, 2016). Furthermore, production of alcohol in KTM fermented with *S. boulardii* is consistent with a previous study in which this yeast strain was used in a beer production (Mulero Cerezo et al, 2019). The differences in concentration between 24 and 48 h fermentation could be due to the attainment of stationary growth phase resulting in constant metabolic activities at 24 h (Mulero Cerezo et al., 2019), and the likelihood of loss due to evaporation during fermentation. Lack of bacteriostatic effect by 2% ethanol control showed that ethanol in the KTM may not be responsible for bacteriostatic effect observed, an indication of presence of other potential antimicrobial metabolites. Suppression of *Salmonella* growth by KTM fermented with SB48, SB49, *K. lactis* and *S. unisporus* after evaporation suggested presence of non volatile molecules, possibly antimicrobial proteins which showed anti salmonella activities. Antimicrobial proteins produced by yeasts (*Saccharomyces* and *Kluyveromyces* species) have been documented to be effective against bacteria (Branco et al., 2017; Rima et al., 2012; Al Sahlany et al., 2020; Hasan et al., 2019; Liu et al., 2018). The shotgun proteomics analysis of KTM CFS confirmed the presence of such proteins which have been shown to have antimicrobial properties. Previous studies have reported antimicrobial activity of cathelicidin (Xia et al., 2015), xanthine dehydrogenase (Okamura et al., 2018), lactotransferrin and mucin 1 (Gut et al., 2018), lactadherin (Sabha et al., 2018), lactoperoxidase (Bafort et al., 2014) and serum amyloid A protein (Kagan et al.,

2012). The mechanisms of these proteins against susceptible bac teria have been reported to involve DNA, RNA, ATP synthesis, or protein synthesis inhibition, as well as disruption in membrane and ionic potential of the cell membrane (Biadała et al., 2020). The proteins marked with asterisk in Table 3 may have inhibited *Salmonella* growth in the current study however, further studies are needed.

5. Conclusion

Kefir yeast isolates obtained from traditional kefir grains showed comparable anti salmonella effect to that of *S. boulardii* with respect to adhesion as well as growth inhibition due to antimicrobial metabolites production. Shot gun proteomics analy sis showed presence of cathelicidin, xanthine dehydrogenase, mucin 1, lactadherin, lactoperoxidase, serum amyloid A protein and lactotransferrin in yeast fermented killer toxin medium which have anti bacterial properties. These proteins in KTM may be responsible for bacteriostatic effect observed in this study. *K. lactis* and *S. unisporus* have potential to be used prophylactically and therapeutically in control of *Salmonella* infection. However, further studies involving cell lines, animals as well as human trials are needed to prove these kefir isolates efficacy in prevention and treatment of *Salmonella* infection.

Credit authorship contribution statement

Abraham Majak Gut: Writing original draft, Writing review & editing. **Todor Vasiljevic:** Supervision, Writing review & edit ing. **Thomas Yeager:** Supervision, Writing review & editing. **Osaana N. Donkor:** Supervision, Writing review & editing.

Declaration of competing interest

The authors declare that they have no known competing finan cial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 8: Conclusions and future research directions

This chapter concludes the thesis by restating the aim of the thesis, highlighting the major findings, and providing future research directions.

8.1. Conclusions

Salmonella has been a global problem both in infection and drug resistance especially in developing countries, thus the need for alternative infection control and prevention measures such as the use of fermented products and their probiotic components.

Salmonellae were rapidly eradicated in kefir due to the action of lactic acid as kefir cell-free supernatant contained a high concentration of lactic acid ranging from 83.59 to 229.92 mM. Other organic acids, ethanol, and proteins detected by HPLC, GC, and shotgun proteomics may have contributed in the eradication of salmonellae.

K. lactis and *S. unisporus* were isolated from the two traditional kefir grains and characterized for potential probiotic properties including survival in the gastrointestinal tract, auto-aggregation, hydrophobicity, and hydrolytic enzymes production. GIT survival, hydrophobicity, auto-aggregation, and hydrolytic enzymes production of kefir yeast isolates were comparable to *S. boulardii* strains. Both kefir yeast isolates, and *S. boulardii* strains used as control produced weak alpha-hemolytic and proteolytic activities, but none produced phospholipases at 30 °C. Thus, the kefir yeast isolates showed potential probiotic properties.

The two kefir yeast isolates were analyzed for anti-salmonella properties. Adhesion and sedimentation assay, slide agglutination, microscopy, and turbidimetry assay were used to analyze the adhesion of *S.* Arizonae and *S.* Typhimurium onto yeast cells. *Salmonella* growth inhibition due to the antimicrobial metabolites produced by yeasts in KTM was analyzed by a slab on the lawn, turbidimetry, tube dilution, and solid agar plating assays. Alcohol and antimicrobial proteins production by yeasts in KTM were analyzed using gas chromatography and shotgun proteomics,

respectively. *Salmonella* adherence onto viable and non-viable yeast isolates cell wall was visually observed under SEM and quantitative analysis showed significant differences between the control and the yeasts. Furthermore, yeast-fermented KTM showed *Salmonella* growth inhibition possibly due to the presence of antimicrobial proteins in synergy with ethanol. These results showed that kefir yeast isolates have anti-salmonella properties.

In congruence with the aim of the thesis and specific objectives, the two traditional kefir drinks as well as their yeast isolates showed anti-salmonella properties and therefore have the potential to be used in the control and prevention of *Salmonella* infection.

8.2. Future research direction

This thesis screened two traditional kefir grains and their yeast isolates for anti-salmonella properties in *in vitro* experiments, and anti-salmonella properties have been confirmed. However, the following future studies are recommended:

1. A comprehensive study to identify and characterize bacterial microbiota in the two traditional kefir gains using various growth media and conditions, and analyze them for probiotic properties including a direct effect on *Salmonella*
2. Isolate, purify and sequence the antimicrobial proteins/peptides detected in kefir and yeast fermented KTM and optimize their productions. Analyze pure forms of the proteins/peptides antagonistic effects on *Salmonella* and other enteric bacterial pathogens.

3. Comprehensive studies involving cell lines, animals as well as human trials to prove these two traditional kefirs and their yeast isolates efficacy in control and prevention of *Salmonella* infection.