

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**ORAL DELIVERY OF MICROBIOME-MODULATING
SYNBIOTICS DERIVED FROM KEFIR VIA
ALGINATE ENCAPSULATION SYSTEMS**

TAN LI LING

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2023

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ALGINATE ENCAPSULATION SYSTEMS**

TAN LI LING

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2023

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Prof Joachim Loo

Authorship Attribution Statement

This thesis contains material from four peer-reviewed publications (in which I am listed as first author) and a patent article (in which I am one of the co-inventors).

Chapter 3 was substantially published in the article:

1. L.L. Tan, C.H. Tan, N.K.J. Ng, Y.H. Tan, P.L. Conway, and S.C.J. Loo. Potential probiotic strains from milk and water kefir grains in Singapore – Use for defense against enteric bacterial pathogens. *Frontiers in Microbiology*, 13 (2022).
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Liquorilactobacillus satsumensis from water kefir yields α -glucan polysaccharides with prebiotic and synbiotic qualities. *Carbohydrate Polymers*, 290, 119515 (2022). <https://doi.org/10.1016/J.CARBPOL.2022.119515>

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5. L.L. Tan, M. Mahotra, S.Y. Chan, and S.C.J. Loo. In situ alginate crosslinking during spray-drying of lactobacilli probiotics promotes gastrointestinal-targeted delivery. *Carbohydrate Polymers*, 286, 119279 (2022).
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The contributions of the co-authors for article 1 are as follows:

- Prof S.C.J. Loo and Dr. C.H. Tan provided the initial project direction.
- I co-designed the study with Dr. C.H. Tan and performed all the laboratory work, with assistance from Ms. N.K.J. Ng, Ms. Y.H. Tan, and Ms. L.F. Tay, at SCELSE. I also analysed the data.
- Prof K. Kline provided various bacterial strains used in this study.
- I prepared the manuscript drafts. The manuscript was revised by Prof S.C.J. Loo and Prof P.L. Conway.

The contributions of the co-authors for article 2 are as follows:

- Prof S.C.J. Loo and I conceived the initial project direction.
- I co-designed the study with Prof P.L. Conway and performed all the laboratory work, with assistance from Mr. E.S.Z. Sim and Mr. W.J. Kong, at the NTU MSE and SCELSE. I also analysed the data.
- Mr. J.J. Ngiam performed genomic analyses of the gut microcosm.
- Proton nuclear magnetic resonance spectroscopy was conducted by Ms. C. Lee at the NTU Centre of High Field NMR Spectroscopy and Imaging.
- Prof K. Kline provided various bacterial strains used in this study.
- I prepared the manuscript drafts. The manuscript was revised by Prof S.C.J. Loo and Prof P.L. Conway.

The contributions of the co-authors in article 3 are as follows:

- Prof S.C.J. Loo and I conceived the initial project direction.
- I co-designed the study with Prof S.C.J. Loo.
- I performed all the laboratory work and analysed all data, with assistance from K.L. Ang.
- I prepared the manuscript drafts. The manuscript was revised by Prof S.C.J. Loo.

The contributions of the co-authors in articles 4 and 5 are as follows:

- I conceived the initial project direction, together with Prof S.C.J. Loo and Mr. M. Mahotra.
- I co-designed the study with Mr. M. Mahotra. I performed all laboratory work, with assistance by Ms. S.Y. Chan, and Mr. M. Mahotra conducted the spray-drying experiments, at SCELSE and NTU MSE. I also analysed the data.
- Scanning electron microscopy was conducted Mr. M. Mahotra at the NTU FACTS.
- I prepared the manuscript and patent drafts. The manuscript was revised by Mr. M. Mahotra and Prof S.C.J. Loo. The patent was reviewed by NTUitive, NTU's innovation and enterprise company.

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Abstract

Probiotics and prebiotics, collectively known as synbiotics, have become increasingly popular in recent years as preventative health nutritive ingredients, due to their ability to modulate the human gut microbiota. A search for novel probiotics and prebiotics exists to add diversity to existing candidates and unlock different mechanisms for health efficacies. This research zooms in on the fermented beverage, kefir, as a source of novel probiotics and prebiotics, and is interested in the characterisation of these components for use as functional health ingredients. Various phenotypic and genotypic screening assays were conducted to identify kefir probiotic strains with highest probiotic potential, while material extraction, structural analysis, and screening methods were employed to derive potential prebiotic materials from kefir. The synergistic effects of kefir prebiotics and probiotics was also studied, and the resultant synbiotics formulation was evaluated for its inhibitory properties against enteric pathogens, effects on simulated large bowel microbiota communities, and short chain fatty acids production. In addition, this research explores methods of encapsulating probiotics to facilitate their delivery to the human intestine and incorporation into food and beverage products. Two techniques, extrusion and spray-drying were used for probiotics encapsulation. These encapsulation systems were found to improve the acid survivability, bile resistance, and shelf-life stability of encapsulated probiotics, and facilitated incorporation of probiotics into beverages, such as carbonated sodas and beers. This thesis has established several interesting probiotic strains and prebiotic materials, and demonstrated the utility of probiotic encapsulation systems based on extrusion and spray-drying techniques. Moving forward, *in vivo* animal testing and clinical trials are required to further realise the commercial potential of these kefir-derived synbiotics and probiotics delivery systems in various industrial applications, including in the pharmaceuticals, dietary supplements, food and beverages, and animal feeds sectors.

Lay Summary

In recent years, scientific studies have shown that the health of an individual is highly dependent on the “good bugs” in our gut. With a paradigm shift towards disease prevention rather than treatment, probiotics and prebiotics may therefore hold the key in shaping one’s health, keeping one in the pink of health. In this thesis, novel types of probiotics and prebiotics were identified from the popular and healthy fermented beverage, kefir. Best performing probiotics and prebiotics were combined in a synbiotics formulation, and the kefir synbiotics showed the ability to reduce pathogenicity of disease-causing microorganisms which infect the human gastrointestinal tract. Kefir synbiotics also demonstrated the ability to influence large bowel microbial communities and generated beneficial short chain fatty acids as metabolic by-products. Another aspect of research in this thesis is in the development of encapsulation systems for probiotics. Probiotics, being delicate, living microorganisms, are susceptible to harsh environments, such as in our stomach, in certain food and beverage matrices, and during shelf-storage. To increase the efficacy of probiotics, encapsulation systems were developed in this thesis to protect probiotics and deliver them to specific sites of the intestine where they function best. Two types of encapsulation systems were covered in this research, a “wet” encapsulation method, which is useful for incorporating probiotics into food and beverage matrices, and a “dry” encapsulation method, which can be versatily incorporated into various dietary supplement or food formats. The “wet” encapsulation system demonstrated utility for the incorporation of probiotics into novel beverage formats, such as carbonated sodas and beers. The “dry” encapsulation technique was useful in enhancing acid and bile resistance and shelf-life stability of encapsulated probiotics. Overall, findings from this work have helped to advance research in the use of probiotics and prebiotics as functional dietary ingredients. Future work would focus on maturing the technologies and formulations developed in this thesis, to facilitate their translation into commercially viable products.

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Abbreviations

¹ H-NMR	Proton nuclear magnetic resonance
AA	Antioxidant activity
ABV	Alcohol by volume
ACN	Acetonitrile
Alg	Spray-dried alginate formulation
Alg-Suc	Spray-dried alginate with sucrose
AMR	Antimicrobial resistance
ANI	Average nucleotide identity
ASV	Amplicon sequence variant
a _w	Water activity
BA	Biogenic amine
BL	<i>Bifidobacterium longum</i>
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSH	Bile salt hydrolase
CAGR	Compound annual growth rate
Calac-Alg-Suc-Tre	Spray-dried calcium lactate-alginate-sucrose-trehalose
Calac-Alg-Suc-Tre-Cys	Spray-dried calcium lactate-alginate-sucrose-trehalose-L-cysteine
Calac-Alg-Suc-Tre-Asc	Spray-dried calcium lactate-alginate-sucrose-trehalose-ascorbate
Ca-Alg	Spray-dried calcium-alginate
Ca-Alg-Suc	Spray-dried calcium-alginate-sucrose
Ca-Alg-Suc-IFC	Spray-dried calcium-alginate-sucrose, using inverted feed channels
Ca-Alg-Suc-Tre	Spray-dried calcium-alginate-sucrose-trehalose
Ca-Alg-Tre	Spray-dried calcium-alginate-trehalose
CDI	<i>Clostridium difficile</i> infections
CF-MRS	Carbon-free de Man, Rogosa and Sharpe
CF-TS	Carbon-free tryptic soy
CFU	Colony forming units

DFAST	DDBJ Fast Annotation and Submission Tool
DI	Deionised
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DP	Degree of polymerisation
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSC	Differential scanning calorimetry
ECN	<i>Escherichia coli</i> Nissle 1917
EDTA	Ethylenediaminetetraacetic acid
EE	Encapsulation efficiency
EFSA	European Food Safety Authority
EPS	Exopolysaccharides
ESI	Electrospray ionisation
FACTS	Facility for Analysis, Characterisation, Testing and Simulation
FAO	Food and Agricultural Organisation
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FISH	Fluorescent <i>in situ</i> hybridisation
FMT	Fecal microbiota transplant
FOS	Fructo-oligosaccharides
FTIR	Fourier transform infrared spectrometry
GABA	γ -aminobutyric acid
GBDP	Genome BLAST Distance Phylogeny
GC	Gas chromatography
GIT	Gastrointestinal tract
GLOS	Gluko-oligosaccharides
Glu-MRS	Glucose-supplemented de Man, Rogosa and Sharpe
GOS	Galacto-oligosaccharides
GPC	Gel permeation chromatography
GRAS	Generally recognised as safe
G_ac	High guluronate alginate, autoclaved
G_ac_suc	High guluronate alginate with sucrose, autoclaved

G_fil	High guluronate alginate, filtered
G_fil_suc	High guluronate alginate with sucrose, filtered
HMP	Human Microbiome Project
HP	Hydrolysed polysaccharide
HPLC	High performance liquid chromatography
HPMC	Hydroxypropyl methylcellulose
HPMCP	Hypromellose phthalate
IBS	Inflammatory bowel syndrome
ISAPP	International Scientific Association of Probiotics and Prebiotics
ITS	Internal transcribed spacer
Kef	Kefir probiotics
LAB	Lactic acid bacteria
Lac-MRS	Lactose-supplemented de Man, Rogosa and Sharpe
LB	Luria–Bertani
LC	Liquid chromatography
LDH	Lactate dehydrogenase
LGG	<i>Lactocaseibacillus rhamnosus</i> GG
LPS	Lipopolysaccharides
M/G	Mannuronate/Guluronate
MRS	De Man, Rogosa and Sharpe
MS	Mass spectrometry
MSE	Materials Science and Engineering
M _w	Molecular weight
MYP	Mannitol-yeast extract peptone
M_ac	High mannuronate alginate, autoclaved
M_ac_suc	High mannuronate alginate with sucrose, autoclaved
M_fil	High mannuronate alginate, filtered
M_fil_suc	High mannuronate alginate with sucrose, filtered
NA	Nutrient agar
NB	Nutrient broth
NCBI	National Centre for Biotechnology Information
NMR	Nuclear magnetic resonance

NTU	Nanyang Technological University
OD	Optical density
PAS	Periodic acid Schiff's
PBS	Phosphate buffered solution
PCA	Principal coordinate analysis
PCR	Polymerase chain reaction
PDI	Polydispersity index
PGAP	Prokaryotic genome annotation pipeline
PI	Propidium iodide
QPS	Qualified Presumption of Safety
RID	Refractive index detector
rRNA	Ribosomal ribonucleic acid
SCELSE	Singapore Centre for Environmental Life Sciences Engineering
SCFA	Short chain fatty acid
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
Suc	Spray-dried sucrose formulation
Suc-MRS	Sucrose-supplemented de Man, Rogosa and Sharpe
TCA	Trichloroacetic acid
TDC	Taurodeoxycholate
TFA	Trifluoroacetic acid
T _g	Glass transition temperature
TGA	Thermogravimetric analysis
TLC	Thin layer chromatography
TMSP	3-(trimethylsilyl) propionic-2,2,3,3-d ₄ acid sodium salt
TS	Tryptic soy
TYGS	Type (Strain) Genome Server
UV	Ultraviolet
WC	Wilkins-Chalgren anaerobe
WGS	Whole genome sequencing
WHO	World Health Organisation

WT	Wild type
YPD	Yeast extract-peptone-dextrose
YPER	Yeast protein extract reagent
YPG	Yeast extract-peptone-glycerol

1. Chapter 1

Introduction

This opening chapter provides an introduction, the hypotheses, the objectives, the scope, and the novelty of this thesis. In recent years, probiotics and prebiotics have become increasingly popular as functional dietary ingredients that can modulate the human gut microbiota, sparking interest in the search for novel probiotic strains and prebiotic materials. This research explores the fermented beverage kefir as a source of probiotics and prebiotics and is interested in the characterisation of these components to derive a functional synbiotic formulation. This research also concerns the development of alginate-based encapsulation systems, using the techniques of extrusion and spray-drying, to produce highly viable encapsulated probiotics which are shelf-stable, resistant to gastrointestinal tract conditions, and can be stably incorporated in food and beverage matrices.

1.1. Introduction

The human body is an ecosystem home to trillions of microbes. In the recent two decades, large-scale human microbiome investigations such as the Human Microbiome Project (HMP) have elucidated the intricate interactions which exist between the human microbiome and our human health [1], [2]. It is now known that over 10 trillion microbial cells make up the human microbiota, contributing to approximately half of all the cells in the human body [3]. Most of the human microbiota can be found in the human gut, and dysbiosis of the human gut microbiota, which indicates a state where microbiota richness and diversity is significantly deviated from a healthy control state, has been correlated with numerous gastrointestinal tract (GIT) diseases, including inflammatory bowel disease, inflammatory bowel syndrome, *Clostridium difficile* infections, and colon cancer [4]–[9]. Beyond the gut, dysbiosis of the gut microbiota has also been associated with other chronic health issues, such as obesity, type II diabetes, immune diseases, cancer, and psychoneurological disorders [10]–[15].

The idea that perturbations to the human microbiome may cause disease sparked considerable interest and the question posed: Could there be healthy microbes supplemented to the human body that reverse the disease state? These “healthy microbes” are precisely probiotics. Defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” [16], probiotics have seen a rapidly rising demand in recent years, with a market estimate projecting a compound annual growth rate (CAGR) of 7.5% from 2021 through 2028 [17]. Most existing commercial probiotic bacteria strains are of lactobacilli, *Streptococcus*, *Bacillus*, *Bifidobacterium*, or *Saccharomyces* genera, and have been sourced from the native microbiota in human faeces, human breast milk as well as from fermented food sources.

Prebiotics represent another major class of ingredients capable of modulating the human gut microbiota [18]. The most recent definition of prebiotics is from the International Scientific Association of Probiotics and Prebiotics (ISAPP) consensus panel in 2017: “a substrate that is selectively utilised by host microorganisms thereby conferring a health

benefit” [19]. In simpler terms, prebiotics are non-viable materials which act as food for specific microbes. Co-administered with probiotics, prebiotics can potentially enhance the efficacy of probiotics, by improving its survival, colonisation, and growth [20]. The combination of probiotics and prebiotics is known as synbiotics, a term alluding to the synergistic interactions which exist between probiotics and prebiotics.

Diverse types of probiotics and prebiotics serve different functions in modulating the gut microbiome, such as stimulating growth of different genera of gut microbiota, or inducing different gut metabolite profiles [18]. As such, there exists an ongoing global research interest to identify novel and promising probiotic strains and prebiotic materials to supplement the gut microbiota function. In this thesis, *kefir* is studied as a potential source of probiotics and prebiotics. Kefir is a fermented beverage produced from grains that comprise a symbiotic mixture of bacteria, yeasts, and their extracellular polymeric substances. In recent years, kefir has gained increasing attention worldwide by consumers and researchers for the numerous health benefits that have been associated with the consumption of kefir. These health benefits include antimicrobial, cholesterol assimilation, antioxidant, tumour suppression, gastrointestinal immunity and modification of gut microbiota [21]–[27]. While some of these putative health benefits have been studied academically and clinically, there remains more to be unravelled about the microbiology of kefir, and the potential of harvesting useful, health-benefiting materials from it.

In the development of probiotic and synbiotic formulations, another important aspect is in the design of a suitable cargo vehicle to facilitate their delivery to the intended intestinal site. Encapsulation is a process which can facilitate the delivery of probiotics and synbiotics, by utilising the principle of encasing active ingredients in polymeric matrices which can resist harsh environmental conditions. For oral consumption, the encapsulation matrices are expected to protect the cargo from human GIT insults, such as the acidic pH in the gastric fluid, and bile salts in the small intestinal region. The encapsulation matrices and techniques used should also not compromise the effectiveness of the active ingredient during the manufacturing stage and should yield sufficient shelf-life stability. Encapsulation systems are particularly important for the oral delivery of probiotics, as

probiotics easily lose viability in the manufacturing, storage, and ingestion phases. Viability is considered a key requirement for probiotics effectuation [28], hence well-designed encapsulation systems aimed at maintaining high probiotic viability throughout its life cycle are critical. Additionally, with consideration to designing an encapsulation system suitable for commercialisation, having reasonable manufacturing throughput, manufacturing consistency, good shelf-life at ambient temperature, non-toxicity, and cost-effectiveness are important functional criteria to be met.

In this thesis, the oral delivery of synbiotics derived from kefir via encapsulation systems is explored. An overview of this thesis is provided in **Figure 1.1**. This research seeks to isolate and characterise probiotic strains and prebiotic materials from kefir to constitute a kefir synbiotics formulation. This exploration of kefir as a source of synbiotics is a novel concept reported for the first time. In addition, this research seeks to incorporate the derived kefir synbiotics within robust encapsulation systems to enhance their survival until they are delivered to the human intestine. With these encapsulation systems, this research aims to overcome the typical high viability losses of probiotics in the manufacturing, storage, and ingestion stages, thereby enhancing the health-promoting benefits of kefir probiotics and synbiotics to human hosts.

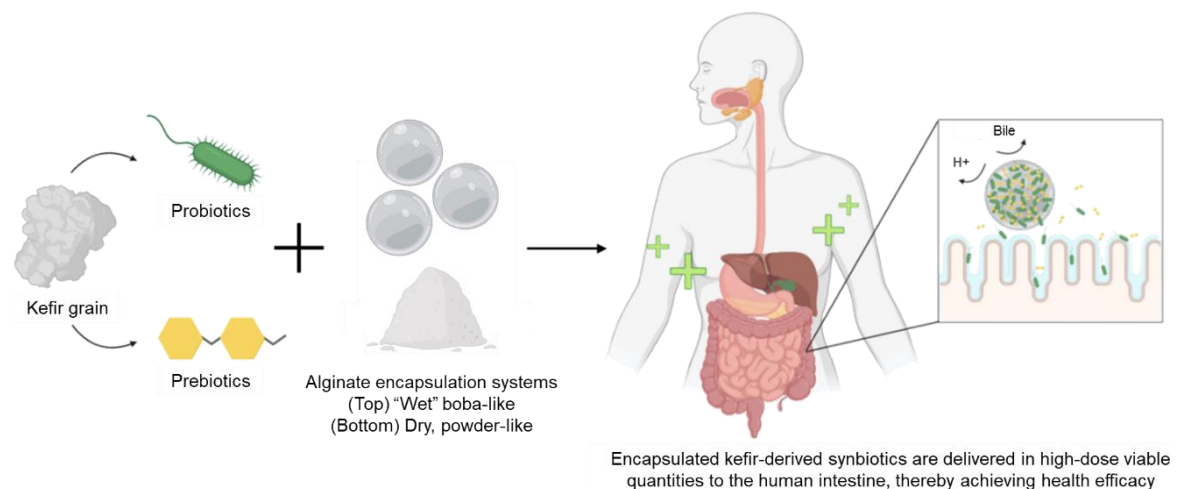


Figure 1.1: Overview of thesis, “Oral delivery of microbiome-modulating synbiotics derived from kefir via alginate encapsulation systems”. See Section 2.4.3 for a thorough review of alginate and the extrusion and spray-drying techniques used to produce the “wet” and “dry” encapsulation systems. Image created with Biorender.

1.2. Hypotheses

In this thesis, the idea of developing kefir-derived synbiotics as microbiome-modulating dietary ingredients for human consumption is explored. Despite numerous academic and clinical studies reporting the health benefits of kefir consumption, the mode of kefir consumption is so far limited to drinking the kefir beverage itself, while kefir-derived health supplements are sparse, if not non-existent in the market (discussed in Section 2.1 and 2.3 of literature review). It is hypothesised that the health-promoting properties of kefir can be attributed to its unique microbiota profile, and that it is possible to isolate novel probiotic strains from the kefir grains consortia to benefit human health. Besides probiotics, exopolysaccharides (EPS) derived from kefir are another potentially useful food-grade material. This thesis hypothesizes that certain types of EPS secreted by kefir strains have prebiotic properties, as similar materials (e.g., dextrans and levans) from other sources have displayed prebiotic properties (discussed in Section 2.2 of literature review). It is hypothesized that the concept of kefir synbiotics, which consist of kefir-derived probiotics and prebiotics, can yield a more efficacious product. Through derivation of this kefir synbiotic formulation, this research can help to provide an alternative consumption format for kefir, thereby providing more convenience and versatility for consumers to reap the health benefits of kefir.

In addition, this research seeks to develop encapsulation systems to deliver high viable doses of kefir probiotics to the human intestine (also applicable for other probiotics). Focus is placed on utilising the encapsulation material, alginate, while employing the techniques of extrusion and spray-drying. Alginate is selected due to its food-grade, natural source, and acid buffering properties, which make it favourable for the encapsulation of probiotics [29], [30]. Extrusion refers to a technique of dropping alginate-probiotic suspensions into a crosslinking solution, which yields spherical “wet” bead-like structures similar to “boba” that is popularly consumed in bubble tea [31]. Spray-drying, on the contrary, yields “dry” powder particles which are produced from liquid feeds via the combined action of atomisation, heat, and vacuum drying [32]. For the extrusion technique, it is hypothesised that the “wet” encapsulated probiotics are suitable for incorporation into food and beverage

systems to improve their functional attributes. For the spray-drying technique, the major challenge lies in developing a technique or formulation that can retain high probiotic survival during the deleterious spray-drying process. This research hypothesises that certain formulation or technique optimisations can be done to make spray-drying suitable for the encapsulation of probiotics, and the resultant method will be a commercially viable method to produce dried encapsulated probiotics powder.

1.3. Objectives and Scope

This research aims to test the hypotheses described in the earlier section, and the five key research objectives are as detailed:

1. To isolate, identify and characterise probiotic strains isolated from locally sourced milk and water kefir.
 - This research is interested in the isolation of probiotics from two types of kefir – milk kefir and water kefir. These milk and water kefir grains are obtained from a local home brewer based in Singapore.
 - This research focuses on the screening of probiotics for enteric health benefits, as most of the prior reported health benefits surrounding kefir were based on the consumption of kefir as a beverage. High-throughput *in vitro* screening approaches are planned to screen kefir-derived isolates, to identify the best strains which show highest probiotic efficacy. Probiotics screening tests such as screening for inhibition against enteric pathogens, bile salt hydrolase (BSH) activity, antioxidant activity, acid and bile resistance, mucoadhesion to *in vitro* epithelial cells, and whole genome analysis will be explored. These tests are selected based on prior reported health efficacies of the kefir beverage, as well as on the general functional attributes that are preferred for probiotics.
2. To identify EPS-producing strains from kefir, extract EPS, hydrolyse EPS, and characterise their chemical structures and prebiotic potential.
 - This research chooses to work with pure EPS-producing strains rather than the bulk

kefir grain material, as the latter is known to generate inconsistent EPS structures and yields with subsequent fermentation cycles [33]. In comparison, pure microbial strains can be better controlled and optimised to yield consistent EPS products.

- This research is firstly concerned with identifying EPS-producing strains from kefir which can produce high yield of EPS, so that any potentially useful prebiotic materials derived downstream would be economically feasible to produce. Various yield optimisation strategies, such as by utilising different incubation conditions (e.g., anaerobic/aerobic, liquid/agar culture for EPS production), are to be attempted to increase EPS yields from EPS-producing strains.
 - Besides EPS, it is also of interest to identify if hydrolysed kefir-derived EPS with lower molecular weights may be suitable as prebiotics, given the fact that shorter chain polysaccharides or oligosaccharides are generally more digestible and better utilised as substrates by microorganisms [34]. As such, an acid-assisted partial hydrolysis method will be employed to generate lower molecular weight polysaccharides.
 - Chemical structures of kefir-derived EPS and hydrolysed EPS are to be determined, e.g., molecular weight, monosaccharide composition, branching analysis. Prebiotic properties of EPS and hydrolysed EPS will be evaluated based on their ability to be selectively utilised by probiotics over pathogens, their digestibility by human gastric pH and digestive enzymes, and their ability to yield short chain fatty acids (SCFAs).
3. To combine kefir probiotics and kefir prebiotics in a kefir synbiotic formulation and demonstrate its synergistic effects.
- Selected kefir-derived probiotics (derived in **Objective 1**) will be combined with the polysaccharides with best prebiotic properties (derived in **Objective 2**) to form a kefir synbiotic formulation. This research seeks to evaluate derived kefir synbiotics using an *ex vivo* large bowel simulated model to determine their effects on gut microbial communities and SCFAs content. The effects of kefir synbiotics treatment will be compared with the treatments of kefir probiotics or kefir prebiotics alone to determine if synergistic effects exist with the co-

supplementation of kefir probiotics with prebiotics. Kefir synbiotics treatment will also be compared with treatments using commercial probiotics and prebiotic materials to determine if kefir synbiotics can outperform these controls.

4. To develop a probiotic encapsulation system by alginate extrusion, for enhanced GIT resistance of probiotics and functionalisation of beverages.

- This will be broken down into two sub-objectives:
 - a) Firstly, an optimisation of the alginate extrusion process will be conducted to determine the method that can provide highest protection against GIT conditions to the encapsulated probiotics.
 - Eight different alginate formulations, differing in terms of mannuronate/guluronate (M/G) ratio of alginate, whether alginates are sterilised by autoclave or filtration, and the presence of sucrose in the formulation, have been identified to be of interest in this study. This formulation will be optimised using the model probiotic, *Lactocaseibacillus rhamnosus* GG (LGG).
 - *In vitro* simulated GIT conditions, i.e., simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), will be used to evaluate the acid/bile-resistant properties of the alginate formulations.
 - Rheological and thermal properties of the alginate formulations will be evaluated to provide mechanistic insights on the acid protective effects of certain formulations.
 - b) Secondly, the optimised formulation for alginate extrusion (from **Objective 4a**) will be used for encapsulating probiotics for addition to carbonated sodas and beers.
 - This research is inspired by the “boba-like” features of extruded alginate encapsulation systems and seeks to determine if the extruded alginate-probiotic spheres could enhance probiotics viability in certain beverages.
 - Carbonated sodas and beers are selected as beverage systems to study as these pose highly deleterious conditions for probiotics due to their acidic pH, hops-derived compounds, and ethanolic content. There has also so far

been a dearth of studies regarding the functionalisation of carbonated sodas and beers with probiotics. The beverages selected for testing include Coca-Cola, 7-Up, Tiger Beer, and Guinness.

- Four probiotic candidates, namely LGG, *Escherichia coli* Nissle 1917 (ECN), *Bifidobacterium longum* (BL), and the kefir probiotics (derived in **Objective 1**), will be tested. These probiotics in the encapsulated and unencapsulated forms will be exposed to the beverages to determine if alginate encapsulation by extrusion could improve their survival.
- Various probiotics will be exposed at refrigerated and room temperature conditions for up to 14 days, and the colony forming units (CFU) of probiotics will be determined at stipulated time intervals.
- Encapsulated probiotics will also be exposed to combinations of beverages and simulated gastric fluid to determine the survival of probiotics in a gastric environment following oral consumption of sodas or beers.

5. To develop a probiotic encapsulation system using spray-drying, aimed at delivering high viable doses of encapsulated probiotics which are shelf-stable and resistant to GIT conditions.

- This research seeks to explore the feasibility of the coaxial spray-drying technique, which simultaneously sprays alginate-probiotics and crosslinking agents through inner and outer nozzles of the spray-dryer respectively, to produce dry crosslinked alginate probiotics particles. This technique was inspired by Kharel *et al* [35], who utilised coaxial spraying to encapsulated beta-carotene in okara and zein matrices.
- Various functional aspects are required to be fulfilled by the resultant powder particles. Firstly, crosslinking of alginate should be successfully achieved. The extent of crosslinking in the final spray-dried powder could be analysed by the Periodic acid schiff's (PAS) assay [36], [37].
- Secondly, high CFU counts of probiotics should survive after the spray-drying process. Due to the high temperature and rapid dehydration process involved in spray-drying, probiotic viability losses during the spray-drying process can be anticipated. Hence, protective additives are required to improve probiotics survival.

In prior studies, protective agents including sucrose, trehalose, L-cysteine and sodium ascorbate [38], [39] have been proposed to enhance spray-drying survival of probiotics. This research shall evaluate the effectiveness of these protective agents in improving viability of probiotics after spray-drying.

- Thirdly, spray-dried encapsulated probiotics should have improved GIT-resistant properties, i.e., able to resist SGF and SIF conditions. These GIT-resistant properties should also be retained over a period of shelf storage. Shelf storage of refrigerated and ambient conditions will be tested.
- Finally, this technique of spray-drying encapsulation should be suitable for the encapsulation of various probiotic strains. This research aims to first optimise the spray-drying encapsulation system for LGG, later then adapting the optimised formulation for *Lactocaseibacillus paracasei*, *Lactiplantibacillus plantarum*, ECN, and the kefir probiotics (derived in **Objective 1**). These four key functional aspects will be systemically investigated to identify the best-performing formulation for spray-drying encapsulation of probiotics using alginate.

1.4. Dissertation Overview

This thesis is structured into seven chapters:

Chapter 1 provides the rationale for this research and outlines the hypotheses, objectives, scope, and novelty.

“This thesis describes the derivation of probiotics and prebiotics from kefir, which when combined, yield synbiotic efficacies. This thesis also develops alginate encapsulation systems based on the extrusion and spray-drying techniques to improve the viability and functionality of probiotics. Overall, this thesis seeks to adapt the optimised encapsulation systems for delivery of kefir probiotics and synbiotics, to facilitate their application as beneficial microbiome-modulating ingredients in the food and supplement industries.”

Chapter 2 reviews the literature concerning the main topics covered in this thesis – probiotics, prebiotics, kefir, and probiotic encapsulation systems. Various types of existing probiotics strains and prebiotics materials are evaluated, and a review of clinical studies regarding probiotics and prebiotics health efficacies is conducted. The mechanisms of action by which probiotics and prebiotics act to elicit health benefits are also detailed. In addition, *in vitro* and *in vivo* screening methods commonly employed in characterising novel probiotics and prebiotics are described, to identify the key requirements which will help determine the best-performing probiotics and prebiotics. The microbiological and EPS compositions of milk and water kefir are reviewed, and a study of existing kefir-related commercial products is done to highlight the lack of kefir-derived dietary supplement products. Various probiotic encapsulation systems are reviewed, and the advantages and limitations of each technique are detailed. Finally, industry trends and consumer perceptions of probiotics, prebiotics, kefir, and encapsulation systems are discussed, to provide a recognition of the commercial potential and value of this research work.

Chapter 3 describes the identification of probiotic strains from locally-sourced milk and water kefir (fulfils **Objective 1**). Various phenotypic and genotypic screening assays were conducted to identify kefir-isolates with potential probiotic properties. Through this systematic investigation, seven bacterial strains were ultimately identified to demonstrate probiotic efficacies and were combined in a “kefir probiotics” formulation.

Results for this chapter have been published in:

- L.L. Tan, C.H. Tan, N.K.J. Ng, Y.H. Tan, P.L. Conway, and S.C.J. Loo. Potential probiotic strains from milk and water kefir grains in Singapore – Use for defense against enteric bacterial pathogens. *Frontiers in Microbiology*, 13 (2022).
<https://doi.org/10.3389/fmicb.2022.857720>

Chapter 4 details the characterisation of prebiotic materials from kefir (fulfils **Objectives 2 and 3**). EPS from various EPS-producing kefir microbial strains were derived and characterised for their structural and chemical properties. Hydrolysed EPS from water kefir-derived *Liquorilactobacillus satsumensis* were found to demonstrate prebiotic effects, and showed synbiotic properties when combined with the kefir probiotics.

Results for this chapter have been published in:

- L.L. Tan, J.J. Ngiam, E.S.Z. Sim, P.L. Conway, and S.C.J. Loo. *Liquorilactobacillus satsumensis* from water kefir yields α -glucan polysaccharides with prebiotic and synbiotic qualities. *Carbohydrate Polymers*, 290, 119515 (2022).
<https://doi.org/10.1016/J.CARBPOL.2022.119515>

Chapter 5 discusses the encapsulation of probiotics by alginate extrusion (fulfils **Objective 4**). Various alginates, differing in terms of their M/G ratios, sterilisation method, and with or without sucrose added, were compared to determine which yielded best gastric acid protection for encapsulated probiotics. The optimised formulation was used to encapsulate various probiotics, including LGG, ECN, BL, and the kefir probiotics, for incorporation in carbonated sodas and beers, to test the feasibility of developing probiotic-sodas and beers through an encapsulation technique.

Results for this chapter have been published in:

- L.L. Tan, K.L. Ang, and S.C.J. Loo. Alginate encapsulation improves probiotics survival in carbonated sodas and beers. *PLOS One*. (Accepted, Dec 2022).

Chapter 6 describes the spray-drying encapsulation of probiotics (fulfils **Objective 5**). A novel coaxial spray-drying technique for probiotics encapsulation using alginate is disclosed in this chapter. Various alginate formulations, which incorporate different crosslinking agents and protective additives, were evaluated to determine the formulation which yielded best shelf-life stability and resistance to GIT conditions. The final optimised formulation was adapted for encapsulation of the kefir probiotics.

Results for this chapter have been published in these articles:

- L.L. Tan, S.C.J. Loo, and M. Mahotra. (2022). Encapsulation of live microorganisms for gastrointestinal-targeted delivery. International Patent No. WO 2022/177508 A1. WIPO PCT.
- L.L. Tan, M. Mahotra, S.Y. Chan and S.C.J. Loo. In situ alginate crosslinking during spray-drying of lactobacilli probiotics promotes gastrointestinal-targeted delivery.

Carbohydrate Polymers, 286, 119279 (2022).

<https://doi.org/10.1016/j.carbpol.2022.119279>

Chapter 7 draws this thesis to a close. A critical appraisal of the extent by which the hypotheses in this thesis are proven or disproven is conducted.

1.5. Findings and Originality

The novel findings of this thesis include:

1. Novel probiotic strains with enteric pathogen inhibitory effects were identified from locally sourced milk and water kefir.
 - These kefir isolates exhibited several of the beneficial properties associated with the kefir beverage itself, such as enteric pathogen inhibition effects, antioxidant properties and potential for cholesterol management through BSH activity. These strains also showed good safety profile and desirable probiotic characteristics, including good survival in acid and bile environments, non-cytotoxicity and high adhesion to Caco-2 cells, the lack of virulence or antimicrobial resistance (AMR) genes, and could be consistently produced through batch fermentations.
 - Seven best-performing kefir strains were combined to yield a “kefir probiotics” formulation. This kefir probiotics formulation showed the capacity to out-compete or exclude the attachment of enteric pathogens to Caco-2 cells.
 - The kefir probiotics derived in this study could potentially be incorporated into other product formats, such as dietary supplements or within food and beverages. These alternative product formats may provide consumers with a greater variety and convenience to attain the health benefits of kefir, thereby facilitating consumers to incorporate diverse microbiome-modulating products into their diet regime.
 - These findings are elaborated in **Chapter 3**.
2. Hydrolysed kefir-derived EPS were found to demonstrate prebiotic effects and synbiotic effects when co-administered with kefir-derived probiotics.

- Water kefir-derived *Liquorilactobacillus satsumensis* Kef-w1 bacterial strain produced high yields of branched dextran-like EPS. Upon acid hydrolysis, the hydrolysed EPS from Kef-w1 exhibited prebiotic qualities, including the ability to be selectively utilised by probiotics over pathogens, and non-digestibility by human digestive enzymes. The hydrolysed kefir-derived EPS was also observed to be fermentable by kefir-derived probiotics, and these were combined in a novel kefir synbiotic formulation.
 - *Ex-vivo* gut simulated conditions demonstrated a propensity of hydrolysed kefir-derived EPS and kefir synbiotics to promote *Bacteroidetes* abundance, and generate SCFAs, including acetate, propionate, and butyrate, as by-products. Overall, this research reveals Kef-w1 hydrolysed EPS material to be a novel ingredient with prebiotic potential. This research also demonstrates, for the first instance, that the concept of kefir-derived synbiotics is feasible, and the kefir-derived synbiotics can serve as useful microbiome-modulating ingredients.
 - These findings are elaborated in **Chapter 4**.
3. Alginate encapsulation by extrusion was found to improve probiotics survival in carbonated sodas and beers.
- Carbonated sodas and beers are known to pose highly deleterious conditions for the survival of probiotics due to the acidic pH, hops-derived compounds, and ethanol present. Prior to this research, there were few literatures regarding the incorporation of encapsulated probiotics in carbonated sodas and beers. This research showed that the viabilities of LGG, ECN, BL, and kefir probiotics improved upon alginate encapsulation when exposed to Coke, 7-Up, Tiger Beer and Guinness. Alginate encapsulation was found to improve probiotics survival most significantly in Coke.
 - Better survival of probiotics was achieved in refrigerated storage conditions than at room temperature. Lower concentrations of lactate were also produced by LGG in carbonated sodas, Coke and 7-Up, at refrigerated conditions.
 - These findings can provide beverage manufacturers with a potential method of incorporating viable probiotics into their products through alginate encapsulation. Based on this study, a general recommendation would be to store encapsulated

probiotics under refrigerated conditions to reduce viability losses, and to avoid flavour alterations that could arise due to probiotic metabolism.

- These findings are elaborated in **Chapter 5**.

4. A novel spray-drying technique was developed to produce dry encapsulated lactobacilli probiotics powder with GIT-resistant properties and good shelf-life properties.

- A coaxial spray-drying technique for *in situ* alginate crosslinking was optimised for the encapsulation of probiotics. This technique could achieve more than 90% alginate crosslinking. Among various formulations investigated, the crosslinked calcium lactate-alginate with sucrose, trehalose, and sodium ascorbate [Calac-Alg-Suc-Tre-Asc] formulation was most promising for probiotics encapsulation via the described spray-drying method. High CFU/g ($>10^9$) of LGG and kefir probiotics were retained after spray-drying and exposure to simulated gastric/intestinal fluids, highlighting the ability of the Calac-Alg-Suc-Tre-Asc formulation to protect entrapped probiotics against desiccation and thermal stresses during spray-drying, as well as confer GIT-protective effects. Encapsulated LGG and kefir probiotics powder were also relatively stable when stored at room temperature or refrigerated conditions, with $>10^9$ CFU/g retained after eight weeks of storage. Overall, this study has yielded an industrially scalable method to produce shelf-stable, gastrointestinal resistant probiotic powders. A patent was filed for this work to pursue the commercialisation potential of this probiotics encapsulation technique.
- These findings are elaborated in **Chapter 6**.

2. Chapter 2

Literature Review

This chapter reviews existing literature on the major topics of this research work – probiotics, prebiotics, kefir, and encapsulation systems. Various types of existing probiotic strains and prebiotic materials are evaluated, and a review of clinical studies regarding probiotics and prebiotics health efficacies is conducted. The mechanisms of action by which probiotics and prebiotics act to elicit health benefits are also detailed. In addition, in vitro and in vivo screening methods commonly employed in characterising novel probiotics and prebiotics are described, to identify the key requirements which will help determine the best-performing probiotics and prebiotics. The microbiological and EPS compositions of milk and water kefir are reviewed, and a study of existing kefir-related commercial products is done to highlight the lack of kefir-derived dietary supplement products. Various probiotic encapsulation systems are reviewed, and the advantages and limitations of each technique are detailed. Finally, industry trends and consumer perceptions of probiotics, prebiotics, kefir, and encapsulation systems are discussed, to provide a recognition of the commercial potential and value of this research work. The last part of this chapter provides a summary of the various research gaps ascertained from the literature review that this thesis addresses.

2.1. Probiotics

The most widely accepted definition of probiotics has been provided by the United Nations Food and Agricultural Organisation (FAO) and the World Health Organisation (WHO) in 2001: ‘live microorganisms which when administered in adequate amounts confer a health benefit on the host’ [16]. Although this definition was originally in relation to food, FAO-WHO was cognisant of the possibility of probiotics to be used in applications beyond food-related. Indeed, probiotics today are used in various hosts such as humans, livestock, fish as well as pets, and probiotics may be administered by various routes, such as via ingestion, topical application or injection [40]. For this thesis however, we are mainly concerned with the administration of probiotics to humans via the oral delivery route.

To some, the idea of using microbes to benefit human health might not be obvious. Indeed, for much of history, microbes have been regarded as harmful, infection-causing agents due to their association with numerous diseases, such as anthrax, typhoid fever, cholera, and tuberculosis. However, many beneficial microbes do exist, and in fact, have been consumed by humankind from as far back as 6,000 B.C. in the form of fermented foods [16]. Healthy benefits of fermented foods have been documented in ancient scripts, such as in the Bible, where Abraham owed his longevity and fecundity to yogurt consumption [41]. It was only in 1910, that the health benefits associated with fermented foods have been traced to microbes living in the foods. Russian scientist, Elie Metchnikoff, also known as the “father of probiotics”, observed that the regular consumption of lactic acid bacteria in fermented dairy products, such as yogurt, was associated with enhanced health and longevity in Bulgarian peasant populations. He suggested that “the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes” [42]. With that, Metchnikoff kickstarted the study of probiotics and numerous health benefits have been studied and proven since. Today, the study of probiotics continues to garner increasing interest by international academics and corporations (**Figure 2.1**).

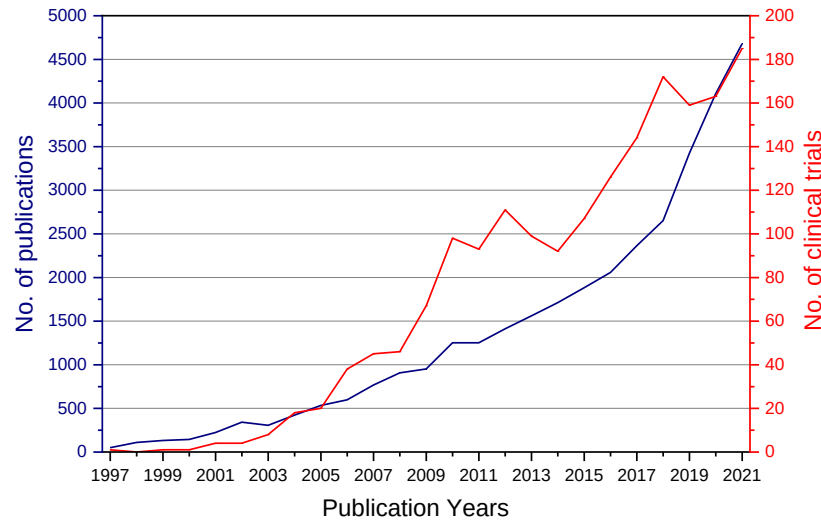


Figure 2.1: Number of scientific publications and clinical trials based on search keyword “probiotics” from 1997-2021. Data for number of publications is derived from Web of Science, while data for number of clinical trials is derived from Clinicaltrials.gov (accessed 30-Mar-2022).

2.1.1. The human microbiome

The term “human microbiome” refers to the genetic material of all microorganisms which populate surfaces on and within our human bodies. Microbiota refers to microorganisms themselves. In the recent two decades, various microbiome-themed projects (**Table 2.1**) have sought to understand what the human microbiome is, and how it influences our human health. These projects were enabled by advances in high-throughput sequencing and mass spectrometry technologies which have only become widely available in the last two decades. Genomic, transcriptomic, proteomic, and metabolic characterisation methods, also called “omics” technologies, can now be applied to understand the operations of complex microorganism communities, such as in the various sites of the human body.

Table 2.1: Large population microbiome studies.

Name of project	Year of initiation	Leading institution(s)	Ref
Human Microbiome Project	2007	National Institutes of Health	[1]
METAgonomics of the Human Intestinal Tract	2008	European Commission	[2]
Asian Microbiome Project	2009	Asian Federation of Societies for Lactic Acid Bacteria	[43]
Flemish Gut Flora Project	2012	Vlaams Instituut voor Biotechnologie	[44]
The Microsetta Initiative	2013	University of California San Diego	[45]
Dutch Microbiome Project	2015	University Medical Center Groningen	[46]
Million Microbiome of Humans Project	2019	BGI Genomics	[47]

The human microbiota is currently known to comprise of over 10 trillion microorganisms, and most of these reside in the colon [3]. Faeces sampling is the most common proxy method used to study the gut microbiota due to its convenience and non-invasiveness. There are more than 1000 species of colonic bacteria and 90% belong to just 2 of the 70 known phyla: the *Firmicutes* (Gram-positive) and the *Bacteroidetes* (Gram-negative) [1]. Considerable differences in richness and diversity indices of microbiota between individuals have been observed, and microbiomes are also known to change over time and according to diet [48]. Richness refers to the number of taxa (e.g., microbial phyla/species) present in a microbial community, whereas diversity indices, such as the Shannon index, consider the relative abundances of different taxa, and takes both richness and evenness into consideration (**Figure 2.2**).

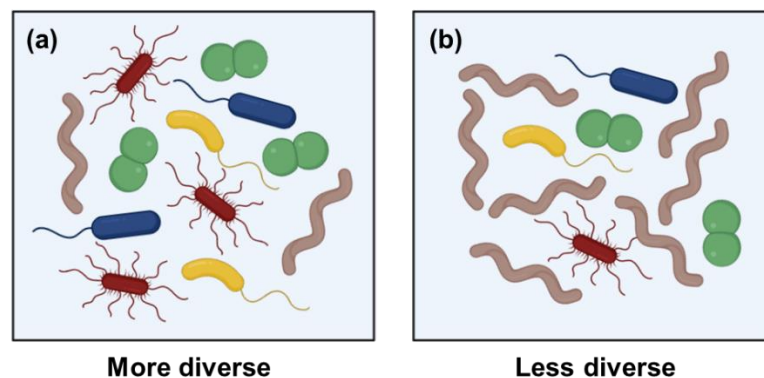


Figure 2.2: Illustration of the concepts “richness” and “diversity”. In both boxes (a) and (b), the species richness is equal (5 species). However, box (b) has a lower diversity index than (a), as the number of each species is less even. Created with Biorender.

Dysbiosis occurs when the richness or diversity of the gut microbiota changes to become differentiated from a healthy control state [49]. Dysbiosis of the gut microbiome has been correlated with numerous gut diseases, including inflammatory bowel disease [7], inflammatory bowel syndrome (IBS) [8], *Clostridium difficile* infections (CDI) [4]–[6], and colon cancer [9]. CDI is a prime example of a human disease which develops because of gut microbiota dysbiosis and is effectively treated by a microbiota-based therapy – faecal microbiota transplant (FMT). So far, FMT remains the most clinically effective method of treating recurrent CDI. Antibiotics are discouraged and are considered one of the risk factors behind CDI development [50]. Dysbiosis of gut microbiota have also been associated with chronic health issues that are not local to the gut, such as obesity [10], type II diabetes [11], [12], immune diseases [13], [51], [52], cancer [14], and psychoneurological disorders [15].

One of the most drastic ways to diminish the gut microbiome is with antibiotics. Gut microbiota diversity decreases immediately during the course of antibiotic ingestion and only begins to recover after the treatment, although often the original state does not fully return [48]. Even short-term doses of antibiotics can perturb the gut microbial composition for years [53]. AMR genes preferentially selected during antibiotic treatment can persist in the microbial community long after the therapy has ended. These microbiome alterations induced by antibiotics can pose threats to human health, for instance through the selection of resistant opportunistic pathogens that can cause acute disease [54]. With increasing public health concern on antimicrobial resistance globally, more attention has been diverted to probiotics to supplement and modify, rather than destroy, the human gut microbiome.

2.1.2. Health benefits of probiotics and mechanisms of action

So far, the most clinically accepted uses of oral probiotics are towards alleviating, treating, or preventing GIT diseases. A systematic study by Hungin *et al* via a Delphi consensus method shows that clinical experts support the use of probiotics for the treatment of these

symptoms (IBS, antibiotic associated diarrhoea, and *Helicobacter pylori* infections) [7]. Probiotics have also been used prophylactically to reduce incidences of *C. difficile*-associated diarrhoea in elderly hospitalised patients [55]. Spore-forming *Bacillus* probiotics also showed improved clinical outcomes in treatment of functional dyspepsia [56]. In infants, probiotics have been used to treat necrotising enterocolitis, a serious neonatal disease causing inflammation and necrosis of areas of the bowel with mortality of up to 30% [57]. The use of probiotics in neonates has been incorporated into clinical guidelines in multiple UK hospital trusts and is recommended by the South West Neonatal Network [58].

Increasing evidence also show the efficacies of probiotics in treating or managing symptoms of obesity [59], diabetes [60], psychoneurological diseases [61], respiratory diseases [62], cancer [14], and immune system diseases [63]. Further research is needed for probiotics to gain clinical acceptance in treating these pathologies [64].

Probiotic effects on “healthy adults”, i.e., adults without specific health pathologies, have also been studied. Khaledi *et al* reviewed experimental trials using probiotics on healthy adults, published between 1990 and 2017 [65]. Results suggest that probiotic supplementation in healthy adults can lead to transient improvement in gut microbiota concentration of supplement-specific bacteria. Evidence also supports the role of probiotics in improving immune system responses, stool consistency, bowel movement, and vaginal lactobacilli concentration.

Most clinical studies in probiotics have been performed with viable probiotics, and it is widely acknowledged that, in general, viable probiotics are more effective than non-viable/dead probiotics [28]. Viability is also an inherent requirement of probiotics in the current definition of probiotics. Various mechanisms have been proposed regarding probiotic effectuation, and the actual mechanisms depend on the strain of the microorganism and the disease targeted. These include pathogen exclusion by colonisation of the epithelial layer (**Figure 2.3a**), enhancing mucus secretion by goblet cells (**Figure 2.3b**), modulation of epithelial barrier function (**Figure 2.3c**), secretion of antimicrobial

factors like bacteriocins or acids (**Figure 2.3d**), effecting the host immune system and nervous system (**Figure 2.3e and 2.3f**) [66] and reduction of low-density lipoprotein cholesterol and total cholesterol in serum by modulating lipid metabolism [67]. Probiotics can also secrete beneficial metabolites, such as SCFAs, bacteriocins, vitamins, and other bioactive compounds, to modulate the gut function [18]. The viability of probiotics is important in facilitating these mechanisms of action. Viable probiotics are more likely to form viable colonies in the gut mucosa, hence can better achieve prolonged colonisation, and can accordingly better effectuate via pathogen exclusion, stimulation of goblet cells and tight junction proteins, and cross-talk with the immune and nervous systems [28]. Secretion of antimicrobial factors or other beneficial metabolites is also a property only conferred by viable bacteria [28].

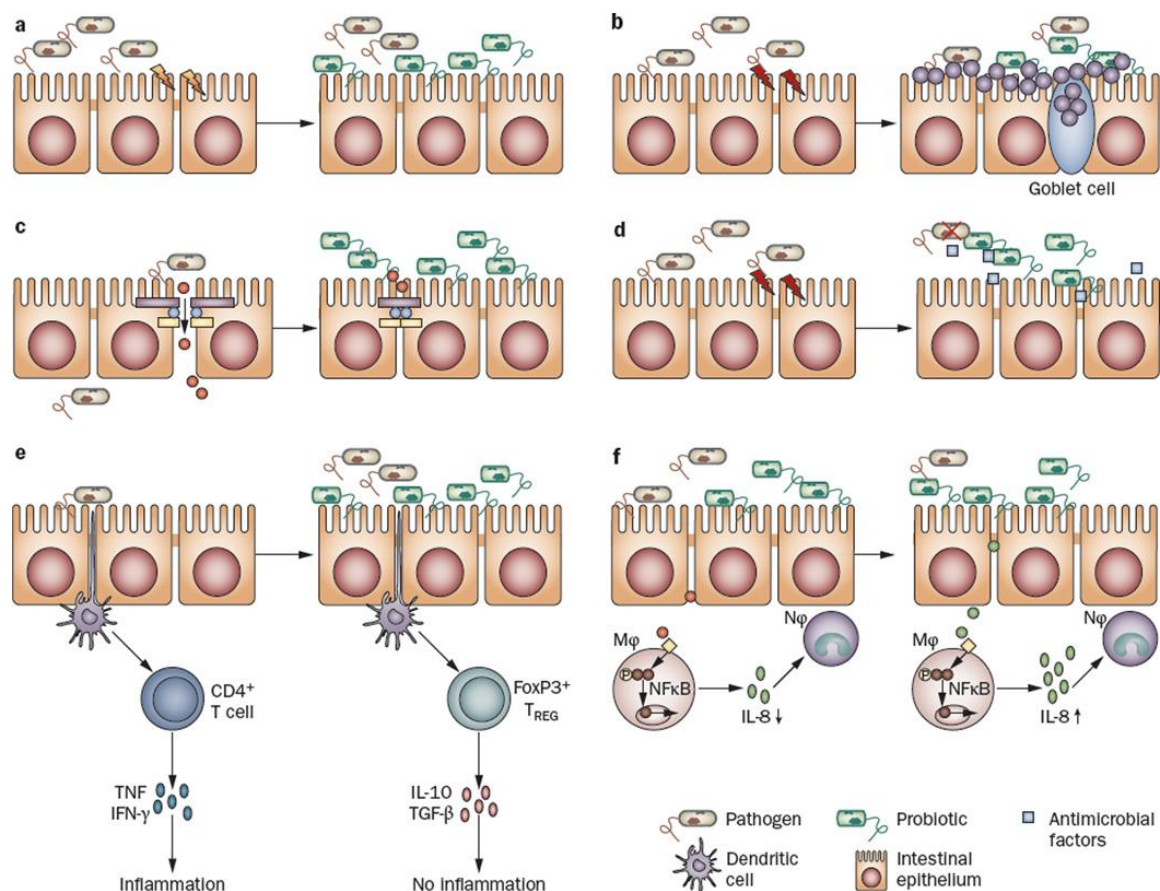


Figure 2.3: Potential mechanisms of action of probiotics – (a) Block pathogen entry by providing a physical barrier, also known as colonisation resistance; (b) Stimulating mucus release by goblet cells; (c) Maintain intestinal permeability by regulating intracellular tight junction proteins, thereby

preventing infections associated with a leaky gut; (d) Produce antimicrobial factors; I Stimulate the innate immune system by signalling dendritic cells, which lead to the induction of regulatory T cells and the production of anti-inflammatory cytokines, including interleukin-10 (IL-10) and transforming growth factor- β (TGF- β); (f) Prevent or trigger immune response by inhibiting (or activating) macrophages (M ϕ) and by influencing the production of IL-8 and subsequent recruitment of neutrophils (N ϕ) to sites of intestinal injury. Obtained with permission from [66].

2.1.3. Types of probiotics

There are many different types of bacteria and fungi which have been coined as probiotics. These are microorganisms in the size range of 0.2 – 50 μm and are generally not observable by the naked eye. The guideline by FAO-WHO requires probiotics to be specified by the International Code of Nomenclature to ensure understanding on an international basis [68], [69]. This includes defining probiotics by both their species and strain names, as probiotic properties are generally strain related and are known to differ even within the same species. A single strain is defined as the descendants of a single, pure microbial culture. To date, various over 1730 probiotics strains showing varying extents of efficacy in promoting human health have been identified [70].

For application in humans and animals, most probiotics fall under the phyla *Firmicutes*. Specifically, *Lactobacillaceae*, *Streptococcus*, *Bacillus*, which all fall under *Firmicutes*, have been used extensively as probiotics. *Bifidobacterium* of the *Actinobacteria* phyla and *Saccharomyces* which are a type of yeast have also been used. The various types of commonly used probiotics species are listed in **Table 2.2**. Many of these bacteria genera are lactic acid-producing, such as lactobacilli¹, *Streptococcus*, *Bifidobacterium*, and *Enterococcus*. Hence, a bulk of probiotic bacteria have been referred to as lactic acid bacteria (LAB). Many species of *Lactobacillaceae* and *Bifidobacterium* probiotics have been accepted as having Generally regarded as safe (GRAS) status in the United States Food and Drug Administration (FDA) [71], or have been granted Qualified Presumption

¹ Note that lactobacilli is a generic term to designate all organisms that were classified as *Lactobacillaceae* until 2020 [411].

of Safety (QPS) status by the European Food Safety Authority (EFSA) [72], hence are generally approved for use as dietary supplements.

Table 2.2: Commonly used probiotics in humans [73]

Kingdom					
Bacteria				Fungi	
Phyla					
Firmicutes		Actinobacteria		Ascomycota	
Genera	Species	Genera	Species	Genera	Species
Lactobacillus	L. acidophilus L. delbrueckii L. lactis	Bifidobacterium	B. adolescentis B. animalis B. bifidum B. infantis B. longum B. thermophilum	Saccharomyces	S. cerevisiae S. boulardii
Lacticaseibacillus	L. casei L. paracasei L. rhamnosus		B. lactis B. breve		
Lactiplantibacillus	L. plantarum				
Levilactobacillus	L. brevis				
Limosilactobacillus	L. fermentum L. reuteri				
Leuconostoc	L. mesenteroides L. lactis				
Streptococcus	S. cremoris S. diacetylactis S. intermedius S. salivarius S. thermophilus				
Bacillus	B. coagulans B. subtilis				

Interesting potential next-generation probiotics have also been proposed [74], including *Akkermansia muciniphila* [75]–[77], *Clostridium butyricum* [78], *Bacteroides fragilis* [79], *Faecalibacterium prausnitzii* [80], *Butyricicoccus pullicaecorum* [81]. These are typically commensal bacteria of the gut microbiota which are selected for their probiotic properties. However, regulatory approval of these strains faces a much more complex situation, due to safety concerns regarding their use. So far, *Akkermansia muciniphila* and *Clostridium butyricum* are not recommended for QPS status by the EFSA [72], despite no safety or tolerance issues being observed by these strains in a randomised control trial [78].

2.1.4. Sources of probiotics

Probiotics have been isolated from different types of sources, such as from the native microbiota of an organism, like from the faeces and breast milk of healthy humans. These

strains have the ability to persist at the body site without causing adverse effects, and some of them may also be necessary for maintaining the well-being of their host [16]. Probiotics derived from human breast milk [82]–[84] and human GIT [85] have been reviewed respectively. Numerous start-up companies have spun off, marketing native microbiota-derived probiotics to enhance human health. Interestingly, FitBiomics (Boston, USA) is a microbiome biotechnology company incorporated in 2019 which isolates probiotic strains from elite athletes to cater to next-generation athletes [86].

Probiotics may also be isolated from fermented foods, such as fermented dairy, fermented vegetables and fermented meats [87]–[89]. Fermented foods are foods that have been subjected to the action of microorganisms or enzymes so that desirable biochemical changes cause significant modifications to the food [90]. Fermentation is an ancient process of food preparation that dates to the Neolithic period approximately 10,000 years ago [91]. Numerous fermented foods have been reputed to possess beneficial health effects, which is attributable to the probiotic effects of microbes present, and the metabolites which these microbes secrete. In various dietary intervention or large cohort studies, fermented food-consumers were found to have higher microbiome diversity and decreased markers of inflammation as compared to non-consumers [92], [93]. Microbes typically found in fermented foods, such as various lactobacilli species, were also found to be significantly higher in the gut microbiome of fermented food-consumers [92], [94], [95].

The wide diversity of fermented foods from different cultures around the world represents potential for novel strains to be discovered from foods yet to be extensively studied [88], [96]. Most bacteria derived from fermented foods are lactic acid bacteria, such as *Lactobacillaceae*, *Pediococcus*, and *Enterococcus*. Bacteria derived from fermented foods can be regarded to have higher safety profile, as they have a long history of safe consumption.

2.1.5. Probiotic industry trends

End-consumers and businesses have shown an increasing interest towards probiotics. Especially in the post-COVID-19 pandemic era, modern consumers are increasingly keen to utilise probiotics for boosting their immunity and gut health for prevention against diseases. The global probiotics market (inclusive of probiotic-containing foods, probiotic supplements and agricultural/animal probiotics) in 2021 is estimated at US\$58.17 billion with a projected CAGR of 7.5% through 2028 [17]. Within the dietary supplements market, the market size for probiotics is estimated at US\$6.12 billion, and expected to grow at CAGR of 7% through 2028 [97]. Thus far, probiotics are mainly consumed via dairy-based yogurts (74% of global retail value of probiotics), while ~11% is attributed towards probiotics supplements [98].

Growing applications of probiotics include in functional food, infant formula, dietary supplements, pharmaceuticals, and for agricultural or animal use. In the dietary supplements space, innovative probiotic products have arisen in recent years, some of which target specific ailments, others offered in different delivery formats, or are targeted at different customer segments (**Figure 2.4**). Dixit *et al* reviews patents which have been filed for probiotic formulations [99]. Commercial encapsulated probiotics formulations, which are of special interest in this paper, are further discussed in **Section 2.4.4**.



Figure 2.4: Segregation of probiotic products in the dietary supplements space

2.1.6. Characterisation of probiotics

To recognise a microbe as a probiotic, a series of studies need to be done to ascertain their taxonomic identity, safety, fitness, and efficacy (**Table 2.3**). These tests are typically conducted with an initial *in vitro* assessment of probiotic potential, following which, promising probiotic strains can be progressed to suitable *in vivo* tests. *In vitro* and *in vivo* tests may be selected based on the desired application outcome, e.g., for treatment of certain diseases, or for prophylactic, general well-being uses. The most advanced test for probiotics will be in double-blind, placebo-controlled clinical trials to determine their safety and efficacy in human hosts.

Table 2.3: Desirable probiotic characteristics and methods to screen for them. Criteria highlighted in purple, orange, blue, and green relate to taxonomic identification, safety aspects, fitness, and functional aspects of probiotics respectively.

Probiotic criteria	Method	Ref.
Well-defined taxonomic identity	16S ribosomal ribonucleic acid (rRNA) gene analysis is useful for species level identification, whole genome sequencing (WGS) required for strain-level definition.	[100]
Absence of virulence or toxin genes	Perform WGS and compare gene sequences with virulence finder databases.	[101]
Absence of AMR genes	Perform WGS and compare gene sequences with ResFinder and PlasmidFinder databases. Alternatively, perform antibiotic susceptibility tests.	[101], [102]
Non-cytotoxic	Commercial kits available, such as CytoTox 96 (Promega), which measures release of lactate dehydrogenase upon Caco-2 cell lysis	[101]
Does not produce concerning biogenic amines (BAs)	Detect concentration of BAs (e.g., histamine, tyrosine) secreted, and reference to recommended limits. Alternatively, detect BAs genes in whole genome sequence of strains.	[103]–[105]
Genetically stable	Sub-culture microbe for many generations (e.g., 100), check for phenotypic or genetic variation.	[106]
Tolerant to gastric acid	Expose microbes to SGF, measuring for their survivability.	[107]–[109]
Tolerant to bile	Expose microbes to SIF, measuring for their survivability.	[107]–[109]
Storage stable	Dried cells, or suspended in preferred media, are stored at designed conditions over time, and measured for survivability.	[110], [111]
Heat stable	Incubate microbes at desired temperature media, measuring for survivability	[30], [111]
Good mucoadhesive and colonisation properties	Can be tested using <i>in vitro</i> cell culture models, or with <i>ex vivo</i> intestinal models. Expose microbes to epithelial cells/lining, wash away unattached cells, and measure number of adherent microbes.	[30], [112]–[114]
Pathogen inhibition activity	<i>In vitro</i> co-culture with pathogenic microorganisms, checking for inhibition (e.g., zone of inhibition in agar diffusion assay, or cell counts in selective media). Also, can be tested via competitive exclusion studies with pathogens, i.e., whether probiotics can exclude the mucoadhesion/colonisation of pathogens on epithelial cell cultures or intestinal lining. <i>In vivo</i> pathogen challenge can also be conducted, i.e., infect animal host with pathogen, and see if probiotics can alleviate symptoms.	[101], [115]–[117]

Table continued in next page.

Cholesterol reduction	Incubate microbes with cholesterol <i>in vitro</i> , measuring concentration of cholesterol pre- and post-incubation by spectrophotometric method. Can also measure serum levels of cholesterol <i>in vivo</i> before and after probiotic administration	[30], [118]
BSH activity	Plate microbes on agars supplemented with bile salts (e.g., taurodeoxycholate (TDC)), check for white precipitate (deconjugated bile salt) after incubation.	[118]
Antioxidant activity	Use 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay to determine antioxidant activity in cell suspension or cell-free supernatant.	[116], [119]
Intestinal cell tight junction integrity	Measure transepithelial resistance of cell cultures or measure fluorescein isothiocyanate-labelled dextran permeability.	[120], [121]
Immune response	Measure pro-/anti-inflammatory factors in serum such as IL-4, IL-10, IgA, IgG, or IgM.	[122]
Secrete psychotherapeutic compounds	Detect neurotransmitters, such as γ -aminobutyric acid (GABA) and tryptophan, in faecal fermentation vessels by mass spectrometry (MS) or liquid chromatography (LC). Alternatively, check whole genome for genes which produce these metabolites. Could also perform analysis in the blood serum of <i>in vivo</i> candidates.	[123]
SCFAs production	Detect SCFAs, e.g., acetic, propanoic, and butyric acids, by MS or LC techniques.	[124]
Vitamin production	Detect vitamins by LC techniques. Also, can check whole genome for vitamin biosynthesis genes.	[125]

2.2. Prebiotics

The prebiotics concept was introduced for the first time in 1995 by Glenn Gibson and Marcel Roberfroid, and was originally defined as “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health” [126]. The ISAPP redefined prebiotics in 2017, as “substrates which are selectively utilised by host microorganisms thereby conferring a health benefit” [19]. In the following sections, the different types of prebiotics, their sources, and methods of production, as well as methods to characterise materials as potential prebiotics, are discussed.

2.2.1. Types of prebiotics

Current prebiotics are predominantly carbohydrate-based, with inulin, fructo-oligosaccharides (FOS), and galacto-oligosaccharides (GOS) being the most widely utilised [19]. Oligosaccharides are short-chained carbohydrates, typically between degree of polymerisation (DP) of 3-30 [19]. While fructose and galactose-containing oligosaccharides are the most extensively studied, oligosaccharides comprising other monomer sugar units such as glucose [127], mannose [128], xylose, arabinose and rhamnose [129], [130] have also been reported with potential prebiotic properties. Polysaccharides, which are longer chain carbohydrate polymers with $DP > 30$, have also exhibited useful prebiotic properties [131]–[133]. In general, oligosaccharides tend to be more easily fermentable than complex polysaccharides due to their lower DP [34], [134]. **Figure 2.5** illustrates the chemical structures of some oligosaccharides and polysaccharides with prior reported prebiotic properties. Besides carbohydrates, other compounds such as flavanols, poly-unsaturated fatty acids, conjugated linoleic acids have also exhibited prebiotic properties [19], but these will not be discussed in this thesis as we only plan to derive carbohydrate prebiotics from kefir.

Diverse types of prebiotics serve different functions in modulating the gut microbiome, such as stimulating growth of different groups of commensal microorganisms, or inducing

different gut metabolite profiles [18]. There exists an ongoing interest to identify novel prebiotics with different functionalities, particularly prebiotics which can be produced sustainably and cost-efficiently [18]. Another area of research interest is in identifying prebiotics that are synergistically paired with probiotic formulations to form synbiotics, wherein the prebiotic substrate can be selectively utilized by the co-administered microorganisms [135].

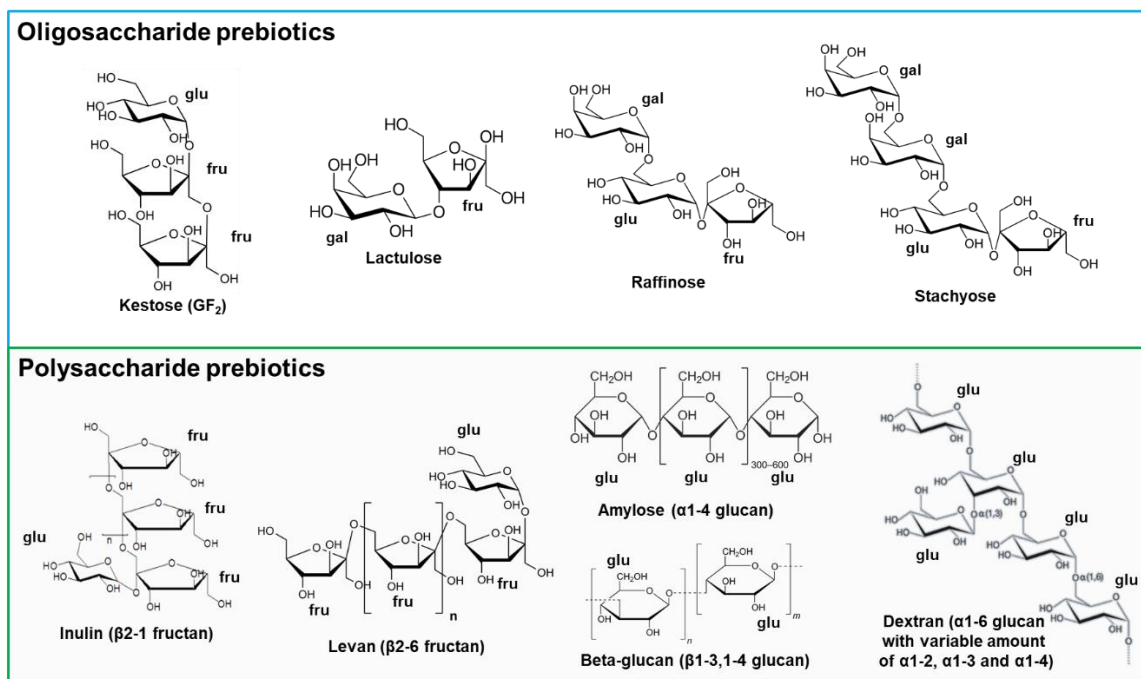


Figure 2.5: Chemical structures of some oligosaccharide and polysaccharide prebiotics. Abbreviations: Glu – glucose, Fru – fructose, Gal – galactose.

2.2.2. Sources and production of prebiotics

Prebiotics have been sourced from plants, such as chicory, garlic, soybean, and apples also from animal/human milk and honey [136]. Carbohydrate prebiotics within these sources can be extracted by a variety of methods, including hot water extraction, alcohol precipitation and enzymatic extraction [137]. Further purification typically follows, using techniques such as dialysis [137] and filtration [138] to remove low molecular weight impurities, the use of trichloroacetic acid (TCA) or enzymes to remove proteins [137], and addition of *Saccharomyces cerevisiae* to remove mono-/disaccharides [139]. Extracted

oligosaccharides and polysaccharides may be used directly, or polysaccharides can be further hydrolysed to form shorter-chain oligosaccharides. Hydrolysis methods can involve the use of heat, acid [140], microwave-assisted [141], or by enzymatic degradation using glucanases or pectinases [142].

Prebiotics have also been derived from microbial sources (**Table 2.4**). Numerous bacteria, yeasts and fungi secrete EPS with prebiotic properties. For instance, the fungi *Aspergillus niger* has been used commercially in the production of inulin and inulin-type FOS. The bacteria *Zymomonas mobilis* has been known to produce levans as EPS which can be further hydrolysed to form FOS [141]. Lactobacilli-derived EPS are also of particular interest as potential prebiotics, due to the GRAS status of lactobacilli, which contribute to overall safety and acceptance of their EPS as food or medical ingredients, and high EPS production capacity of certain lactobacilli strains [143]. For instance, levans produced by *Fructilactobacillus sanfranciscensis* and kefiran produced by *Lactobacillus* sp. have been studied for their prebiotic properties [143]. Depending on the strain and fermentation conditions, different types of oligosaccharides and polysaccharides at different yields can be achieved. The formation of these oligosaccharides and polysaccharides is achieved via the action of enzymes with transglycosylating activity. The enzymes may be isolated and purified to be directly used in prebiotic synthesis. Some examples of transglycosylating enzymes include fructosyl-transferases, which synthesise FOS from sucrose substrates [144], galactosidases and galactosyl-transferases which have been utilised to produce GOS from lactose [145].

Table 2.4: Prebiotics from microbial sources

Prebiotic	Source
Fructo-oligosaccharides (FOS)	Fungi - <i>Aspergillus japonicus</i> , <i>A. niger</i> , <i>A. sydowi</i> , <i>A. foetidus</i> , <i>A. oryzae</i> , <i>A. pullulans</i> , <i>Penicillium citrinum</i> , <i>P. frequentans</i> , and <i>Fusarium oxysporum</i> , <i>Aureobasidium</i> sp., <i>Kluyveromyces</i> , <i>Candida</i> , <i>Saccharomyces</i> ; Bacter-a - <i>Arthrobacter</i> sp., <i>Zymomonas mobilis</i> , <i>Limosilactobacillus reuteri</i> , and <i>Bacillus macerans</i> [144];
Galacto-oligosaccharides (GOS)	Transgalactosylation by galactosidases from <i>Aspergillus oryzae</i> , <i>Sterigmatomyces elviae</i> , <i>Bifidobacteria</i> , and <i>Lactobacillaceae</i> [136], engineered <i>Saccharomyces cerevisiae</i> [146]
Gluko-oligosaccharides (GLOS)	<i>Leuconostoc mesenteroides</i> [141], <i>Leuconostoc lactis</i> [147]
Inulin	<i>Aspergillus</i> , <i>Kluyveromyces</i> [144]
Levan	Bacter-a - <i>Bacillus subtilis</i> , <i>Bacillus megaterium</i> , <i>Streptococcus salivarius</i> , <i>Gluconacetobacter diazotrophicus</i> , <i>Zymomonas mobilis</i> , <i>Erwinia amylovora</i> , <i>Fructilactobacillus sanfranciscensis</i> , and <i>Pseudomonas syringae</i> [133]
Dextran	L-B - <i>Leuconostoc</i> , <i>Lactobacillaceae</i> , <i>Streptococcus</i> , <i>Weissella</i> , and <i>Pediococcus</i> [148]
Kefiran	Kefir-derived <i>Lactobacillaceae</i> and yeasts [149]

2.2.3. Prebiotic industry trends

The global prebiotics market demand was US\$4.7 billion in 2019 and is expected to grow at a CAGR of 11.2% from 2020 to 2026 [150]. Inulin accounted for greatest market revenue (38.06% in 2015) in the global prebiotic industry [151]. Compared with the probiotics market (US\$58 billion in 2021), the prebiotics market is approximately 10 times smaller. However, the rising demand of combinatory synbiotics is expected to drive higher demand for prebiotics [151]. The synbiotic market size is projected to reach US\$1.8 billion by 2026, growing at a CAGR of 8.9% from 2021 to 2026 [152].

2.2.4. Characterisation of prebiotics

Various methods have been described for the analysis of prebiotic carbohydrates. In the following sub-sections, the material characterisation techniques used to elucidate the chemical structure of prebiotics, as well as assays to determine the extent of prebiotic efficacy of extracted carbohydrates, are discussed.

2.2.4.1. Materials characterisation

As described earlier in **Section 2.2.1**, there are numerous types of carbohydrate prebiotics that differ in their chemical structures, hence yielding different prebiotic efficacies. These structural differences are described in **Table 2.5**. Besides chemical differences of the extracted poly-/oligosaccharides, the purity of the extracted carbohydrates can also be probed, such as by a spectrometry method or endotoxin assays [153].

Table 2.5: Structural differences of different carbohydrates and their corresponding characterisation methods

Structural difference	Examples	Characterisation methods
Monomer type	Fructose, glucose, galactose, xylose, or mannose	Complete hydrolysis by trifluoroacetic acid (TFA), followed by gas chromatography (GC) [154], high performance liquid chromatography with refractive index detector (HPLC-RID) [155], or Fourier transform infrared spectrometry (FTIR) [154]
Degree of branching	Linear vs Branched, Levans ($\beta 2 \rightarrow 6$ Fructan) vs Inulin ($\beta 1 \rightarrow 2$ Fructan)	Nuclear magnetic resonance (NMR) [34], [156]
Molecular weight/degree of polymerisation (DP)	High DP >30 (considered polysaccharides), or low DP <30 (oligosaccharides)	Gel permeation chromatography (GPC) with RID [154], thin layer chromatography (TLC) [157], electrospray ionisation mass spectrometry (ESI-MS) [157]

2.2.4.2. Prebiotic properties

The following criteria are required to classify a material as human oral prebiotics: 1) resistant to acidic pH of stomach and cannot be hydrolysed by digestive enzymes; 2) able to be fermented by intestinal microbiota; 3) selectively modulates the growth and/or activity of intestinal bacteria, thereby improving the host health [136]. A series of assays can be employed to evaluate the degree to which these criteria are fulfilled (**Table 2.6**).

Table 2.6: Assays to evaluate prebiotic properties of a material

Prebiotic criteria	<i>In vitro</i> assay methods
Resistant to acid and digestive enzymes	Expose material to SGF and SIF, followed by quantitation of reducing sugars and total sugars to determine % hydrolysis [158].
Selective modulation of microbiota – using pure cultures	Expose selected pure microbial cultures to broth with the tested material as the carbon substrate, perform optical density (OD) or CFU measurement to determine extent of microbial growth. Compare growth of probiotics and commensals relative to pathogen growth [158], [159].
Selective modulation of microbiota – using mixed cultures	Expose material to faecal inoculum, and determine microbiota composition changes by shotgun sequencing techniques, quantitative polymerase chain reaction (PCR) [160], or by use of fluorescent <i>in situ</i> hybridisation (FISH) with 16S rRNA gene oligonucleotide probes [156] Typically, the stimulation of lactobacilli and bifidobacterial populations is considered a desirable modulation of intestinal/colonic microbiota [19].
Evolution of SCFAs	Determine amount of SCFAs, such as lactate, formate, acetate, propionate, isobutyrate, butyrate, isovalerate and valerate, in fermentation supernatants via HPLC-RID [34].
Does not result in excess gas production	Measure amount of gas produced in faecal inoculum using specific apparatus [34]

While the use of pure microbial cultures for determination of selective modulatory effect is useful for preliminary screening of prebiotic materials due to its higher experimental throughput, it is necessary that the selective promotion of intestinal bacteria is verified in mixed microbial ecosystems containing the full microbiota of interest. In mixed microbial ecosystems, cross-feeding effects can arise, such as the degradation of complex polysaccharides by certain bacteria species, and the corresponding use of shorter-chain degradation by-products by other species [19]. It is necessary to determine the consequent net effect on overall microbiota population.

SCFAs (<6 carbons) are the main by-products of prebiotics fermentation and the production of SCFAs has been correlated with many health benefits in humans [161]. Acetate, propionate, and butyrate are the most abundant (90 – 95%) SCFAs in the human colon and stool, and are present in an approximate molar ratio of 60:20:20 [162]. SCFAs can modulate certain aspects of health including improved colonocyte and enterocytes functions, prevention of diarrhoea, alleviation of leaky gut and modulation of other important factors including energy and lipid metabolism and the immune system [161].

Prebiotics for human oral consumption should not form gas distension issues, e.g., flatulence, after ingestion; hence, their fermentation must be selective and preferably exclude gas-forming genera (such as *Clostridium*) [19].

2.3. Kefir

Kefir is an ancient, fermented beverage which has been consumed for centuries. This fermented food is reputed to originate from before the Bronze age, in the regions of Eastern Eurasia and the Caucasus mountains. The earliest archaeological evidence of kefir has been found to be in a kefir cheese form, within mummies found in a Xiaohe cemetery (1450 BC), Xinjiang, China [163]. Today, kefir grains have been distributed throughout the world, and is most popular in the regions of Russia, southwestern Asia and Eastern and Northern Europe [21]. In Singapore, kefir has also become increasingly popular, as evidenced by a 40-thousand-member kefir interest group on Facebook [164].

There are two types of kefir – milk kefir and water kefir, each of them being fermented by placing respective grains (**Figure 2.6**) in milk or sugary water and incubating at ambient temperature. Milk kefir is also known as “Tibetan mushroom kefir”, “gıyıp ayran”, “qundəps”, or “búlgaros”, depending on the region. Other names for water kefir include “sugary kefir”, “acquakefir”, “tibicos”, “water crystals”, and “ginger bees”. Kefir grains are cauliflower-like granules between a few millimetres and 3 cm in diameter. Milk kefir grains are opaque white in appearance, while water kefir grains are translucent. Both types of kefir grains comprise a symbiotic mixture of bacteria, yeasts and their EPS, and the microbial composition of milk and kefir grains are known to differ slightly [165].

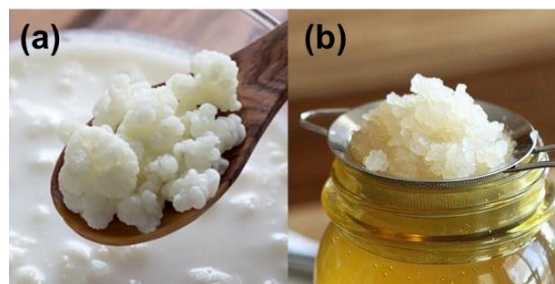


Figure 2.6: Images of kefir grains. (a) Milk kefir grains [166]; (b) Water kefir grains [167]

Kefir has gained increasing attention from researchers worldwide (**Figure 2.7**) due to its health-promoting efficacies. Most research interest in kefir has been from the countries, Turkey (13.2%), China (9.33%), Brazil (9.33%), Argentina (6.35%), Greece (5.72%) and USA (5.66%) (data from Web of Science, % indicates number of publications with keyword “kefir” affiliated to that country). A total of 15 clinical studies have been reported for kefir to date (based on the search query “kefir” on Clinicaltrials.gov).

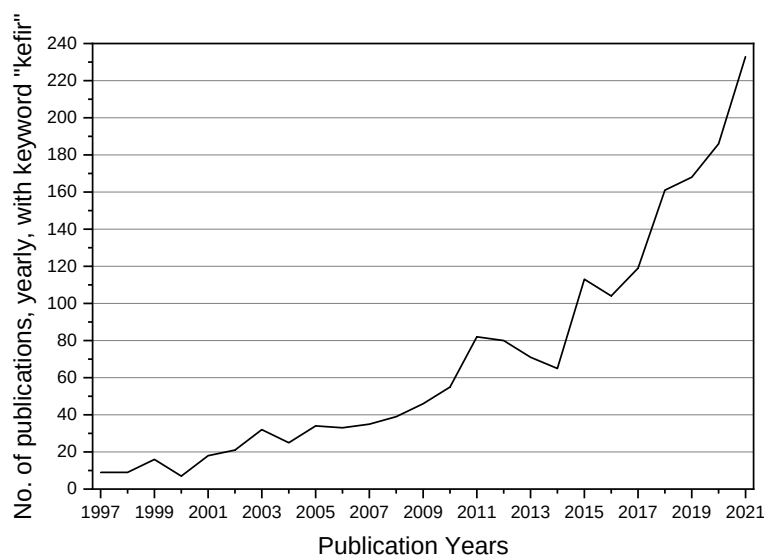


Figure 2.7: Number of scientific publications based on search keyword “kefir” from 1997-2021. Data for number of publications is derived from Web of Science (accessed 30-March-2022).

2.3.1. Kefir benefits and microbial composition

Since the ancient times, kefir has been rumoured to yield many health benefits, such as longevity and curative effects, on its drinkers [163]. In recent decades, animal and clinical studies have been conducted to seek a scientific understanding of the actual health benefits that can be associated with kefir. Some of the studied health benefits include antipathogenic, cholesterol assimilation, antioxidant, tumour suppression, wound healing, gastrointestinal immunity, anti-allergenic, and modification of gut microbiota (**Figure 2.8**) (extensively reviewed in [21]–[27]).

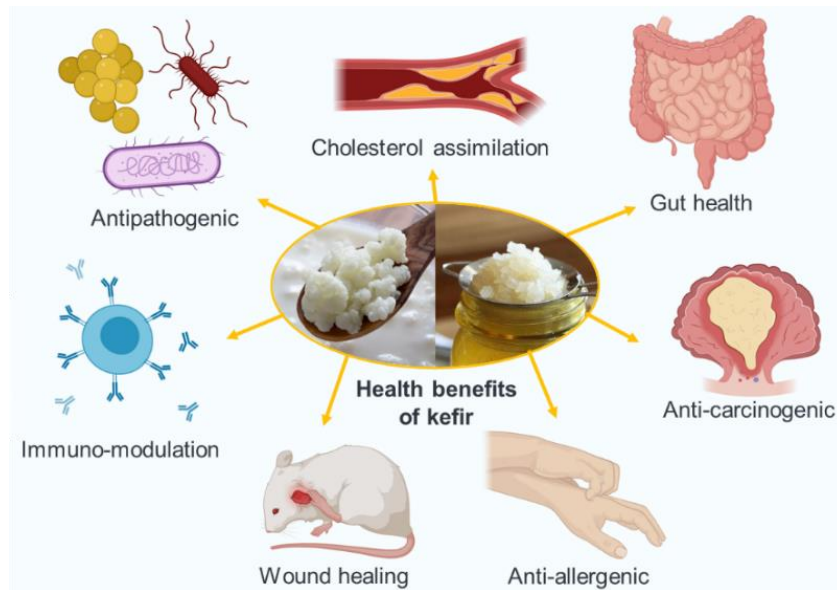


Figure 2.8: The health benefits of kefir [21], [168]. Image created using Biorender.

The health-promoting properties of kefir are likely attributable to its unique microbiota profile. The primary bacterial and fungal genera in kefir include lactobacilli, *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Acetobacter*, *Saccharomyces*, *Kluyveromyces*, and *Candida* [21], [169]. Several species isolated from kefir have been found to exhibit probiotic properties. For instance, numerous lactobacilli and yeast strains from kefir exhibited antipathogenic activities, inhibiting the growth of *Salmonella typhimurium* and enteropathogenic *Escherichia coli* O157:H7 [170], [171]. *Lactobacillus acidophilus*, *Lactiplantibacillus plantarum*, and *Lentilactobacillus kefiri* strains from milk kefir were found to lower serum total cholesterol, low-density lipoprotein and triglyceride levels in Sprague Dawley rats fed with a high cholesterol diet [172]. In a study by Vinderola *et al* [173], kefir containing viable microorganisms was found to be more effective in modulating the gut mucosal immune system than pasteurised kefir. These examples suggest that the health-promoting properties of the kefir beverage can be attributed to the properties of strains present within kefir.

The microbial composition of kefir is known to differ depending on various factors. As kefir grains are brought from one geographical region to another, the microbial compositions within these grains can change, owing to differences such as in water quality,

type of milk or sugar used, temperature, humidity, altitude or hygiene conditions [174]. Gao *et al* [175], Nejati *et al* [176], and Bengoa *et al* [177] have reviewed various species isolated from milk and water kefir of different geographical origins. Different cultivation conditions, such as the grain to substrate ratio and incubation times, are also known to affect the kefir microbiota [176]. Kefir grains and the bulk kefir beverage have also been observed to have differing microbial communities [21].

2.3.2. Kefir EPS

A variety of polysaccharide materials have been isolated from kefir. These materials may be isolated by precipitating directly from crushed or homogenised kefir grains, or by extracting from kefir-derived pure strain cultures. Polysaccharides, namely kefiran, dextran, and levan, have been isolated from kefir (**Table 2.7**). So far, the prebiotic effects of kefir-derived polysaccharides have not been investigated, although dextrans [34], [131] and levans [141] from other sources have showed prebiotic activities.

Table 2.7: Polysaccharides from kefir and their health properties

Polysaccharide/structure	Source	Effects on health
Kefiran (equal amounts of glucose and galactose)	<i>Lactobacillus kefiranofaciens</i> [178], [179], milk kefir grains [180]	Cholesterol and lipid modulation [178]; Anti-inflammatory [181], [182]; Prebiotic properties [149];
Dextran (glucan)	<i>Liquorilactobacillus hilgardii</i> [183], [184], <i>Liquorilactobacillus satsumensis</i> [185], [186], <i>Liquorilactobacillus hordei</i> [187], <i>Levilactobacillus brevis</i> [188], water kefir grains [189]	Prebiotic properties [34], [131], [190], [191];
Levan (fructan)	<i>Zymomonas</i> ssp. [192], water kefir grains [189]	Antitumor [193]; Immunostimulatory [194];

2.3.3. Kefir market trends and commercial products

The global kefir market is projected to grow at a CAGR of 6.4% from 2020 to 2028 and reach US\$2.48 billion by the end of 2028 [195]. This indicates an increasing awareness of

the health benefits of kefir and high demand for kefir products. Most of the kefir market share comes from sale of kefir beverages, specifically milk kefir (80% of global kefir market share in 2019) [196]. Commercially-produced kefir beverages are fermented via two main methods – using a combination of starter cultures, or by backslopping, which uses the fermented kefir beverage itself as a starter for the next batch [25], [33]. The use of starter cultures have become popular as it is considered more consistent than the use of grains and sensory attributes like taste, aroma and texture may also be optimised by tweaking the start culture composition [197]. However, such kefir beverages consist of a limited variety of strains, hence, are not comparable to the complex microbiota of naturally fermented kefir. Backslopping allows for large-scale production of kefir as it is not dependent on the heterogenous spatial distribution of kefir grains in the milk or sugary water substrate. However, backslopping is known to produce microbiota and sensory shifts in kefir in subsequent batches [33].

Besides beverages, kefir has also been adapted into confectionary and facial products (**Figure 2.9**). To the best of our knowledge, there have not been any kefir-derived probiotic or synbiotic supplements in the market. The closest product utilises kefir together with other probiotic cultures, but it appears the incorporated strains were not isolated from kefir [198].



Figure 2.9: Types of kefir products. From left – Milk kefir, water kefir, kefir cheese, kefir ice-cream, home-brew kefir starter cultures, kefir confectionary bar, kefir face mask.

2.4. Probiotics encapsulation systems

Encapsulation refers to a process of entrapping a cargo within a material to confer certain functionalities. The field of probiotics encapsulation systems, also commonly referred to as probiotic delivery systems, has been growing in the recent two decades, following the growing interest in the gut microbiome and probiotics (**Figure 2.10**). In the following subsections, the challenges involved in probiotics administration which warrant the development of encapsulation systems are described. Various probiotic encapsulation techniques and the commercially available probiotic encapsulation technologies are also analysed.

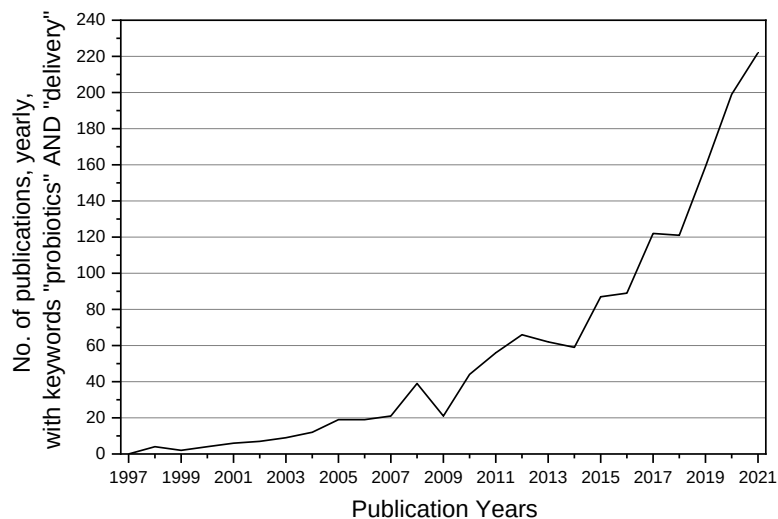


Figure 2.10: Number of scientific publications based on search keywords “probiotics” and “delivery” from 1997-2021. Data for number of publications is derived from Web of Science (accessed 4-April-2022).

2.4.1. Challenges in probiotics administration – GIT insults

Probiotics face numerous challenges in their administration to the lower human GIT. The gastric environment is considered most hostile for probiotics, as acidic and enzymatic stomach juices (pH 1–2.5, contains proteases, half gastric emptying time around 80.5 min [199]) readily kill off most living organisms. In the duodenum, probiotics are exposed to bile salts from the gall bladder. Bile functions as a biological detergent that emulsifies and

solubilises lipids, hence it has the ability to disrupt the phospholipids of probiotic cell membranes [200]. The typical site of action of probiotics is in the small and large intestine.

Dodoo *et al* recently assessed the viability of some commercial probiotics during GIT transit [201]. The results showed that all the tested commercial products suffered over six logs reduction in CFU within five minutes incubation in gastric acid. The poor viability of commercial probiotics against gastric acid insults suggests that the actual dosage of viable probiotics delivered to the intestinal region are much lower than the claimed probiotic count on the product label. This brings to question whether sufficient dosages of probiotics are supplemented to be able to incur a significant health benefit. There is, therefore, a need to utilise encapsulation systems to confer protection to probiotics against GIT insults, thereby increasing delivered probiotic dosage to the target site of action.

2.4.2. Challenges in probiotics administration – Shelf-life stability

Besides conferring gastroprotective effects, encapsulation systems can also enhance other functional benefits of probiotic formulations. These include, extension of shelf life, better probiotics survival in room temperature storage, and enhanced heat, freezing or desiccation resistance. These factors have important implications on the viability and efficacy of probiotics and are crucial to consider in the development of a probiotic product.

Generally, indicative shelf lives of probiotic food and beverages, such as yogurts and Yakult, range from 38 to 53 days [202], [203]. For probiotic supplements, typical shelf lives range between one to two years [204]. The relatively shorter shelf life of probiotic beverages can be associated with sensory changes due to metabolic activity of probiotics (e.g. accumulation of acids leading to “off-flavours”) [202], as well as lower survivability of probiotics in “wet” foods as compared to dried supplement formats [205]. A dry probiotic product typically achieves better shelf-life stability, as molecular mobility tends to be lower and cellular components of microorganisms are preserved [206]. Hence, microorganisms can enter a dormant state which allows them to remain stable for a long period of time.

Fajardo-Cavazos and Nicholson evaluated the ambient storage (~20 °C) stability of three commercial probiotic supplements – *Bifidobacterium longum* BB536 (Life Extension, Ft. Lauderdale, USA), *Lactobacillus acidophilus* DDS-1 (Nutricost, Orem, USA), and *Bacillus subtilis* HU58 (Microbiome labs, St. Augustine, USA) [207]. Significant viability losses between 2 to 3 logCFU per capsule were observed for *B. longum* and *L. acidophilus* over 100 days of storage, while *B. subtilis* remained viable with > 9.5 logCFU per capsule after 500 days of storage. This demonstrates that stability properties differ based on probiotic strains, and generally spore-forming microbes such as *Bacillus* or *Saccharomyces* tend to have better shelf-life stability [208].

An analysis of the viability of various expired probiotics supplements was also conducted by Wilcox *et al* [204]. Of 33 expired probiotic products evaluated, 11 products lost all probiotics viability, and 5 were within or above the original product cell count claim (**Table 2.8**). This suggests that it remains a challenge to extend shelf lives of probiotic supplements beyond 1 to 2 years, but it is realistically possible to have probiotics viable for long storage periods beyond ten years. From **Table 2.8**, it can also be seen that certain bacteria species, particularly *Bifidobacterium* spp., are less shelf stable and unlikely to retain viability over a long storage period. In the same article [204], Wilcox *et al* also observed that probiotic capsules stored in metal containers had higher viabilities than those stored in glass or plastic containers, with plastic containers showing worst probiotic viabilities. In the shelf storage phase, various environmental factors including humidity, ambient heat and oxidative stress can greatly reduce viability of microorganisms [204]. Different containers or encapsulation matrices can confer varying degrees of protection to prolong probiotics shelf life.

Table 2.8: Expired probiotics accessed in Wilcox *et al* [204]². Reproduced with permission.

No.	Labelled organisms	Species identified	Years expired	% Viability
A	<i>Streptococcus salivarius</i>	<i>S. salivarius</i>	4	80.4
B	<i>Lactobacillus paracasei</i> ST11	<i>L. paracasei</i>	7	108.3
C	<i>Lactobacillus acidophilus</i> , <i>Bifidobacterium lactis</i>	<i>L. acidophilus</i>	16	75.8
D	<i>Lactobacillus casei</i> , <i>Streptococcus faecalis</i>	<i>L. casei</i> , <i>Enterococcus faecalis</i>	13	107.5 (<i>L. casei</i>), 100.4 (<i>S. faecalis</i>)
E	<i>Lactobacillus rhamnosus</i> GG	N/A	17	0.0
F	<i>L. acidophilus</i> NAS super strain	N/A	15	0.0
G	<i>L. casei</i> HA-108, <i>L. rhamnosus</i> HA-111, <i>Bifidobacterium breve</i> HA-129, <i>Bifidobacterium longum</i> subsp. <i>longum</i> HA-135, <i>L. acidophilus</i> HA-122, <i>Lactobacillus plantarum</i> HA-119, <i>L. rhamnosus</i> (bifidus) HA-114, <i>Bifidobacterium bifidum</i> HA-132, <i>Lactobacillus fermentum</i> HA-179, <i>Lactobacillus salivarius</i> HA-118, <i>L. paracasei</i> HA-196, <i>Bifidobacterium animalis</i> subsp. <i>lactis</i> HA-194	<i>L. casei</i> , <i>L. fermentum</i> , <i>L. salivarius</i>	6	87.8
H	<i>L. acidophilus</i>	N/A	22	0.0
I	<i>L. rhamnosus</i>	<i>L. rhamnosus</i>	16	65.7

Table continued in next page.

² NA: not available. % viability is the proportion of cells present at expiry that remained viable at testing, shown here after logarithmic transformation.

A—Dr. Milk Probiotic Milk, Blis Technologies Ltd, Otago, New Zealand; B—DS, Innēov France, France; C—Bio-Tura, Pharma Nord, Marlton, New Jersey, United States; D—Yakult RI, Yakult Honsha Co., Tokyo, Japan; E—Culturelle, CAG Functional Foods, Omaha, Nebraska, United States; F—GY-NA-TREN FLORA, Natren, Westlake Village, California, United States; G—Ultimate Multi Probiotic, Natural Factors, Canada; H—Milk-Free Acidophilus, Schiff Vitamins, Salt Lake City, Utah, United States; I—Florgynal vaginal capsule, Laboratories IPRAD, Paris, France; J—Ultimate Flora, Renew Life, Oakville, Ontario, Canada, K—Plantadophilus, Transformation Enzyme Corp., Houston, Texas, USA, L—Yogurt, Institut Rosell, Montreal, Quebec, Canada; M—Women’s Multi Probiotic, Natural Factors, Canada; N—Reuterin, BioGaia AB, Stockholm, Sweden; O—Theralac, Therabiotics, Inc., Victoria, Minnesota, United States; P—Trevis, Chr. Hansen Biosystems A/S, Hørsholm, Tanska, Denmark; Q—Entero-Dophilus Globetrotter, Instuit Rosell, Montreal, Quebec, Canada; R—Investigational drug, Chr. Hansen Biosystems A/S, Hørsholm, Tanska, Denmark; S—Florastor, MFI Pharma, Richmond Hill, Ontario, Canada; T—Lactipan, EMS Sigma Pharma, Hortolândia, São Paulo, Brazil; U—Floratil 200, Merck, Kenilworth, New Jersey, United States; V1–5—UREX-cap-5, manufactured by Chr. Hansen, Hoersholm, Denmark, received for clinical studies; W—RepHresh Pro-B, manufactured by Chr. Hansen, Hoersholm, Denmark, distributed by Lil’ Drug Store Products, Inc., Cedar Rapids, IA; X—BiO-LiFE PRO-UTlx, manufactured by FA Herbs Sdn Bhd, Kuala Selangor, distributed by BiO-LiFE Marketing Sdn Bhd, Kuala Lumpur, Malaysia; Y1–2—Femdophilus, manufactured by Chr. Hansen, Hoersholm, Denmark, distributed by Jarrow Formulas, Los Angeles, CA; Z1–2—Ecoflora, manufactured by Tablets (India) Ltd, Pondicherry, India; AA—LaciBios femina, manufactured by Exeltis, Poland.

J	<i>L. rhamnosus</i> HA-111, <i>L. casei</i> HA-108, <i>L. plantarum</i> HA-119, <i>L. acidophilus</i> HA-122, <i>B. bifidum</i> HA-132, <i>Bifidobacterium longum</i> HA-135, <i>L. fermentum</i> HA-179, <i>L. salivarius</i> HA-118, <i>Lactobacillus bulgaricus</i> HA-137	<i>L. rhamnosus</i> , <i>L. fermentum</i>	6	92.9
K	<i>L. plantarum</i>	N/A	20	0.0
L	<i>L. rhamnosus</i> , <i>Streptococcus thermophilus</i> , <i>Lactobacillus delbrueckii</i>	N/A	10	0.0
M	<i>L. acidophilus</i> , <i>L. casei</i> , <i>L. rhamnosus</i> , <i>B. longum</i> , <i>L. plantarum</i> , <i>L. fermentum</i> , <i>L. lactis</i> , <i>S. thermophilus</i> , <i>B. breve</i> , <i>B. bifidum</i>	<i>L. rhamnosus</i> , <i>L. lactis</i>	6	92.2 (<i>S. thermophilus</i>), 98.1 (other strains)
N	<i>L. reuteri</i> ATCC55730	N/A	15	0.0
O	<i>L. paracasei</i> (F-19), <i>L. rhamnosus</i> (HN001), <i>L. acidophilus</i> (LA-1), <i>B. lactis</i> (HN019), <i>B. bifidum</i> (TB-12)	N/A	10	0.0
P	<i>L. acidophilus</i> , <i>B. lactis</i> , <i>L. bulgaricus</i> , <i>S. thermophilus</i>	<i>L. acidophilus</i> , <i>S. thermophilus</i>	18	89.9
Q	<i>L. rhamnosus</i> Rosell-11, <i>L. acidophilus</i> Rosell-52	N/A	15	0.0
R	<i>L. reuteri</i> RC14, <i>L. rhamnosus</i> GR1	<i>L. reuteri</i> , <i>L. rhamnosus</i>	1	86.7
S	<i>Saccharomyces boulardii</i>	<i>S. boulardii</i>	11	81.1
T	<i>S. boulardii</i> 17	<i>S. boulardii</i>	13	0.0
U	<i>S. boulardii</i> 17	<i>S. boulardii</i>	13	98.3
V1	<i>L. reuteri</i> RC14, <i>L. rhamnosus</i> GR1	NA	14	0.0
V2	<i>L. reuteri</i> RC14, <i>L. rhamnosus</i> GR1	<i>L. reuteri</i> , <i>L. rhamnosus</i>	11	95.9 (<i>L. reuteri</i>), 94.4 (<i>L. rhamnosus</i>)
V3	<i>L. reuteri</i> RC14, <i>L. rhamnosus</i> GR1	<i>L. reuteri</i> , <i>L. rhamnosus</i>	8	98.4 (<i>L. reuteri</i>), 90.0 (<i>L. rhamnosus</i>)
V4	<i>L. reuteri</i> RC14, <i>L. rhamnosus</i> GR1	<i>L. reuteri</i> , <i>L. rhamnosus</i>	6	91.9 (<i>L. reuteri</i>), 91.3 (<i>L. rhamnosus</i>)
V5	<i>L. reuteri</i> RC14, <i>L. rhamnosus</i> GR1	<i>L. reuteri</i> , <i>L. rhamnosus</i>	11	90.6 (<i>L. reuteri</i>), 93.0 (<i>L. rhamnosus</i>)
W	<i>L. reuteri</i> RC14, <i>L. rhamnosus</i> GR1	<i>L. reuteri</i> , <i>L. rhamnosus</i>	8	84.6 (<i>L. reuteri</i>), 85.5 (<i>L. rhamnosus</i>)
X	<i>L. reuteri</i> RC14, <i>L. rhamnosus</i> GR1	<i>L. reuteri</i> , <i>L. rhamnosus</i>	13	101.0
Y1	<i>L. reuteri</i> RC14, <i>L. rhamnosus</i> GR1	<i>L. reuteri</i> , <i>L. rhamnosus</i>	12	99.1 (<i>L. reuteri</i>), 94.6 (<i>L. rhamnosus</i>)
Y2	<i>L. reuteri</i> RC14, <i>L. rhamnosus</i> GR1	NA	12	0.0
Z1	<i>L. reuteri</i> RC14, <i>L. rhamnosus</i> GR1	<i>L. reuteri</i> , <i>L. rhamnosus</i>	13	92.7
Z2	<i>L. reuteri</i> RC14, <i>L. rhamnosus</i> GR1	<i>L. reuteri</i> , <i>L. rhamnosus</i>	13	100.8
AA	<i>L. reuteri</i> RC14, <i>L. rhamnosus</i> GR1	NA	11	0.0

2.4.3. Encapsulation techniques and materials

Various encapsulation technologies have been extensively reviewed in [199], [209]–[214]. These techniques can be broadly divided into three categories – 1) Wet, 2) Dry, and 3) Oil-based. **Table 2.9** describes and compares various probiotic encapsulation techniques, while **Figure 2.11** provides a matrix representation of these.

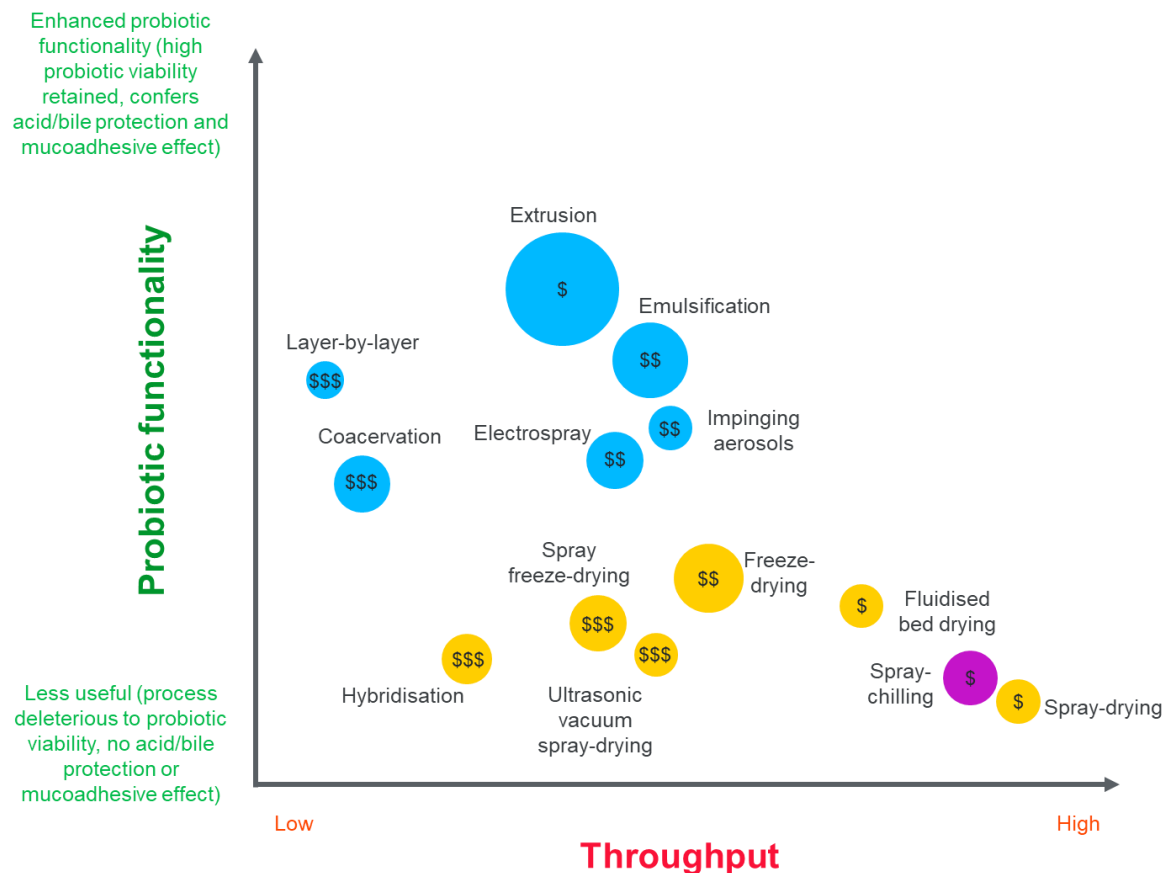


Figure 2.11: Matrix comparison of probiotic encapsulation techniques for particulate formation (excludes film or fibre forming processes). Techniques indicated with blue circles generate “wet” particles at the end of the process, yellow for “dry” particles, and purple for oil-based particles. Size of circles indicates the particle size yielded by each method. Dollar (\$) signs indicate the relative cost of each encapsulation technique.

Table 2.9: Techniques used to encapsulate probiotics

Encapsulation techniques	Particle size	Encapsulation materials	Major steps in processes	Advantages	Disadvantages
Extrusion (wet)	500 μm - 3 mm	Alginate [108], [215], κ -carrageenan [215], pectin [216], locust bean gum [217], cellulose [107], chitosan [215]	(i) Disperse probiotics in a polymer solution. (ii) Drop mixture via a syringe needle or atomiser into hardening solution. (iii) Collect particles by sedimentation.	- Simple and cheap method - Mild process can maintain high probiotic viability - Monodisperse particle sizes - Can be scaled up by using multiple nozzles - Well studied, many reference literature	- Low throughput for viscous polymer/probiotic mixtures - Difficult to produce particles smaller than 500 μm
Electrospray (wet)	20 - 200 μm	Alginate, acacia gum [214]	(i) Disperse probiotics in a polymer solution. (ii) Apply voltage difference between probiotic solution and hardening solution. Spray mixture into hardening solution.	- Smaller particle sizes than extrusion - Monodisperse particle sizes - Application of electric field generally causes no harm on microbes [218]	- Dripping of solution may occur if conditions not well optimised [214] - Difficult to scale up
Emulsification (wet)	1 μm - 1 mm	Alginate [219], chitosan [219], xanthan [220], locust bean gum [220], κ -carrageenan [220]	(i) Disperse probiotics in an aqueous polymer solution. (ii) Add probiotics suspension to an excess volume of oil. (iii) Homogenise the oil/water mixture to form water-in-oil emulsions. (iv) Add aqueous hardening solution to solidify probiotic beads. (v) Collect particles by sedimentation or filtration.	- Possible to form very small (1 μm) capsules - Scalable process	- High size polydispersity - Possible negative influence of high stirring rate on probiotics viability - Remnant oil might be present on beads surface - Large amount of oil used is considered a waste
Coacervation (wet)	20 - 200 μm	Gelatin, whey protein, pectin, caseinate, gum arabic, carrageenan, chitosan [209]	(i) Disperse probiotics in solution containing two or more hydrocolloids. (ii) Change pH of solution. pH change triggers polymers to yield opposite charges, hence the polymers link together and form a layer around probiotic cells. (iii) Collect particles by sedimentation.	- Mild process can maintain high probiotic viability	- Difficult to scale up

Table continued in next page.

Table 2.9: Techniques used to encapsulate probiotics (cont.)

Encapsulation techniques	Particle size	Encapsulation materials	Major steps in processes	Advantages	Disadvantages
Freeze-drying (dry)	Depends on upstream or down-stream (e.g., micronisation) processes	Sucrose, lactose, trehalose, skim milk, maltodextrin [214], cellulose sulfate [221], dextranomer [113]	(i) Disperse probiotics in cryoprotectant solution. (ii) Freeze solution. (iii) Freeze-dry by decreasing chamber pressure, enabling the frozen water to sublime.	- One of the mildest processes for drying probiotics and can retain high probiotic viability - Can yield probiotics with good shelf stability - Most common method used to produce shelf-stable dried probiotics	- Costly (mostly energy cost) - Long processing time - Requires careful optimisation of parameters (e.g., freezing rate, cryoprotectants, reconstitution media)
Spray-drying (dry)	2 - 100 μm	Resistant starch [222], skim milk [223], hydroxypropyl methylcellulose (HPMC) [224], chitosan [224], alginate [224]	(i) Disperse probiotics in a polymer solution. (ii) Spray-dry feed. Combined action of atomisation, vacuum and heat rapidly forms dried powder. (iii) Collect particles at spray-dryer collector.	- Directly produces dry powder from liquid feed - High throughput, can be operated on a continuous basis - Scalable - Low operational cost - Monodisperse particle sizes - High viability has been achieved for some strains [225]	- High temperature used in the process is deleterious to many probiotic strains
Fluidised bed drying (dry)	15 - 40 μm	CELLETS® [226], Whey protein, shellac [218]	(i) Add solid starter beads into fluidised bed dryer. (ii) Add probiotic with polymer solution into dryer. Probiotics layer and dry on surface of starter beads, drying is facilitated by air flow and mild heating. (iii) Add additional polymer layers and continue drying. (iv) Repeat wetting/drying cycles. (v) Collect dried particles when complete.	- Scalable - Able to incorporate multilayer coatings	- Agglomeration of particles frequently occur - Difficult to master this technique
Spray-chilling (oil)	20 - 200 μm	Cocoa butter, Palm oil	i) Disperse probiotics in a molten oil matrix by homogenisation (ii) Spray-chill feed. Simultaneous atomisation and chilling rapidly forms solid lipid particles. (iii) Collect particles at spray-chiller collector.	- High throughput, can be operated on a continuous basis - Scalable - Low operational cost	- Elevated temperature (usually >50 °C) of molten oil can be deleterious to probiotics [227] - Low encapsulation capacity as many probiotics tend to be on surface of beads. [214]

Table continued in next page.

Table 2.9: Techniques used to encapsulate probiotics (cont.)

Encapsulation techniques	Particle size	Encapsulation materials	Major steps in processes	Advantages	Disadvantages
Layer-by-layer (wet)	0–1 - 6 μm	Alginate, chitosan [114], carboxymethyl cellulose [228], dextran sulfate [229]	(i) Suspend probiotic in an aqueous polymer solution, intended to be the first layer (usually cationic). (ii) Wash the probiotic precipitate to remove excess polymer. (iii) Add second polymer (usually anionic) to probiotics (iii) Repeat steps i and ii, for further layering (iv) Collect particles by centrifugation	- Uses minimal polymer - Retains probiotic original morphology - May impart mucoadhesive properties to probiotic cells	- Cationic chitosan layer is often antibacterial and antagonistic towards probiotics - Demonstrates acid protection towards <i>Bacillus coagulans</i> [114], but not for <i>Lactobacillus acidophilus</i> [228] and <i>Lactobacillus pentosus</i> [111] - Generally limited studies
Impinging aerosols (wet)	10 - 40 μm [230]	Alginate [230]	(i) Disperse probiotics in a polymer solution. (ii) Spray aerosols of probiotic solution and crosslinking agent simultaneously into a chamber. (iii) Collect encapsulated particles from base of vessel.	- Smaller particle sizes achieved compared to alginate extrusion	- Limited studies
Spray freeze-drying (dry)	10 - 500 μm	Trehalose, maltodextrin [231]	(i) Disperse probiotics in a cryoprotectant solution. (ii) Spray solution into a cryogenic liquid, e.g., liquid nitrogen. (iii) Transfer frozen particles to freeze-dryer. (iv) Collect particles from freeze-dryer.	- More efficient freezing than freeze-drying method - Produces freeze-dried particulates	- Rapid freezing can be deleterious to cell viability - Limited studies
Ultrasonic vacuum spray-drying (dry)	20 - 50 μm [232]	Trehalose, maltodextrin [232]	(i) Disperse probiotics into a polymer solution. (ii) Ultrasonic vacuum spray-dry feed. Combined action of ultrasonic atomisation and vacuum forms dried powder without heating. (iii) Collect particles at collector.	- Improves on spray-drying method by using mild temperature conditions	- Costly as the equipment is highly niche currently - Limited studies
Hybridisation (dry)	20 - 100 μm [233]	FOS, lactulose [233]	(i) Disperse probiotics in a polymer solution. (ii) Dry solution in hybridisation equipment. Impaction and air stream generated by a high speed rotating blade achieves dry encapsulation. (iii) Collect particles at collector.	- Improves on spray-drying method by using mild temperature conditions	- Costly as the equipment is highly niche currently - Limited studies

In this thesis, the extrusion and spray-drying techniques are studied in greater detail, in combination with alginate as the encapsulation material. The following sub-chapters discuss alginate, the extrusion method, and the spray-drying method respectively.

2.4.3.1. Alginate as a probiotic encapsulation material

Alginate is a negatively-charged linear polysaccharide consisting of (1,4)-linked β -D-mannuronate (M) and α -L-guluronate (G) (**Figure 2.12a**), and is derived from brown algae or bacterial sources. Alginate is amongst the most commonly used material for probiotics encapsulation due to its biocompatibility, non-toxicity, natural source, and relatively low cost [29]. Alginate is also classified as safe for use in the European Union (E 401 and E 404) and in the United States (GRAS, 21 CFR 184.1187 and GRAS, 21 CFR 184.1724), and therefore, it can be used as a food-grade ingredient for probiotics encapsulation [234].

Ionic crosslinking of alginate is achieved by exposure to multivalent cation crosslinkers, typically Ca^{2+} , as divalent cations become ionically bonded to the carboxyl groups of the G residues of two neighbouring alginate chains in an “egg-box”-like structure (**Figure 2.12b**). Crosslinked alginate hydrogels are known to confer gastric acid-protective effects on entrapped microorganisms [30], [108], [215], [235]. Crosslinked alginates also facilitate intestine-targeted release of encapsulated microorganisms, as higher concentration of phosphate ions in the small intestine sequesters cation crosslinkers [236], hence “un-crosslinking” the alginate matrix.

Various properties of alginate, such as its M/G ratio, sequence of M and G units, G-block length, and molecular weight, are known to influence resultant properties of crosslinked alginate gels [29]. G residues are believed to have more affinity to crosslinking cations than M. Hence, alginates with higher M/G ratios tend to create more permeable alginate gel matrices, while lower M/G ratios lead to stiffer structures [237]. Higher molecular weight alginates are known to produce more viscous and mechanically stiff crosslinked alginates. The use of different cations also influence crosslinking, and the order of alginate affinity

to various cations has been found to decrease in the order: $\text{Pb} > \text{Cu} > \text{Cd} > \text{Ba} > \text{Ba} > \text{Sr} > \text{Sr} > \text{Ca} > \text{Co, Ni, Zn, Mn} > \text{Mg}$ [238].

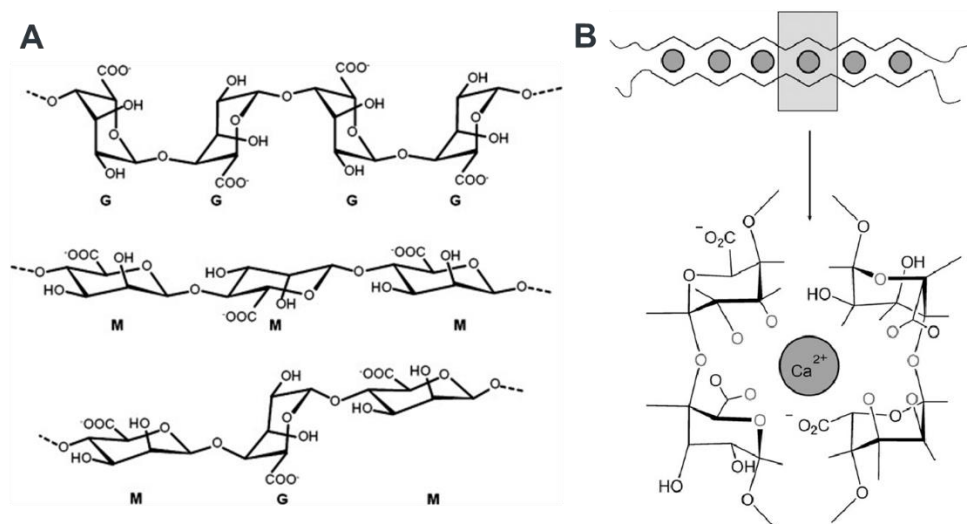


Figure 2.12: Chemical structures of alginate and crosslinked calcium-alginate. (a) Mannuronate (M) and guluronate (G) residues in alginate, presented as consecutive G-blocks (top), consecutive M-blocks (middle), and alternating M/G blocks (bottom) [29]. (b) Ionic crosslinking of alginate. G blocks participate in the formation of a crosslinked “egg-box”-like structure with interstices in which calcium ions are placed [239]. Reproduced with permission from various sources.

2.4.3.2. Extrusion encapsulation using alginate

Alginate extrusion refers to a technique of dropping alginate-probiotic suspensions into a crosslinking solution, such as calcium chloride. Alginate extrusion is by far the most well-studied encapsulation technique for probiotics encapsulation, due to its relative ease of implementation and mild gelation conditions involved. Spherical, “wet”, bead-like structures similar to “boba” that is popularly consumed in Taiwanese bubble tea are yielded from alginate extrusion. Numerous studies have demonstrated the success of alginate extrusion technique in protecting probiotics, including lactobacilli [30], [215], [235], *Lactococcus* [108], *Escherichia* [116], *Bifidobacterium* [235], and yeasts [240] species, against simulated gastric acid. Various studies have also explored the incorporation of these systems in food and beverages, such as in yogurts, ice cream, chocolate, and juices, , and showed that alginate encapsulation could improve cell viability in these food matrices

over a period of shelf life [241].

The limitation of alginate is, however, its large size and relatively low production throughput. It is difficult to produce extruded alginate particles smaller than 500 μm by a simple syringe pump and needle setup, due to surface tension forces which occur at the tip of the extruded needle. However, some extruders have been devised, such as the BUCHI B-390 encapsulator, which utilise vibration units to break up the flow of droplets from the needle, thereby achieving smaller particle sizes (down to 150 μm) [242]. To scale up production of extruded alginate beads, it is possible to utilise multiple nozzles droplet generation devices to improve production throughput.

2.4.3.3. Spray-drying encapsulation using alginate

Spray-drying is a high-throughput, easily scaled-up method of powder production which has been commonly employed in the industrial setting. Via the combined action of atomization, heat and vacuum drying, spray-drying generates dried powder particles rapidly in a single-step, making spray-drying a highly efficient and economical technique [32]. The resultant dry powder product is additionally advantageous in ways such as having prolonged shelf life, able to be versatily incorporated into different product types, and able to be transported more efficiently.

Despite the advantages of spray-drying, the main challenge associated with this technique for the encapsulation of probiotics is its high temperature and rapid dehydration process, which can greatly reduce bacteria viability [32], [39]. Previous studies have sought to overcome this issue, by proposing the use of protective agents, such as sucrose, inulin, trehalose, maltodextrin, skim milk and whey protein [38], [39]. These materials showed varying extents of improvement on probiotics survivability; however, few investigations were done with regards to the gastroprotective effect of these materials.

Regarding the spray-drying of probiotics with alginate, there are also several challenges associated. Mainly, the method of crosslinking alginate *in situ* within a spray-dryer is tricky.

Since spray-drying directly produces dry powder, it is counter-productive to spray-dry an alginate-probiotic slurry followed by crosslinking in an aqueous solution, as an extra step would still be required to dry the formulation. Feeding a crosslinked alginate formulation directly into the spray-dryer inlet would also be infeasible as high viscosity of the crosslinked gel may result in nozzle clogging. A method of *in situ* alginate crosslinking during spray-drying has been described by Jeoh's lab (**Figure 2.13**) [37], [243]–[247]; however, this method involves the use of a volatile base to facilitate the crosslinking process, which is potentially deleterious to many pH-sensitive probiotic microorganisms. As such, it appears that there have been no previously described methods of spray-drying with *in situ* alginate crosslinking suitable for encapsulating probiotics.

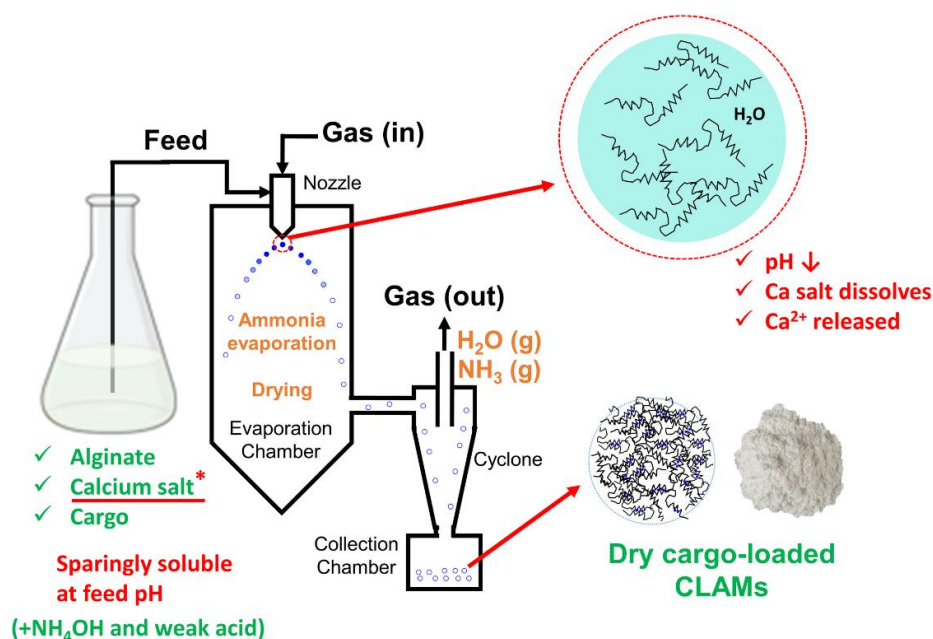


Figure 2.13: Method of spray-drying crosslinked alginate microparticles produced by Jeoh's lab [248]. Alginate formulation containing insoluble calcium and a weak acid titrated with a volatile base is atomized in a spray dryer to produce crosslinked alginate microcapsules. The solution pH drops upon volatilisation of the base after atomization, solubilizing calcium to interact with and crosslink negatively charged alginates. Reproduced under Creative Commons Attribution license.

2.4.4. Commercial encapsulated probiotic formulations

Corporations and consumers have grown increasingly aware of encapsulation technologies, and the added functionality that they can imbue to probiotic formulations. The market for encapsulated probiotics and prebiotics has been projected to record a 7.8% CAGR from 2021 – 2027 and reach US\$10.8 Billion by 2027 [249]. Presently, encapsulation technologies have been applied just 9% of probiotic supplement products [250]. A majority of probiotic supplement products incorporate prebiotics (inulin, FOS, GOS), anti-caking agents (magnesium stearate, silicon dioxide), antioxidants (ascorbic acid) within cellulose, gelatin, or hydroxypropyl methylcellulose (HPMC, also called hypromellose) capsules, with no explicit use of a protective encapsulation system. Commercial probiotic encapsulation brands and patents which have been released are described in **Table 2.10**.

HPMC capsules are typically water-soluble and do not confer acid protective effects to probiotics, but some manufacturers have incorporated additional enteric coatings or enteric polymer excipients to imbue “delayed-response” characteristics to the capsules (e.g., Capsugel® DRcaps®, Eudraguard® biotic, **Table 2.10**). However, the safety of these enteric coatings is in question as enteric polymers are typically synthetic phthalates, such as hypromellose phthalates (HPMCP), or synthetic methacrylates. Various studies have indicated health risks associated with accumulated phthalates exposure [251], [252], making such enteric coated capsules less suitable for probiotics supplement products, which are typically non-prescription-based, can be purchased over the counter, and require a high safety profile. A growing number of consumers also value dietary supplements made entirely from natural materials. As the probiotic supplement market becomes more competitive, delivery systems which have good safety profile and do not involve synthetic materials can act as a tool to improve functionality of probiotic products and differentiate them from other competition. Alginate-based encapsulation systems may provide a food-grade, natural solution suitable for the delivery of probiotics.

Table 2.10: Commercial encapsulated probiotic formulations

Product/Company	Description of encapsulation technology	Ref.
Duolac™ (Cell Biotech, South Korea), Nutrilite (Amway, USA)	Double coating LAB with a protein (skim milk/soy protein) and a polysaccharide (xanthan/cellulose/levan)	[253]
Bac-in-a-Box® (Austrinova Singapore Pte. Ltd., Singapore)	Freeze-dried cellulose sulfate microcapsules with probiotics	[221], [254], [255]
Capsugel® DRcaps® (Lonza Group AG, Switzerland)	HPMC with HPMCP capsules	[256], [257]
Eudraguard® biotic (Evonik Industries, Germany)	Poly(methyl acrylate-co-methyl methacrylate-co-methacrylic acid), a fully polymerised anionic copolymer of methyl acrylate, methyl methacrylate and methacrylic acid, in a 7:3:1 ratio respectively, as a pill coating	[258], [259]
Protein-based encapsulation (NIZO food research, Netherlands)	Core-shell spray-drying of probiotics. Core contains probiotic mix; shell contains crosslinked proteins	[260]
Dextranomer microspheres (Scioto Biosciences, USA)	Dextranomer microspheres containing sucrose/maltose are incubated with <i>L. reuteri</i> . Mechanism of attachment is due to glucosyltransferase protein, which binds the bacterial cell to exo-glucans. Dextranomer spheres can contain prebiotics, promoting LAB growth and/or secretion of antimicrobials.	[113]
BIO-tract® (Probi, Sweden)	Homogenous tablet formulation comprised of hydrophilic and hydrophobic components. Hydrophilic agents include swelling, viscosity increasing, gel strength enhancing agents. Hydrophobic agents include waxes and other inert materials. Tablets were prepared using a method of dry blending and direct compression using a Carver hydraulic press or a rotary tablet press.	[261]
Intelicaps® (Gencor, USA)	Production of spherical alginate beads in industrial scale using vibrating nozzle system	[262]
EnCaptimus™ (AnaBio Technologies, Ireland)	Gelled whey protein microencapsulation	[263]
Jintan probiotics capsule (Morishita Jintan Co. Ltd, Japan)	Soft capsule with gelatin and alginate in outer layer and an inner layer comprising hardened oil	[264]
Zeta probiotics (Cosmax NBT, Korea)	Lysine-based mixture for rehydration of freeze-dried probiotics	[265]

2.5. Relevance of research objectives in context of prior literature

This literature review revealed several interesting opportunities pertinent for this thesis. First, there is an ongoing search for novel probiotic strains. Despite a myriad of probiotic strains already existing for human consumption as supplement products, novel probiotic strains continue to be of interest, as these novel strains add diversity and may provide health efficacies via different mechanisms. The isolation of probiotics from kefir has been studied prior, focusing mostly on lactobacilli [171], [172], [266], [267], lactococci [268], and *Kluyveromyces* spp. [269]. Other species which have been identified in kefir by culture-independent techniques but were not successfully isolated or have yet to be studied for their potential probiotic qualities (e.g., *Liquorilactobacillus hilgardii* [270]). Additionally, microbial isolates from locally sourced (Singapore) kefir grains have never been prior reported, and there is potential for novel strains to be derived from this new source. Hence, this information adds to the importance and novelty of **Objective 1** of this thesis, that is *to isolate, identify and characterise probiotic strains isolated from locally sourced milk and water kefir*.

Next, the prebiotic effects of kefir-derived poly-/oligosaccharides have not yet been studied. Prior articles studying kefir-derived polysaccharides have focused mainly on optimising its yield [183] and its structural characterisation [189], while their potential applications in health remain to be further elucidated. There exists a plausible chance that kefir-derived carbohydrates have prebiotic properties, as similar materials (e.g., dextrans and levans) from other sources have displayed prebiotic properties [34], [131], [141]. Theoretically, it has also been proposed that EPS can serve as a nutrient carbon source for microbes residing in the biofilm community, hence there exists a possibility that kefir-derived probiotic strains could selectively utilise the kefir-derived EPS [271]. **Objective 2** of this thesis aims *to identify EPS-producing strains from kefir, extract EPS, hydrolyse EPS, and characterise their chemical structures and prebiotic potential*, and can help to relate the structural understanding of kefir-derived polysaccharides and their prebiotic properties.

In addition, this literature review has highlighted an increasing consumer demand for probiotics and synbiotics as dietary supplements as well as for kefir products. Currently, consumption of kefir is primarily via drinking the kefir beverage itself, while kefir-derived supplements and functional food products have yet to commercialise extensively. The concept of kefir-derived synbiotics has also not been explored in prior literature. Combining these areas of interest leads to **Objective 3** of this project – *to combine kefir probiotics and kefir prebiotics in a kefir synbiotic formulation*, which can serve as an alternative consumption format of the nutritious and popular kefir beverage.

Finally, this thesis has reviewed various encapsulation systems which can enhance functionality of probiotic formulations, by protecting the susceptible probiotic cargo against GIT insults and improving shelf-life stability. Alginate is a good choice of material for probiotics encapsulation due to its food-grade, natural source, mild gelation properties, and gastroprotective effects. In the choice of suitable encapsulation techniques, a variety of “wet” and “dry” methods, such as extrusion, emulsification, spray-drying, layer-by-layer, etc., can be used. Alginate extrusion techniques are well-studied and are well-suited for preliminary studies of the effectiveness of certain encapsulation materials. Alginate extrusion techniques are also suitable to incorporate encapsulated probiotics in food and beverage matrices, and the “boba-like” structure of extruded alginate beads make it particularly suitable for beverage applications. This leads to **Objective 4** of this study, *to develop a probiotic encapsulation system by alginate extrusion, for enhanced GIT resistance of probiotics and functionalisation of beverages*.

For spray-drying probiotics encapsulation, despite the industrial and economic advantages associated with the use of spray-drying, there appears to be a lack of suitable systems that can maintain high viable probiotic doses in the powder product and confer gastroprotection to probiotics. Innovations to spray-drying probiotics technologies can potentially provide a highly scalable and cost-effective way of producing encapsulated probiotics which are shelf-stable and resistant to GIT conditions, paving way to cheaper probiotics protection strategies. This indicates an important need for solutions that are represented by **Objective 5** of this study, *to develop a probiotic encapsulation system using spray-drying, aimed at*

delivering high viable doses of encapsulated probiotics which are shelf-stable and resistant to GIT conditions.

Overall, the five key objectives of this thesis address important unmet needs in the probiotics encapsulation field. In the following chapters, the research conducted to fulfil the five objectives of this study are detailed.

3. Chapter 3*

Identification of probiotic strains from kefir

Kefir grains are reported to impart numerous health-boosting properties to milk and water kefir beverages. The objective of this work was to investigate the microbial communities in kefir grains and explore the possibility of deriving useful probiotic strains from them. A total of 158 microbial strains, representing 6 fungal and 17 bacterial species, were isolated from milk and water kefir grains collected from a Singapore-based homebrewer. Seven bacterial strains were ultimately identified as potential probiotics, and combined to form a “kefir probiotics” formulation. These strains exhibited desirable probiotic characteristics, including enteric pathogen inhibitory property, good survival in acid and bile environments, bile salt hydrolase activity, antioxidant activity, non-cytotoxicity and high adhesion to Caco-2 cells, and a lack of virulence or antimicrobial resistance genes. Overall, probiotic candidates derived in this study are well-characterised strains with a good safety profile which can serve as novel agents to combat enteric diseases. These kefir-derived probiotics also add diversity to the existing repertoire of probiotic strains and may provide consumers with alternative product formats to attain the health benefits of kefir.

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3.1. Introduction

In this chapter, a systematic investigation of novel probiotic strains derived from locally-sourced milk and water kefir grains was performed, in pursuit of **Objective 1** of this thesis.

A series of phenotypic and genotypic screening assays were conducted to identify strains with the highest probiotic potential. These assays include agar well diffusion for detection of antipathogenic effects, cholesterol assimilation activity, BSH activity, tolerance to acidic pH/bile salts, antioxidant activity, non-cytotoxicity, adhesion to Caco-2 cells properties and whole genome analysis.

Based on these assays, best performing probiotic strains were identified and combined to form a “kefir probiotic blend”. The pathogen inhibition property of the “kefir probiotic blend” was finally evaluated using a competitive adhesion/exclusion assay to determine the ability of kefir probiotics to out-compete or exclude attachment of pathogens to Caco-2 cells.

3.2. Materials and Methods

3.2.1. Materials

Milk and water kefir grains used in this study were active grains donated by a homebrewer in Singapore. *Lactocaseibacillus rhamnosus* GG (LGG) (i-Health Inc., Cromwell, USA) and *Lactiplantibacillus plantarum* (wild type (WT) strain), were used as positive probiotic controls in various assays. *Escherichia coli* K-12, provided by colleagues at SCELSE, was used as a negative control for the well diffusion assay. Pathogens used in this study were enteric pathogens that infect the human gastrointestinal tract, as detailed in **Table 3.1**. All enteric pathogens were purchased from ATCC, USA, with exception of *K. pneumoniae* and *S. aureus* which were provided by colleagues at SCELSE. Human intestinal Caco-2 cell lines HTB-37™ were purchased from ATCC, USA.

Table 3.1: List of pathogens for screening

Species	Strain	Afflicted targets	Transmission routes
<i>Bacillus cereus</i>	ATCC 11778	Human intestine, respiratory tract, wounds, eye	Food [272]
<i>Salmonella enterica</i>	ATCC-BAA-190	Human intestine	Food [272]
<i>Vibrio parahaemolyticus</i>	ATCC 17802	Human intestine	Raw aquaculture [272]
<i>Clostridium perfringens</i>	ATCC 13124	Human intestine, domestic animal intestine	Food [272]
<i>Clostridium difficile</i>	ATCC 9689-FZ	Human intestine	Faeces from symptomatic CDI patients [273]
<i>Klebsiella pneumonia</i>	KP-1	Human wounds, urinary tract, intestine	Respiratory fluids from infected persons, person-to-person contact [274]
<i>Escherichia coli</i> O157:H7	ATCC 43888	Human intestine	Undercooked beef, contaminated produce, person-to-person contact [272]
<i>Staphylococcus aureus</i>	USA300	Human skin, respiratory tract, intestine, blood	Respiratory fluids, person-to-person contact, contaminated objects and surfaces [272]

De Man, Rogosa and Sharpe (MRS), Mannitol-yeast extract peptone (MYP), Yeast extract-peptone-glycerol (YPG), Yeast extract-peptone-dextrose (YPD), M17 and Nutrient broth/agar (NB/NA) media, were used for isolation of strains from the kefir granules. MRS, M17 and NB media were purchased from Thermo Fisher Scientific, USA. YPD was purchased from Sigma-Aldrich, USA. MYP medium was prepared with 25 g/l mannitol, 5 g/l yeast extract, 3 g/l peptone [275]. YPG contained 10 g/l peptone, 5 g/l yeast extract, 0.01 g/l bromophenol blue. 150 µg/ml of cycloheximide were added to MRS, MYP and NB media to inhibit yeast growth; 100 mg/l of chloramphenicol was added into YPG and YPD media to inhibit bacteria growth. Antibiotics were filtered and added into respective media after autoclaving. Tryptic soy (TS) medium, used for culturing of enteric pathogens, was purchased from Thermo Fisher Scientific, USA. Bacto agar was purchased from BD, USA. Yeast extract, Yeast protein extract reagent (YPER), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Dulb'cco's Modified Eagle Medium (DMEM), foetal bovine serum (FBS), trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) were purchased from Thermo Fisher Scientific, USA. Q5 High Fidelity PCR kit was purchased from New England Biolabs, USA. The CytoTox 96® non-radioactive cytotoxicity kit was purchased from

Promega, USA. DNeasy Ultraclean Microbial Kit was purchased from QIAGEN, Germany. All other chemicals used in this experiment were purchased from Sigma Aldrich, USA. Sterilisation by autoclaving at 121 °C for 15 minutes was done for every media, chemical solutions, or apparatus prior to use, where necessary.

3.2.2. Methods

Figure 3.1 shows an overview of the “funnel approach” used to screen the kefir-derived isolates. Amongst all isolates initially derived from milk and water kefir (158 strains in total, from 6 fungal and 17 bacterial species), 36 strains (includes 23 strains representing the 23 distinct species isolated and 13 more strains selected to test for strain-specific activity) were selected to evaluate the pathogen inhibition potential of various species and strains. After which, strains with probiotic potential were further evaluated for their BSH activity, antioxidant activity, tolerance to acidic and bile salts conditions, non-cytotoxicity, adhesion properties and whole genome properties. Kefir isolates with highest probiotic potential were finally combined in a “kefir probiotics blend”, which can be likened to a blend of probiotics and a competitive adhesion/exclusion assay was done to determine the ability of the “kefir probiotics blend” to out-compete or exclude attachment of enteric pathogens to Caco-2 cells.

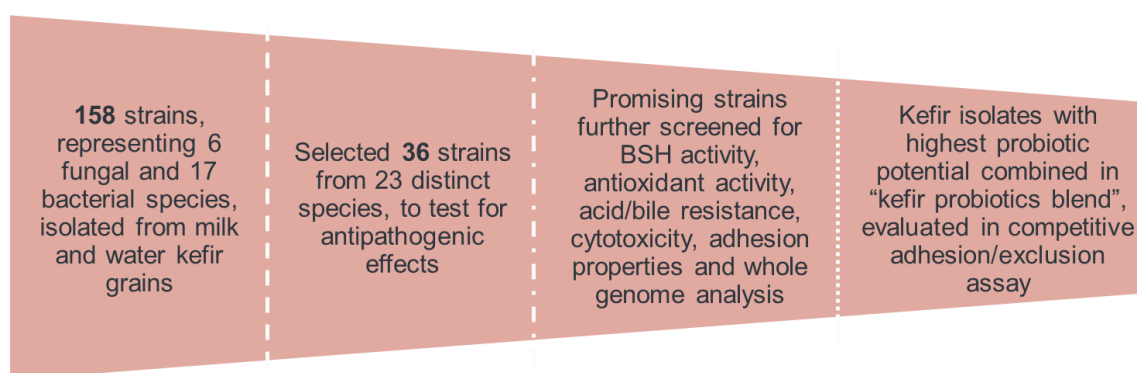


Figure 3.1: Funnel approach used to identify kefir strains with highest probiotic potential.

3.2.2.1. Isolation of microbes from milk and water kefir

Milk and water kefir grains were ground with mortar and pestle, then serially diluted in phosphate buffered solution (PBS), before spreading on various media, including MRS, MYP, YPG, YPD, NA and M17 agar (1.5% agar used). These media were selected in attempt to isolate of a variety of kefir microorganisms; MRS was used to isolate lactobacilli; MYP for acetic acid bacteria [275]; YPG and YPD for yeast [276]; NA as a general growth medium, and M17, for lactic streptococci [277]. Plates were incubated at 30 °C for three to seven days at both aerobic (air) and anaerobic (4% H₂, 5% CO₂, 91% N₂) conditions. Anaerobic handling of strains was done within the Bactron 300 anaerobic chamber (Sheldon Manufacturing, USA), and culture media used for these anaerobic strains were also pre-reduced by placing in the anaerobic chamber overnight. Representative colonies were selected for isolation and purified by streaking onto fresh agar plates. Purified colonies were then cultured in corresponding broths and aliquots were stored at -80 °C with 20% (v/v) glycerol as cryoprotectant.

3.2.2.2. Species identification by 16S rRNA gene and ITS sequencing

The 16S rRNA gene and internal transcribed spacer (ITS) segments were sequenced to determine the species identity of kefir-derived microbial strains. Individual isolates were cultured in respective broths and 100 µl of cell cultures were lysed by addition of 20 µl YPER and heating at 98 °C for 5 min [278]. The cell lysate was centrifuged at 14,000 xg for 10 min, and the supernatant was collected for PCR. Q5 High Fidelity PCR kit (New England Biolabs, MA, USA) was used with universal primers 27' (5' AGAGTTTGATCMTGGCTC'G 3'), and 1492' (5' TACGGYTACCTTGTTACGAC'T 3') to amplify 16S rRNA gene for bacteria. For yeast, primers used were ITS ' (5' TCCGTAGGTGAACCTGC'G 3') and ITS ' (5' TCCTCCGCTTATTGATAT'C 3'). The PCR reaction mix consisted of 10 µl 5X Q5 reaction buffer, 1 µl 10 mM dNTPs, 25 µM forward primer, 25 µM reverse primer, 0.5 µl Q5 High Fidelity deoxyribonucleic acid (DNA) polymerase, 5 µl DNA template and 28.5 µl nuclease-free water, totaling 50 µl.

PCR amplification was carried out with following parameters: 98 °C for 3 min, 30 cycles of 98 °C for 10 s, 57 °C for 15 s, 72 °C for 1 min, 72 °C for 2 min and holding at 12 °C. Quality of PCR products was checked by gel electrophoresis, performed at 100 V for 50 min with ethidium bromide staining for 30 min. Gels were viewed under ultraviolet (UV) light with Universal Hood II Gel Doc system (Bio-Rad Laboratories, USA) to obtain the band image. Samples that produced defined bands were sent to an external vendor (1st base, Singapore) for sequencing. Briefly, PCR products were purified using FavorPrep™ GEL/PCR Purification Kit (Favorgen Biotech Corporation, Taiwan). Purified DNA templates were then subjected to cycle sequencing following standard protocol from BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Products were purified by MagBio HighPrep™ DTR Clean-up System (Magbio Genomics, USA) before loading into ABI 3730xl DNA Analyser (Applied Biosystems, USA) for DNA Sanger Sequencing. Obtained nucleotide sequences were analysed using the ApE plasmid editor software [279] and species assignment of kefir isolates was done using the National Centre for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) platform, based on the BLAST result which yielded highest total score.

3.2.2.3. Agar well diffusion assay

Antimicrobial activities of isolates from kefir strains against pathogens were assessed using agar well diffusion [101]. 36 kefir strains representing 23 different species were selected for this assay. Kefir isolates were cultured in MRS broth at 30 °C, static conditions for 3 days at the respective oxygenated/anaerobic conditions as described in **Table S1**. LGG was used as a positive probiotic control (culture conditions indicated in **Table S2**). Pathogen cultures were inoculated in respective media and growth conditions as described in **Table S3**, before adjustment to Optical Density at 600 nm (OD₆₀₀) of 0.1 (except KP1, which was adjusted to OD 0.01), and spreading 100 µl onto respective agars (TS agar, NA, or NA with 3%). Wells 6 mm in diameter were formed in the agar and filled with 50 µl of cultures (contains microbes) or culture supernatants (without microbes), with duplicates performed for each kefir isolate strain. Cell-free supernatants were obtained by centrifuging the incubated cultures at 10,000 xg for 5 minutes. Plates were incubated in growth conditions

according to the growth requirements of each pathogen. Finally, plates were observed for the presence of inhibition zones around individual wells. Inhibition zones are clear sites that have no visible pathogen growth, indicating that the kefir isolates have successfully inhibited growth of the enteric pathogen.

3.2.2.4. Bile salt hydrolase (BSH) activity

BSH activity is a desirable probiotic trait which has been postulated to increase survival and persistence of microbes in the intestinal tract. BSH activity of probiotics has also been reported to induce cholesterol-lowering/fat-lowering efficacies via the following mechanisms: 1) Reduce fat emulsification ability of bile salts and 2) Deconjugated bile salts are more hydrophobic than their conjugated counterparts, thus are less reabsorbed through the intestines resulting in higher excretion into the faeces. Replacement of new bile salts from cholesterol as a precursor subsequently leads to decreased serum cholesterol levels. [200]

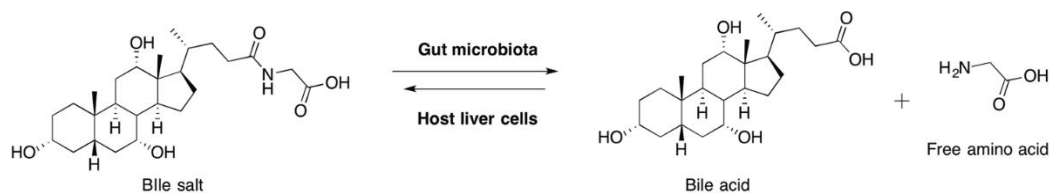


Figure 3.2: Some members of the gut microbiota deconjugates bile salts, which are produced by the liver [280].

BSH activities of kefir isolates were evaluated as previously reported by Michael *et al* [118]. Aliquots (5 µl) of the cultures of the kefir isolates were inoculated onto two sets of MRS agars, one containing 0.5% (w/v) taurodeoxycholate hydrate (TDC), another without. Plates were incubated at 37 °C under anaerobic conditions for 24 hours. BSH activity was indicated by the development of a white precipitate (the deconjugated bile acid, **Figure 3.2**) on TDC-supplemented agars after 48 hours. *Lactiplantibacillus plantarum* WT (culture conditions indicated in **Table S2**) was used as a positive control in this assay [118].

3.2.2.5. Acid and bile resistance

The protocol of Chandel *et al* [281] was used to examine the acid and bile resistance of the kefir isolates to determine their survival capacity during exposure to acidic pH and bile typical of the human GIT. In this assay, MRS broths were adjusted to pH 2, pH 3 or with 0.3% (w/v) ox-bile added. Aliquots (100 µl) of cultures of the kefir isolate were added to 4.9 ml of each of the adjusted MRS broths in duplicates and incubated at 37 °C for 2 hours under 200 rpm shaking conditions in an Ecotron shaking incubator (Infors AG, Switzerland). Ten-fold serial dilutions and drop plating (100 µl aliquots) was done to enumerate the colony-forming units (CFU/ml) before and after acid/bile exposure.

3.2.2.6. Antioxidant activity assay

Antioxidant activities are known to be present in several probiotics and constitute another potentially desirable probiotic attribute. Antioxidant activities of kefir isolates were determined using the DPPH free radical assay [282]. Aliquots (0.5 ml) of cultures of kefir isolates were added to 3 ml of 0.05 mM DPPH in ethanol in duplicate. Controls were prepared by mixing MRS broth (0.5 ml) with 3 ml of absolute ethanol. The reaction mixture was then incubated in the dark at room temperature for 30 minutes. A colour change from deep violet to light yellow could be observed if antioxidant activity was present. After incubation, the absorbance at 517 nm was measured with a spectrophotometer. The antioxidant activity percentage (AA%) was determined according to:

$$AA\% = [1 - (A_{sample}/A_{control})],$$

where A_{sample} is the average of measured absorbance at 517 nm of the sample with DPPH added minus the absorbance of MRS broth without DPPH added, and $A_{control}$ is the measured absorbance at 517 nm of MRS broth with DPPH added minus the absorbance of MRS broth without DPPH added.

3.2.2.7. Cytotoxicity of kefir isolates using Caco-2

The *in vitro* cytotoxicity of kefir isolates was determined using Caco-2 cells. The Caco-2 cells were maintained in DMEM containing 20% (v/v) FBS, 100 units/ml of penicillin and 100 µg/ml of streptomycin, at 37 °C in a humidified 5% CO₂ atmosphere. Caco-2 cells were seeded at a concentration of 2.8×10^4 cells/cm² in 24-well tissue culture plates and grown for 7 days until confluence was reached. For the last medium change, DMEM without antibiotics was used. Duplicate wells with confluent Caco-2 cells were inoculated with 0.5 ml of 1×10^7 CFU/ml (as determined by optical density from a standard culture of OD and CFU) kefir isolates, which were prepared by washing respective cultures of kefir isolates in PBS and resuspending in DMEM. Inoculated Caco-2 plates were then incubated for 24 hours in 37 °C, 5% CO₂ conditions. Following incubation, Caco-2 cells were washed twice with PBS, and the number of Caco-2 cells that remained viable in each well was determined with the CytoTox 96® non-radioactive cytotoxicity kit, using a protocol from Van den Bossche *et al* [283] which reduces bacterial interference with lactate dehydrogenase (LDH) quantification. Caco-2 cells were completely lysed with 200 µl of 1x Lysis solution (from kit) and incubated at 37 °C for 45 minutes. Efficient lysis of cells was further achieved through vigorous pipetting. Lysed Caco-2 cells were then centrifuged at 5,000 xg for 10 minutes, and 50 µl of the supernatant was added to 50 µl CytoTox 96® Reagent. Following a 30-minute incubation at room temperature in dark conditions, 50 µl of Stop Solution (from kit) was added to each sample, and absorbance at 490 nm was measured in a microplate reader (Infinite M200, Tecan Group Ltd, Switzerland). Un-inoculated Caco-2 wells were used as maximum lysis controls, and 1x Lysis solution was used to determine the background absorbance. Background absorbance was subtracted from all measured absorbance values with Caco-2 cells. Percent cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = 100 - 100 * \frac{\text{LDH from remaining viable cell fraction}}{\text{Maximum LDH release from no bacteria control}}$$

3.2.2.8. Adhesion of kefir isolates to Caco-2

The ability of kefir isolates to adhere to an intestinal surface was determined by an *in vitro* adhesion assay using the human epithelial cell line Caco-2 [101]. Caco-2 cells were seeded at 2.8×10^4 cells/cm² in 12-well tissue culture plates and the culture medium was changed daily for 21 days to allow growth into the late post confluence stage. For the last medium change, DMEM without antibiotics was used. Duplicate confluent Caco-2 cell monolayers were inoculated with 1 ml of 1×10^8 CFU/ml kefir isolates, which were prepared by washing cultures of the kefir isolates in PBS and re-suspending in DMEM. Inoculated Caco-2 plates were then incubated for 2 hours in 37 °C, 5% CO₂ conditions to allow for microbial attachment. Following incubation, non-attached or loosely adherent microbes were removed by washing Caco-2 monolayers three times with sterile PBS. To release adherent microbes, 200 µl of 0.25% (w/v) trypsin with 0.53 mM EDTA were added to each well, and incubated for 10 min in 37 °C, 5% CO₂ conditions. PBS (800 µl) was then added per well to dilute the trypsin-EDTA, and ten-fold serial dilutions and drop-plating was done to enumerate CFU of attached kefir microbes. Percentage adhesion was calculated by taking the ratio of attached microbes to the CFU of microbes added.

3.2.2.9. Whole genome sequencing (WGS) and genotypic characterisation

Selected kefir isolates were genotypically characterised by WGS. Genomic DNA was extracted from kefir isolates using a DNeasy Ultraclean Microbial Kit, according to manufacturer's instructions. The quality and concentration of extracted DNA concentration was checked via gel electrophoresis and a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, USA), respectively, prior to sequencing. Library preparation was performed using a TruSeq DNA HT Library Preparation Kit (Illumina, USA), and sequencing was done on the Illumina MiSeq platform with 300 base pairs (bp) paired end reads. Raw reads were cleaned using Trimmomatic version 0.39 [284], and quality of reads was checked with FastQC [285]. De novo genome assembly was done using SPAdes version 3.14.1 [286], and the quality of resultant assembled contigs was checked with the DDBJ Fast Annotation

and Submission Tool (DFAST) [287]. DFAST was also used to confirm the species and taxonomy of kefir isolates via average nucleotide identity (ANI) analysis [288]. Assembled contigs of kefir isolates Kef-w/m1-21 were submitted to GenBank under the BioProject ID PRJNA721546, with accession numbers JAGPZD0000000000 to JAGPZX0000000000. Functional gene annotation of assembled contigs was performed using the NCBI prokaryotic genome annotation pipeline (PGAP). Bacteriocins, virulence factors and AMR genes were identified using the BAGEL4 [289], Virulence Finder v2.0.3 [290] and ResFinder 4.1 pipelines [291] respectively. Plasmids were identified using PlasmidFinder 2.1 [292]. BAs and toxins production genes (including genes related to production of histidine decarboxylase, tyrosine decarboxylase, ornithine decarboxylase, agmatine dehydrolase, l-lysine decarboxylase, agmatine deiminase, hemolysin, cytotoxin, fengygin, surfactin, lichenisin and lipopolysaccharides) were manually searched for among annotated genes of each kefir isolate. Vitamins and GABA biosynthesis genes were also searched for manually. Taxonomic analysis was performed using the Type (Strain) Genome Server (TYGS), a free bioinformatics platform available under <https://tygs.dsmz.de> [293]. Briefly, the kefir isolate genomes were compared against all type strain genomes available in the TYGS database via the MASH algorithm to determine closely related type strains. These strains were compared pairwise to derive their intergenomic distances, then used to infer a balanced minimum evolution tree with branch support via FASTME 2.1.6.1 including SPR postprocessing.

3.2.2.10. Preparation of “kefir probiotic blend”

Based on the above phenotypic and genotypic characterisation assays, seven best performing kefir bacterial strains were selected for combination in a multi-strain “kefir probiotic blend” (**Table 3.2**). This multi-strain probiotics blend is hypothesised to provide a broader range of effects via more mechanisms of action [294], and may better simulate the health efficacies of consuming kefir as a whole. The kefir probiotics blend was prepared by adjusting the cultures of the selected kefir isolates to indicated OD600 values and concentrating each of the isolates into the same volume by centrifugation and resuspending in PBS. Total CFU/ml of kefir probiotics blend was approximately 3×10^9 CFU/ml.

Table 3.2: Composition of “kefir probiotics blend”

Kefir probiotic species	Strain	OD600 used in kefir probiotic blend	CFU in 1ml of kefir probiotic blend	CFU ratio
<i>Lentilactobacillus hilgardii</i>	Kef-w9	5.2	6.03E+07	0.02
<i>Lactacaseibacillus paracasei</i>	Kef-w14	6	8.57E+08	0.28
<i>Lactacaseibacillus paracasei</i>	Kef-w19	6	8.25E+08	0.27
<i>Liquorilactobacillus satsumensis</i>	Kef-w13	5.6	4.13E+08	0.13
<i>Liquorilactobacillus satsumensis</i>	Kef-w11	5.6	5.56E+08	0.18
<i>Lactobacillus helveticus</i>	Kef-m4	6	1.75E+08	0.06
<i>Lentilactobacillus kefir</i>	Kef-m15	2.5	2.06E+08	0.07

3.2.2.11. Competitive adhesion and exclusion of enteric pathogens by the kefir probiotic blend

An important probiotic mechanism of action is through adhesion to host cells, which competitively blocks the adherence of, or excludes, pathogens from host cell binding sites [295]. The ability of kefir isolates to compete for adhesion sites with or exclude pathogens from Caco-2 epithelial cells, was tested using a modification of an established method [117], [296]. Briefly, Caco-2 cells were seeded at a concentration of 2.8×10^4 cells/cm² in 12-well tissue culture plates and grown for 21 days to allow growth into the late post confluence stage. For the last medium change, DMEM without antibiotics was used. Cultures of the eight human enteric pathogens included in **Table S3** and the kefir probiotics blend as indicated in **Table 3.2** were used. For this assay, pathogens and kefir isolates were prepared by washing cultures in PBS and re-suspending in DMEM, to yield 1×10^8 CFU/ml respectively. Duplicate confluent Caco-2 cells were inoculated with either: 1) 1 ml of a mixture of kefir isolates and individual pathogens, and incubated for 2 hours, for the competitive adhesion assay, or 2) 1 ml of kefir isolates, incubated for the first hour, prior to the removal of kefir isolates and the addition of 1 ml of individual pathogens and incubated for the next hour, for the competitive exclusion assay. Incubation was done under 37 °C, 5% CO₂ conditions to allow for probiotics/pathogens attachment. Following incubation, non-attached or loosely adherent bacteria were removed by washing Caco-2 monolayers three times with sterile PBS. To release adherent bacteria, 200 µl of trypsin-

EDTA were added to each well, and incubated for 10 min in 37 °C, 5% CO₂ conditions. PBS (800 µl per well) was then added to dilute the trypsin-EDTA, and drop-plating of aliquots from ten-fold serial dilutions onto selective agars (e.g., NA or TS media were used to enumerate enteric pathogens, while MRS was used to enumerate kefir isolates) to enumerate attached probiotics/pathogens as CFU. The rationale of the competitive adhesion method is to determine if the kefir isolates could reduce pathogen adhesion to Caco-2 when added in combination, while competitive exclusion determines if pre-exposure to kefir microbes can reduce attachment of pathogens. The CFU data of attached probiotics/pathogens in the competitive adhesion and exclusion methods were hence compared to the adhesion data of individual pathogens to Caco-2 cells. Normalised adhesion was calculated as the percentage of pathogens adhered (competitive adhesion or competitive exclusion method) divided by the percentage of pathogens adhered when added alone.

3.2.2.12. Statistical analysis

Results for the competitive adhesion/exclusion assay were expressed as mean of duplicates with standard deviation bars. Letters on bars were determined by Dunnett test based on a pairwise-comparison with pathogen-only treatment, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns – no significant difference.

3.3. Results

3.3.1. List of isolated strains

In total, 158 strains representing 6 fungal and 17 bacterial species, were isolated from milk and water kefir (**Table 3.3**). Isolated genera include *Lactobacillus*, *Liquorilactobacillus*, *Lacticaseibacillus*, *Lentilactobacillus*, *Leuconostoc*, *Lactococcus*, *Acetobacter*, *Gluconobacter*, *Oenococcus*, *Clostridium*, *Zymomonas*, *Saccharomyces*, *Kluyveromyces*, *Pichia*, *Lachancea*, *Candida* and *Brettanomyces*. Most species were isolated from milk or water kefir exclusively, with exception of *Pichia fermentans*, *Saccharomyces cerevisiae* and *Clostridium beijerinckii*, which were isolated from both milk and water kefir.

Table 3.3: List of microbial strains isolated from milk and water kefir

No.	Microorganism Species	Kingdom	Number of strains isolated	Strain names	Source	Isolation media	Previously isolated from kefir	Previously identified in kefir by metagenomic techniques
1	<i>Liquorilactobacillus satsumensis</i>	Bacteria	20	Kef-w1, Kef-w2, Kef-w11, Kef-w13, Kef-w18, Kef-w22-36	Water Kefir	MRS, MYP, NA, YPD, M17	[270], [297], [298]	[299]
2	<i>Lactobacillus kefiranoferiens</i>	Bacteria	4	Kef-m3, Kef-m37-39	Milk Kefir	MRS, MYP	[171]	[300]–[302]
3	<i>Lactobacillus helveticus</i>	Bacteria	1	Kef-m4	Milk Kefir	MYP	[297], [303]	[301], [302]
4	<i>Acetobacter persici</i>	Bacteria	4	Kef-w5, Kef-w40-42	Water Kefir	MYP	[304]	-
5	<i>Leuconostoc mesenteroides</i>	Bacteria	5	Kef-m6, Kef-m16, Kef-m43-45	Milk Kefir	MRS, M17	[184], [270], [305]	[300], [301], [306]
6	<i>Lacticaseibacillus paracasei</i>	Bacteria	12	Kef-w7, Kef-w14, Kef-w17, Kef-w19-20, Kef-w46-52	Water Kefir	MRS, NA, YPD, M17	[171], [297], [298], [307]	[300], [302], [308]
7	<i>Liquorilactobacillus hilgardii</i>	Bacteria	3	Kef-w8-10	Water Kefir	MRS	[184], [298]	[299], [308], [309]
8	<i>Zymomonas mobilis</i>	Bacteria	10	Kef-w12, Kef-w53-61	Water Kefir	MRS, YPD, YPG	-	[309]

Table continued in next page.

9	<i>Lentilactobacillus kefir</i>	Bacteria	2	Kef-m15, Kef-m62	Milk Kefir	MRS, YPD	[172], [270], [310], [311]	[300], [301], [306]
10	<i>Liquorilactobacillus nagelii</i>	Bacteria	1	Kef-w21	Water Kefir	YPD	[298], [305]	[308]
11	<i>Lactobacillus delbrueckii</i>	Bacteria	1	Kef-m63	Milk Kefir	MYP	[303]	[306]
12	<i>Lactococcus lactis</i>	Bacteria	31	Kef-m64-93	Milk Kefir	MRS, MYP, NA, YPD, M17	[270], [303], [310], [311]	[300], [301]
13	<i>Acetobacter fabarum</i>	Bacteria	7	Kef-m94-100	Milk Kefir	MRS, MYP, YPD	[305], [311]	-
14	<i>Kluyveromyces marxianus</i>	Fungi	8	Kef-m101-108	Milk Kefir	MYP, YPG	[303], [311]–[313]	[302], [306]
15	<i>Acetobacter orientalis</i>	Bacteria	3	Kef-m109-111	Milk Kefir	MYP, YPD	[305]	[300]
16	<i>Pichia fermentans</i>	Fungi	3	Kef-m112-113, Kef-w114	Milk and Water Kefir	YPG	[297]	-
17	<i>Saccharomyces cerevisiae</i>	Fungi	19	Kef-m115, Kef-w116-125, Kef-m126-129, Kef-w130-133	Milk and Water Kefir	YPD, YPG	[297], [298], [303], [311], [312]	[306], [308]
18	<i>Clostridium beijerinckii</i>	Bacteria	3	Kef-w134, Kef-m135-136	Milk and Water Kefir	MYP, YPD	-	[302]
19	<i>Lachancea fermentati</i>	Fungi	11	Kef-w137-147	Water Kefir	YPD, YPG	[305]	[192]
20	<i>Candida ethanolica</i>	Fungi	4	Kef-w148-151	Water Kefir	YPG	-	[314]
21	<i>Gluconobacter oxydans</i>	Bacteria	1	Kef-w152	Water Kefir	YPD	[298]	[299]
22	<i>Oenococcus oeni</i>	Bacteria	3	Kef-w153-155	Water Kefir	MYP	[270]	[299], [301]
23	<i>Brettanomyces anomalus</i>	Fungi	3	Kef-w156-158	Water Kefir	YPG	[313]	-

3.3.2. Pathogen inhibition using well diffusion assay

Amongst the 158 kefir-derived isolates, 23 strains representing the 23 distinct species isolated, together with 13 more strains selected to test for strain-specific activity, a total of 36 strains, were selected for a detailed screening. Of these 36 tested strains, 24 kefir-isolates showed inhibitory activities towards at least one of the tested pathogens (**Table 3.4**). Photos of agar wells with inhibition zones are included in **Figure 3.3**. These kefir-isolates were from the *Lactobacillus*, *Liquorilactobacillus*, *Lentilactobacillus*,

Lacticaseibacillus, *Leuconostoc*, *Lactococcus* and *Gluconobacter* genera. None of the tested kefir-fungal strains showed antimicrobial properties. The growth of the common food borne pathogens *V. parahaemolyticus*, *B. cereus* and *S. enterica* were most inhibited by multiple kefir isolates. Inhibition of *C. difficile*, a bacterium which causes diarrhea and colon inflammation in humans [315], was observed for the isolates *Lacticaseibacillus paracasei*, *Liquorilactobacillus satsumensis* and LGG. Enteropathogenic *E. coli* O157:H7, a particular virulent serotype of *E. coli* known to cause diarrhea, abdominal cramps and other complications [316], was found to be inhibited by several *Lacticaseibacillus paracasei* strains. *K. pneumoniae* and *S. aureus* were also inhibited by *Liquorilactobacillus satsumensis*, *Lacticaseibacillus paracasei* and *Liquorilactobacillus hilgardii* kefir isolates. The kefir isolates inhibited both Gram-positive (*B. cereus*, *C. difficile*, *S. aureus*) and Gram-negative (*V. parahaemolyticus*, *S. enterica*, *E. coli*, *K. pneumoniae*) pathogens. Inhibitory mechanisms of kefir-isolates against these pathogens are most likely attributed to production of antimicrobial metabolites, such as organic acids, hydrogen peroxide and bacteriocins [317]. Notably, the pathogen inhibition capacity of several kefir isolates, particularly *Lacticaseibacillus paracasei* and *Liquorilactobacillus satsumensis*, outperformed the reference probiotic LGG. Comparing between different strains of the same species, some slight differences in inhibitory activities can be observed. Overall, *Liquorilactobacillus satsumensis*, *Lactobacillus helveticus*, *Leuconostoc mesenteroides*, *Lacticaseibacillus paracasei*, *Liquorilactobacillus hilgardii*, *Lentilactobacillus kefir*, *Liquorilactobacillus nagelii*, *Lacticaseibacillus paracasei* and *Gluconobacter oxydans* showed most promising antimicrobial efficacies. These promising strains were selected for further phenotypic screening.

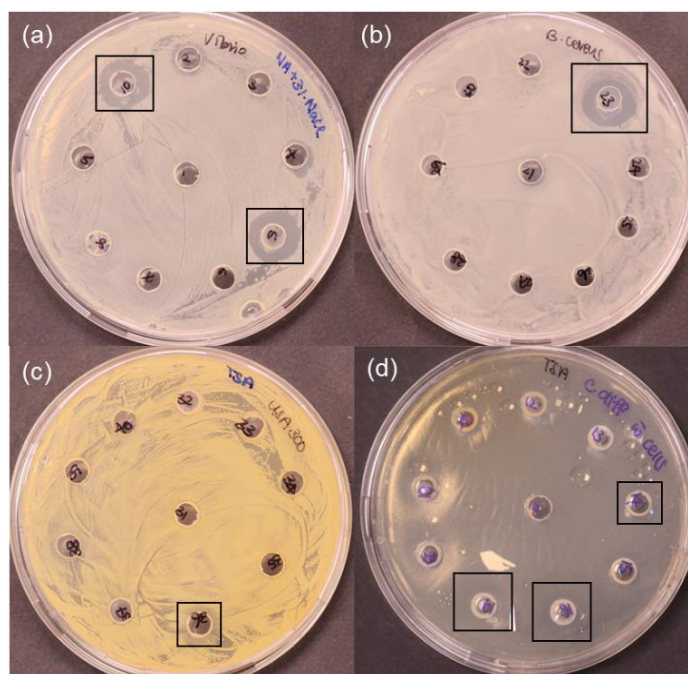


Figure 3.3: Image of various agars with (a) *V. parahaemolyticus*, (b) *B. cereus*, (c) *S. aureus* and (d) *C. difficile* in the well diffusion assay. Black boxes indicate that cultures/cell-free supernatants within indicated wells that demonstrated inhibitory activity towards the enteric pathogens.

Table 3.4: Inhibitory activities of kefir isolates and LGG against human enteric pathogens³.

Presence of zone of inhibition										
No.	Kefir isolate species	Strain	Pathogens							
			<i>B. cereus</i>	<i>S. enterica</i>	<i>V. parahaemolyticus</i>	<i>C. perfringens</i>	<i>C. difficile</i>	<i>K. pneumoniae</i>	<i>S. aureus</i>	<i>E. coli</i> O157: H7
1	<i>Liquorilactobacillus satsumensis</i>	Kef-w1	+++	++	+++	+++	-	-	-	-
2		Kef-w2	+++	++	+++	-	+	+	+	-
3		Kef-w11	+++	+	+++	-	++	-	-	-
4		Kef-w13	+++	+++	+++	-	-	-	-	-
5		Kef-w18	+++	-	+++	-	+	-	-	-

Table continued in next page.

³ The bottom-left triangle indicates inhibition by kefir isolate cultures, while the top-right triangle indicates inhibition by the cell-free supernatant component. Inhibition zones of > 4 mm, 2-4 mm and < 2 mm were reviewed as strong (+++), intermediate (++) and weak inhibition (+), respectively. Cells indicated with (-) are without any zone of inhibition observed. Only kefir isolates with inhibitory activities towards at least one tested pathogen are listed. Zones of inhibition were averaged from duplicate wells.

6	<i>Lactobacillus kefiranoferiens</i>	Kef-m3	-	-	+	-	-	-	-	-	-
7	<i>Lactobacillus helveticus</i>	Kef-m4	-	-	++	++	-	-	-	-	-
8	<i>Leuconostoc mesenteroides</i>	Kef-m16	+	-	++	+	-	-	-	-	-
9		Kef-m45	+	-	+	+	-	-	-	-	-
10	<i>Lacticaseibacillus paracasei</i>	Kef-w7	+++	+++	+++	+++	-	++	+	-	++
11		Kef-w14	+++	+++	++	++	+++	++	+	-	-
12		Kef-w17	+++	-	+	+	+++	-	+	-	+
13		Kef-w19	+++	+++	+++	+++	-	+	-	-	++
14		Kef-w20	++	-	+	+	++	+++	-	-	+
15		Kef-w8	+	-	-	++	-	-	+	+	-
16	<i>Lentilactobacillus hilgardii</i>	Kef-w9	++	+	+	++	+	-	-	-	-
17		Kef-w10	+	+	++	+	++	-	-	-	-
18	<i>Lentilactobacillus kefir</i>	Kef-m15	-	-	-	++	-	-	-	-	-
19	<i>Liquorilactobacillus nagelii</i>	Kef-w21	+	+++	-	++	+++	-	-	-	-
20	<i>Lactobacillus delbrueckii</i>	Kef-m63	-	-	-	+	-	-	-	-	-
21	<i>Lactococcus lactis</i>	Kef-m64	-	-	-	+	+	-	-	-	-
22		Kef-m68	-	-	-	+	-	-	-	-	-
23		Kef-m89	-	-	+	+	-	-	-	-	-
24	<i>Gluconobacter oxydans</i>	Kef-w152	++	-	-	+	+	-	-	-	-
25	<i>Lacticaseibacillus rhamnosus</i>	LGG	++	-	++	-	+++	-	++	-	-

3.3.3. Phenotypic characteristics of kefir isolates

From the well diffusion assay results, 24 kefir isolates were selected for further screening and results are presented in **Table 3.5**. For acid/bile tolerance, all selected kefir-isolates showed susceptibility to pH 2, similar to the control probiotic LGG. Twelve strains, namely *Liquorilactobacillus satsumensis* Kef-w1, Kef-w2, Kef-w13, *Lactobacillus helveticus* Kef-m4, *Lacticaseibacillus paracasei* Kef-w7, Kef-w14, Kef-w17, Kef-w19, *Liquorilactobacillus hilgardii* Kef-w8-10, *Lentilactobacillus kefir* Kef-m15 showed the ability to survive 2-hour exposure to pH 3 and 0.3% (w/v) ox-bile with < 1 logCFU reduction, suggesting their intrinsic resistances to acid and bile exposure typical of the

conditions in the human GIT. For BSH activity, 7 kefir isolates of species *Liquorilactobacillus hilgardii*, *Lentilactobacillus kefir*, *Lactobacillus delbrueckii* and *Lactobacillus lactis* demonstrated BSH activity. Strains showing positive BSH activity were identified by the presence of a white precipitate on the colonies grown on TDC-supplemented agar (**Figure 3.4**). Antioxidant activities, ranging from 23% to 78%, were observed of the kefir isolates. Highest antioxidant activity was observed of *Lacticaseibacillus paracasei* Kef-w19, notably higher than that of the reference strain LGG [318]. Adhesion of tested kefir isolates to Caco-2 cells were notably higher than that of LGG, measured at 0.9%. The adhesion of kefir isolates ranged from 1.2% to 18.1%, with *Liquorilactobacillus satsumensis* Kef-w11, *Liquorilactobacillus hilgardii* Kef-w8-10, *Lentilactobacillus kefir* Kef-m15 and *Liquorilactobacillus nagelii* Kef-w21 having adhesion percentages of more than 10%, indicative of good adhesion properties [319]. All tested kefir isolates were found to be non-cytotoxic to Caco-2 cells, showing percentage cytotoxicities of < 1.21%.

Table 3.5: Phenotypic properties of selected kefir isolates, including results for acid and bile resistance, BSH activity, antioxidant activity, adhesion, and cytotoxicity to Caco-2 cells⁴.

No.	Kefir isolate species	Strain	Acid and bile resistance, Log ₁₀ CFU surviving				BSH activity	Anti-oxidant activity (AA %)	% adhered to Caco-2 (%)	Cyto-toxicity to Caco-2 (%)
			Initial	After pH2	After pH3	After bile				
1	<i>Liquorilactobacillus satsumensis</i>	Kef-w1	7.55	0.00	7.54	6.70	-	56%	7.6%	0.53%
2		Kef-w2	7.51	0.00	7.53	6.83	-	51%	1.2%	-0.37%
3		Kef-w11	7.70	0.00	7.57	5.28	-	32%	10.0%	-0.05%
4		Kef-w13	7.44	0.00	7.31	7.11	-	58%	3.3%	-0.20%
5		Kef-w18	7.55	2.74	7.55	5.54	-	62%	8.9%	1.21%
6	<i>Lactobacillus kefiranofaciens</i>	Kef-m3	6.58	3.22	4.11	6.42	-	Nil	Nil	Nil
7	<i>Lactobacillus helveticus</i>	Kef-m4	7.37	3.35	7.24	6.58	-	60%	1.2%	0.81%
8	<i>Leuconostoc mesenteroides</i>	Kef-m16	6.10	2.74	3.59	5.82	-	Nil	Nil	Nil
9		Kef-m45	6.90	0.00	2.74	6.44	-	Nil	Nil	Nil

Table continued in next page.

⁴ All results were calculated as an average of duplicate experiments (detailed results in **Table S4**). Nil indicates that the kefir isolate was not studied for the particular assay.

10	<i>Lactocaseibacillus paracasei</i>	Kef-w7	8.02	0.00	7.74	8.21	-	56%	4.0%	-0.66%
11		Kef-w14	6.32	3.22	6.36	5.85	-	68%	4.8%	0.23%
12		Kef-w17	8.03	0.00	8.09	7.86	-	63%	3.0%	0.07%
13		Kef-w19	7.49	0.00	7.61	7.43	-	78%	5.4%	0.03%
14		Kef-w20	6.90	0.00	5.76	7.09	-	Nil	Nil	Nil
15	<i>Lentilactobacillus hilgardii</i>	Kef-w8	7.49	0.00	7.14	7.48	-	23%	17.6%	-2.14%
16		Kef-w9	7.69	0.00	7.12	7.89	+	37%	17.2%	-3.24%
17		Kef-w10	7.64	0.00	7.02	7.48	+	37%	13.3%	-1.62%
18	<i>Lentilactobacillus kefiri</i>	Kef-m15	7.28	0.00	6.92	8.24	+	69%	10.2%	0.81%
19	<i>Liquorilactobacillus nagelii</i>	Kef-w21	7.58	0.00	7.29	5.35	-	72%	18.1%	1.11%
20	<i>Lactobacillus delbrueckii</i>	Kef-m63	6.70	0.00	2.74	6.43	+	Nil	Nil	Nil
21	<i>Lactococcus lactis</i>	Kef-m64	6.49	0.00	0.00	6.43	+	Nil	Nil	Nil
22		Kef-m68	7.14	0.00	0.00	6.86	+	Nil	Nil	Nil
23		Kef-m89	7.59	0.00	0.00	7.29	+	Nil	Nil	Nil
24	<i>Gluconobacter oxydans</i>	Kef-w152	5.86	0.00	4.91	5.60	-	Nil	Nil	Nil
25	<i>Lactocaseibacillus rhamnosus</i>	LGG	7.51	0.00	7.48	7.47	-	73%	0.9%	0.84%
26	<i>Lactiplantibacillus plantarum</i>	Wild Type	Nil				+	Nil	Nil	Nil

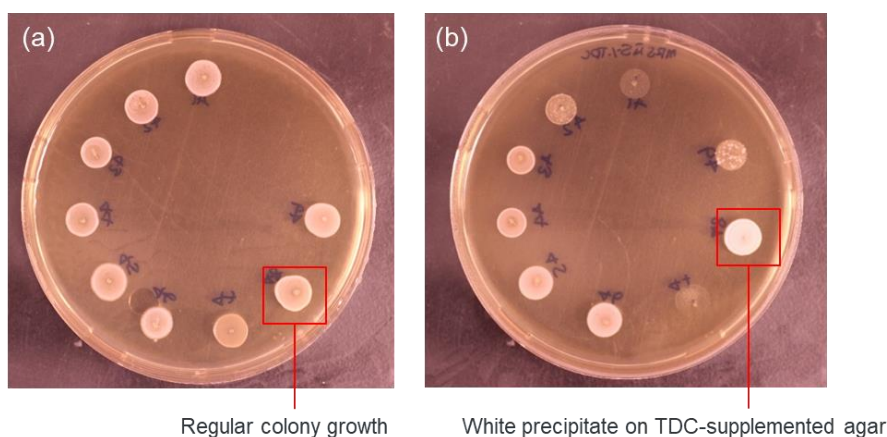


Figure 3.4: An example of positive BSH activity, as indicated by *L. plantarum* WT. (a) shows regular colony growth on MRS agar, while (b) shows growth on 5% TDC-supplemented agar.

3.3.4. Genotypic characteristics of kefir isolates

WGS was carried out for 15 selected kefir isolates, and the collated data of bacteriocins, virulence, AMR, BAs, toxins, vitamins, GABA genes and plasmids for each kefir isolate are presented in **Table 3.6**. Antibiotic resistance and virulence genes were absent for all

kefir isolates. Plasmids, previously identified in *Lactiplantibacillus plantarum* [320] and *Lentilactobacillus buchneri* [321], were found present in *Liquorilactobacillus satsumensis* Kef-w2, Kef-w13, *Lactobacillus helveticus* Kef-m4 and *Lentilactobacillus kefir* Kef-m15. Genes encoding for production of bacteriocins, which are antimicrobial peptides produced by bacteria and serve as natural alternatives to antibiotics, were identified in *Liquorilactobacillus satsumensis* Kef-w13, *Lactobacillus helveticus* Kef-m4, *Lacticaseibacillus paracasei* Kef-w7, Kef-w14, Kef-w17 and Kef-w19.

The toxin protein, hemolysin III, a common hemolysin found in lactobacilli bacteria, was found in all kefir isolates. There are numerous reports about hemolysin III and its safety in lactobacilli, hence this toxin protein is not considered of significant concern [322], [323]. *Liquorilactobacillus nagelii* Kef-w21 was found to harbour lipopolysaccharide (LPS) endotoxin proteins, which are potentially harmful as LPS can trigger intestinal inflammation and increased permeability. LPS are generally known to be present in Gram-negative bacteria, and the only Gram-positive bacteria so far known to contain an LPS is *Listeria monocytogenes* [324]. To err on the safe side, *Liquorilactobacillus nagelii* Kef-w21 was excluded from the kefir probiotic blend.

The production of BAs was investigated, as lactobacilli, particularly those involved in fermented foods, often produce them. Notably, synthesis genes associated with histamine and tyramine, two most concerning BAs which have higher incidences of intolerance upon ingestion [325], were found to be absent in the kefir isolates. Agmatine decarboxylase and ornithine decarboxylase genes, which are involved in the biosynthesis of putrescine, were identified in the kefir isolates. Since agmatine decarboxylase and ornithine decarboxylase genes have been previously been identified in many known probiotics and starter cultures [326], [327], this was not considered to be a major safety concern.

All kefir isolates were found to contain genes for biosynthesis of riboflavin (vitamin B2), *Lactobacillus helveticus*, *Lacticaseibacillus paracasei*, *Liquorilactobacillus hilgardii* were found to additionally contain cobalamin (vitamin B12) synthesis genes and *Liquorilactobacillus hilgardii* was found to also contain pyridoxal (vitamin B6) synthesis

genes. Genes for biosynthesis of GABA, a neurotransmitter which reduces anxiety and stress [123], was identified in *Liquorilactobacillus hilgardii* strains, consistent with other reports [328]. Phylogenetic analysis revealed several highly similar strains amongst the kefir isolates (**Figure 3.5**). *Lacticaseibacillus paracasei* Kef-w7, Kef-17, Kef-w19, Kef-w20 were highly similar while *Liquorilactobacillus satsumensis* Kef-w11 and Kef-w18 were similar to each other. Accordingly, only one isolate of very similar strains was selected for the kefir probiotic blend formulation. Ultimately, seven kefir isolates (**Table 3.2**) were selected to constitute the kefir probiotic blend based on the phenotypic and genotypic data analyses.

Table 3.6: Genetic features of selected kefir isolates

No.	Kefir isolate species	Strain	AMR genes	Virulence genes	Plasmids	Bacteriocin genes	Toxin synthesis genes	BA synthesis genes	Vitamin synthesis genes	GABA synthesis genes
1	<i>Liquorilactobacillus satsumensis</i>	Kef-w1	Absent	Absent	Absent	0	Hemolysin III protein	Ornithine decarboxylase	Riboflavin	Absent
2		Kef-w2	Absent	Absent	CP-005948	0	Hemolysin III protein	Ornithine decarboxylase	Riboflavin	Absent
3		Kef-w11	Absent	Absent	Absent	0	Hemolysin III protein	Ornithine decarboxylase	Riboflavin	Absent
4		Kef-w13	Absent	Absent	CP-005948	1	Hemolysin III protein	Ornithine decarboxylase	Riboflavin	Absent
5		Kef-w18	Absent	Absent	Absent	0	Hemolysin III protein	Ornithine decarboxylase	Riboflavin	Absent
6	<i>Lactobacillus helveticus</i>	Kef-m4	Absent	Absent	CP-002655, CP-002654	5	Hemolysin III protein	Ornithine decarboxylase	Riboflavin, Cobalamin	Absent
7	<i>Lactocaseibacillus paracasei</i>	Kef-w7	Absent	Absent	Absent	2	Hemolysin III protein	Ornithine decarboxylase	Riboflavin, Cobalamin	Absent
8		Kef-w14	Absent	Absent	Absent	4	Hemolysin III protein	Ornithine decarboxylase	Riboflavin, Cobalamin	Absent
9		Kef-w17	Absent	Absent	Absent	2	Hemolysin III protein	Ornithine decarboxylase	Riboflavin, Cobalamin	Absent
10		Kef-w19	Absent	Absent	Absent	2	Hemolysin III protein	Ornithine decarboxylase	Riboflavin, Cobalamin	Absent
11	<i>Lentilactobacillus hilgardii</i>	Kef-w8	Absent	Absent	Absent	0	Hemolysin III protein	Agmatine deiminase	Riboflavin, Cobalamin, Pyridoxal	Present
12		Kef-w9	Absent	Absent	Absent	0	Hemolysin III protein	Agmatine deiminase	Riboflavin, Cobalamin, Pyridoxal	Present
13		Kef-w10	Absent	Absent	Absent	0	Hemolysin III protein	Agmatine deiminase	Riboflavin, Cobalamin, Pyridoxal	Present
14	<i>Lentilactobacillus kefir</i>	Kef-m15	Absent	Absent	CP-002654	0	Hemolysin III protein	Agmatine deiminase	Riboflavin	Absent
15	<i>Liquorilactobacillus nagelii</i>	Kef-w21	Absent	Absent	Absent	0	Hemolysin III protein, LPS protein	Ornithine decarboxylase	Riboflavin	Absent
16	<i>Lactocaseibacillus rhamnosus</i>	LGG	Absent	Absent	Absent	1	Absent	Ornithine decarboxylase	Riboflavin, Cobalamin	Absent

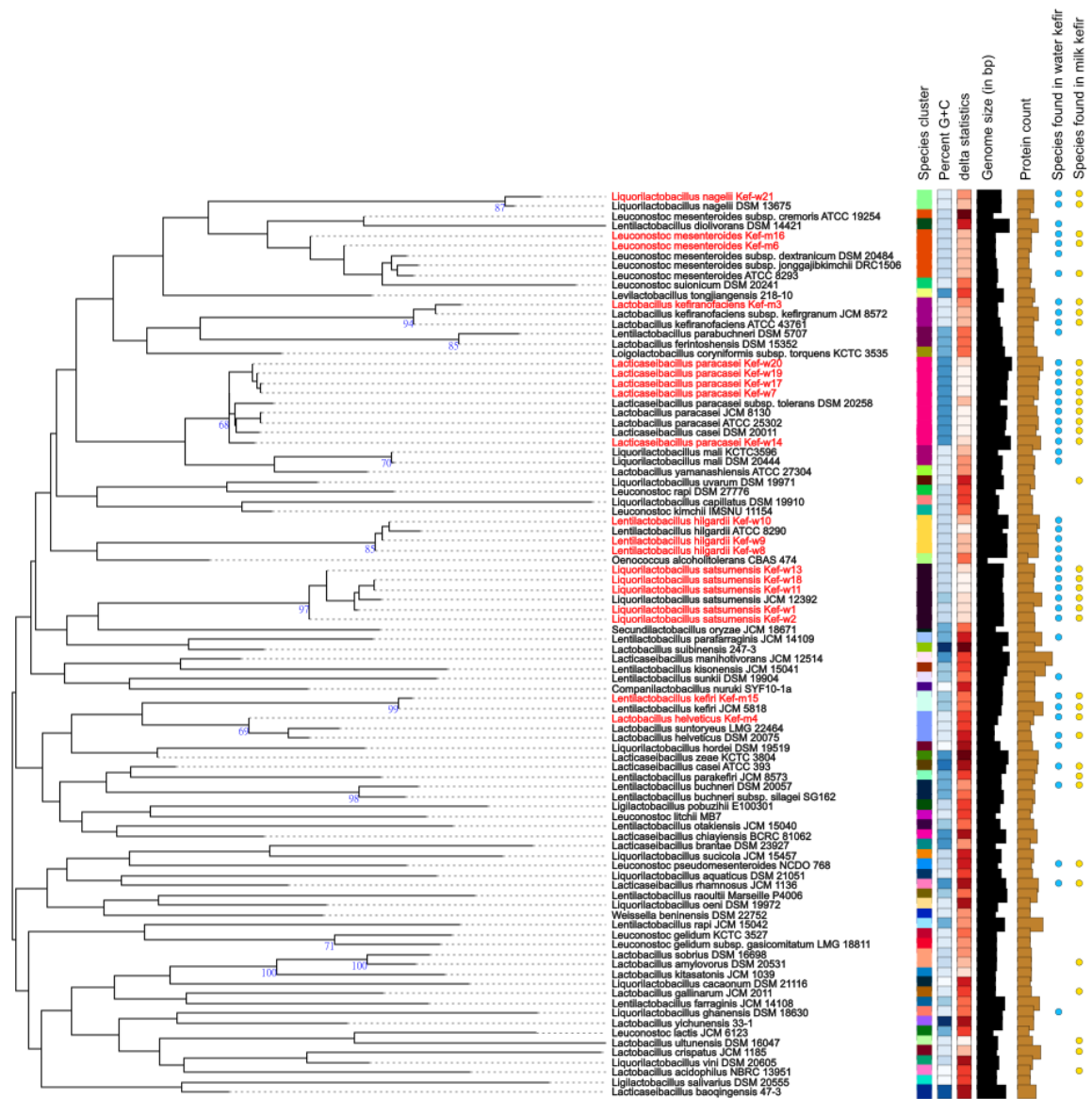


Figure 3.5: Phylogenetic tree of selected kefir isolates (highlighted in red) and other related strains. The branch lengths are scaled in terms of Genome BLAST Distance Phylogeny (GBDP) approach distance formula d_5 .

3.3.5. Competitive adhesion/exclusion

The kefir probiotics blend demonstrated reduction in pathogen adhesion to Caco-2 cells for seven of the eight tested human enteric pathogens, except *E. coli* O157:H7 (**Figure 3.6a**). Statistically significant reduction in pathogen adhesion was observed for *B. cereus*, *S. enterica*, *S. aureus*, *C. difficile* and *C. perfringens*. Highest reduction in pathogen adhesion was observed for *C. perfringens*, in which $< 0.05\%$, indicating around 3.5 log CFU reduction, of *C. perfringens* cells adhered when kefir probiotics were administered in combination. Notably, a significant reduction in pathogen adhesion ($> 50\%$) was also observed for *S. aureus* and *C. difficile*. In comparison with LGG (**Figure 3.6b**), the kefir probiotics blend showed greater inhibition of adhesion of *V. parahaemolyticus* and *C. perfringens*, and similar inhibition of adhesion of the other enteric pathogens. Competitive exclusion appeared to reduce pathogen adhesion more effectively than competitive adhesion, especially for pathogens *B. cereus* and *S. enterica*. This suggests that pre-consumption of kefir probiotics may prophylactically protect epithelial cells against pathogen adhesion better than consumption at the time of exposure to the pathogen.

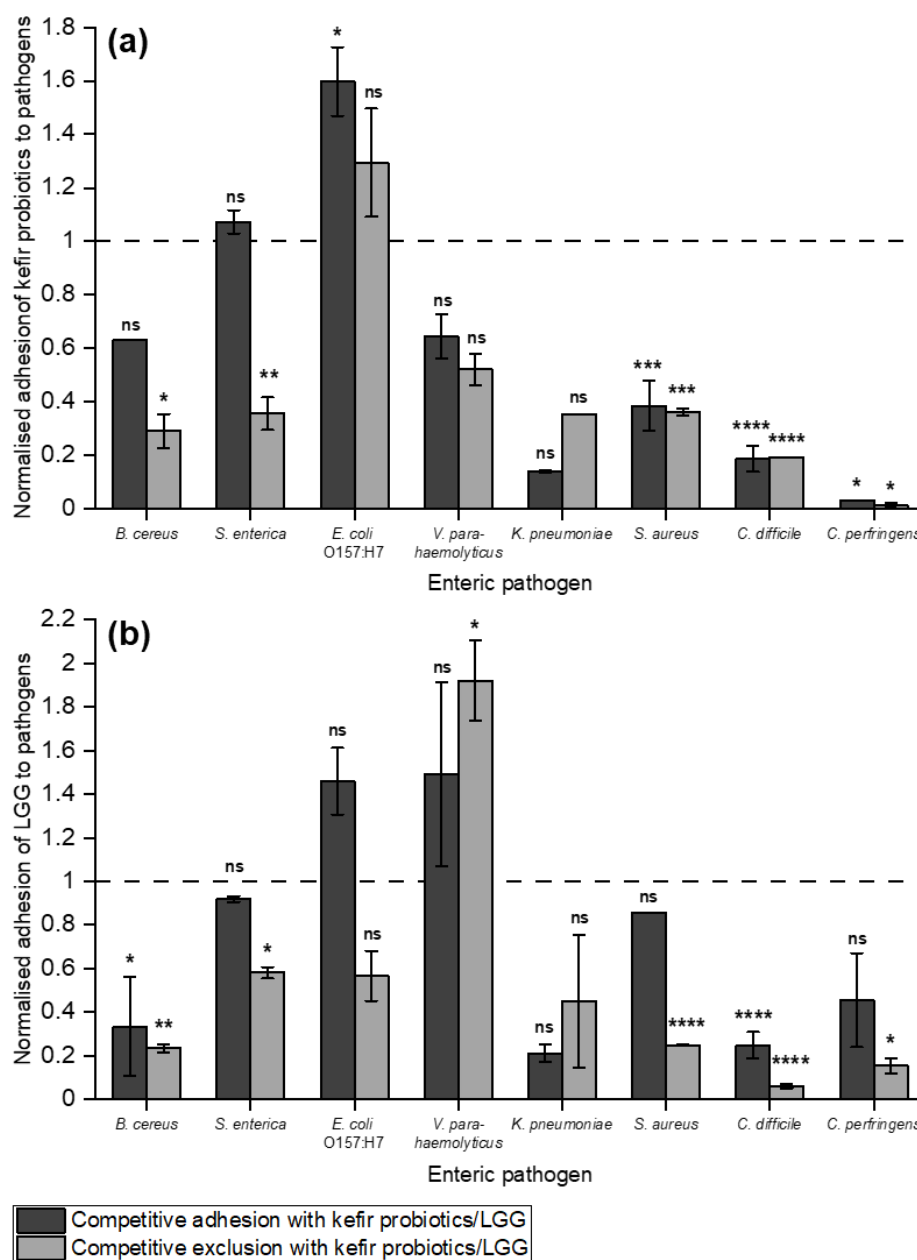


Figure 3.6: Ability of (a) kefir probiotics or (b) LGG to compete with enteric pathogens for adhesion sites (referred to as competitive exclusion) or exclude adhesion of enteric pathogens when introduced before exposure to the pathogen (referred to as competitive exclusion). Normalized adhesion of 1 indicates that the number of pathogens adhered in competitive adhesion/exclusion with kefir probiotics, was the same as when the pathogens were added alone. Data are presented as an average of duplicates with standard deviation bars. Letters on bars were determined by Dunnett test based on a pairwise-comparison with pathogen-only treatment, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns – no significant difference. Refer to **Table S5** for statistical data.

3.4. Discussion

Bacteria and fungi were isolated from locally-sourced milk and water kefir grains. Isolates were identified to the species level. This process yielded 158 isolates representing 23 microbial species, indicating the wide diversity of culturable microbes from both milk and water kefir. Most of these species have been previously reported to be isolated from kefir, while several were only identified in kefir by culture independent techniques. Notably, *Zymomonas mobilis*, *Clostridium beijerinckii* and *Candida ethanolica* have not been previously isolated from kefir, but have been purified from beer, soil, vinegar, and apple surfaces, respectively [329]–[333]. While most of these species have been identified in other kefir from different geographical origins, it is worth noting that the microbial species derived in this study were not completely identical to any prior publications. This may be due to differing culture techniques used for the isolation of the microbes from the granules. For instance, Miguel *et al* [297] used only MRS and malt yeast glucose peptone media to isolate bacteria and yeasts respectively under aerobic conditions, while in this study, MRS, MYC, NA and M17 media were used for bacteria isolation, YPG and YPD for yeasts isolation, under both aerobic and anaerobic conditions. Inherent differences in microbiota composition in kefir grains of different origins also explain the unique microbiota profile observed in this study. Microbial compositions within kefir grains are known to change between geographical regions, owing to differences in temperature, humidity, altitude conditions, water quality, type of milk/sugar available and hygiene conditions [174]. Singapore has a tropical climate, with year-round temperatures ranging from 24 °C to 32 °C. As such, “room temperature” here is considerably higher and more consistent than other climates, which may give rise to microbiota differences in the complex community of the kefir granules. Findings in this study can prove valuable for comparison with studies of the diversity of the kefir microbiota from different geographic origins. Overall, the initial library of 158 strains provided a substantial sample size for this study to proceed, and various assays were conducted to identify the most promising probiotic candidates.

This study adopted a funnel approach to identify kefir-derived microbial strains with highest probiotic potential. Through a series of screening assays, including antimicrobial

well diffusion, acid and bile resistance, BSH activity, antioxidant activity, cytotoxicity, adhesion to Caco-2, and WGS, the seven best performing kefir isolates (**Table 3.2**) were identified from the initial repertoire of 158. These seven kefir isolates showed good survival (i.e. $< 2 \log(\text{CFU})$ reduction) in pH 3 acid and bile solutions, non-cytotoxicity and good adhesion to Caco-2, suggesting their suitability for use as oral probiotics. Whole genome analysis revealed an absence of AMR genes and virulence genes, corroborating the safety of these potential probiotic strains. Other qualities identified in the kefir isolates, including BSH activity, high antioxidant activity, presence of vitamins and GABA synthesis genes, add potential functionality of these strains in for general well-being, cholesterol management and mental health aspects. Since these kefir isolates are lactobacilli, and are also isolated from a fermented food source with a safe history of use, these can likely be considered GRAS by the United States FDA [71], or have QPS status granted by the EFSA [334], facilitating their use as potentially novel probiotic agents.

These kefir isolates also demonstrated potent antimicrobial effects against the enteric pathogens *B. cereus*, *S. enterica*, *V. parahaemolyticus*, *E. coli* O157:H7 and *C. difficile*, as demonstrated using the well diffusion assay. Generally, larger zones of inhibition were observed for the wells containing cultures of the kefir isolates, as compared to the cell-free supernatant of the same isolates (**Table 3.4**). This suggests that the activity of live cells contributed to the anti-pathogenic property of the kefir isolates, possibly due to additional production of antimicrobial compounds during the period of incubation. From WGS results, bacteriocin genes were identified in various kefir isolate strains, suggesting that bacteriocins production may have been the reason for strong pathogen inhibitory activity demonstrated by these kefir isolates. The seven kefir isolates applied in combination showed the ability to outcompete pathogens *B. cereus*, *S. enterica*, *V. parahaemolyticus*, *K. pneumoniae*, *S. aureus*, *C. difficile* and *C. perfringens* for epithelial cell adhesion sites and can potentially reduce pathogenesis of these pathogens. It is known that adhesion to intestinal lining plays an important role in pathogenesis of the tested pathogen species [335]–[342]. Some of the reduction in pathogen adhesion may also be attributed to the growth inhibitory effects noted for the kefir isolates. As described, enteric bacterial diseases pose a significant global health burden and are an urgent problem to be tackled

[316]. In particular, non-typhoidal *S. enterica* and enteropathogenic *E. coli*, both which were found to be inhibited by kefir isolates, accounted for > 50% of enteric pathogen associated deaths [316]. Other tested enteric pathogens, including *B. cereus* (causes 63,400 foodborne disease cases per year in U.S. alone [343]), *V. parahaemolyticus* (leading cause of seafood-associated infections in U.S. and Japan [344]), *C. perfringens* (second most frequent cause of bacterial foodborne outbreaks in U.S. [344]) and *C. difficile* (caused an estimated half a million infections and 29 000 deaths in 2012 in U.S. [315]), also pose major health concerns and urgently require treatment and prophylactic solutions. The demonstrated impact of the kefir isolates against these pathogens suggests that kefir-derived probiotics are alternative, non-antibiotic, naturally derived candidates for prophylactic and therapeutic applications targeting these enteric pathogens. Further studies will be required to validate the effectiveness of kefir probiotics against enteric bacterial diseases, including examining the effects of kefir probiotics on the intestinal epithelial barrier, investigating the *in vivo* persistence of kefir probiotics in human gut and *in vivo* inhibition of the pathogens in model systems.

The findings from this study can be used to develop alternative means to reap the health benefits of kefir. Kefir is a well-known superfood with numerous purported beneficial qualities, including antipathogenic effects, antioxidant properties, cholesterol assimilation, tumor suppression and gastrointestinal immunity [21]. Currently, consumption of kefir is primarily via drinking the kefir beverage itself, while kefir-derived supplements and functional food products have yet to commercialize extensively. In this study, probiotic candidates isolated from kefir were found to exhibit several of the beneficial properties associated with the kefir beverage itself, such as enteric pathogen inhibition effects, antioxidant properties and some potential for cholesterol management. The probiotic isolates from kefir were also shown to be safe and could be consistently produced through batch fermentations. This suggests a potential avenue for kefir probiotics to be supplemented directly in other product formats to consumers yet allowing them to receive the desired health benefits. Some of these formats include dietary supplements, such as capsules, tablets, or sachets, or within functional foods and beverages to enhance their nutritional profile. Kefir probiotics may also be encapsulated in polymeric matrices, to

imbue additional functional qualities such as protection from the rigors of the GIT and shelf-life stability. These alternative product formats may provide consumers with a greater variety and convenience to attain the health benefits of kefir, thereby facilitating consumers to incorporate diverse microbiome-modulating products into their diet regime.

3.5. Conclusion

Microbes were isolated from Singapore-sourced kefir grains, and through a series of screening assays, seven bacterial isolates with the highest probiotic potential were identified. These kefir isolates demonstrate desirable probiotic characteristics, including good survival in acid and bile environments, BSH activity, antioxidant activity, non-cytotoxicity and high adhesion to Caco-2 cells, lack of virulence or AMR genes. Notably, kefir isolates also demonstrate antimicrobial activity against enteric pathogens in the well diffusion assay and showed the capacity to out-compete or exclude the attachment of enteric pathogens to Caco-2 cells. Overall, research conducted in this chapter achieves **Objective 1** of this thesis, and the derived kefir isolates represent novel probiotic candidates which can mitigate the burden of enteric pathogen associated diseases and benefit human health. Further studies via *in vitro* and *in vivo* models and clinical studies are required to ascertain the safety and efficacy of these kefir-derived probiotics.

4. Chapter 4*

Extraction and characterisation of polysaccharides from kefir with prebiotic and synbiotic properties

*EPS from kefir grains are a potential source of novel, food-safe and functional materials. Herein, prebiotic properties of polysaccharides produced by kefir-derived bacteria were explored. Strains were cultured in sucrose-supplemented media for EPS production. Partial hydrolysis of EPS from the highest yield strain, *Liquorilactobacillus satsumensis* Kef-w1, was performed to yield shorter chain polysaccharides. Structural characterisation revealed that hydrolysed polysaccharides were branched glucans comprising α -1,3/ α -1,4 and α -1,6 bonds, with molecular weight of ~ 10 kDa. Hydrolysed polysaccharides showed selective utilisation by probiotics, but not by pathogens, and were non-digestible by human digestive enzymes. Particularly, hydrolysed polysaccharides were fermentable by kefir-derived probiotics, and these were combined in a kefir synbiotic formulation. Using ex vivo large bowel simulated conditions, hydrolysed polysaccharides and kefir synbiotics promoted *Bacteroidetes* abundance and increased short chain fatty acid concentrations. Overall, hydrolysed glucans from *L. satsumensis* Kef-w1 have prebiotic properties with enhanced benefits in a synbiotic when combined with kefir probiotics.*

*This section is published substantially as:

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<https://doi.org/10.1016/J.CARBPOL.2022.119515>. No permission is required from Elsevier for thesis purposes.

4.1. Introduction

In this chapter, the prebiotic and synbiotic properties of these EPS from kefir-derived microbial strains were characterised, in pursuit of **Objectives 2 and 3** of this thesis.

This chapter describes the identification of EPS-producing bacterial strains from kefir, and the extraction of polysaccharides from these strains. The polysaccharide yields were determined and their material structures, such as monosaccharide composition, molecular weight (M_w) and degree of branching, were analysed. Acid-assisted partial hydrolysis was also conducted to derive lower M_w polysaccharides, as shorter chain polysaccharides or oligosaccharides are generally more digestible and better utilised as substrates by microorganisms [34]. Prebiotic characteristics of hydrolysed polysaccharides were then evaluated, such as the capacity to resist digestion by acidic pH and digestive enzymes typical of the human GIT. The selective substrate utilisation assay was next conducted to determine if derived polysaccharides could be selectively utilised by beneficial probiotics, specifically kefir-derived probiotics [169], but were not utilised by, or could inhibit, the growth of pathogens. Polysaccharides which demonstrated prebiotic efficacies were combined with kefir probiotics (**Chapter 3**) and tested in an *ex-vivo* model which simulates the large bowel to determine their effects on gut microbial communities and SCFA content.

4.2. Materials and Methods

4.2.1. Materials

EPS-producing kefir-isolates used in this study were isolated by the described protocol in **Section 4.2.2.1**. Kefir probiotics, comprising a blend of seven strains including, *Lentilactobacillus hilgardii* Kef-w9, *Lacticaseibacillus paracasei* Kef-w14/19, *Liquorilactobacillus satsumensis* Kef-w11/13, *Lactobacillus helveticus* Kef-m4 and *Lentilactobacillus kefiri* Kef-m15, used for the selective substrate utilisation and faecal fermentation assays, were identified as in **Chapter 3** of this thesis. LGG (i-Health Inc., Crownwell, USA) was used as a positive probiotic control in the faecal fermentation assay.

Enteric pathogens used in this study include *Bacillus cereus* ATCC 11778, *Salmonella enterica* ATCC BAA-190, *Vibrio parahaemolyticus* ATCC 17802, *Clostridium perfringens* ATCC 13124, *Clostridium difficile* ATCC 9689-FZ, *Klebsiella pneumoniae* KP-1, *Escherichia coli* O157:H7 ATCC 43888, *Staphylococcus aureus* USA300. All enteric pathogens were purchased from ATCC, USA, with exception of *K. pneumoniae* and *S. aureus* which were provided by colleagues at SCELSE.

MRS, TS, NB, and Wilkins-Chalgren Anaerobe (WC) media were purchased from Thermo Fisher Scientific, USA. Sucrose-supplemented MRS media (Suc-MRS) was prepared using the following recipe (**Table S6**), modified from [345]. Glucose or lactose-supplemented MRS (Glu-MRS/Lac-MRS) was prepared similar to **Table S6**, but with glucose/lactose added instead of sucrose. Carbon-free MRS media (CF-MRS) was also prepared similar to **Table S6**, but without added sucrose. Carbon-free TS media (CF-TS) was prepared according to **Table S7**. Bacto agar was purchased from BD, USA. Lab-Lemco powder and yeast extract, were purchased from Thermo Fisher Scientific, USA. Pullulan standards (ReadyCal-Kit Pullulan high) for GPC was purchased from PSS Polymer Standards Service GmbH, Germany. Deuterium oxide and 3-(trimethylsilyl) propionic-2,2,3,3-d₄ acid sodium salt (TMSP) used for proton nuclear magnetic resonance (¹H-NMR) analysis, were purchased from Cambridge Isotope Laboratories, USA. QIAamp PowerFecal Pro DNA Kit was purchased from QIAgen, Germany. All other chemicals used in this work were purchased from Sigma Aldrich, USA. Sterilisation by autoclaving at 121 °C for 15 min was done for all media, chemical or apparatus prior to use, where necessary.

4.2.2. Methods

The overall plan for the identification of prebiotics from kefir is as shown in **Figure 4.1**. First, EPS-producing strains from kefir were selected and growth conditions were optimised for high yield polysaccharide production. Next, polysaccharides were partially acid-hydrolysed to yield short chain polysaccharides which tend to be more digestible and better utilised as substrates by microorganisms [34]. The chemical structures of both native and hydrolysed polysaccharides were characterised by various techniques, including

differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), HPLC, GPC, and ^1H -NMR. Prebiotic properties were assessed using high-throughput assays to determine if the polysaccharides show any selective utilisation by beneficial probiotics, but were not utilised by, or could inhibit, the growth of pathogens. Kefir-derived polysaccharides showing selective modulatory properties were further exposed to SGF and SIF to determine if they have resistance to low pH and digestive enzymes. Selected kefir-derived polysaccharides with promising prebiotic properties were finally combined with kefir probiotics and tested in an *ex-vivo* large bowel model to determine their effects on gut microbial communities and SCFA content.

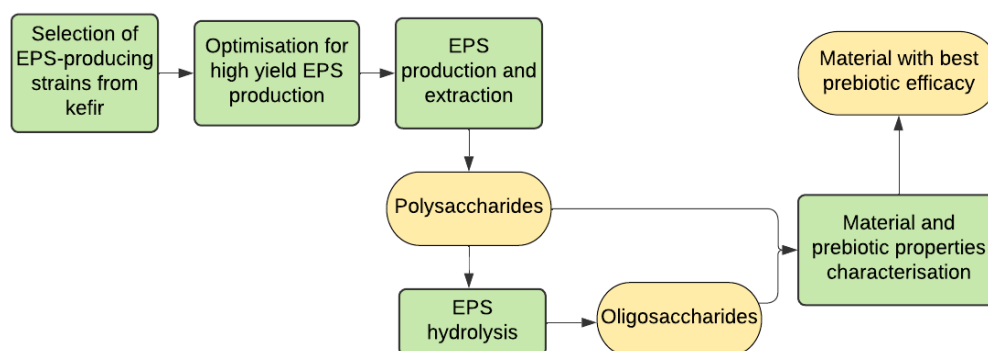


Figure 4.1: Overview of prebiotics screening work.

4.2.2.1. Identification of EPS-producing strains

EPS-producing kefir isolates were identified based on a morphology appearance method. A preliminary evaluation to identify the EPS-producing kefir isolates was performed by Dr. Tan Chuan Hao, Ms. Noele Ng Kai Jing, and Ms. Tay Lai Foong [346]. Based on the previous evaluation by Tan *et al* [346], only water kefir strains, of species *Liquorilactobacillus satsumensis*, *Liquorilactobacillus hilgardii*, *Liquorilactobacillus nagelii* and *Zymomonas mobilis*, showed the ability to produce EPS in Suc-MRS. Unfortunately, isolated *Lactobacillus kefiranofaciens* strains were not found to be EPS-producing, hence production of the polysaccharide kefiran was not expected. Various kefir isolates also did not demonstrate EPS-producing properties in Glu-MRS or Lac-MRS

media. Hence, only the stated water kefir isolates and their EPS derived from Suc-MRS were studied in this chapter.

To verify the EPS-producing ability of these strains, the selected kefir strains were streaked onto Suc-MRS agars and incubated for three days at 30 °C in both aerobic (air) and anaerobic (4% H₂, 5% CO₂, 91% N₂) conditions. Anaerobic handling of strains was done within the Bactron 300 anaerobic chamber (Sheldon Manufacturing, USA). Strains with observable EPS production, i.e., had raised, mucoid or slimy colony morphologies, were identified as EPS-producing strains. An EPS score, ranging from 0 – 5, where 5 indicates the highest amount of EPS produced based on visual inspection of the colonies, was assigned to isolates.

4.2.2.2. Culturing of microbes for EPS growth

EPS-producing kefir isolates were inoculated in Suc-MRS broths and incubated at 30 °C in anaerobic conditions to develop the liquid culture for later EPS-production. The production of EPS by the kefir isolates was done using two types of media – liquid or agar. For EPS production in liquid, 200 µl of culture was added to 25 ml of Suc-MRS broth and incubated at 30 °C in anaerobic conditions for 72 h. For EPS production on agar, 200 µl of culture was spread on a 25 ml Suc-MRS agar plate, followed by incubation.

4.2.2.3. EPS extraction and purification

Native EPS were extracted from the Suc-MRS fermentate using ethanol precipitation and a modification of a previously described method [347]. For EPS on agar, EPS was scraped off from the agar using a scraper and dissolved in 15 ml deionised (DI) water before heating at 90 °C for 30 min to lyse all cells. For liquid culture, the culture was directly heated at 90 °C for 30 min. The heated solution was then cooled, and cell debris was removed by centrifugation (10,000 xg, 4 °C, 10 min). After centrifugation, 84% (w/v) of trichloroacetic acid (TCA) was added to the supernatant at a volume ratio of 1:5, such that final concentration of TCA was 14% (w/v). The mixture was incubated under 50 rpm shaking

conditions at 23 °C for 40 min, to precipitate proteins. These precipitated proteins were removed by centrifugation (10,000 xg, 4 °C, 10 min). Chilled absolute ethanol was then added to the supernatant in a 1:1 volume ratio and the mixture was incubated at -20 °C overnight for polysaccharide precipitation. Following precipitation, the mixture was thawed and centrifuged to collect the precipitated polysaccharide pellet. The pellet was resuspended in water and ethanol precipitation was carried out for an additional two cycles to enhance sample purity. After the third cycle, the polysaccharide was frozen overnight and freeze-dried for three days (0.027 mbar, -84 °C) (FreeZone 4.5 Plus Labconco, USA) to obtain the purified native EPS.

4.2.2.4. Acid-assisted partial hydrolysis of EPS

To derive polysaccharides of smaller M_w , an acid-assisted partial hydrolysis technique was used to break down the native EPS materials. The native EPS (5 mg/ml) were dissolved in a range of trifluoroacetic acid (TFA) concentrations, ranging from 0.2 M to 3 M. The mixture was then heated at 80 °C for 2 h to allow hydrolysis to occur. Following heating, the mixture was cooled and frozen at -80 °C, then freeze-dried. To ensure thorough removal of TFA, the derived polysaccharides were resuspended in water and freeze-dried for two additional cycles to obtain the final hydrolysed polysaccharide product.

4.2.2.5. Thermal characterisation

DSC and TGA were used to characterise the melting point and decomposition temperature of native EPS. The Q10 DSC (TA Instruments, USA) was used, and EPS samples were heated at 10 °C/min from 25 to 250 °C under nitrogen gas. Heat flow was determined relative to a reference pan and endothermic peaks were interpreted as the melting peaks. For TGA, measurements were performed using Q500 TGA (TA Instruments, USA). Samples were placed in aluminium pans and heated from 25 to 800 °C under nitrogen gas.

4.2.2.6. Monosaccharide composition determination by HPLC-RID

To analyse the monosaccharide constituents in the extracted EPS, HPLC-RID was used. The native EPS (10 mg) were first completely hydrolysed by addition of 1 ml 3 M TFA and heating at 130 °C for 2 h. Samples were then freeze-dried to completely remove residual TFA and rehydrated in 1 ml of DI water. The dissolved hydrolysed samples in water (200 µl) were then added to 800 µl of HPLC-grade acetonitrile (ACN) and filtered (0.45 µm membrane), thereby yielding a final solution of 2 mg/ml constituent monosaccharides in an ACN:H₂O (80:20, v:v) solvent. The resultant monosaccharides were analysed on Shimadzu Prominence HPLC with RID-10A detector (Shimadzu, Japan) and a NH2P-50 4E hydrophilic interaction chromatography column (Shodex, Japan). The column was maintained at a temperature of 30 °C, and 20 µl of each sample was eluted with filtered, degassed ACN:H₂O (80:20, v:v) solvent at 1 ml/min. Various monosaccharides, including arabinose, fructose, galactose, glucose, mannose, rhamnose and xylose, at concentrations of 1 mg/ml each, were used as standards.

4.2.2.7. Molecular weight estimation by GPC

GPC analysis was done using the OHpak SB-806M HQ GPC column (Shodex, Japan) in a Shimadzu Prominence HPLC with RID-10A detector system (Shimadzu, Japan). ReadyCal-Kit Pullulan high (PSS Polymer Standards Service GmbH, Germany), which includes pullulan of M_w ranging from 180 – 1,330,000 Da, was used as a calibration standard. Polysaccharides were dissolved in DI water at 5 mg/ml concentration, then filtered using a 0.45 µm membrane. The column was maintained at a temperature of 25 °C, and 50 µl of each sample was eluted with filtered, degassed DI water at 1 ml/min for 15 min.

4.2.2.8. Degree of branching determination by ^1H -NMR

^1H -NMR was used to analyse the branching structure of the polysaccharides. Polysaccharides were dissolved in deuterium oxide at 5 mg/ml concentration with 0.1% (w/v) TMSP. ^1H -NMR spectra were recorded on Avance III HD 800 MHz NMR spectrometer (Bruker, USA) using a 5 mm z-gradient TBI probe. The data was acquired and processed using XWINNMR 3.5 software. The temperature was 343 K. ^1H -NMR spectra were acquired by using a 30° pulse, 800 MHz sweep width, 2.5 s relaxation delay and 2.0 s acquisition time. A total of 64 scans were recorded to attain the ^1H -NMR spectra.

4.2.2.9. Impact of pH and digestive enzymes on EPS samples

The stability when exposed to low pH and digestive enzymes was investigated because prebiotics are required to be resistant to the acidic pH of stomach and are not hydrolysed by digestive enzymes [136]. Polysaccharide samples were added at 10 mg/ml concentration to SGF and SIF. SGF was prepared with 0.2 M NaCl, 2000 U/ml porcine pepsin and adjusted to pH 2 with hydrochloric acid. SIF was prepared with 200 U/ml α -amylase from porcine pancreas in PBS pH 7.4. Polysaccharide samples were incubated with SGF or SIF for 3 or 5 hours respectively, under 37 °C, 200 rpm shaking conditions, with aliquots taken every hour. The aliquots were neutralised and filtered, before GPC was done to determine the M_w of products.

4.2.2.10. Selective substrate utilisation assay

An important feature of a prebiotic is that it selectively modulates growth of microorganisms, whereby it stimulates growth of beneficial bacteria while suppressing growth of pathogens [136]. A selective substrate utilisation assay, adapted from a published method [159], was performed using the hydrolysed polysaccharides. The kefir probiotics blend [169] and eight human enteric pathogens, including *B. cereus*, *S. enterica*, *V. parahaemolyticus*, *C. perfringens*, *C. difficile*, *K. pneumoniae*, *E. coli* O157:H7, *S. aureus*, were tested for their ability to utilise the hydrolysed polysaccharides. Known prebiotics,

inulin and FOS, were used as positive controls. Polysaccharides were added to CF-TS and CF-MRS media at 2% (w/v) concentration and sterilised by autoclaving. The kefir probiotics blend was prepared by culturing individual strains in MRS at 30 °C, anaerobic conditions for 72 h, after which, respective kefir isolate cultures were adjusted to indicated OD600 values (**Table 3.2**) and concentrated into the same volume (same as protocol in **Section 3.1.2.10**). The kefir probiotics blend culture was then diluted in PBS by a factor of 10^{-3} . Enteric pathogens were inoculated in various broths and incubation conditions/dilution factors for respective strains are indicated in **Table S8**. Diluted probiotics/pathogen cultures were then added to the polysaccharide-containing MRS/TSB media in duplicates, at final polysaccharide concentrations of 0.8%, 0.4%, 0.2%, 0.1% and 0% (w/v). Incubation of the probiotics and pathogens was conducted at 37 °C, anaerobic conditions for up to 48 h, simulating conditions in the human intestinal environment. Growth of probiotics and pathogens was monitored by measuring OD600, using a microplate reader, at 24-h intervals. Growth of probiotics and pathogens in respective polysaccharide-containing media was calculated with this equation: $\Delta\text{Growth} = \frac{OD600_{final} - OD600_{initial}}{\Delta\text{Growth (no substrate control)}}$. ΔGrowth values of <1 indicate that presence of the substrate inhibited bacterial growth relative to the no substrate control, while ΔGrowth values of >1 indicate additional bacterial growth due to the presence of polysaccharide substrate.

4.2.2.11. Fermentation of kefir-derived prebiotic and synbiotics in large bowel simulated conditions using *ex-vivo* model

Polysaccharides with demonstrated prebiotic efficacies for the pure cultures were further selected for their effects on microbial communities and SCFA content within the gut using an *ex-vivo* model to simulate the large bowel [348]. The kefir probiotics blend [169] and kefir-derived synbiotics, which are combinations of the kefir probiotics blend and the selected polysaccharide substrate, were also tested in this assay. Three healthy volunteers who complied with the inclusion criteria were recruited at NTU, Singapore. The inclusion criteria were adult, non-smokers, not obese with body mass index $< 27.5 \text{ kg/m}^2$, had not taken a course of antibiotics in the last 3 months, and with no underlying diseases. Participants were required to provide metadata including a 3-day dietary recall, and a

Global Physical Activity Questionnaire [349] which screened the participant's physical activity level. All participants gave their written informed consent. Anonymised donor details can be found in **Table S9**. The study was approved by the Institutional Review Board of NTU (IRB approval number: IRB-2018-08-022). Faecal samples were collected from donors and stored anaerobically until use. Three separate *ex-vivo* microcosm experiments were carried out with different faecal samples used for each run. LGG was used as a control probiotic, while inulin was used as a control prebiotic. Kefir probiotics (**Table 3.2**), selected polysaccharides and kefir synbiotics, comprising a combination of both probiotics and the polysaccharides, were tested. To begin, 15 g of each faecal sample was anaerobically added to 60 ml of WC broth and mixed thoroughly by vortex to obtain a 20% faecal slurry. Probiotics and polysaccharides were added to WC broth, then mixed in a 1:1 (v/v) ratio with the faecal slurry in duplicates, to yield 5 ml of each treatment group with respective probiotics and/or polysaccharide concentrations as listed in **Table 4.1**. Polysaccharide-containing WC broth were filtered (0.2 µm membranes) to ensure sterility before use. The *ex-vivo* microcosms were next incubated in anaerobic conditions at 37 °C, with rotary agitation of 120 rpm for 48 h. After 48 h of incubation, aliquots of (1 ml) of each duplicate sample were harvested for quantification of SCFAs and 16S rRNA gene sequencing.

Table 4.1: Treatment groups for *ex-vivo* microcosm experiment

No.	Treatments	Probiotics added (CFU/ml)	Prebiotics added (mg/ml)	Total volume (ml)
1	Faecal sample (negative control)	-	-	5
2	LGG, 2E6 CFU/ml (probiotic control)	2E6	-	5
3	LGG, 2E7 CFU/ml (probiotic control)	2E7	-	5
4	Inulin, 10 mg/ml (prebiotic control)	-	10	5
5	Kefir probiotics blend, 2E6 CFU/ml	2E6	-	5
6	Kefir probiotics blend, 2E7 CFU/ml	2E7	-	5
7	Glucan polysaccharide, 10 mg/ml	-	10	5
8	Kefir synbiotics, 2E6 CFU/ml probiotics with 10 mg/ml polysaccharide	2E6	10	5
9	Kefir synbiotics, 2E7 CFU/ml probiotics with 10 mg/ml polysaccharide	2E7	10	5

4.2.2.12. SCFA analysis

SCFAs were extracted followed by gas chromatography mass spectrometry (GC-MS) analysis using a modification of a published method [124]. In the extraction step, 25 μl of 10% phosphoric acid solution was added to 500 μl sample, then homogenised by vortexing for 10 min. The suspension was centrifuged (10 min, 17949 $\times g$), and 500 μl of the supernatant was transferred to a clean tube. Next, 5 μl of 50 mM 4-methyl valeric acid was added to the supernatant such that a final concentration of 500 μM was achieved. The 4-methyl valeric acid was added as an internal standard to correct for extraction and injection variability between samples and minor changes in the instrument response. Next, 0.5 ml of ethyl acetate solvent was added with vortexing for 10 min. The samples were then centrifuged (10 min, 17949 $\times g$), and a separation of the organic and aqueous layers could be observed at this point. The upper organic phase layer (300 – 400 μl) was transferred into a clean tube and stored at $-20\text{ }^{\circ}\text{C}$ prior to analysis. Acetic acid, propionic acid, and butyric acid, ranging in concentrations from 0.15625 mM to 10 mM were prepared as calibration standards. For GC-MS analysis, the Agilent 7890A gas chromatograph coupled with a 5977B mass selective detector (Agilent Technologies, USA) was used. The GC was fitted with a HP-FFAP GC column (30 m, 0.25 mm ID, 0.25 μm , Agilent) and helium was used as the carrier gas at 1 ml/min. Injection was made in split mode with a ratio of 30:1, and injection volume was 1 μl . A glass liner with a glass wool plug at the lower end of the liner was used to avoid the contamination of the GC column with non-volatile faecal material. The column temperature was initially 80 $^{\circ}\text{C}$ (1 min), then increased to 120 $^{\circ}\text{C}$ at 20 $^{\circ}\text{C}/\text{min}$, and finally to 210 $^{\circ}\text{C}$ at 6.13 $^{\circ}\text{C}/\text{min}$ and kept at this temperature for 2 min (total time 18.8 min). Solvent delay was 3.5 min. The detector was operated in electron impact ionisation mode (electron energy 70 eV), scanning the 30 – 250 m/z range using SIM mode. The temperatures of the injector, ion source, quadrupole, and interface were 250, 230, 150, and 280 $^{\circ}\text{C}$, respectively. Identification of the SCFAs was based on the retention time of standard compounds and the NIST 17 MS database. Quantifications were performed in MassHunter Quantitative software with base peak ion selected as quantifier for each SCFA.

4.2.2.13. Microbiome analysis

For 16S rRNA gene microbiome analysis, microbial DNA from the samples were extracted using the QIAamp PowerFecal Pro DNA Kit, according to manufacturer's instructions. The purity and concentration of extracted DNA concentration was checked via a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) and a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, USA) respectively prior to sequencing. Sequencing of the 16S rRNA gene was performed by an external vendor (Genewiz, USA). Briefly, PCR was done using the primers 338F (5' ACTCCTACGGGAGGCAGCAG 3') and 806R (5' GGACTACHVGGGTWTCTAAT 3') to amplify the V3 and V4 regions of 16S rRNA gene. Next, Illumina adaptors were ligated to generate a standard Illumina library, and sequencing was done on the Illumina NovaSeq™ 6000 platform with SP flowcell and 250 bp paired end reads. The 16s rRNA gene data was preprocessed using fastp [350] with default settings. Reads with more than 40% of bases of Phred33 score < 15 were discarded. Trimmed reads were processed using the DADA2 pipeline [351] version 1.22 to merge forward and reverse primers, detect and remove chimeras, to obtain amplicon sequence variants (ASVs). Taxonomic assignment was performed using the RDP classifier [352] version 2.12. Classification level of all ASVs were assigned to the species level using the Silva [353] release 138 Reference Taxonomy Database. Alpha diversity measures for community abundance include Chao1, Shannon, and Simpson indices. Ordination analyses were conducted using principal coordinate analysis (PCA), using Bray-Curtis similarity distance matrix. All statistical analyses and graphical representations were performed in R version 4.1.2, using the packages vegan, phyloseq, dada2, ggplot2, tidyverse and ggrepel.

4.3. Results and Discussion

4.3.1. Derivation of EPS-producing strains

Twelve water kefir-derived bacterial strains of species *Liquorilactobacillus satsumensis*, *Liquorilactobacillus hilgardii*, *Liquorilactobacillus nagelii* and *Zymomonas mobilis*, were identified as EPS-producers. From literature, *L. satsumensis*, *L. hilgardii* and *L. nagelii* are known to yield dextran-like EPS (reviewed in **Section 2.3.2**), which are glucan polysaccharides with predominately α -1,6 linkages and a variable amount of α -1,2-, α -1,3-, and α -1,4-branched linkages [189]. *Z. mobilis* are known to secrete levans [192]. From **Figure 4.2**, these strains showed formation of mucoid, gelatinous, whitish colonies on Suc-MRS agar. Two broad colony morphologies were identified, indicated as smooth (*L. satsumensis* Kef-w1, w2, w13, *L. hilgardii*, *L. nagelii* and *Z. mobilis*) and irregular (*L. satsumensis* Kef-w18, w11).

EPS production was visibly higher for all strains when strains were incubated in anaerobic conditions (**Table 4.2**). Lactobacilli are generally catalase-negative (**Table S10**), and exposure to oxygen may be harmful to bacteria cells due to an inability to scavenge radical oxygen species. The additional production of EPS by water kefir strains in anaerobic conditions may be a protective mechanism to generate a hypoxic microenvironment for bacterial better growth [354]. *Z. mobilis* is catalase-positive (**Table S10**), but has been reported to grow better under anaerobic conditions [355].

Higher amounts of EPS were produced by *L. satsumensis* (smooth morphology), *L. nagelii* and *L. hilgardii* under anaerobic incubation conditions (**Table 4.2**). The low yield of EPS generated by *Zymomonas mobilis* makes it economically inefficient to continue extracting the polysaccharide material, hence *Z. mobilis* EPS were not further studied. In addition, from the analysis of whole genome sequences of these water kefir-derived bacterial strains (protocol covered in **Section 3.2.2.9**), LPS genes, which produce potentially harmful endotoxin proteins, were identified in *L. nagelii* and *Z. mobilis* (**Table S10**). As such, only

EPS from *L. satsumensis* and *L. hilgardii* were studied in the next sections, and the anaerobic incubation condition was used to increase EPS yields.

L. satsumensis strains with irregular morphologies yielded lower EPS amount (**Table 4.2**). These observed morphologies were likely due to strain specific differences in EPS-related genes (**Table S12**). *L. satsumensis* strains which showed irregular morphologies were absent in the EPS biosynthesis polyprenyl glycosylphosphotransferase protein [356] and EPS transporter PelG protein [357], otherwise present in *L. satsumensis* strains with smooth colony morphologies. This suggests a critical role of the said EPS-related genes in affecting EPS secretion by *L. satsumensis*.

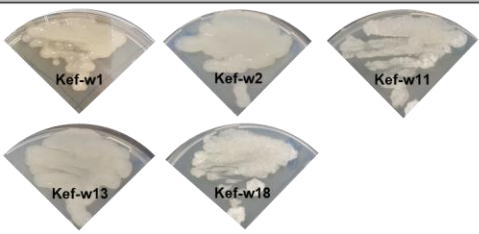
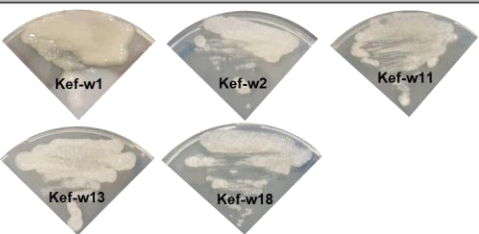
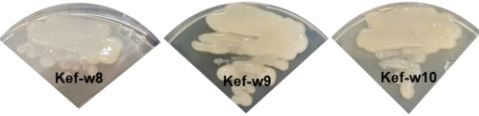
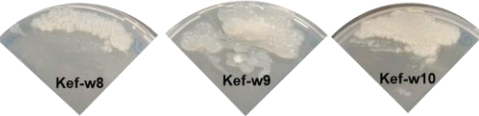
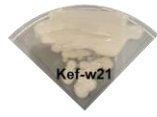
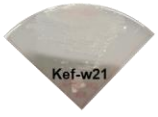
Species	Colony morphology on Suc-MRS (Anaerobic)	Colony morphology on Suc-MRS (Aerobic)
<i>L. satsumensis</i>		
<i>L. hilgardii</i>		
<i>L. nagelii</i>		
<i>Z. mobilis</i>		

Figure 4.2: Colony morphologies of various water kefir-derived EPS-producers on Suc-MRS agars, under anaerobic and aerobic conditions.

Table 4.2: EPS scores (0-5) and description of colony morphologies of various water kefir-derived *L. satsumensis* EPS-producers on Suc-MRS agars, under aerobic (OX) and anaerobic (ANA) conditions

No.	Kefir isolate species	Strain	Substrate	EPS score (1-5)		Colony morphology	
				OX	ANA	OX	ANA
1	<i>Liquorilactobacillus satsumensis</i>	Kef-w1	SucMRS	4	5	Smooth	Smooth
2		Kef-w2	SucMRS	3	5	Smooth	Smooth
3		Kef-w11	SucMRS	3	4	Irregular	Irregular
4		Kef-w13	SucMRS	4	5	Smooth	Smooth
5		Kef-w18	SucMRS	3	4	Irregular	Irregular
6	<i>Lactobacillus hilgardii</i>	Kef-w8	SucMRS	2	5	Irregular	Smooth
7		Kef-w9	SucMRS	4	5	Irregular	Smooth
8		Kef-w10	SucMRS	2	5	Irregular	Smooth
9	<i>Lactobacillus nagelii</i>	Kef-w21	SucMRS	1	5	-	Smooth
10	<i>Zymomonas mobilis</i>	Kef-w12	SucMRS	0	2	-	Smooth
11		Kef-w57	SucMRS	0	2	-	Smooth
12		Kef-w60	SucMRS	0	2	-	Smooth

4.3.2. EPS yield optimisation

EPS were extracted and purified from the *L. satsumensis* and *L. hilgardii* strains after incubating them in liquid Suc-MRS broths or on solid agar substrates in anaerobic conditions. From **Table 4.3**, higher EPS yields were attained using liquid culture for all eight strains, possibly due to better accessibility of nutrients in a liquid medium. EPS yield data was consistent with the observed EPS scores as presented in **Table 4.2**. For *L. satsumensis*, higher EPS yield was attained for strains with smooth colony morphologies (Kef-w1, w2, w13), and *L. satsumensis* Kef-w1 yielded highest EPS yield of 22 g/l from Suc-MRS liquid substrate, equivalent to 27.5 g/g of sucrose converted into EPS material. This yield is notably higher than that reported by Côté *et al* [185], which reports ~ 4.5% g/g of soluble EPS produced by *L. satsumensis* NRRL B-59839. The higher yield obtained for *L. satsumensis* Kef-w1 compared to Kef-w2, w11 and w13 may be due to the presence of dextransucrase gene in Kef-w1, which increases its production of dextran-like EPS (**Table S10**). For *L. hilgardii*, highest yield was observed for the Kef-w9 strain, with 8.3 g/l EPS produced from Suc-MRS liquid substrate. Listed yields were based on a laboratory-

scale production technique, within small 25 ml batch-vessels. Yields can potentially be further scaled up through use of batch reactors.

Table 4.3: Yields of EPS extracted from various water kefir bacterial strains

No.	Kefir isolate species	Strain	EPS yield (g/l substrate)		EPS yield (g/g sucrose)	
			Agar	Liq	Agar	Liq
1	<i>Liquorilactobacillus satsumensis</i>	Kef-w1	18.9	22.0	0.236	0.275
2		Kef-w2	4.2	9.8	0.052	0.122
3		Kef-w11	2.6	5.9	0.033	0.074
4		Kef-w13	15.9	16.0	0.199	0.201
5		Kef-w18	4.5	7.0	0.056	0.088
6	<i>Liquorilactobacillus hilgardii</i>	Kef-w8	1.9	6.3	0.024	0.079
7		Kef-w9	3.7	8.3	0.046	0.104
8		Kef-w10	3.4	7.5	0.043	0.094

4.3.3. Characterisation of native EPS

The native EPS derived from *L. satsumensis* Kef-w1 and *L. hilgardii* Kef-w9 were characterised via DSC, TGA, HPLC-RID, GPC and ¹H-NMR to determine their thermal and chemical properties. Prior to these experiments, however, EPS from *L. hilgardii* Kef-w9 was found to be insoluble in DI water, and this made the analysis of Kef-w9 EPS difficult (GPC, ¹H-NMR, and selective substrate utilisation assay for prebiotic efficacy were difficult to execute for insoluble materials). Accordingly, the EPS from *L. hilgardii* Kef-w9 was not further studied, and only *L. satsumensis* Kef-w1 EPS were studied in greater detail.

Melting temperature of the *L. satsumensis* Kef-w1 native EPS was determined to be 118 °C, while onset of decomposition occurred at 260 °C (**Figure 4.3**). The native EPS was water-soluble and determined to be comprised of only glucose monomers (**Figures 4.4a and b**). Extracted EPS had M_w of 960 kDa, with polydispersity index (PDI) of 1.2 (**Figure 4.5**). This high M_w of Kef-w1 native EPS was similar to that observed de Paiva *et al* [186], who reported ~ 800 kDa EPS derived from *L. satsumensis* strains. A slight reduction of M_w in extracted EPS may have occurred due to the use of TCA in proteins precipitation (**Section**

4.2.2.3). ^1H -NMR showed the presence of α -1,3 or α -1,4 (corresponds to δ 5.36 and 5.40 ppm respectively, which are close by hence difficult to determine which bond might be present) and α -1,6 bonds (corresponds to δ 5.01 ppm) in the native EPS [358] (**Figures 4.4c and d**). Comparing the ^1H -NMR spectra of Kef-w1 EPS with commercial dextran (**Figure S1**), they are highly similar, hence this reaffirms Kef-w1 EPS to be a dextran-like glucan polysaccharide. The ratio of α -1,3/ α -1,4 and α -1,6 bonds in the Kef-w1 EPS were determined to be 11.8% and 88.2%, respectively, suggesting that the native EPS material is a dextran-like polysaccharide with a linear α -1,6 backbone and some α -1,3 and/or α -1,4 branching (**Figure 4.4e**).

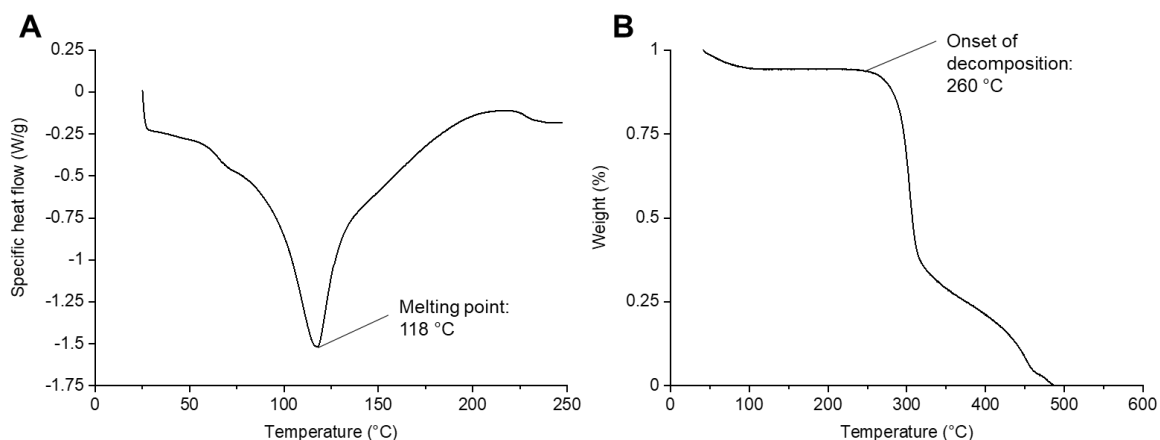


Figure 4.3: Thermal characterisation graphs of native EPS extracted from *L. satsumensis* Kef-w1. (a) DSC thermogram; (b) TGA thermogram.

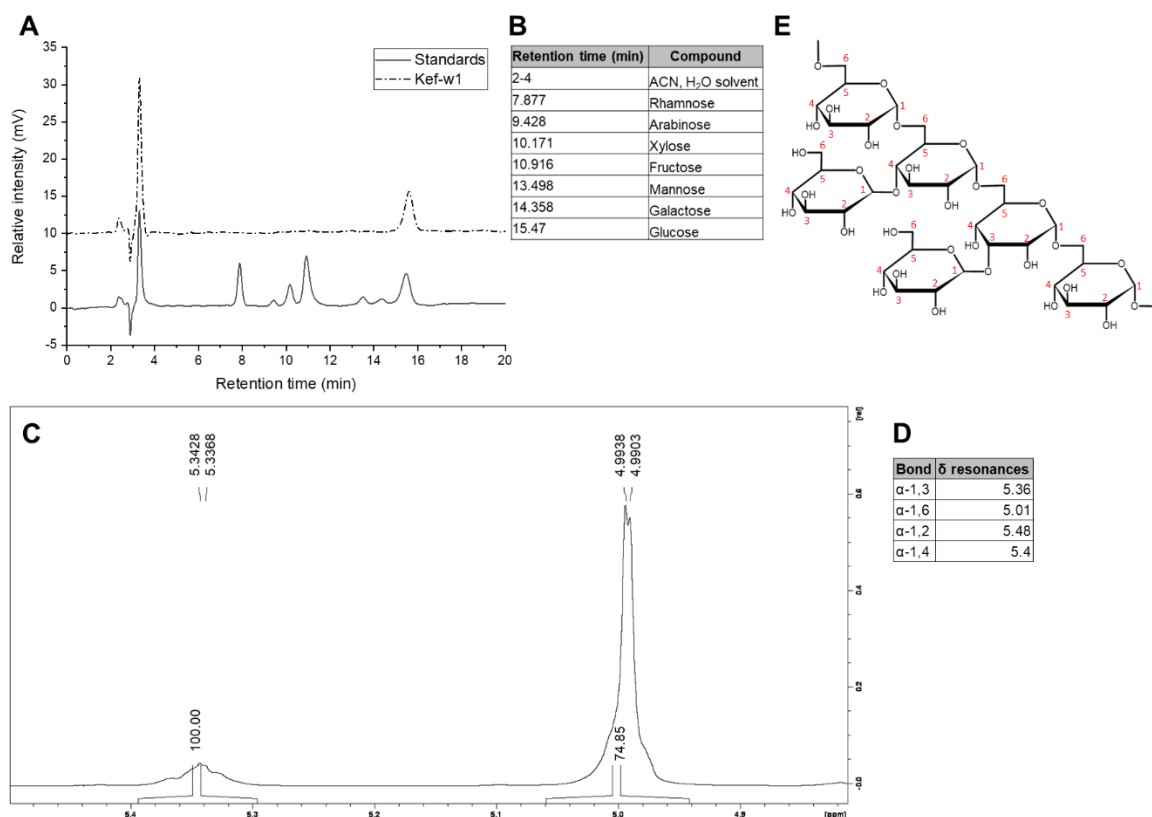


Figure 4.4: Structural characterisation *L. satsumensis* Kef-w1 derived EPS. (a) HPLC-RID chromatogram of Kef-w1 EPS and monosaccharide standards. (b) Retention times for monosaccharide standards. (c) ^1H -NMR spectrum of Kef-w1 EPS. (d) ^1H -NMR δ resonances for various bonds of interest [358]. (e) Proposed chemical structure for Kef-w1 native EPS

4.3.4. Partial hydrolysis of native EPS

A range of TFA concentrations were attempted to achieve partial hydrolysis of the *L. satsumensis* Kef-w1 EPS, and GPC was used to evaluate the M_w distribution of various hydrolysed products. The GPC calibration curve can be referenced in **Figure S3**. From **Figure 4.5**, increasing concentrations of TFA facilitated greater extent of EPS hydrolysis. At TFA concentrations of 0.2 M to 1 M used, the high M_w fraction of Kef-w1 EPS (at retention time of ~ 6.6 min, corresponding to $\sim 1,400$ kDa) was increasingly hydrolysed to form lower M_w polysaccharides (at retention time of $\sim 10.0 - 10.4$ min, corresponding to $\sim 9 - 23$ kDa). Beyond 1 M TFA, lower M_w fractions, at retention times of ~ 11 min, corresponding to ~ 1 kDa, were obtained. At TFA concentrations of 2.4 M and above,

further hydrolysis of EPS appeared to occur, with broader peaks covering up to ~ 11.8 min identified, indicating hydrolysed fractions of ~120 Da, likely corresponding to fully hydrolysed glucose residues. TFA concentration of 1 M was identified as best suited for partial hydrolysis of native EPS, such that a monodisperse lower M_w polysaccharide product was yielded, without risking complete hydrolysis. Following descriptions of the hydrolysed polysaccharide material (Kef-w1 HP) are based on 1 M TFA hydrolysis.

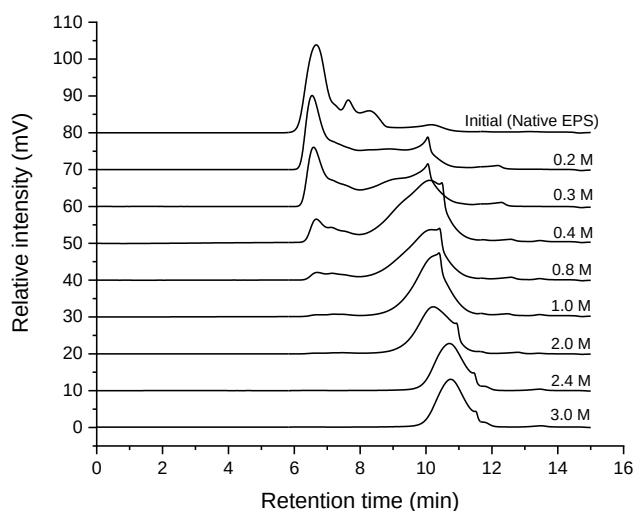


Figure 4.5: GPC chromatogram of *L. satsumensis* Kef-w1 EPS hydrolysed using different concentrations of TFA.

4.3.5. Characterisation of hydrolysed polysaccharide (HP)

The Kef-w1 HP has a M_w ranging from 8,000 – 12,000 Da, corresponding to DP of 50 – 75. Some variances in M_w were observed between batches, likely due to temperature variances in the hydrolysis process. Observed PDIs were in the range of 4 – 5. Based on $^1\text{H-NMR}$ (**Figure S2**), Kef-w1 HP was determined to be comprised of 14% α -1,3/ α -1,4 and 86% α -1,6 glucan bonds, indicating a slight increase in degree of branching following hydrolysis. Low quantities of monosaccharides or low molecular weight oligosaccharides are also present in Kef-w1 HP, this can be seen from the corresponding signals (5.22 / 5.24 ppm) in the $^1\text{H-NMR}$ spectrum. Kef-w1 HP was also determined to be resistant to acidic pH and digestive enzymes. As seen in **Figure 4.6**, M_w of Kef-w1 HP remains unchanged

following incubation in SGF and SIF (retention times remain at 10.4 min), indicating that Kef-w1 HP was not hydrolysed by acidic pH and amylase enzymes.

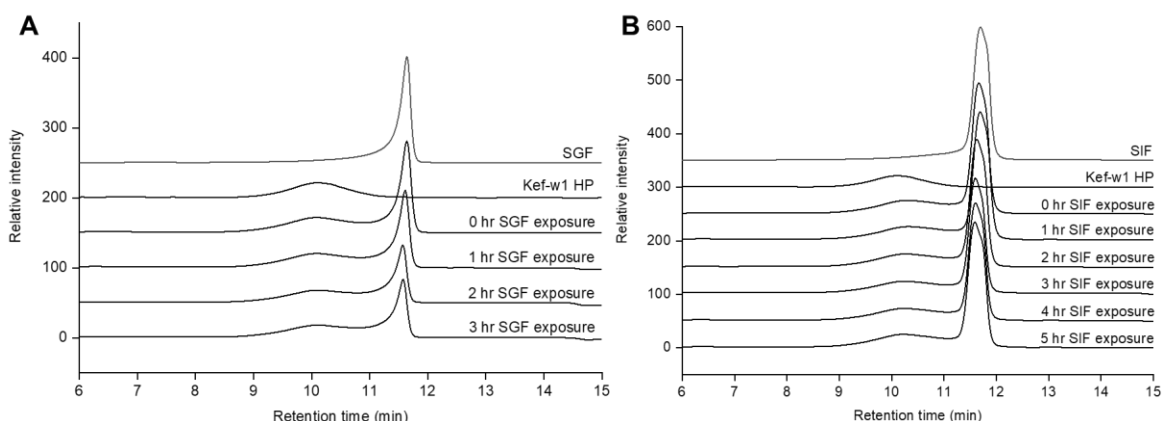


Figure 4.6: GPC chromatograms of Kef-w1 HP when exposed to (a) SGF and (b) SIF

4.3.6. Selective utilisation of hydrolysed polysaccharide by kefir-derived probiotics

The *L. satsumensis* Kef-w1-derived native EPS and hydrolysed polysaccharide were tested for their fermentability by kefir probiotics (**Chapter 3**) and pathogenic bacterial strains. From **Figure 4.7a**, inulin and FOS yielded highest growth of the kefir probiotics blend, as these fructan prebiotics are generally known to promote lactobacilli growth [136]. Interestingly, Δ growth of the kefir probiotics blend was observed to increase with increasing concentrations of Kef-w1 HP, indicating that Kef-w1 HP was also utilised by kefir probiotics. In contrast with the native EPS, Kef-w1 HP were more utilisable by kefir probiotics, suggesting that the acid-assisted hydrolysis step increased the prebiotic efficacy of the material. This is consistent with the understanding that lower M_w carbohydrates are generally more easily fermented by microbes, as longer chain carbohydrates require more enzymatic hydrolysis steps for complete fermentation [34]. Δ Growth values of individual kefir probiotic strains within the kefir probiotics blend are shown in **Figure 4.8**. In this figure it can be seen that *L. hilgardii* Kef-w9 and *L. satsumensis* Kef-w11/13 were best able to utilise Kef-w1 HP, with Δ growth values of 2.5, 8.7 and 9.8 respectively at 0.8% substrate supplementation. *L. paracasei* Kef-w14/19, *L. helveticus* Kef-m4 and *L. kefir* Kef-m15 showed some additional growth when supplemented with Kef-w1 HP, with

Δ growth values of 1.9, 2.2, 1.3, 1.8, respectively, at 0.8% substrate concentration. In the probiotics blend formulation, it is possible that cross-feeding effects could have occurred, as *L. hilgardii* and *L. satsumensis* strains may first degrade the complex polysaccharide, after which other strains in the probiotics blend could use the shorter-chain degradation by-products more efficiently [19].

For pathogenic strains, all eight tested pathogens showed Δ growth values of approximately 1 or less, indicating that they were unable to utilise Kef-w1 HP (**Figure 4.7**). Inhibitory effects were exhibited towards the growth of enteric pathogens *B. cereus*, *C. perfringens*, *E. coli* O157:H7 and *S. aureus*, with Δ growth values of < 1 , indicating less growth than the no substrate control. Growth of *C. perfringens* was found to be most inhibited, with there being nearly zero growth at $\geq 0.4\%$ polysaccharide supplementation. Compared to the control prebiotics, inulin and FOS, Kef-w1 HP performed better in inhibiting/limiting the growth of *B. cereus*, *S. enterica*, *C. perfringens*, *C. difficile*, and *E. coli* O157:H7, as inulin and FOS showed various extents of being utilised by these pathogens. This is likely due to the lower M_w of inulin and FOS, which make them more digestible by various bacterial strains. From **Figure S4**, M_w of inulin and FOS were measured to be 1,700 and 3,200, corresponding to DP 10 and 19 respectively, which is lower than the DP of 50 – 75 for Kef-w1 HP.

The specific utilisation of Kef-w1 HP by kefir probiotics, but not by pathogens, may be attributed to pathogens not having the necessary enzymes (e.g., dextranase or other glucanases) to hydrolyse these long-chain polysaccharides and utilise them as carbon substrates. In contrast, kefir probiotics appear able to utilise the polysaccharide material, possibly due to an environmental adaptation of these strains to the water kefir grain environment, which allows them to utilise dextran-like glucans as an energy source. In a prior review by Lynch *et al* [333], it has been proposed that dextran utilisation is likely a niche adaptation in water kefir isolates. Dextranases were reported to be present in *Liquorilactobacillus hordei* isolated from water kefir, but absent in a *L. hordei* strain isolated from barley [359]. Such adaptation may have arisen as a mechanism to obtain nutrients from the dextran-like EPS material, which is abundantly available in water kefir

grains. Kefir probiotic strains utilised in this study potentially contained dextranase enzymes as well, which enabled their use of the Kef-w1 HP substrate. It was reported that the kefir probiotics blend demonstrated antipathogenic effects towards enteric pathogens, and could serve as a natural candidate to treat enteric bacterial infections [169]. The specific utilisation of Kef-w1 HP by kefir probiotics suggests that co-administration of this α -glucan substrate with the kefir probiotics blend in a synbiotic formulation can potentially enhance its antipathogenic efficacy.

An inhibitory effect of Kef-w1 HP has also been observed in this study, however, there appears to be no prior reported mechanisms regarding the direct antagonism of α -glucans to enteric pathogens. It is possible that the Kef-w1 glucan has a specific branching structure, perhaps as a natural defence against foreign microbes non-native to water kefir grains, which could have contributed to its inhibitory effects against pathogens. This antipathogenic potential of Kef-w1 HP requires further study to understand its mechanisms of action. Overall, Kef-w1 HP demonstrated the selective utilisation criterion, such that it could be utilised by probiotics, but was not utilised by, or could even inhibit, the growth of pathogens. The ability of kefir probiotics to utilise the hydrolysed polysaccharide also add to the potential of applying the kefir probiotics and Kef-w1 HP in combination for a synergistic effect.

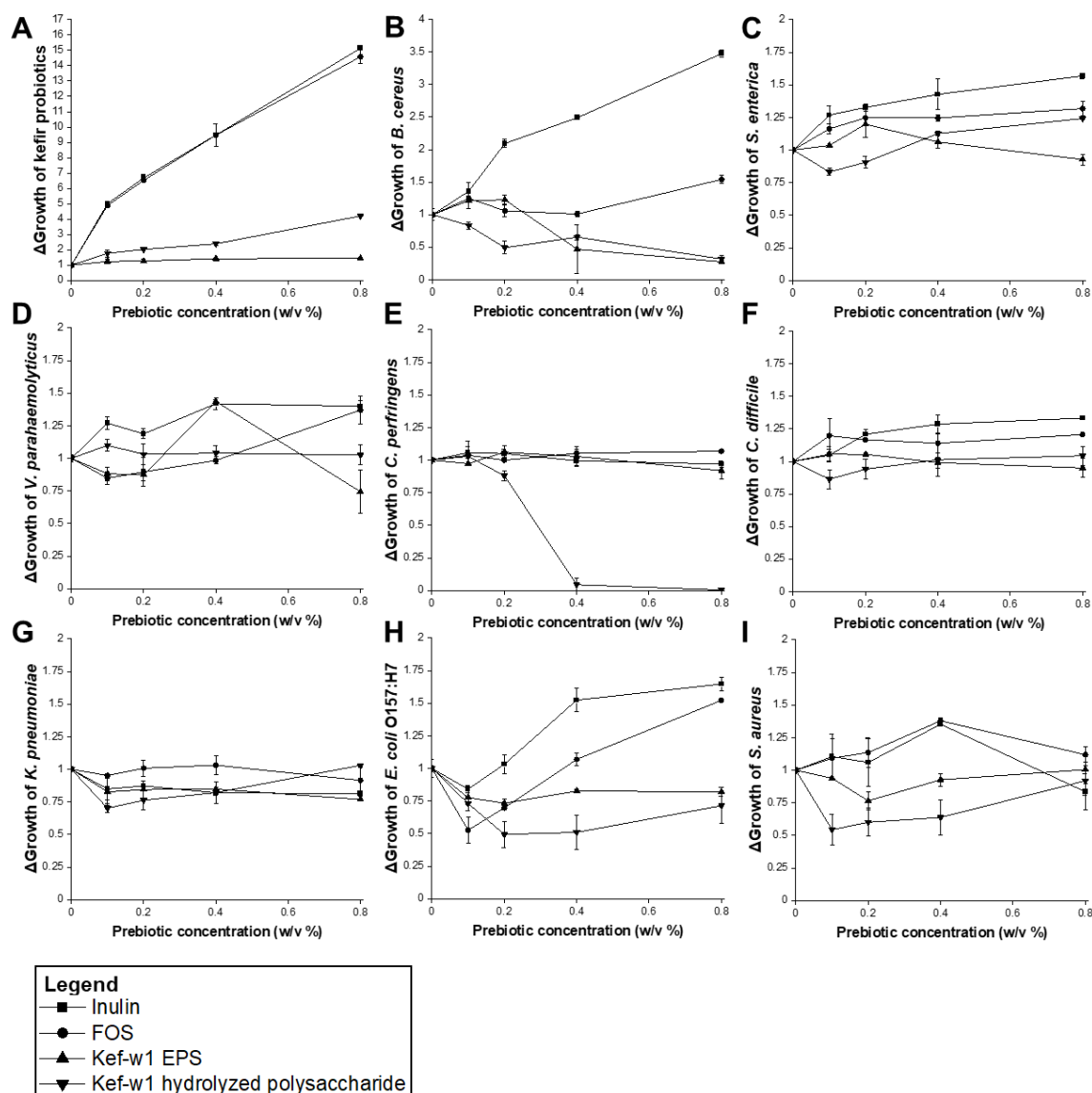


Figure 4.7: Δ Growth values of probiotics/pathogen cultures supplemented with varying concentrations of *L. satsumensis* Kef-w1 native EPS and Kef-w1 HP substrates. Tested microbes include (a) kefir probiotics blend, (b) *B. cereus*, (c) *S. enterica*, (d) *V. parahaemolyticus*, (e) *C. perfringens*, (f) *C. difficile*, (g) *K. pneumoniae*, (h) *E. coli* O157:H7, and (i) *S. aureus*. Data presented as an average of technical duplicates with standard deviation bars.

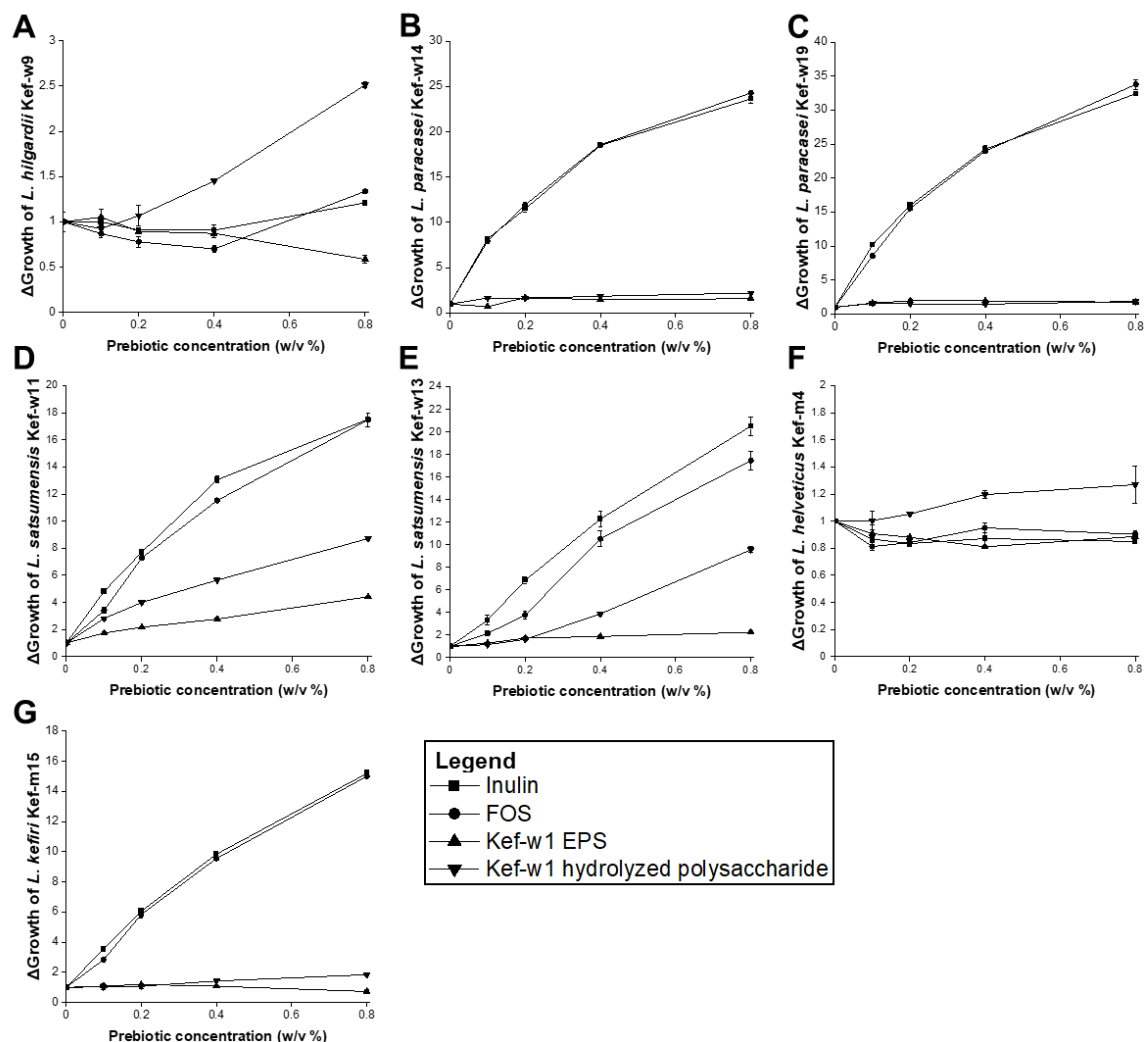


Figure 4.8: Δ Growth values of individual kefir-derived probiotics (which collectively make up the kefir probiotics blend) upon supplementation with varying concentrations of *L. satsumensis* Kef-w1 native EPS and hydrolysed polysaccharide substrates. Tested probiotics include (a) *L. hilgardii* Kef-w9, (b) *L. paracasei* Kef-w14, (c) *L. paracasei* Kef-w19, (d) *L. satsumensis* Kef-w11, (e) *L. satsumensis* Kef-w13, (f) *L. helveticus* Kef-m4, and (g) *L. kefiri* Kef-m15. Individual cultures of these probiotics were diluted in PBS by a factor of 10^{-3} before the start of incubation.

4.3.7. *Ex-vivo* large bowel simulated fermentation of Kef-w1 and kefir synbiotics

Kef-w1 HP has by far demonstrated potential prebiotic effects, and was further evaluated, in combination with the kefir probiotics blend, for its effects on *ex-vivo* large bowel microbial communities and SCFA content. From **Figure 4.9**, SCFAs including acetate, propionate, and butyrate, generally increased in concentrations following Kef-w1 HP, kefir synbiotics and inulin supplementation for all three donors. Differences in SCFAs content were observed between different donors, indicating significant host-to-host variability in the faecal samples obtained. Acetate increased by 3 to 4-folds with Kef-w1 HP supplementation for all three donors. Propionate increased by 2 to 4-folds with Kef-w1 HP supplementation for donors 1 and 2 but was relatively unchanged for donor 3. Butyrate increased by 1.5 to 2-folds for all donors but was reduced for the kefir synbiotics formulation containing 1E8 CFU of kefir probiotics for donor 3. Probiotics supplementation alone, such as LGG or kefir probiotics alone, did not yield significantly higher SCFAs relative to the control set for all three donors. SCFAs are known by-products of prebiotics fermentation and increased SCFAs have been correlated with many health benefits in humans, including improved colonocyte and enterocytes functions, prevention of diarrhoea, alleviation of leaky gut and modulation of other important factors including energy and lipid metabolism and the immune system [161]. This result demonstrates that Kef-w1 is fermentable by native gut microbiota and generates useful SCFAs as by-products, thereby further supporting its utility as potential prebiotics.

Microbiome compositions in the simulated bowel conditions following various treatments are illustrated in **Figure 4.10** and **Figure 4.11**. Significant microbiome variation was observed between the three donors, best illustrated in **Figure 4.10a**, as differences in microbiome were most strongly correlated with donor, rather than associated with various treatments. Within each donor, significant shifts in microbiome were observed for Kef-w1 HP, kefir synbiotics and inulin samples as compared to the control set for all three donors (**Figure 4.10b-d**). Inulin and Kef-w1 HP supplementation were observed to yield disparate microbiome profiles, suggesting that these substrates were fermented by different communities of gut microbiota. Probiotic supplementation alone, did not yield significant

shifts in the microbiome for donors 1 and 2, whereas supplementation of 1E8 CFU kefir probiotics and kefir synbiotics resulted in significant microbiome shifts for donor 3.

The phyla breakdown of microbiome following various treatments are shown in **Figure 4.11a**. For all three donors, *Firmicutes* constitute the majority microbiota component, while *Actinobacteria*, *Bacteroidetes* and *Proteobacteria* were also present. For donor 1, inulin treatment increased abundance of *Actinobacteria* and reduced *Bacteroidetes*, while Kef-w1 HP and kefir synbiotics increased *Bacteroidetes* relative to the control. For donor 2, Kef-w1 HP and kefir synbiotics treatment increased *Firmicutes* ratio. For donor 3, inulin treatment increased *Actinobacteria* percentage and reduced *Bacteroidetes* and *Proteobacteria*, while Kef-w1 HP increased *Bacteroidetes* ratio and decreased *Proteobacteria*. For donor 3, *Firmicutes* ratio was also increased upon supplementation of 1E8 CFU LGG or kefir probiotics, suggesting a persistence of these probiotics in the *ex-vivo* environment. Comparing kefir probiotics alone and kefir synbiotics treatment for donor 3, *Firmicutes* ratio was higher for synbiotics treatment, suggesting that the co-administration of Kef-w1 HP with kefir probiotics possibly promoted growth of kefir probiotics in the *ex-vivo* environment.

As a whole, there appears a general trend that inulin tends to increase abundance of *Actinobacteria*, likely due to promotion of *Bifidobacterium* in the microbiome (**Figure S5**), while Kef-w1 HP supplementation tends to promote *Bacteroidetes*, mostly *Bacteroides* (**Figure S5**), growth. The promotion of *Bifidobacterium* by inulin has been well studied in literature, many *Bifidobacterium* species and strains are able to degrade and utilise fructan oligosaccharides (Ramirez-Farias et al., 2008). Prior studies have shown a propensity for dextran-like substrates to stimulate *Bacteroidetes* or *Bacteroides-Prevotella* populations in faecal inoculums [34], [191]. *Bacteroidetes* are known to be fermenters of complex carbohydrates, and release SCFAs (mainly acetate, propionate and butyrate) as by-products [360]. *Bacteroidetes* also play key roles in immunomodulation of the human immune system and assist with prevention of bacterial and viral infections [361]. Diversity metrics are shown in **Figure 4.11b**. Generally, α -diversity of microbiomes decreased upon inulin, Kef-w1 HP, and kefir synbiotics supplementation, likely due to an over-promotion of

specific genera which were adept at utilising the prebiotic substrates. Overall, Kef-w1 HP demonstrated a tendency to promote *Bacteroidetes* growth and evolve SCFAs, which could provide an advantage for its use as a functional prebiotic ingredient.

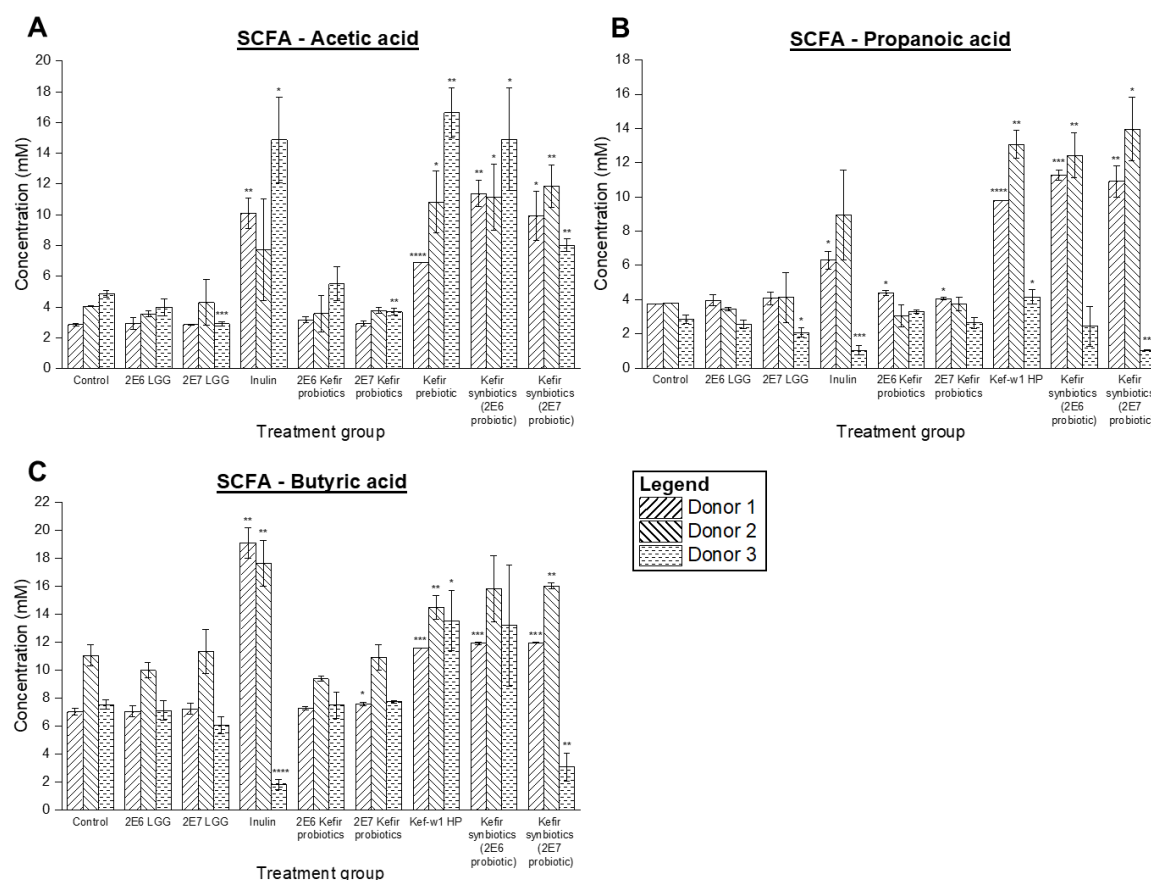


Figure 4.9: SCFAs content in *ex-vivo* microcosms (large bowel simulated model) with different probiotic/prebiotic/synbiotic treatments. Synbiotic was a mix of Kef-w1 HP and Kefir probiotic. Data presented as an average of technical triplicates with standard deviation bars. Asterisks on bars based on t-test comparisons with the control group for respective donors - * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

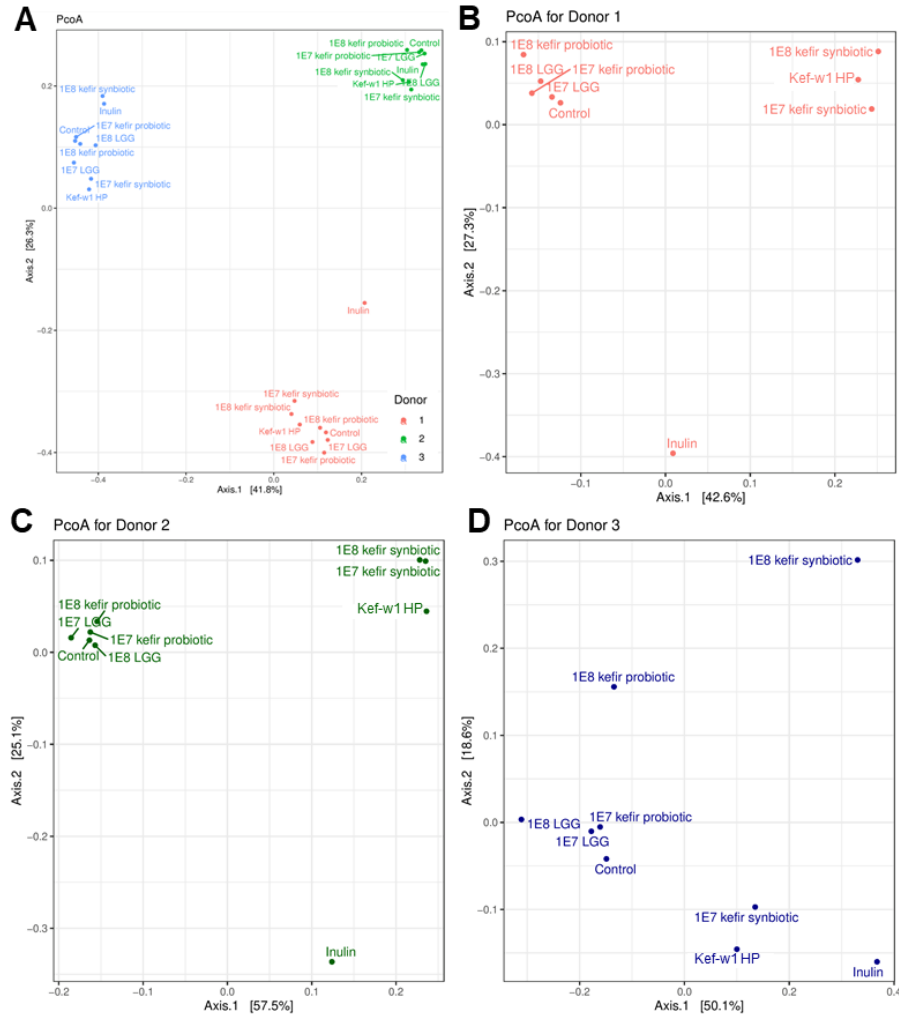


Figure 4.10: PCA analysis of microbiome shifts in *ex-vivo* microcosms (large bowel simulated model) upon supplementation of various probiotics, Kef-w1 HP, and kefir synbiotics (combination of probiotic and Kef-w1 HP) for (a) different treatments and donors, (b) donor 1, (c) donor 2, and (d) donor 3.

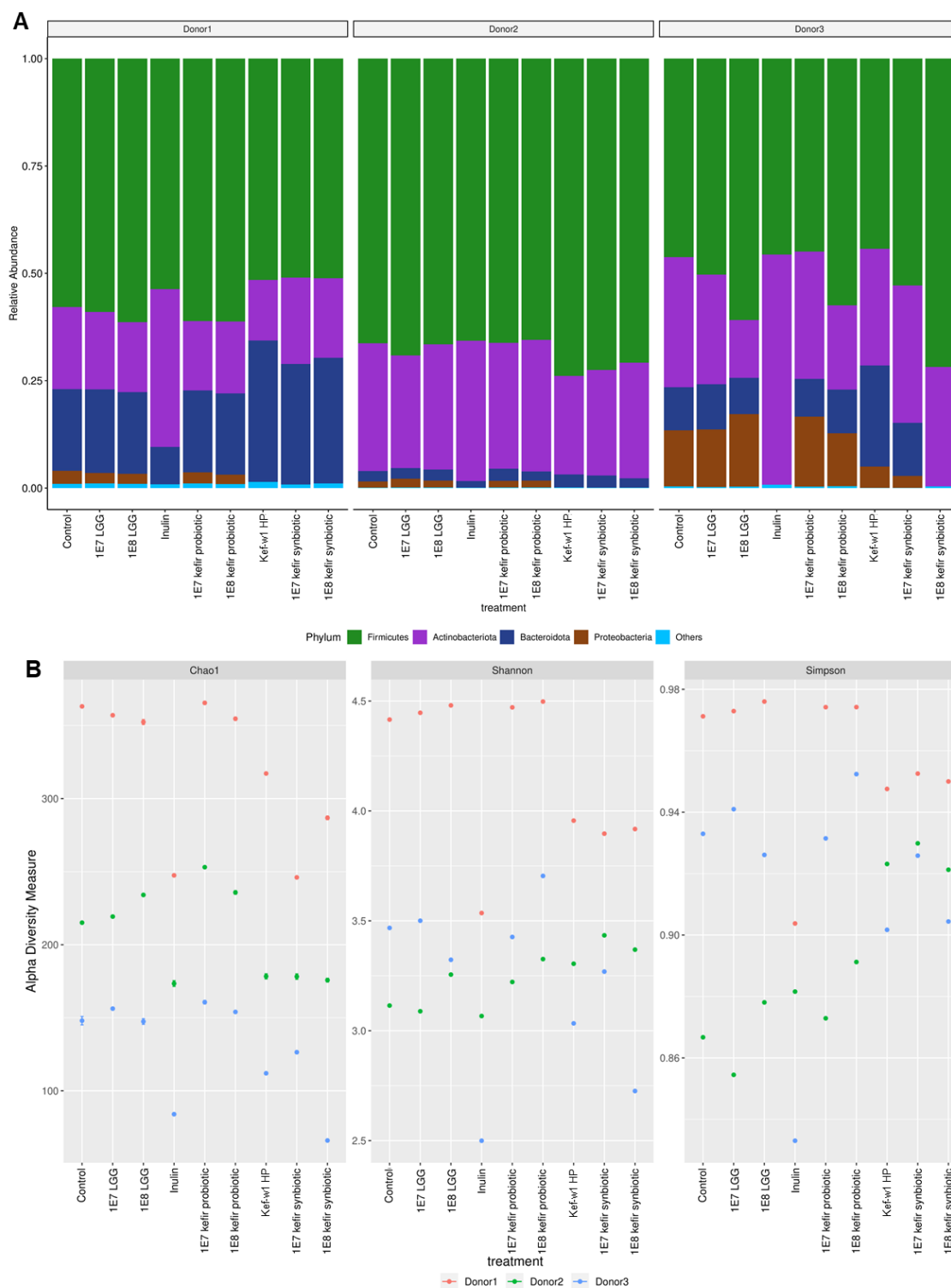


Figure 4.11: Microbiome composition and diversity (large bowel simulated model) upon supplementation of various probiotics, Kef-w1 HP, and kefir synbiotics (combination of probiotic and Kef-w1 HP). (a) Phyla-level community composition of *ex-vivo* microcosms. Phyla with abundance <1% were combined into Others. (b) Diversity metrics of *ex-vivo* microcosms.

4.4. Conclusion

Liquorilactobacillus satsumensis, *Liquorilactobacillus hilgardii*, *Liquorilactobacillus nagelii* and *Zymomonas mobilis* strains derived from water kefir demonstrated EPS-producing properties, of which exopolysaccharides from the *L. satsumensis* Kef-w1 strain provided high yields of soluble, LPS-free, α -glucan EPS from Suc-MRS media. The extracted EPS material from *L. satsumensis* Kef-w1 consisted of a mixture of α -1,3/ α -1,4 and α -1,6 glucan bonds, indicating a branched dextran-like material structure. Upon acid hydrolysis, the native EPS from Kef-w1 yielded a shorter chain α -glucan polysaccharide, termed Kef-w1 HP, with DP 50 – 75. Kef-w1 HP exhibited prebiotic qualities, including the ability to be selectively utilised by probiotics, but not by pathogens, and non-digestibility by human digestive enzymes. Kef-w1 HP was also observed to be fermentable by kefir-derived probiotics, and these were combined in a novel kefir synbiotic formulation. *Ex-vivo* gut simulated conditions with added Kef-w1 HP and kefir synbiotics demonstrated a propensity of Kef-w1 HP to promote *Bacteroidetes* abundance, and generate SCFAs, including acetate, propionate, and butyrate, as by-products. Co-administration of Kef-w1 HP with kefir probiotics also showed evidence of growth promotion of kefir probiotics in the ex-vivo environment, suggesting synergistic effects for the co-supplementation of kefir-derived probiotics and prebiotics. Overall, this chapter has derived a novel ingredient with prebiotic potential, Kef-w1 HP, and this material may be applied in combination with kefir probiotics to yield a synbiotic formulation or incorporated individually as a functional food additive. Thereby, **Objectives 2 and 3** of this thesis are fulfilled in this chapter.

5. Chapter 5*

Encapsulation of probiotics by alginate extrusion

*Alginate extrusion is amongst the most studied probiotic encapsulation methods in the literature, preferred for its mild and simple process, as well as the ability of alginate to protect entrapped probiotics against acidic pH. The performance of alginate, however, depends on its structural and chemical characteristics. Here, various factors, such as alginate type, alginate sterilisation method, and the presence of sucrose, were examined to determine their acid protection effects. The optimised formulation was adapted for encapsulating probiotics to functionalise commercial sodas and beers. Results demonstrate that alginate encapsulation significantly improved the viabilities of probiotics (*Lactobacillus rhamnosus* GG, *Escherichia coli* Nissle 1917, *Bifidobacterium longum*, and kefir probiotics) in various beverages and temperature conditions over a 14-day test duration. Refrigerated storage better preserved probiotic viabilities and reduced the formation of probiotic metabolic by-products than at room temperature storage. Findings here could provide beverage manufacturers with a novel way to develop probiotic-sodas and probiotic-beers through encapsulation.*

*This section is published substantially as:

L.L. Tan, K.L. Ang, and S.C.J. Loo. Alginate encapsulation improves probiotics survival in carbonated sodas and beers. *PLOS One*. (Accepted, Dec 2022). This is an open-access article distributed under the terms of the Creative Commons Attribution License.

5.1. Introduction

In this chapter, a probiotic encapsulation system based on alginate extrusion was developed in pursuit of **Objective 4** of this thesis. This chapter is broadly divided into two parts: 1) Comparison of different alginate formulations and their GIT-protective effects, and 2) Incorporation of alginate-encapsulated probiotics in commercial sodas and beers, corresponding to the two sub-objectives under **Objective 4**.

In the first part, the alginate-extrusion method was optimised to provide maximal survival of LGG probiotics when exposed to SGF or SIF conditions. Variables including the M/G ratio of alginate, whether alginates are sterilised by autoclave or filtration, and the presence of sucrose in the formulation, were studied. The study of these variables in affecting extruded alginate-probiotics systems has so far been limited in literature. It is hypothesised that structural properties of alginate, such as its M/G ratio and molecular weight (which may be affected by autoclaving) have implications on the crosslinking process of calcium-alginate, hence can affect the extent of protection for encapsulated probiotics. Sucrose was also investigated as it is known to be a crosslinking modifier for alginate [362]. Sucrose can also improve palatability and taste of the alginate spheres, which can facilitate its application in beverages for the second part of this study.

The optimised formulation for alginate-extrusion was then used for encapsulating probiotics for addition to carbonated sodas and beers to evaluate whether encapsulation can improve the survival of probiotics in these beverage matrices. The addition of probiotics into popularly consumed carbonated sodas and beers presents an interesting challenge as the presence of acidic pH, hops-derived compounds and ethanol have highly deleterious effects to probiotics viabilities. Carbonated sodas are known to be highly acidic, with pH values ranging from 2 to 3.5, depending on the type of soda [363]. This acidic pH is due to the presence of acids, such as phosphoric acid and citric acid, which are added to impart distinctive tart and tangy flavours to sodas [363]. Dissolved carbonic acids also contribute to acidity in carbonated sodas. These acids provide bactericidal and fungicidal effects, which under usual circumstances, are preferred by soda manufacturers for their

preservative function. However, this low pH property becomes a challenge in attempts to incorporate live probiotic cultures into carbonated sodas. For beers, the main components which affect probiotics viability are ethanol and hops iso- α acids. Ethanol concentration in beer varies between 3 – 9%, depending on the type of beer. Ethanol is known to have antiseptic properties and inhibits bacterial growth by disrupting membrane stability and denaturing proteins [364]. Hops iso- α acids, which are extracted from hops during wort boiling, are potent antimicrobial compounds that can inhibit Gram-positive bacteria [365]. The antibacterial mechanism of hops iso- α acids has been associated with their role as ionophores, which dissipates the pH gradient across bacterial membranes and reduces proton motive force [366]. Hops iso- α acids also contribute mild acidity to beers. Probiotic bacteria, particularly those which were not isolated from alcoholic beverage sources, typically do not have intrinsic resistances to ethanol and hops.

Prior to this study, there were few literatures regarding the incorporation of encapsulated probiotics in carbonated sodas and beers. Past literature mostly discussed the incorporation of alginate-encapsulated probiotics in fruit juices (e.g., orange or apple) [367], [368], the use of probiotics as starter-cultures for beer fermentation [365], or addition of unencapsulated probiotics to low- and non-alcoholic beers [369]. To the best of our knowledge, the addition of alginate-encapsulated probiotics into beers was only once explored by Haffner and Pasc [370], which tested the survivability of LGG in a commercial Lager Beer.

Hence, in the second part of this chapter, the feasibility of adding probiotics into commercial soda and beer beverages, was tested. Four probiotic candidates, namely LGG, *Escherichia coli* Nissle 1917 (ECN), *Bifidobacterium longum* (BL), and kefir probiotics (Kef) (derived in **Chapter 3**), were added to four beverages, which are Coca-Cola, 7-Up, Tiger Beer, and Guinness, under refrigerated, room temperature and SGF conditions. Alginate encapsulation, using the optimised formulation derived in the first part of this chapter, was tested to determine if alginate encapsulation by extrusion could improve probiotics viability in carbonated sodas and beers.

5.2. Materials and Methods

5.2.1. Materials

LGG was isolated from a purchased Culturelle® LGG probiotic pill (i-Health, Inc., USA). ECN was isolated from a purchased Mutaflor® pill (Pharma-Zentrale GmbH, Germany). BL ATCC 15707 was purchased from ATCC, USA. Kefir probiotics, comprising a blend of seven strains including, *Lentilactobacillus hilgardii* Kef-w9, *Lacticaseibacillus paracasei* Kef-w14/19, *Liquorilactobacillus satsumensis* Kef-w11/13, *Lactobacillus helveticus* Kef-m4, and *Lentilactobacillus kefir* Kef-m15, were identified in **Chapter 3** of this thesis.

Protanal® LFR5/60 high G sodium alginate ($M/G = 30/70$, $M_w = 20-60$ kDa) and CR8133 high M alginate ($M/G = 65/35$, $M_w = 90-180$ kDa) were procured from FMC Biopolymer, USA. MRS broth and TS medium were purchased from Thermo Fisher Scientific, USA. Luria–Bertani (LB) Lennox broth and Bacto-agar were purchased from BD, USA. Carbonated sodas and beers, including Coca-Cola Classic (The Coca-Cola Company, USA), 7-Up (Keurig Dr Pepper, USA), Tiger Asian Lager (Heineken Asia Pacific, Singapore) and Guinness Foreign extra stout (Diageo, UK) were purchased from local supermarkets. Glutaraldehyde and sodium cacodylate buffer were purchased from Electron Microscopy Services, USA. All other chemicals used in this experiment were purchased from Sigma Aldrich, USA.

5.2.2. Methods

The following sub-sections describe the characterisation of various alginate materials, the fabrication of alginate-probiotic beads, and subsequent assays to determine the survivability of encapsulated probiotics when subject to simulated GIT conditions, and addition to carbonated sodas and beers. Eight alginate formulations (**Table 5.1**) were evaluated in this study determine if these factors (M/G ratio of alginate, sterilisation method of alginate and presence of 10% sucrose) would affect the extent of protection on

encapsulated probiotics. Autoclaved alginates were sterilised at 121 °C for 15 min, while filtered alginates were sterilised by vacuum filtration through 0.2 µm membrane.

Table 5.1: Abbreviations for tested alginate extrusion formulations

Alginate type	Presence of 10% (w/v) sucrose	
	Without sucrose	With sucrose
High G, filtered	G_fil	G_fil_suc
High G, autoclaved	G_ac	G_ac_suc
High M, filtered	M_fil	M_fil_suc
High M, autoclaved	M_ac	M_ac_suc

5.2.2.1. Rheology characterisation of alginates

Rheological properties of alginates were measured using the Dynamic Rheometer (MCR102, Anton Paar, Singapore), with a cone and plate module. Each alginate sample was placed in the rheometer and at each point of measurement, shear stress was measured at a certain shear rate. 20 points of increasing shear rates from 1 s⁻¹ to 200 s⁻¹ were measured and tabulated.

5.2.2.2. Growth and preparation of probiotics

Single colonies of LGG, ECN, and BL were inoculated in sterile MRS, LB, and TS broths respectively and incubated at 37 °C for 24 h (aerobic incubation for LGG and ECN, anaerobic for BL) to reach the stationary phase of growth. LGG, ECN, and BL were then adjusted to following designated OD600 values; for LGG, 6 (~ 5×10⁸ CFU/ml), for ECN, 1 (~ 5×10⁸ CFU/ml), and for BL, 1 (~ 7×10⁷ CFU/ml). The kefir probiotics blend was prepared by culturing individual strains in MRS at 30 °C, anaerobic conditions for 72 h, after which, respective kefir isolate cultures were adjusted to indicated OD600 values (**Table 3.2**) and concentrated into the same volume (same as protocol in **Section 3.2.2.10**). The probiotic cells were then washed thrice with 0.9% (w/v) NaCl, with centrifugation at 10,000 xg for 5 min between each wash. Next, the probiotic cell pellets were resuspended in 0.9% NaCl at one-tenth of the original volume to obtain a 10x concentrated probiotic

sample. Serial dilution and drop plating on MRS, LB, or TS agar were performed to determine the initial CFU concentration of respective probiotics.

5.2.2.3. Extrusion of alginate beads

The 10x concentrated probiotic was mixed with alginate in a 1:9 (v:v) ratio and thoroughly mixed, yielding a final concentration of 2% (w/v) alginate solution. For formulations containing the sucrose cryoprotectant, 11.11% (w/v) sucrose was added to the alginate solutions in advance, yielding a final concentration of 10% sucrose. Solutions were transferred into a syringe to prepare for extrusion. An extrusion setup was prepared in a biological safety cabinet. The cell suspension was extruded via a syringe pump (KD Scientific, USA) through a 26G needle into 40 ml of sterile 0.1 M calcium chloride crosslinking bath. Needle-to-crosslinking bath distance used was 1 cm and flow rate used was 1 ml/min. The crosslinking bath was kept stirring at 250 rpm during the extrusion process, and particles were left to crosslink for 30 min. Extruded beads were washed thrice with sterile water to remove any unencapsulated probiotics. Calcium lactate was used in replacement of calcium chloride to crosslink alginate-probiotic beads for the exposure to soda and beer experiments, due to its less bitter organoleptic properties than calcium chloride.

5.2.2.4. Imaging

The morphology of extruded beads was characterised using light microscopy (Stereo Discovery V8, ZEISS, Germany) and scanning electron microscopy (SEM). Chemical fixation was done for extruded beads prior to SEM imaging. Probiotics-loaded alginate beads were immersed in 2% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 for 1 h. Beads were then rinsed thrice in 0.1 M sodium cacodylate buffer, then thrice more in DI water. Dehydration was carried out by soaking beads in 30%, 50%, 70%, 90% and 100% (v/v) ethanol sequentially for 5 min each. Ethanol was then removed, and beads were frozen at -80 °C, followed by freeze-drying (0.027 mbar, -84 °C). Freeze-dried beads were observed using a JSM-6360 SEM (JEOL Ltd., Japan) operating in the secondary

electron mode. Freeze-dried powders were mounted to a stub with carbon tape and coated with 10 nm of conductive gold prior to imaging.

5.2.2.5. Thermal characterisation of alginate beads

DSC and TGA are used to characterise the thermal properties of various calcium-alginate without probiotics loading. DSC was used to characterise the melting point distribution of the calcium-alginates. Ca-alginates were heated at 10 °C/min from 20 to 210 °C under nitrogen gas conditions and heat flow was determined relative to a reference pan. TGA was used to examine the thermal stability of the Ca-alginates. TGA was performed under nitrogen gas over a temperature range of 30 to 600 °C at a rate of 10 °C/min using a platinum pan and the mass of sample was measured over time.

5.2.2.6. Compressive strength testing of alginate beads

A universal testing machine (Instron 5567, USA) was used to determine the compressive strength of calcium-alginate beads without probiotics loading. The compressive strength data can serve as an indicator of the extent of crosslinking and the textural properties of alginate beads. The diameter of each bead was first measured using a micrometre screw gauge. Next, each bead was compressed up to a 30% compressive strain, and the corresponding compressive strength was recorded.

5.2.2.7. Alginate bead dissolution

Dissolution of alginate beads was done to determine the encapsulated CFU, thereby the encapsulation efficiency can be derived. The same dissolution method was also performed after exposure to SGF, carbonated sodas, or beers, to determine the remaining encapsulated CFU. Crosslinked alginate beads were dissolved by addition of 0.05 M sodium citrate. Ten-fold serial dilution and drop plating on suitable agars were done to enumerate encapsulated CFU.

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Encapsulated CFU}}{\text{Encapsulated CFU} + \text{CFU in crosslinking bath}} * 100$$

5.2.2.8. Exposure to simulated gastrointestinal fluids

The ability of the various alginate formulations (**Table 5.1**) to protect encapsulated probiotics against GIT insults was studied by exposing the alginate-encapsulated probiotics to SGF/SIF. SGF was prepared by dissolving 2000 U/ml porcine pepsin in 0.2 M NaCl pH 2 solution and sterilised by filtration through a 0.45 µm membrane. SIF was prepared as 0.3% (w/v) ox-bile with 10 mM glucose in PBS 7.4 solution [371]. 500 mg of extruded alginate-probiotic beads was added to 5 ml of SGF and incubated under 37 °C, 200 rpm shaking conditions in an Ecotron shaking incubator (Infors AG, Switzerland) for two hours. Subsequently, excess SGF was removed, and alginate beads were either dissolved to determine remaining surviving CFU, or added to 5 ml of SIF for two additional hours of exposure under 37 °C, 200 rpm shaking conditions. 100 µl of SIF (without any beads) was collected at the 1-hour and 2-hour time points to determine the quantity of viable cells released. The SIF was then removed and the alginate beads were dissolved to determine the residual CFU remaining in the alginate beads after sequential SGF and SIF exposure. The intrinsic acid resistance ability of LGG was determined by adding 100 µl of 10x concentrated LGG to 5 ml of SGF, adjusted to pH 2, pH 2.5 or pH 3, under 37 °C shaking conditions for 2 hours. The survivability of LGG when exposed to bile salts was determined by adding 100 µl of 10x concentrated LGG to 5 ml of SIF, with either 0.3% or 8% (w/v) ox-bile, under similar 37 °C shaking conditions for 2 hours. 8% ox-bile simulates the

concentrated bile acid secreted from the gallbladder, while 0.3% simulates the diluted bile concentration typically present in the small intestine [371].

5.2.2.9. Exposure to soda and beer beverages

Encapsulated probiotics (LGG, ECN, BL, and kefir probiotics) were exposed to various carbonated sodas and beer beverages (Coke, 7-Up, Tiger Beer, and Guinness). 500 ± 10 mg of alginate probiotic beads were added to 5 ml of beverages in triplicates and incubated under 25 °C (room temperature) or 4 °C (refrigerated) conditions, with 50 rpm shaking on an orbital shaker. Probiotic beads were harvested at 1 hour, 2 hour, 1 day, 2 days, 3 days, 7 days, and 14 days time points for beverage exposure. Encapsulated probiotic counts at various time points were enumerated by dissolving beads in 0.05 M sodium citrate followed by serial dilution and drop-plating. The intrinsic survivabilities of various probiotics were also evaluated by adding 50 µl of washed 10x concentrated LGG, ECN, BL, and Kef to 4.95 ml of various beverages in triplicates, under 25 °C or 4 °C shaking conditions, for up to 14 days.

5.2.2.10. Exposure to SGF and beverage combinations

Various encapsulated probiotics were also exposed to SGF and soda/beer beverage combinations, to determine the survivability of probiotics in a gastric environment following oral consumption of sodas or beers. An equal volume of SGF was added to various beverages to form a beverage-SGF solution. 500 ± 10 mg of alginate probiotic beads were added to 5 ml of beverage-SGF solutions, and incubated under 37 °C, 200 rpm shaking conditions. Probiotic beads were harvested at 1 hour and 2 hour time points and dissolved in 0.05 M sodium citrate. Serial dilution and drop-plating were done to evaluate surviving probiotic counts. Unencapsulated probiotics were also tested in various beverage-SGF combinations to determine their intrinsic survivability. 50 µl of 10x concentrated probiotics were added to 4.95 ml of various beverage-SGF combinations, and incubated at 37 °C, 200 rpm shaking conditions for 2 hours.

5.2.2.11. Lactate quantification

Lactic acid is a known by-product of lactobacilli glucose metabolism which can potentially alter beverage taste if accumulated in high concentrations [372], [373]. Lactate concentration was evaluated in various soda and beer beverages with added LGG. Unencapsulated and encapsulated LGG were added to various beverages in a similar method as described in **Section 5.2.2.9** and incubated at 25 °C room temperature or 4 °C refrigerated conditions, with 50 rpm shaking. Aliquots of beverages were removed at 0 h, 2 h, 24 h, 48 h, 72 h, 7 days, and 14 days exposure duration. The aliquots were filtered via a 0.2 µm membrane and analysed using Shimadzu Prominence HPLC (Shimadzu, Japan) equipped with an Aminex HPX-87H organic acid analysis column (Bio-Rad Laboratories, USA). The column was maintained at a temperature of 40 °C, and 30 µl of each sample was eluted with filtered, degassed 0.005 M H₂SO₄ solvent at 0.6 ml/min. Sodium L-lactate in concentrations of 0.01 – 50 mg/ml were used as standards. The lactate peak was identified at retention time of ~ 12.5 min. Chromatograms of various beverages and the standard curve for lactate concentration are provided in **Figure S6**. Concentrations of lactate produced by LGG in various beverages were calculated by normalising concentrations with the 0 h baseline.

5.3. Results and Discussion

This section is presented in two parts – **Section 5.3.1:** Comparison of different alginate formulations and their GIT-protective effects, and **Section 5.3.2:** Incorporation of alginate-encapsulated probiotics in commercial sodas and beers.

5.3.1. Comparison of different alginate formulations and their GIT-protective effects

In this section, various alginate formulations (**Table 5.1**) were compared based on their physical properties and GIT-protective effects. The optimal alginate formulation would be used for the encapsulation of probiotics for addition to sodas and beers, studied in the next section.

5.3.1.1. Rheological properties of alginate

Viscosity properties of various alginate formulations were shown in **Figure 5.1**. All samples with high M alginates were measured to be more viscous than high G alginates. This was within expectation as the high M alginate used had a higher molecular weight (high M alginate $M_w = 90\text{-}180\text{ kDa}$ > high G alginate $M_w = 20\text{-}60\text{ kDa}$). The addition of 10% (w/v) sucrose also increases viscosity of the alginate sample. A slight shear thinning behaviour was observed for all alginate samples. This rheological behaviour is typically observed for polymer solutions due to the increasing orientation of the polymer chains with an increasing shear rate [374]. Autoclaving appears to reduce alginate viscosity significantly. This suggests some degree of depolymerisation or chain scission during the autoclave process. This phenomenon has also been reported in Lorson *et al* [374], where autoclaved alginate solutions and powder showed lower viscosities than native, UV-sterilised, or filtered and lyophilised alginates.

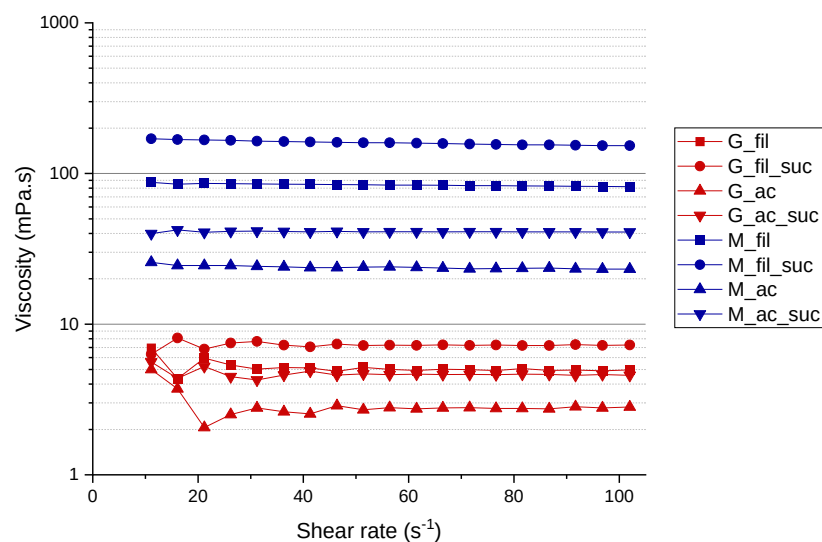


Figure 5.1: Viscosities of various alginate (2% w/v) samples at 25 °C with shear rate sweep from 10 to 102 s^{-1}

5.3.1.2. Morphology of extruded alginate-probiotic beads

Figure 5.2 shows the morphologies of extruded LGG-containing Ca-alginate beads. The shape and average diameters of alginate beads using the various alginate samples are detailed in **Table 5.2**.

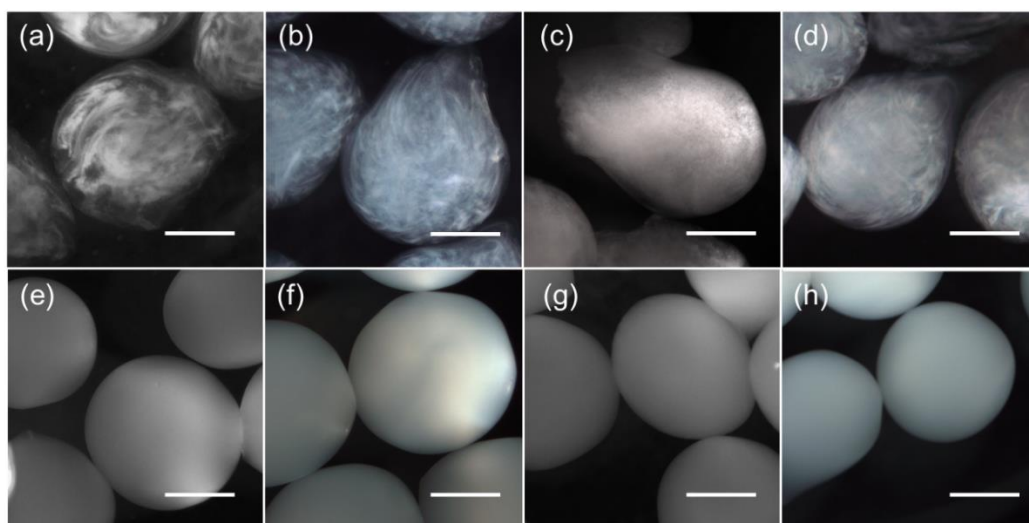


Figure 5.2: Extruded LGG-containing Ca-alginate beads, using various alginate formulations: (a) G_fil, (b) G_fil_suc, (c) G_ac, (d) G_ac_suc, (e) M_fil, (f) M_fil_suc, (g) M_ac, (h) M_ac_suc. Scale bar indicates 1 mm.

Table 5.2: Diameter and shape details of various alginate beads.

Alginate type	Average diameter $\pm$ SD	Shape
G_fil	2.44 ± 0.07 mm	Teardrop shape
G_fil_suc	2.46 ± 0.06 mm	Teardrop shape
G_ac	2.54 ± 0.08 mm	Oddly shaped
G_ac_suc	2.11 ± 0.08 mm	Teardrop shape
M_fil	1.98 ± 0.13 mm	Consistently spherical
M_fil_suc	2.40 ± 0.05 mm	Consistently spherical
M_ac	2.01 ± 0.09 mm	Generally spherical
M_ac_suc	1.94 ± 0.03 mm	Consistently spherical

Extruded Ca-alginate beads were in the range of 1.9 – 2.6 mm in diameter. Spherical or slightly tear-drop shaped beads were obtained for all alginate samples, except G_ac. This was likely due to the very low viscosity of the G_ac alginate (**Figure 5.1**), and it was hence unable to overcome the surface tension forces exerted by the hardening solution, causing the bead to deform [375].

5.3.1.3. Thermal characterisation of alginate beads

Since properties of the alginate samples appeared to change when subject to autoclave heat treatment, the thermal properties of the alginate samples before and after autoclaving were further studied using DSC and TGA. **Figure 5.3** shows the thermal characteristics of freeze-dried calcium-crosslinked alginate beads using the various alginate samples (without any encapsulated probiotics). Only alginates without sucrose were evaluated in this figure.

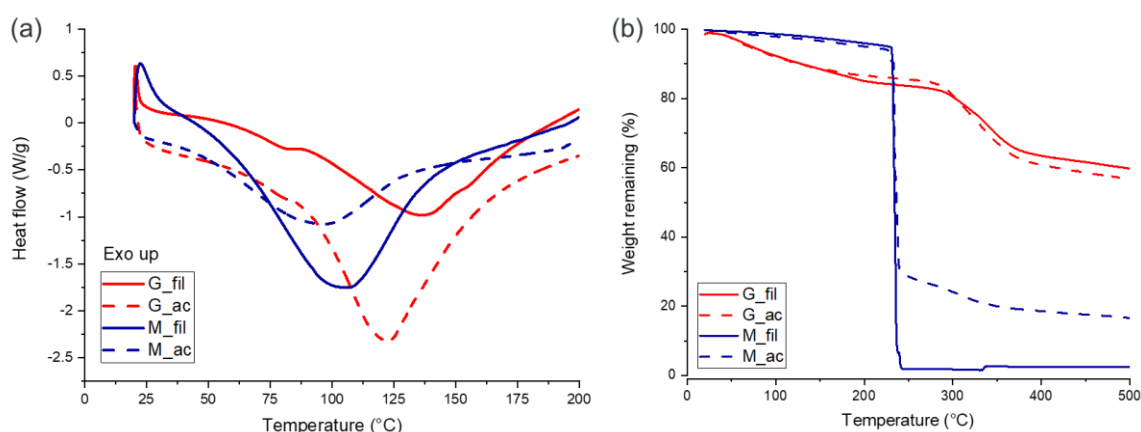


Figure 5.3: (a) DSC and (b) TGA thermographs for various freeze-dried Ca-alginate bead samples.

Melting points of the various Ca-alginate bead samples can be deduced from the characteristic endothermic peaks of DSC curves. From **Figure 5.3a**, the melting points of both high M and high G Ca-alginate beads were noticeably lower after autoclave heat treatment (high M: 96 °C vs. 108 °C, high G: 123 °C vs. 136 °C), adding to the evidence that autoclaving has resulted in a certain degree of depolymerisation of both alginate samples. Also, high G Ca-alginate beads showed higher melting points than the high M Ca-alginate beads, possibly due to a higher degree of crosslinking for the high G alginates. Divalent cations are known to associate more strongly with guluronic acid than mannuronic acid residues, hence result in a higher degree of crosslinking in high G alginates [29]. From **Figure 5.3b**, no significant change in decomposition temperature of both alginates was observed before and after autoclave treatment, suggesting that the chemical structures within the high M and high G alginates were not altered after autoclaving.

5.3.1.4. Compressive strength of crosslinked alginate beads

Compression tests were done on eight calcium-alginate bead samples using the various alginates (without any encapsulated probiotics). The compressive stress at 10, 20 and 30% strain were determined and plotted in **Figure 5.4**.

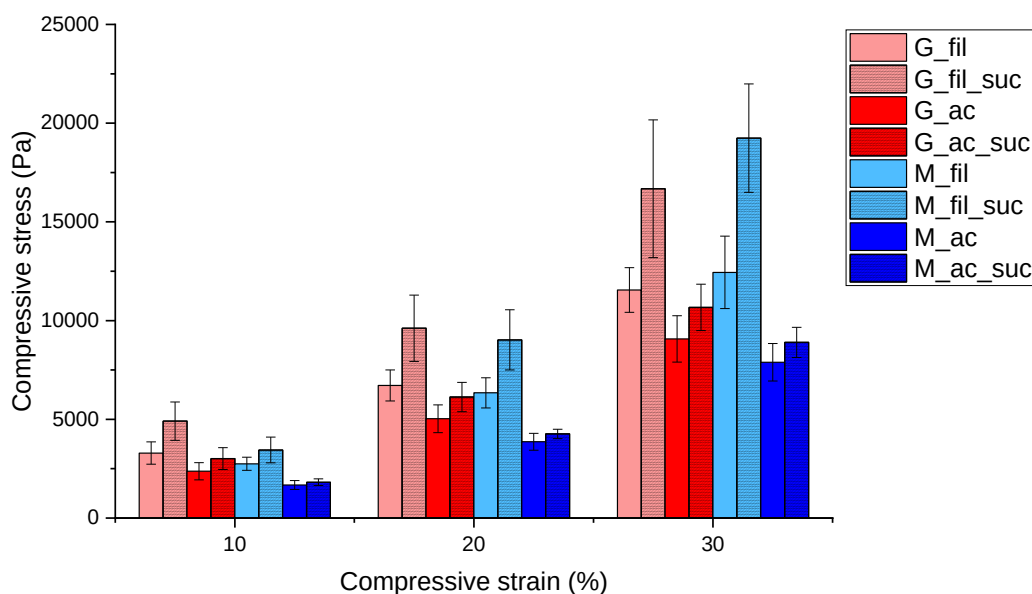


Figure 5.4: Compression testing results for various crosslinked alginate beads.

Beads with autoclaved alginates showed lower compressive stress than filtered alginates, likely due to lower molecular weights of the autoclaved alginates, which contributed to formation of softer gels. Alginate beads containing sucrose exhibited higher compressive strength than beads without sucrose, possibly due to the higher viscosity contributed by sucrose, and the crosslinking modification effects of sucrose [362]. High G alginate beads showed comparable compressive stress values when compared to high M alginate beads, although the viscosities of high G alginates were found to be much lower than high M alginates. This is likely due to a higher degree of crosslinking in high G alginates, as discussed in the previous section.

5.3.1.5. Encapsulation efficiency

Encapsulation efficiency (EE) measures the CFU of probiotics which are successfully encapsulated within the crosslinked alginate matrix. EE close to 100% was achieved via the alginate-extrusion method for all alginates and crosslinkers used (**Table 5.3**).

Table 5.3: Encapsulation efficiencies of LGG in various alginate beads. CFU counts are based on 1 ml of extruded alginate-LGG beads. B.d. indicates that CFU was below detection limit of $2.7 \log_{10}\text{CFU}$.

Alginate type	Encapsulated $\log\text{CFU}$	Unencapsulated $\log\text{CFU}$	EE (%)
G_fil	8.64	b.d.	100.00%
G_fil_suc	8.71	5.36	99.96%
G_ac	8.72	4.60	99.99%
G_ac_suc	8.68	4.60	99.99%
M_fil	8.63	b.d.	100.00%
M_fil_suc	8.65	b.d.	100.00%
M_ac	8.74	b.d.	100.00%
M_ac_suc	8.67	4.49	99.99%

5.3.1.6. Exposure to SGF

The survival of LGG probiotics encapsulated in various Ca-alginate formulations when exposed to SGF was compared to the survival of free LGG cells. From **Figure 5.5a**, a lower pH is more detrimental towards LGG survival and exposure to pH 2 for an hour completely kills LGG cells. Ca-alginate encapsulation using some alginate types was observed to improve LGG survival. M_ac_suc and G_ac_suc resulted in highest and most consistent LGG survival after pH 2 exposure, with 8.52 and 8.18 $\log_{10}\text{CFU}$ surviving per ml of extruded alginate beads (**Figure 5.5b**).

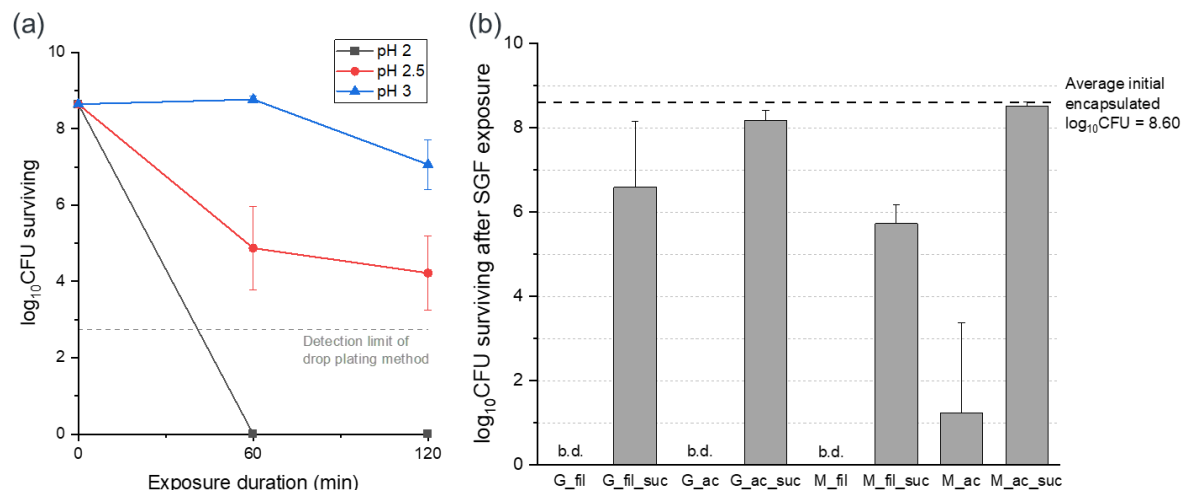


Figure 5.5: Survival of (a) free LGG and (b) Ca-alginate encapsulated LGG when exposed to SGF. For (b), CFU counts are based on 1 ml of extruded Ca-alginate-LGG beads and b.d. indicates that CFU was below detection limit. Data are presented as an average of triplicates with standard deviation bars.

The autoclaved alginates showed higher survivability of LGG than filtered alginates. This may be due to the lower molecular weights of autoclaved alginates, as discussed in **Section 5.3.1.3**, which allows them to better organise and form a less porous bead when crosslinked, conferring greater acid resistance [108].

Sucrose appeared to improve LGG survival in SGF significantly. Several hypotheses are suggested for the protective mechanism of sucrose for LGG against acid stress. Firstly, the protective effect may be due to the adsorptive effects of sucrose on the LGG cell surface through hydrogen bonding to the polar groups, which enhances the stability of the bacterial cell membrane in acidic environment [376]. Secondly, there may be some fermentability of sucrose by LGG which enhances its acid resistance. In most empirical studies, LGG is known not to ferment glucose, although a metabolic pathway for sucrose exists in the LGG genome sequence (**Figure 5.6**) [377]. It is possible that some fermentation of sucrose was achieved by LGG, which contributes to increased intracellular glucose content. Glucose has been shown to increase survivability of LGG in acidic milieu by a proton expulsion mechanism [378]. Thirdly, sucrose may have modified the crosslinking process of calcium alginate, by acting as an intramolecular spacer, thereby allowing for a more evenly-spaced

crosslinked structure of calcium alginate which was preferable for probiotic protection [362]. Fourthly, the addition of sucrose increased the viscosity of alginate, which resulted in more spherical particles (as in the case of G_ac vs. G_ac_suc) that may have conferred better protective effects on the encapsulated probiotics.

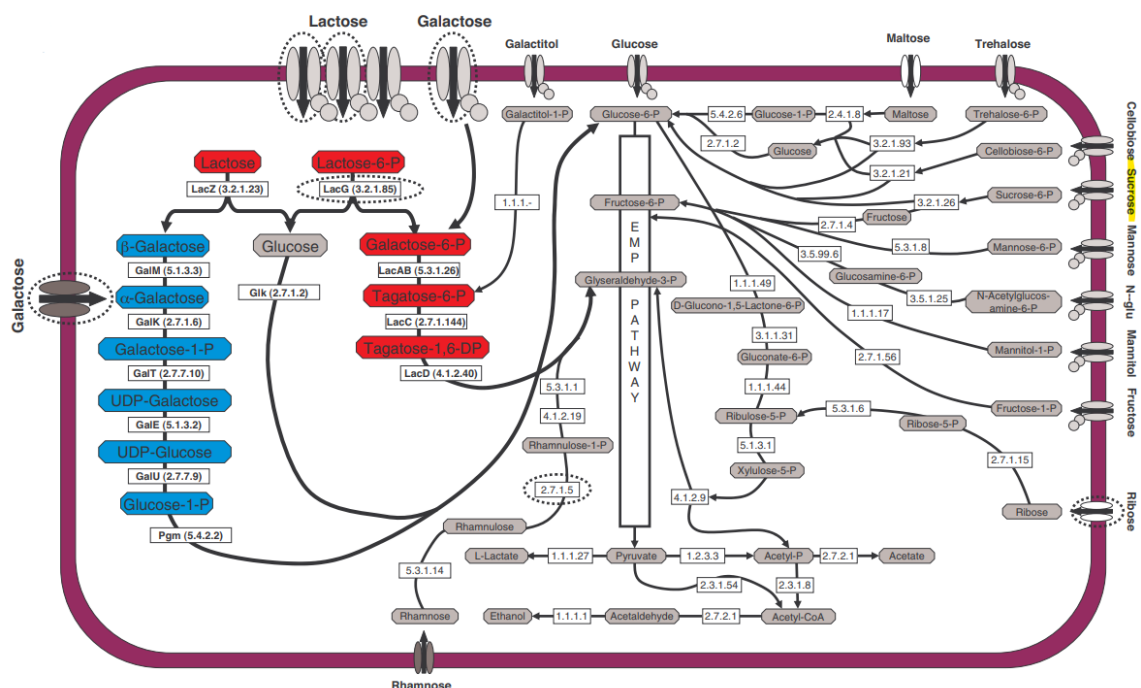


Figure 5.6: Sugar utilisation pathways in *L. rhamnosus*. Transporters, pathways, enzymes, and metabolites predicted to be involved in central carbohydrate utilisation by GG, LC705, and pLC1 strains. Sucrose pathway highlighted in yellow. Reproduced with permission from [377].

5.3.1.7. Exposure to SIF

Since the G_ac_suc and M_ac_suc formulations showed greatest SGF protection, these were further exposed to SIF to determine their degree of protection for LGG probiotics against bile salts.

From **Figure 5.7a**, 0.3% (w/v) ox-bile was not observed to be deleterious to free LGG cells when directly exposed. However, an increased concentration of ox-bile, at 8%, resulted in significant reduction of $\sim 3 \log_{10}$ CFU after 2 hours exposure. **Figure 5.7b** shows the survival of encapsulated LGG when sequentially exposed to SGF followed by SIF with

0.3% (w/v) bile salts. As the alginate beads were transferred into SIF to simulate passage from the stomach into the small intestine, a significant reduction in CFU of LGG in both alginate formulations was observed. In SIF, LGG was released from the alginate matrix due to the sequestering of Ca^{2+} ions by phosphate ions in SIF [379]. $\sim 4 \log_{10}\text{CFU}$ of LGG were released into the SIF media for both alginate formulations over a 2-hour period while $\sim 5 \log_{10}\text{CFU}$ remain encapsulated within the alginate matrices. This adds up to $< 6 \log\text{CFU}$ of LGG surviving after sequential exposure in SGF and SIF, indicating $> 2 \log\text{CFU}$ reduction in the SIF media for both alginate formulations. It appears that LGG, after exposure to the acidic SGF, became more susceptible to antimicrobial effects of bile salts. Both G_ac_suc and M_ac_suc formulations appeared not so effective in protecting encapsulated LGG against bile. It is likely that bile salts are still able to diffuse into the calcium-alginate beads and disrupt lipid cell membranes of probiotic cells.

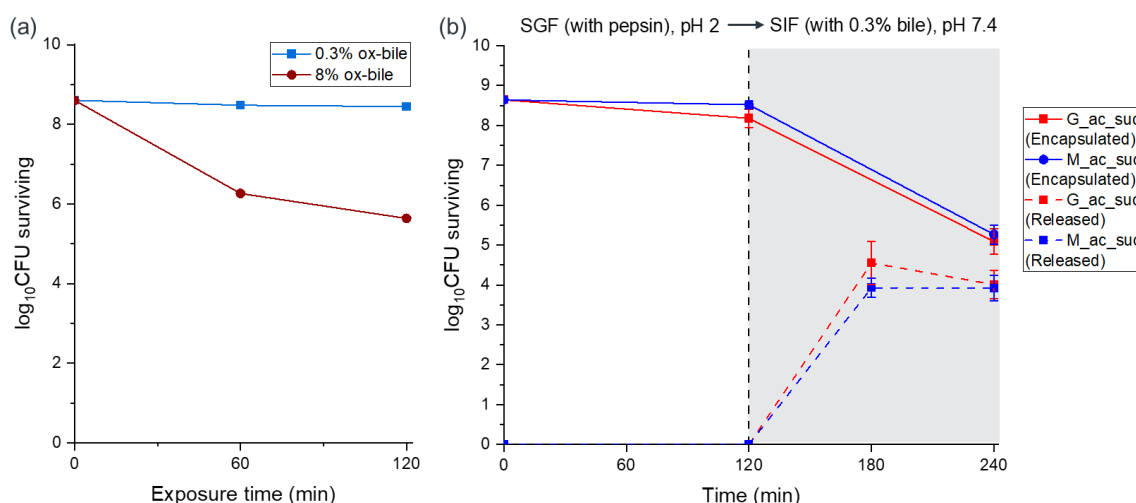


Figure 5.7: Exposure of encapsulated and free LGG to SIF. (a) Survival of free LGG when exposed to SIF with 0.3% or 8% ox-bile for 2 hours. (b) Alginate-encapsulated (G_ac_suc and M_ac_suc) LGG exposed to SGF and SIF sequentially. CFU counts are based on 1 ml of extruded alginate-LGG beads.

In this first phase of study, none of the tested alginate formulations could yield protection of encapsulated probiotics against sequential SGF and SIF exposure. Regardless, it is demonstrated that encapsulation by extrusion could result in an improvement for acid

survivability of probiotics, and the G_ac_suc and M_ac_suc formulations could yield highest protection for encapsulated probiotics against SGF.

5.3.2. Incorporation of alginate-encapsulated probiotics in commercial sodas and beers

In this section, Ca-alginate encapsulation formulations were evaluated to determine if alginate encapsulation by extrusion can improve probiotics viability in carbonated sodas and beers. From the previous section, M_ac_suc and G_ac_suc formulations yielded highest survival of LGG after SGF challenge. Between M_ac_suc and G_ac_suc, M_ac_suc yielded more consistently spherical particles, hence this section utilises the M_ac_suc alginate for encapsulating probiotics for addition to carbonated sodas and beers.

5.3.2.1. Survivability of unencapsulated probiotics in beverage

Various probiotics (LGG, ECN, BL, and Kef) were directly exposed to sodas and beers to determine their intrinsic survivability in these beverages. From **Figures 5.8 and 5.9**, varying extents of cell death were observed for the different probiotics at different exposure temperature conditions. Unencapsulated LGG showed the greatest susceptibility to Coke, with ~ 5 logCFU reduction following two hours of exposure at room temperature and refrigerated conditions (**Figures 5.8a and 5.9a**). LGG was also highly susceptible to Guinness, with complete viability losses observed within < 1 day exposure at room temperature, and < 2 days in refrigerated conditions. For exposure to Tiger Beer, unencapsulated LGG was able to survive up to the 14 days tested in refrigerated conditions with no viability losses but saw complete death within seven days at room temperature conditions. Unencapsulated LGG was able to survive and maintain its viability in 7-Up at both temperature conditions throughout the 14 days of exposure.

ECN similarly showed the greatest susceptibility to Coke and Guinness, with complete viability losses in both beverages at room temperature and refrigerated conditions within < 2 days of exposure (**Figures 5.8b and 5.9b**). 7-Up exposure also demonstrated significant

antagonism to unencapsulated ECN survival, resulting in complete cell death within < 3 days of exposure at both temperature conditions. Amongst the four tested beverages, ECN was most tolerant to the Tiger Beer media, however, 1.6 logCFU losses were observed after 14 days of refrigerated exposure, and at room temperature, ECN did not survive beyond seven days.

Unencapsulated BL experienced significant viability losses in all four tested beverages (**Figures 5.8c and 5.9c**). Coke and Guinness were most antagonistic to BL, with complete cell death observed within 1 hour of exposure at both room temperature and refrigerated conditions. For 7-Up exposure, BL showed complete susceptibility within < 1 day exposure at room temperature, and < 2 days in refrigerated conditions. Tiger Beer resulted in complete death of BL within < 2 days exposure at room temperature, and 3.2 logCFU losses after 14 days of refrigerated exposure.

Kefir probiotics demonstrated similar survivability trends as LGG, and showed complete viability loss in Coke within 2 days of exposure in both temperature conditions (**Figures 5.8d and 5.9d**). Kef was also susceptible to Guinness, but demonstrated greater resistance than LGG at refrigerated conditions. Complete viability losses of Kef were observed within 2 days exposure at room temperature, while 5.16 logCFU losses occurred in refrigerated conditions after 14 days. For exposure to Tiger Beer, unencapsulated Kef survived 14 days tested in refrigerated conditions with no viability losses but saw complete death within seven days at room temperature conditions. Unencapsulated Kef maintained > 8 logCFU in 7-Up at both temperature conditions throughout the 14 days of exposure.

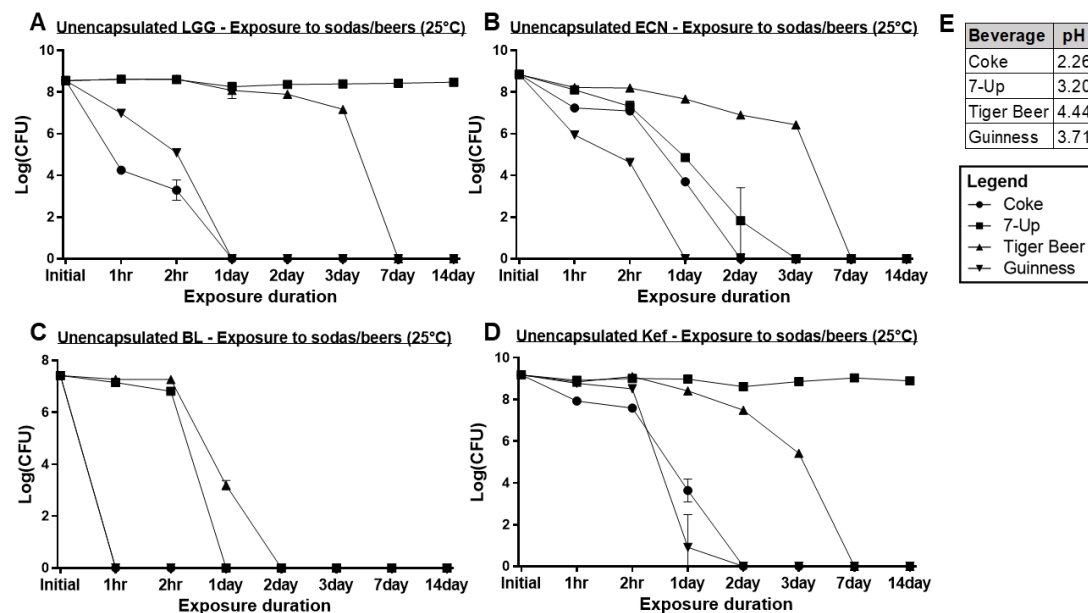


Figure 5.8: LogCFU survival of unencapsulated probiotics, (a) LGG, (b) ECN, (c) BL, and (d) Kef, in various beverages at room temperature conditions for up to 14 days. (e) pH values of various beverages measured at room temperature. Data are presented as an average of triplicates with standard deviation bars.

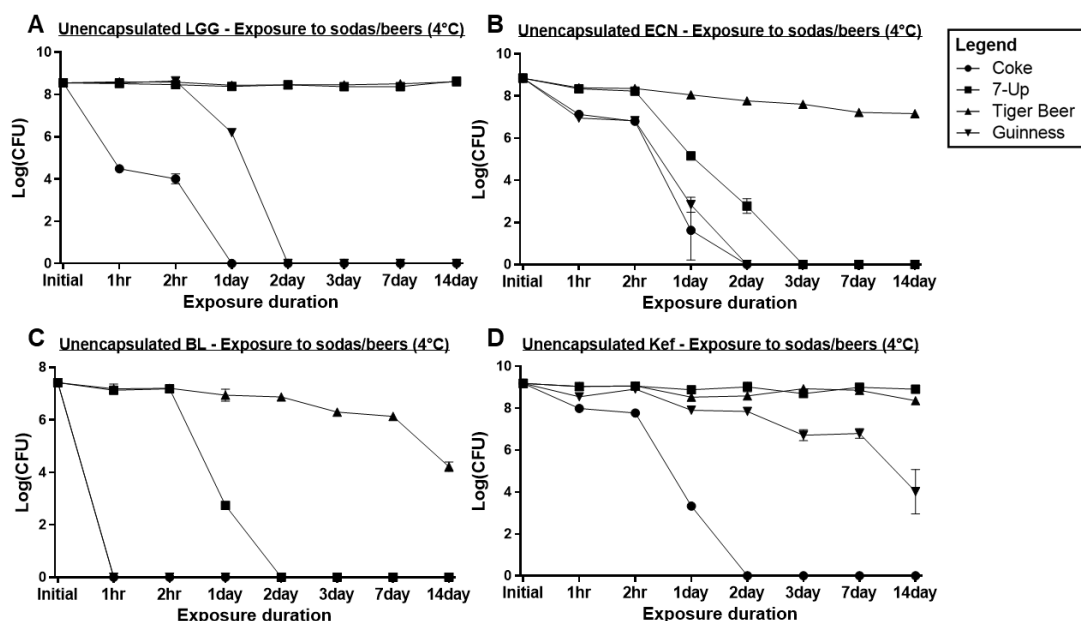


Figure 5.9: LogCFU survival of unencapsulated probiotics, (a) LGG, (b) ECN, (c) BL, and (d) Kef, in various beverages at refrigerated conditions for up to 14 days. Data are presented as an average of triplicates with standard deviation bars.

All unencapsulated probiotics, LGG, ECN, BL, and Kef, showed better survivability in 4 °C refrigeration than at 25 °C room temperature conditions. Lower storage temperatures are known to generally improve microorganism survival due to reduced rates of metabolism and chemical reactions, which can slow the effects of protein degradation or cell membrane oxidation [380], [381]. Comparing between the probiotics, LGG and Kef generally showed better survival in the sodas and beers than ECN and BL. This may be attributed to the better acid and oxygen tolerance of lactobacilli. LGG and Kef were specifically isolated strains with an ability to proliferate at acidic conditions of pH 3 and higher [169], [382], hence has naturally higher resistance to the acidic beverages tested. Lactobacilli, like LGG, are also generally more tolerant to oxygen than *Bifidobacterium*, as the former are aerotolerant anaerobes, while the latter are obligate anaerobes [383].

Comparing the two tested sodas, Coke and 7-Up, Coke was significantly more antagonistic to the survival of all four probiotic candidates than 7-Up. This is likely due to the more acidic pH of Coke, which was measured at 2.26, as compared to 3.20 for 7-Up (**Figure 5.8e**). Between the two tested beers, Tiger Beer and Guinness, Guinness was more detrimental towards the survival of probiotics. Guinness is an ale-stout, which is produced in warmer conditions of 16 to 24 °C by fermentation of *Saccharomyces cerevisiae*. On the contrary, Tiger Beer is a lager, which is produced in colder conditions of around 10 °C by fermentation of *Saccharomyces pastorianus* [384]. Several studies have evaluated the composition of stouts and lagers; stouts are known to present higher concentrations of hops iso- α -acids, such as xanthohumol, isoxanthohumol, 6-prenylnaringenin and 8-prenylnaringenin, than lagers, which likely contributed to stronger bactericidal effects in stout [384]–[386]. Hops iso- α -acids are known to have potent inhibitory effects towards Gram-positive bacteria, while Gram-negative strains tend to be more resistant against these compounds [387]. From **Figures 5.8b and 5.9b**, ECN demonstrated susceptibility particularly to Guinness Beer. This may be due to a combined effect of hops iso- α -acids antimicrobials and the acidified pH of the beverage [388]. Guinness also contained a higher alcohol content (5.5% alcohol by volume (ABV)) as compared to Tiger Beer (5% ABV), which likely presented a more challenging environment for the survival of probiotics.

5.3.2.2. Survival of unencapsulated probiotics in beverages + SGF

The viabilities of unencapsulated probiotics in SGF and beverage combinations are determined to evaluate the survivability of probiotics in a gastric environment following oral consumption of sodas or beers. All four probiotics, LGG, ECN, BL, and Kef, showed worse survivability in beverage-SGF combinations, as compared to beverages alone. LGG showed complete viability losses following two-hour exposure in Coke-, Tiger Beer- and Guinness-SGF solutions (**Figure 5.10a**), indicating that unencapsulated LGG would not survive gastric passage if it were to be consumed alongside the soda and beer beverages. ECN showed significant logCFU losses of 4.1, 8.0, 1.7 and 8.8 for Coke-, 7-Up-, Tiger Beer- and Guinness-SGF solutions respectively, after two hour exposure (**Figure 5.10b**). Unencapsulated BL was completely susceptible towards all beverage-SGF combinations within one hour exposure (**Figure 5.10c**). Kef showed complete viability losses in Guinness-SGF, 4.13 logCFU losses in Coke-SGF and 3.09 logCFU losses in Tiger-SGF after two hour exposure (**Figure 5.10d**). The poor survival of probiotics in beverage-SGF combinations is likely attributed to the lower pH of the simulated gastric environment (**Fig. 5.10e**). Upon comparison of Tiger Beer-SGF (pH 3.44) with 7-Up only (pH 3.20), it was also interesting to note that Tiger Beer-SGF was significantly more antagonistic than 7-Up towards the survival of all three probiotics, although the pH of Tiger Beer-SGF was higher. This suggests that a combinatory effect of bactericidal compounds present in Tiger Beer and the low pH due to the addition of SGF exacerbated survival of the probiotics in the Tiger Beer-SGF solution. Overall, the survival of all three unencapsulated probiotics in beverage-SGF combinations was unsatisfactory, and encapsulation formulations to improve their viability in these beverages and SGF are required.

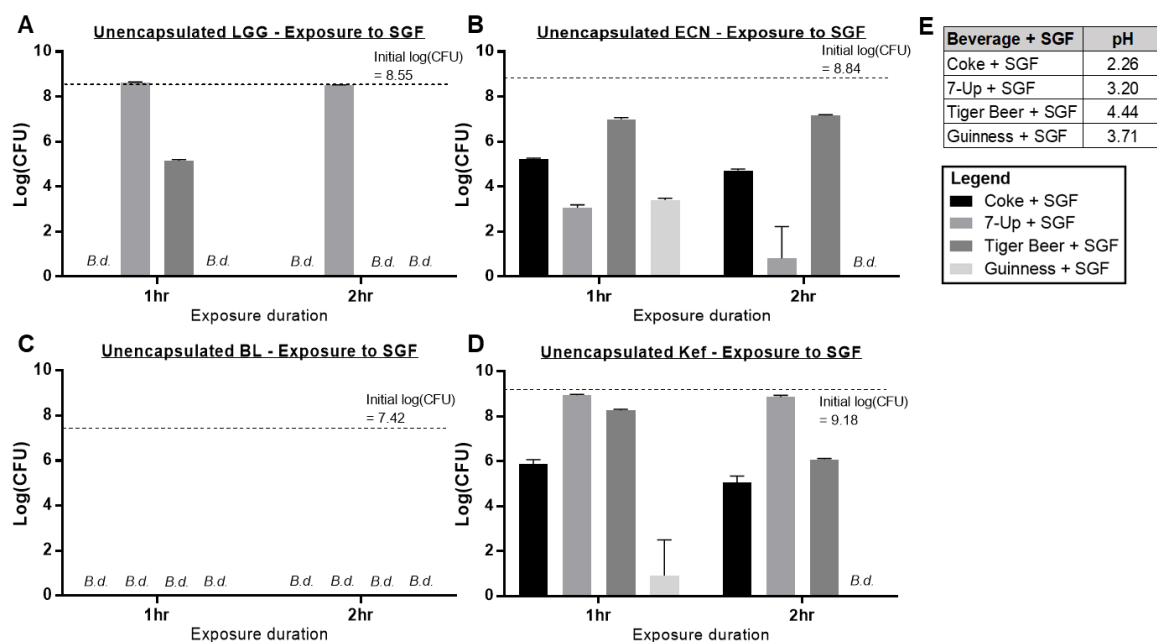


Figure 5.10: LogCFU survival of unencapsulated probiotics, (a) LGG, (b) ECN, (c) BL, and (d) Kef, in various SGF and beverage combinations. (e) pH values of various SGF and beverage combinations at room temperature. B.d. indicates that the cell count is below the detection limit.

5.3.2.3. Morphology of alginate-encapsulated beads

Alginate encapsulation was performed, and **Figure 5.11** shows various images of the extruded probiotic-containing alginate beads. Upon incorporation into various beverages, the alginate-probiotic beads sank and appeared “boba-like” (**Figure 5.11a**). Various probiotics, LGG, ECN, BL, and Kef were observed to be embedded within the crosslinked calcium alginate matrix, suggesting that these probiotic bacteria were physically entrapped within the alginate matrix (**Figures 5.11b – e**).

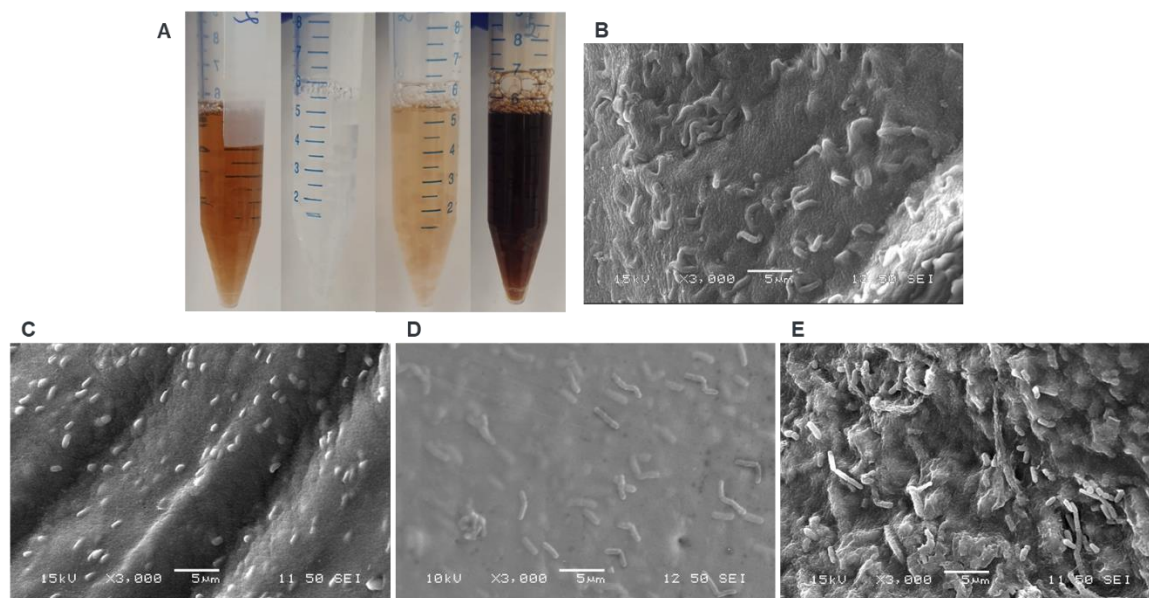


Figure 5.11: Images of alginate-encapsulated probiotic beads. (a) Alginate-probiotic beads added to various beverages, from left to right, Coke, 7-Up, Tiger Beer and Guinness; SEM images of (b) LGG, (c) ECN, (d) BL, and (e) Kef embedded within calcium-alginate (M_{ac}_suc) beads

5.3.2.4. Encapsulated probiotics survival in beverages and SGF

Probiotics encapsulated in Ca-alginate were exposed to various sodas and beers to determine their survivability. From **Figures 5.12, 5.13 and 5.14**, significant improvement in the survivability of all four probiotics, LGG, ECN, BL, and Kef, was observed upon encapsulation. Encapsulated LGG was observed to survive with < 1 logCFU/g losses for all beverages tested after 14 days of exposure in refrigerated conditions (**Figure 5.13a**). At room temperature conditions, the survivability of encapsulated LGG was worse than at 4 °C, but was considerably improved compared to unencapsulated LGG for Coke and Guinness exposure. Encapsulated LGG survived for up to two days of exposure to Coke at room temperature with < 1 logCFU/g loss, while unencapsulated LGG showed 5.3 logCFU losses within two hours of exposure (**Figures 5.8a and 5.12a**). Additionally, encapsulated LGG maintained high viability with < 1 logCFU/g loss within two hours of Guinness exposure at room temperature, as compared to 3.5 logCFU losses for unencapsulated LGG.

For ECN, a significant improvement in survivability was observed for encapsulated ECN exposed to various beverages within two hours of room temperature and refrigerated exposure, with $> 8 \log\text{CFU/g}$ remaining viable (**Figures 5.12b and 5.13b**). At refrigerated conditions, encapsulated ECN also showed high survival ($> 7 \log\text{CFU/g}$) in Coke and Tiger Beer after 14 days and $> 6 \log\text{CFU/g}$ survival in 7-Up and Tiger Beer after 3 days. On the other hand, at room temperature conditions, encapsulated ECN survived with $> 7 \log\text{CFU/g}$ in Coke and Tiger Beer after three days exposure, while encapsulated ECN saw significant viability losses ($< 6 \log\text{CFU/g}$ surviving) in 7-Up and Guinness following one day and three days exposure respectively.

Survivability of BL was also improved significantly upon alginate encapsulation (**Figures 5.12c and 5.13c**). High survivability of encapsulated BL was maintained ($> 6 \log\text{CFU/g}$ surviving) in Coke, 7-Up and Tiger Beer after 14 days exposure at both temperature conditions. For Guinness exposure, high survivability of encapsulated BL was achieved ($> 8 \log\text{CFU/g}$) within two hours exposure at room temperature and one day exposure at refrigerated conditions.

For Kef, survivability was similarly improved upon alginate encapsulation (**Figures 5.12d and 5.13d**). At refrigerated exposure conditions, encapsulated Kef survived with $< 1 \log\text{CFU/g}$ losses for Coke, 7-Up, and Tiger Beer after 14 days of exposure. Encapsulated Kef showed $5.84 \log\text{CFU/g}$ surviving ($3.73 \log\text{CFU/g}$ reduction) after 14 days of refrigerated exposure to Guinness. At room temperature conditions, encapsulated Kef survived with $< 1 \log\text{CFU/g}$ for Coke and 7-Up, and showed $3.8 \log\text{CFU/g}$ reduction for Tiger Beer and Guinness.

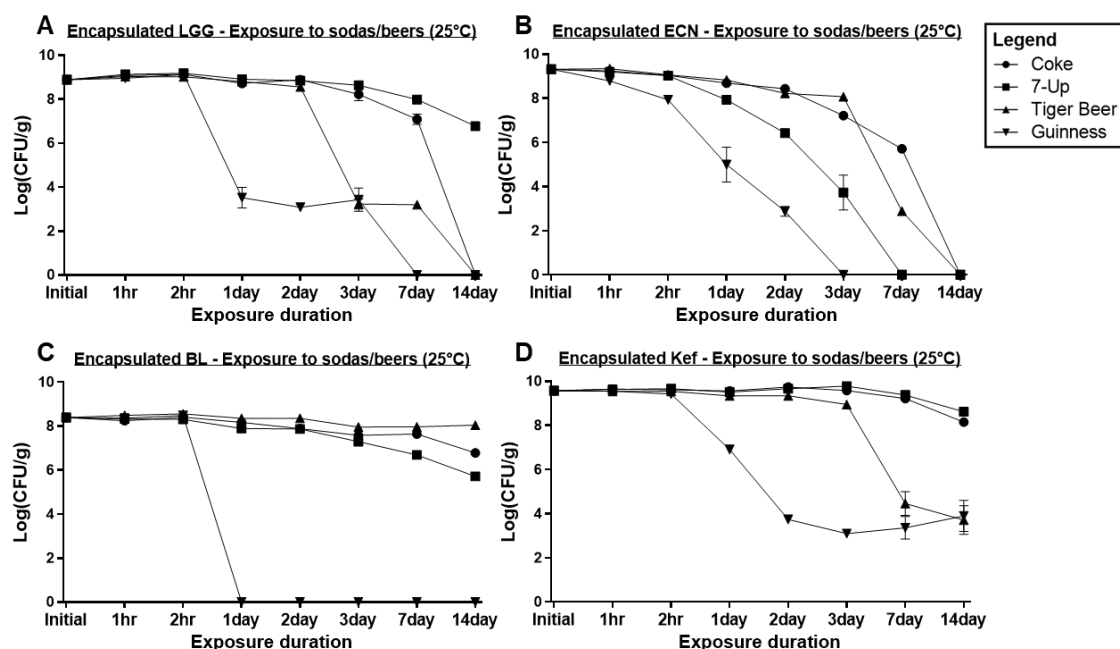


Figure 5.12: LogCFU survival of probiotics, (a) LGG, (b) ECN, (c) BL, and (d) Kef, encapsulated in Ca-alginate (M_{ac}_suc) upon exposure to various beverages at room temperature conditions for up to 14 days.

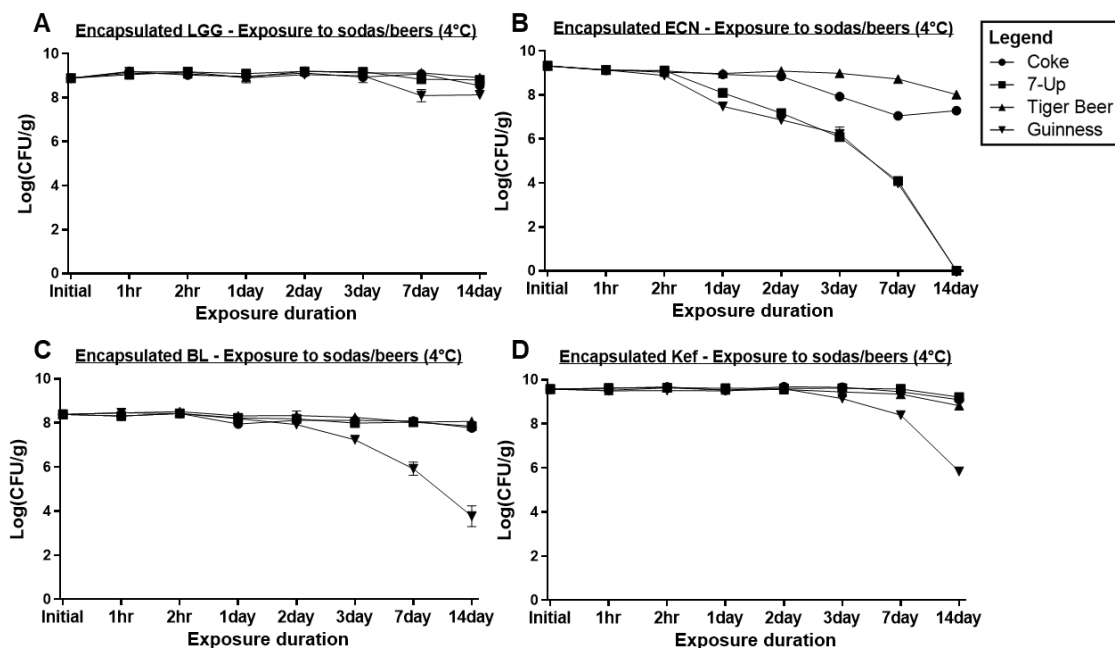


Figure 5.13: LogCFU survival of probiotics, (a) LGG, (b) ECN, (c) BL, and (d) Kef, encapsulated in Ca-alginate (M_{ac}_suc) upon exposure to various beverages at refrigerated conditions for up to 14 days.

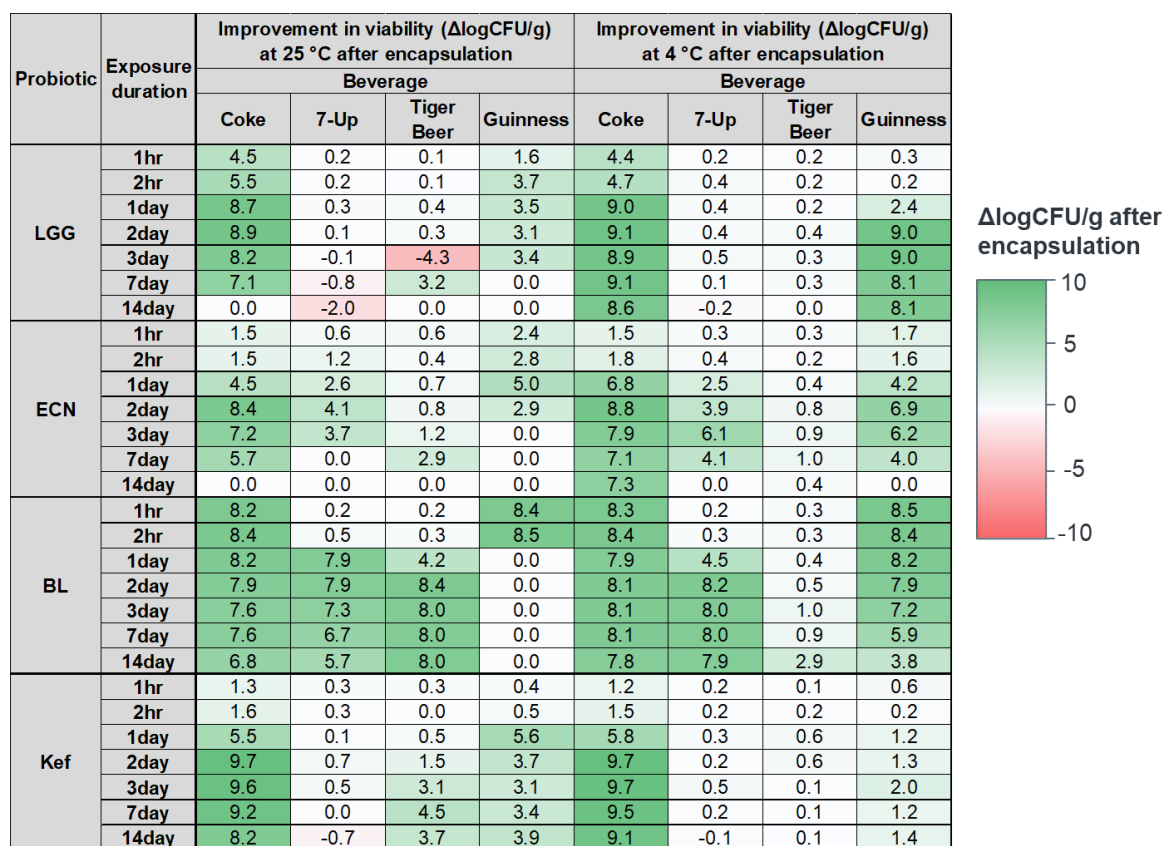


Figure 5.14: Heatmap showing improvement in probiotics viability after alginate encapsulation.

For all four probiotic candidates, the most pronounced improvement brought about by encapsulation was for Coke exposure (**Figure 5.14**), with $> 7 \log\text{CFU/g}$ improvement in viability for all four encapsulated probiotics after 14 days of refrigerated exposure. For 7-Up, significant improvement in survivability was observed for encapsulated BL, with $7.8 \log\text{CFU/g}$ remaining viable after 14 days of refrigerated exposure, as compared to complete viability losses of unencapsulated BL within two days of exposure (**Fig. 5.8c and 5.9c**). The improvement in viability could be attributed to the pH buffering capacity of alginate. In acidic pH, alginate is known to sequester protons and convert to alginic acid. Hence, alginate beads could maintain a localised pH higher than its surroundings, and this promoted survivability of encapsulated probiotics [29], which were otherwise susceptible to the acidic pH in the carbonated sodas. The pH buffering mechanism improved survivability of probiotics in Tiger Beer and Guinness as well. Various studies have demonstrated that the antibacterial effect of hops iso- α -acids increases with decreasing pH

[388], [389]. With a higher localised pH, encapsulated probiotics were expectedly more tolerant to higher concentrations of hops iso- α -acids. Alginate encapsulation also likely reduced diffusivity of iso- α -acids and other inhibitory compounds present in beer, thereby prolonging the viability duration of encapsulated probiotics.

In general, it was observed that the viability of encapsulated probiotics was better maintained in 4 °C refrigerated conditions as compared to at 25 °C room temperature conditions throughout the 14 days test duration. Accordingly, in the development of probiotics encapsulation formulations for functional sodas and beers, the recommendation would be to store probiotics-incorporated beverages in refrigerated conditions to best preserve probiotics viability. Within the 14 days test duration, the best performing encapsulated probiotic and beverage combinations which yielded $> 8 \log\text{CFU/g}$ viable probiotics after refrigerated storage include LGG with all four tested beverages, ECN with Tiger Beer, and BL/Kef with Coke/7-Up/Tiger Beer. Another alternative to incorporating probiotics within these sodas and beverages would be to develop alginate-probiotic beads which are stored separately from the beverages, only to be added into the beverages when ready to be consumed. In this case, the duration of exposure of encapsulated probiotics to the various beverages could be kept within two hours, where high viabilities of encapsulated probiotics ($> 8 \log\text{CFU/g}$) could still be maintained.

5.3.2.5. Encapsulated probiotics survival in beverages and SGF

Survival of probiotics were also significantly improved in SGF exposure conditions following alginate encapsulation. As seen in **Figure 5.15**, high viabilities ($> 8 \log\text{CFU/g}$) were achieved following two hours of beverage-SGF exposure for LGG, ECN, BL, and Kef in Coke-, 7-Up- and Tiger-SGF solutions, indicating that alginate encapsulation was able to protect these strains during gastric passage. For Guinness-SGF, 2.4, 1.6, 2.7, and 0.71 $\log\text{CFU/g}$ reduction were observed for LGG, ECN, BL, and Kef respectively after 2 hours, however this was still a significant improvement as compared to unencapsulated probiotics. Overall, alginate encapsulation has demonstrated its utility in protecting probiotics within beverages, as well as during passage through the gastric environment.

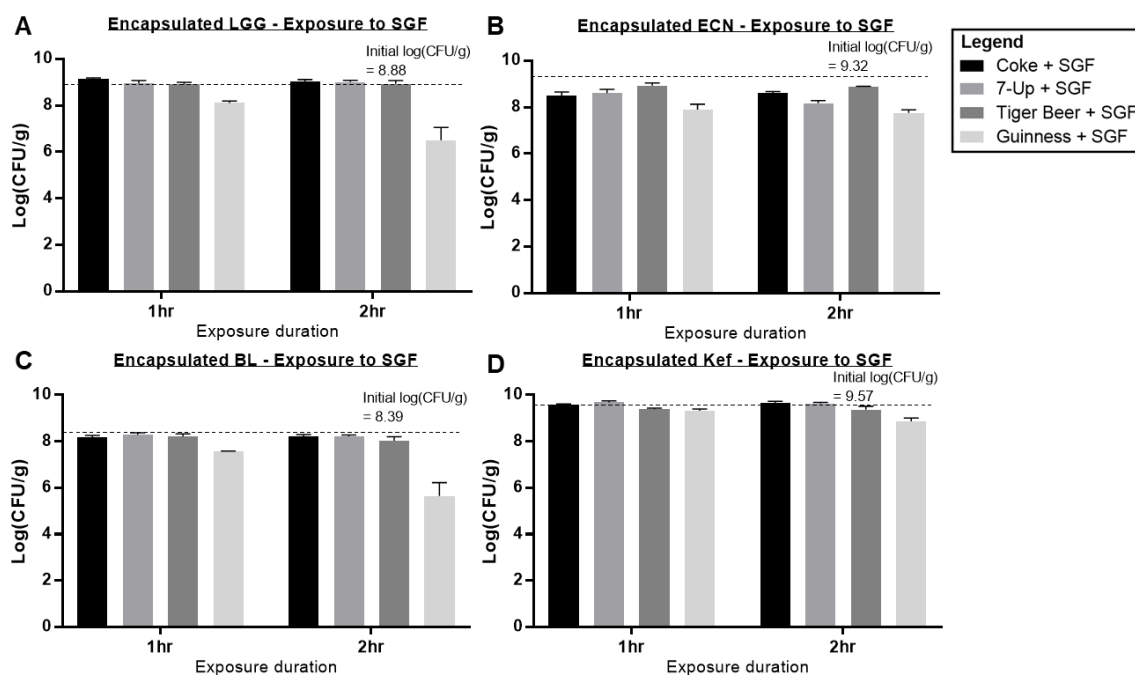


Figure 6.15: LogCFU/g survival of encapsulated probiotics, (a) LGG, (b) ECN, (c) BL, and (d) Kef in various SGF and beverage combinations.

5.3.2.6. Lactate production by LGG

The lactate content within LGG-containing beverages was studied to determine if lactate had accumulated as a by-product of LGG metabolism. An accumulation of lactate can potentially introduce undesirable “off-flavours” to beverages [372], [373]. Here, unencapsulated LGG and alginate-encapsulated LGG were exposed to various beverages and the lactate content was evaluated by HPLC. Increased L-lactate concentrations were detected in LGG-supplemented Coke and 7-Up, but not in Tiger Beer and Guinness. This is likely due to the absence of glucose in Tiger Beer and Guinness, as simple sugars tend to be depleted in beers during the yeast fermentation process [386].

From **Figure 5.16a**, an increase in L-lactate was observed in encapsulated LGG stored at room temperature conditions, with 1.98 mg/ml present following 14 days of exposure. A minor increase in L-lactate of 0.23 mg/ml was detected for encapsulated LGG in Coke stored at refrigerated conditions. No significant concentration of L-lactate was detected for

unencapsulated LGG in Coke at both temperature conditions. For 7-Up, an increase in L-lactate was measured for both unencapsulated and encapsulated LGG at room temperature and refrigerated conditions (**Figure 5.16b**). L-lactate concentration reached 2.57 mg/ml for both unencapsulated and encapsulated LGG at room temperature conditions after 14 days, while encapsulated LGG in refrigerated conditions showed slightly higher L-lactate production of 1.02 mg/ml as compared to 0.84 mg/ml L-lactate produced by unencapsulated LGG.

The data suggests that viable LGG was able to metabolise glucose present in Coke and 7-Up, and had produced a greater amount of L-lactate in 7-Up. With reference to the nutritional labels of these two sodas (**Figure S7**), Coke used in this experiment had a higher concentration of total sugars (10.6 g/100ml) than 7-Up (4.7 g/100ml). Hence, the higher L-lactate concentration in 7-Up was not due to the concentration of sugars but was likely due to the higher pH level in 7-Up, which provided a less hostile environment for LGG metabolism. Presently, there appears to be no released guidelines regarding the concentration of lactate which induces an “off-flavour”, as this depends on the type of beverage, and the relative amounts of other flavour compounds present in the beverage. Overall, further sensory studies are required to determine if the addition of lactobacilli, such as LGG and Kef, to soda beverages would result in significant organoleptic changes. Generally, the recommendation would be to store beverages, particularly those containing simple sugars which are fermentable by viable probiotics, under refrigerated conditions, to minimise unanticipated changes to beverage tastes.

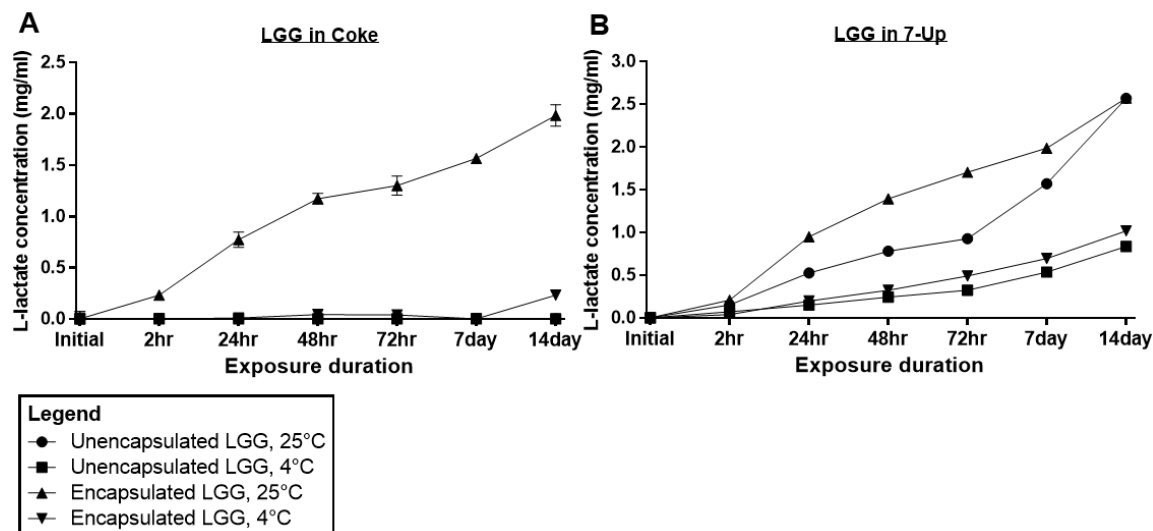


Figure 5.16: L-lactate concentration in various sodas with LGG added at room temperature and refrigerated conditions for up to 14 days. (a) LGG in Coke; (b) LGG in 7-Up.

5.4. Conclusion

This chapter reports the use of alginate to encapsulate probiotics by the extrusion technique. In the first phase of this chapter, various alginate formulations, with different M/G ratios of alginate, different sterilisation treatments, and with or without the addition of sucrose were compared to determine if these factors affect the acid protective effects of the alginate encapsulation system. Results demonstrated that the Ca-alginate formulations which utilised autoclaved alginates with sucrose (G_{ac}_suc and M_{ac}_suc) yielded high survival of encapsulated LGG after SGF exposure, with < 0.5 logCFU loss. However, both G_{ac}_suc and M_{ac}_suc alginate formulations were less effective in protecting LGG after sequential SGF and SIF exposure, and > 2 logCFU loss was observed. Regardless, since the G_{ac}_suc and M_{ac}_suc formulations yielded good protection of encapsulated probiotics against acid, it was interesting to explore if these formulations could similarly protect probiotics against acidic beverages.

In the second phase of this chapter, the exposure of probiotics encapsulated in Ca-alginate by the extrusion technique to various commercial carbonated sodas and beers, including Coke, 7-Up, Tiger Beer, and Guinness was, for the first time, described. Probiotic

candidates, LGG, ECN, and BL, in addition to the kefir probiotics (as derived in **Chapter 3**), in the unencapsulated and encapsulated forms, were exposed to sodas and beers under refrigerated, room temperature and SGF conditions. Results showed that the viabilities of all four probiotic candidates were improved in all tested beverages, most significantly in Coke, under all exposure conditions upon alginate encapsulation. The viabilities of all probiotics, both unencapsulated and encapsulated, were also better maintained in refrigerated conditions as compared to room temperature. Within the 14 days test duration, encapsulated probiotic and beverage combinations which yielded $> 8 \log\text{CFU/g}$ viable probiotics after refrigerated storage include, LGG with all four tested beverages, ECN with Tiger Beer, BL with Coke/7-Up/Tiger Beer, and kefir probiotics with Coke/7-Up/Tiger Beer. The effectiveness of alginate encapsulation in enhancing probiotic viabilities in these beverages can be attributed to the pH buffering effect of alginate in acidic media, which protects encapsulated probiotics from the acidic soda beverage, and mitigates antagonistic effects of acid in combination with bactericidal compounds present in beers (e.g., hops iso- α acids). Results also demonstrated that LGG added to carbonated sodas, Coke and 7-Up, can result in an accumulation of lactate as a by-product of sugar metabolism.

Overall, alginate encapsulation proves to be a useful method that can provide beverage manufacturers a novel way to develop probiotic-sodas and probiotic-beers. It is also recommended to store encapsulated probiotics in these beverages under refrigerated conditions to reduce viability losses, and to minimise flavor alterations that could arise due to probiotic metabolism.

6. Chapter 6*

Spray-drying encapsulation of probiotics

*Alginate formulations have shown desirable functional characteristics for probiotic encapsulation. However, various technologies used to produce these formulations are detrimental to probiotics viability or do not produce dry, shelf-stable products. Herein, a novel spray-drying technique that combines particle formation, alginate crosslinking and drying into a single step was developed for probiotics encapsulation. Among tested formulations, the crosslinked alginate with sucrose [Ca-Alg-Suc] was most promising, achieving $\sim 10^9$ CFU/g of surviving *Lactocaseibacillus rhamnosus* GG (LGG) after spray-drying and exposure to simulated gastric fluid (SGF). Ca-Alg-Suc was evaluated with other probiotics, and high post-spray-drying and post-SGF viabilities were obtained for the lactobacilli strains. Other modifications to Ca-Alg-Suc were attempted, and overall, the crosslinked calcium lactate-alginate with sucrose, trehalose, and sodium ascorbate formulation (Calac-Alg-Suc-Tre-Asc) yielded best shelf-life stability and resistance to simulated gastrointestinal fluids exposure. Successful encapsulation of different lactobacilli probiotics via the proposed spray-drying technique highlights potential of this procedure to be scaled up for commercial applications.*

*This section has been published/filed in:

1) L.L. Tan, M. Mahotra, S.Y. Chan and S.C.J. Loo. In situ alginate crosslinking during spray-drying of lactobacilli probiotics promotes gastrointestinal-targeted delivery. *Carbohydrate Polymers*, 286, 119279 (2022). <https://doi.org/10.1016/j.carbpol.2022.119279>. No permission is required from Elsevier for thesis purposes.

2) L.L. Tan, S.C.J. Loo, and M. Mahotra. (2022). Encapsulation of live microorganisms for gastrointestinal-targeted delivery. International Patent No. WO 2022/177508 A1. WIPO PCT.

6.1. Introduction

In this chapter, a probiotic encapsulation system was developed using the spray-drying technique in pursuit of **Objective 5** of this thesis.

This chapter describes a spray-drying technique for probiotics encapsulation which utilises coaxial spraying for alginate crosslinking. This method combines particle formation, alginate crosslinking and drying into a single step, thereby streamlining the production of encapsulated probiotics powder. LGG encapsulated in six encapsulation formulations were initially characterised and compared. These formulations include: 1) Sucrose only [Suc], 2) Alginate only [Alg], 3) Alginate with sucrose [Alg-Suc], 4) Crosslinked calcium-alginate [Ca-Alg], 5) Crosslinked calcium-alginate with sucrose [Ca-Alg-Suc] and 6) Crosslinked calcium-alginate with sucrose, using inverted feed channels [Ca-Alg-Suc-IFC]. Survivability of probiotics in various formulations after spray-drying, SGF exposure, and storage, were assessed. The resulting best performing formulation was additionally adapted for encapsulating other probiotics, including *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, and ECN.

In the latter part of this study, further adaptations involving the use of calcium lactate to replace calcium chloride as the crosslinking agent, and the use of sucrose, trehalose, L-cysteine, and sodium ascorbate as protective agents were evaluated to improve on sensory qualities, shelf-life stability, and SIF resistance of the spray-dried encapsulated probiotics formulation. The other tested formulations include: 7) Crosslinked calcium-alginate with trehalose [Ca-Alg-Tre], 8) Crosslinked calcium-alginate with sucrose and trehalose [Ca-Alg-Suc-Tre], 9) Crosslinked calcium lactate-alginate with sucrose and trehalose [Calac-Alg-Suc-Tre], 10) Crosslinked calcium lactate-alginate with sucrose, trehalose and L-cysteine [Calac-Alg-Suc-Tre-Cys] and 11) Crosslinked calcium lactate-alginate with sucrose, trehalose and sodium ascorbate [Calac-Alg-Suc-Tre-Asc]. Finally, the most optimal formulation was adapted for encapsulation of kefir probiotics (derived in **Chapter 3**).

6.2. Materials and Methods

6.2.1. Materials

LGG was isolated from a purchased Culturelle® LGG probiotic pill (i-Health, Inc., USA). ECN was isolated from a purchased Mutaflor® pill (Pharma-Zentrale GmbH, Germany). *Lactiplantibacillus plantarum* (wild type strain) was previously isolated by Professor Patricia Conway from fermented food sources. *Lacticaseibacillus paracasei* Kef-w19 was isolated from water kefir (described in **Section 3.2.2.1**). Kefir probiotics, comprising a blend of seven strains including, *Lentilactobacillus hilgardii* Kef-w9, *Lacticaseibacillus paracasei* Kef-w14/19, *Liquorilactobacillus satsumensis* Kef-w11/13, *Lactobacillus helveticus* Kef-m4 and *Lentilactobacillus kefir* Kef-m15, used for the selective substrate utilisation and faecal fermentation assays, were identified in **Chapter 3** of this thesis.

Protanal® LFR5/60 sodium alginate (M/G = 30/70, M_w = 20-60 kDa) was procured from FMC Biopolymer, USA. MRS broth and Live/Dead™ BacLight™ Bacterial Viability Kit were purchased from Thermo Fisher Scientific, USA. LB Lennox broth and Bacto-agar were purchased from BD, USA. All other chemicals used in this experiment were purchased from Sigma Aldrich, USA.

6.2.2. Methods

The following sub-sections describe the encapsulation of probiotics in alginate by spray-drying and subsequent assays to determine the extent of alginate crosslinking, powder morphology, residual moisture, survivability of encapsulated probiotics after spray-drying, survivability of probiotics when subject to gastrointestinal insults, and survivability of probiotics after storage.

6.2.2.1. Growth and preparation of probiotics

Single colonies of LGG, *L. plantarum* and *L. paracasei* were inoculated in sterile MRS broths and incubated aerobically at 37 °C for 24 h (for LGG and *L. plantarum*) and at 30 °C for 72 h (for *L. paracasei*). ECN was inoculated in LB broth and incubated aerobically at 37 °C for 24 h. The kefir probiotics blend was prepared by culturing individual strains in MRS at 30 °C, anaerobic conditions for 72 h, after which, respective kefir isolate cultures were adjusted to indicated OD600 values (**Table 3.2**) and concentrated into the same volume (same as protocol in **Section 3.2.2.10**). Stationary phase cell concentrations of 6E8, 1E9, 1E9, 6E8 and 4E9 colony forming units/ml (CFU/ml) were attained for LGG, *L. plantarum*, *L. paracasei*, ECN, and kefir probiotics respectively. The probiotic cells were then washed thrice with 0.9% (w/v) NaCl, with centrifugation at 10,000 xg for 5 min between each wash. Next, the probiotic cell pellets were resuspended in 0.9% NaCl at one-tenth of the initial inoculated volume to obtain a 10x concentrated probiotic sample. Drop plating on MRS agar was performed to determine the initial CFU concentration.

6.2.2.2. Preparation of alginate-probiotic slurry and crosslinking agent

Various alginate formulations (**Table 6.1**) were prepared by dissolution in DI water and autoclaving (121 °C, 15 min). As an example, the alginate formulation in Alg-Suc was prepared by dissolving 2.22% (w/v) sodium alginate with 11.11% (w/v) sucrose in DI water and autoclaving. The 10x concentrated probiotics suspension was then added in a 1:9 (v:v) ratio with the alginate-sucrose polymeric slurry and thoroughly mixed, thereby yielding a final 2% (w/v) alginate with or without 10% (w/v) sucrose with $10^8 - 10^9$ CFU/ml of probiotics. 10 mM calcium chloride or calcium lactate was used as the crosslinking agent, and similarly prepared by dissolution in DI water and sterilised by autoclaving. The 10 mM concentration was chosen as higher concentrations were observed to cause frequent spray-drier nozzle blockage. 10 mM calcium lactate was also tested as an alternative crosslinking agent, due to its less bitter organoleptic properties than calcium chloride.

Table 6.1: Formulations for spray-drying.

No.	Formulation	Alginate (%)	Sucrose (%)	Trehalose (%)	L-cysteine (%)	Sodium Ascorbate (%)	Crosslinker (fixed at 10mM)	Crosslinker channel
1	Suc	-	10	-	-	-	-	-
2	Alg	2	-	-	-	-	-	-
3	Alg-Suc	2	10	-	-	-	-	-
4	Ca-Alg	2	-	-	-	-	Calcium chloride	Outer
5	Ca-Alg-Suc	2	10	-	-	-	Calcium chloride	Outer
6	Ca-Alg-Suc-IFC	2	10	-	-	-	Calcium chloride	Inner
7	Ca-Alg-Tre	2	-	10	-	-	Calcium chloride	Outer
8	Ca-Alg-Suc-Tre	2	5	5	-	-	Calcium chloride	Outer
9	Calac-Alg-Suc-Tre	2	5	5	-	-	Calcium lactate	Outer
10	Calac-Alg-Suc-Tre-Cys	2	5	5	0.2	-	Calcium lactate	Outer
11	Calac-Alg-Suc-Tre-Asc	2	5	5	-	0.2	Calcium lactate	Outer

6.2.2.3. Encapsulation of probiotics via spray-drying

The Buchi-290 spray-dryer (Buchi AG, Switzerland) operating in co-current mode was set up with assembly of the Buchi three-fluid coaxial nozzle (inner nozzle diameter: 1.4 mm, outer diameter: 2.5 mm), similar to that described in [35]. Nitrogen gas flow was turned on to 0.742 m³/h for nozzle flow and 35 m³/h for aspirator flow. Prior to spraying probiotics, the spray-dryer equipment was pre-heated at 140 °C to sterilise and dry-out any residual moisture in the apparatus. Next, the inlet temperature of the spray-dryer was set to 120 °C, and the alginate-probiotics slurry and calcium chloride crosslinking agent were respectively fed into the inner and outer feed channels. 1.5 ml/min inner channel flow rate and flow rate ratio (inner:outer) of 1:2 was used. For samples Suc, Alg, and Alg-Suc, which did not require crosslinking, no solutions were fed through the outer channel. For the Ca-Alg-Suc-IFC formulation, the feeds of the inner and outer channels were switched, such that the alginate-probiotics slurry was fed to the outer channel while the crosslinking agent was fed to the inner channel, and flow rate ratio (inner:outer) was accordingly 2:1. Batch volumes of 25 – 50 ml of alginate-probiotics slurry were prepared at one go. Dried

probiotics powders were generated in the collection chamber and were maintained at outlet temperatures ranging from 62 – 79 °C for 20 – 35 min before collection.

6.2.2.4. Extent of alginate crosslinking

The extent of alginate crosslinking was determined using the Periodic acid Schiff's (PAS) assay adapted from [36], [37]. The aim of this assay was to determine the ratio of soluble alginate (un-crosslinked) to total alginate. 10 mg of Ca-Alg and 60 mg of Ca-Alg-Suc particles, not containing any probiotics, were added into 5 ml of water and 5 ml of 0.05 M of sodium citrate separately. Particles were rotated at 30 rpm for 1 h at room temperature to dissolve the alginate. Samples were then centrifuged at 13,300 xg for 10 min, and the supernatants were diluted with dilution factors of 2x and 10x for water-dissolved and citrate-dissolved samples respectively. Alginate standards with concentrations of 0.3 to 0.025 mg/ml were also prepared. 200 µl of samples and standards were transferred to a 96-well plate, and 20 µl of 50% (w/v) periodic acid in 7% (v/v) acetic acid was added. Schiff's reagent was prepared by dissolving 16.7 mg of sodium metabisulfite per ml of Schiff's fuchsin-sulfite reagent. Both the plate and Schiff's reagent were then incubated at 37 °C for 1 h. Next, 20 µl of Schiff's reagent was added to each well and the plate was incubated for another 1 h at room temperature to allow colour development. Absorbance of the samples was measured at 550 nm using Infinite M200 microplate reader (Tecan Group Ltd, Switzerland). The extent of crosslinking was computed as follows:

Extent of crosslinking (%)

$$= \left(1 - \frac{\text{Concentration of soluble alginate in water}}{\text{Concentration of soluble alginate in citrate}} \right) \times 100$$

6.2.2.5. Powder yield measurement

Powder yields were determined based on the mass of the spray-dried powder collected relative to the total mass of solids within the formulation, as according to the equation:

$$\text{Powder yield (\%)} = \frac{\text{Mass of spray - dried powder collected}}{\text{Original mass of solids in sample}} \times 100$$

6.2.2.6. Scanning electron microscopy

Morphologies of the spray-dried powders were observed using a JEOL JSM-6360 SEM (JEOL Ltd., Japan), with an accelerating voltage of 5 kV in the secondary electron mode. Spray-dried powders were mounted to a stub with carbon tape and coated with 10 nm of conductive gold prior to imaging.

6.2.2.7. Epifluorescence microscopy

Viability staining of spray-dried probiotic powder was performed using Live/Dead™ BacLight™ Bacterial Viability Kit. This live/dead stain consists of SYTO 9 and propidium iodide (PI) nucleic acid stains. PI penetrates only cells with disrupted membranes, and intercalates with DNA to emit a red fluorescence. SYTO 9 enters both live and dead cells, but when both PI and SYTO 9 are present, SYTO 9 is displaced by PI due to the latter's stronger affinity for nucleic acids [390]. Selected spray-dried samples were rehydrated and washed thrice in 0.9% NaCl to remove any un-crosslinked polymer or probiotics. Each sample was then stained with 500-times diluted SYTO 9 and propidium iodide (PI) in 0.9% NaCl. Samples were stained for 15 min in dark conditions then fluorescently imaged (488 nm excitation, SYTO 9: 498 nm, PI: 617 nm) using the Axio Observer Z1 Inverted Microscope (Carl Zeiss AG, Germany).

6.2.2.8. Residual moisture test

Spray-dried powders were further dehydrated in a VO400 vacuum oven (Mettler GmbH + Co. KG, Germany) for 3 days. Residual moisture content of powders was determined by gravimetric analysis based on the formula below:

$$\begin{aligned} \text{Residual moisture content (\%)} \\ = \frac{\text{Initial weight of powder} - \text{Dried weight of powder}}{\text{Initial weight of powder}} \times 100 \end{aligned}$$

6.2.2.9. Enumeration of probiotics after spray-drying

Viable probiotic counts following spray-drying were evaluated by dissolving the powder in 0.05 M sodium citrate followed by drop-plating on MRS agars. LGG and *L. plantarum* were incubated at 37 °C, aerobic conditions for 48 hours, *L. paracasei* was incubated at 30 °C, aerobic conditions for 72 hours, ECN was incubated at 37 °C, aerobic conditions for 24 hours, and kefir probiotics was incubated at 30 °C, anaerobic conditions for 72 hours. Bacterial colonies at specific dilution factors were counted and surviving probiotics were calculated in CFU/g.

6.2.2.10. Exposure to SGF and SIF

The encapsulated probiotic powders were exposed to SGF and SIF to determine their ability to persist in the human gastric environment. SGF was prepared as 0.2 M NaCl, 2000 units/ml porcine pepsin, adjusted to pH 2 by addition of hydrochloric acid. SIF was prepared as 0.3% (w/v) ox-bile with 10 mM glucose in PBS pH 7.4 solution [371]. 50 ± 2 mg of spray-dried powders were added to 5 ml of SGF or SIF respectively. Other tested powder mass : SGF volume ratios include 25 ± 1 mg : 5 ml and 15 ± 0.6 mg : 7.5 ml. SGF adjusted to pH 2.5 and pH 3, were also tested for the Suc samples, to determine the intrinsic acid resistance of various probiotics. Samples were incubated at 37 °C, 200 rpm shaking conditions in an Ecotron shaking incubator (Infors AG, Switzerland) for 2 hours. After 2

hours, samples were centrifuged (10,000 xg, 5 min), and the SGF/SIF was removed by pipetting. Viable probiotic counts after SGF/SIF exposure were determined by dissolution in 0.05 M sodium citrate and drop-plating.

6.2.2.11. Storage viability testing

Storage viabilities of spray-dried powders were determined over a 4- and 8-week period. To maintain a dry environment, MiniPax absorbent packets (Sigma-Aldrich, USA) were placed as desiccants together with the spray-dried powders. Samples were stored at 4 °C (refrigerated) or 25 °C (room temperature) conditions. Following storage, viable probiotic counts were determined by dissolution in 0.05 M sodium citrate and drop-plating. Samples were also exposed to SGF pH 2 after storage, following the method described in **Section 3.4.2.10.**, to determine if gastroprotective effects of the formulations were retained after storage.

6.2.2.12. Statistical analysis

Results were expressed as mean of triplicates with standard deviation bars. For the statistical evaluation of numerical data, one way analysis of variance and post-hoc Tukey test was used. Letters on bars were based on * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns – no significant difference.

6.3. Results and Discussion

6.3.1. Extent of alginate crosslinking

In this chapter, a novel method of *in situ* alginate crosslinking via coaxial spray-drying was proposed. The efficacy of this alginate crosslinking process was determined using the PAS assay. Based on the concentration of soluble alginate released from Ca-Alg and Ca-Alg-Suc powders, the extent of crosslinking was calculated and expressed as a percentage of insoluble crosslinked alginate that remained in the polymer matrix. Extent of crosslinking were 92.49% and 99.12% for Ca-Alg and Ca-Alg-Suc, respectively, indicating that the majority of alginate polymers were crosslinked via the described spray-drying technique. Addition of sucrose did not hamper the alginate crosslinking process. Instead, better crosslinking was achieved for the Ca-Alg-Suc formulation, possibly due to effects of sucrose in modifying the alginate crosslinking process. Sucrose has been reported in other studies to modify alginate crosslinking by acting as an intermolecular inert spacer [362]. Overall, high degree of alginate crosslinking was achieved by the coaxial spray-drying method for both Ca-Alg and Ca-Alg-Suc formulations.

6.3.2. Physical characterisation of spray-dried powders

Powder yields ranged from 58 – 75%, consistent with typical yields of B-290 spray-driers [391]. SEM morphologies of spray-dried LGG-loaded powders are shown in **Figures 6.1a-f**. Particle sizes ranged between 2 – 34 μm , within the expected size range of powders produced from the B-290 spray-drier [392]. Alg (**Figure 6.1b**) and Ca-Alg (**Figure 6.1d**) powders appeared smaller with textured surfaces, while sucrose-containing formulations were larger and had smoother surfaces. This is likely due to the lower solids content in formulations without sucrose, hence individual atomised droplets of Alg and Ca-Alg had lower solid mass and yielded smaller particles. The Suc formulation (**Figure 6.1a**) showed irregular powder morphologies, seemingly caused by agglomeration or coalescence of several atomised particles. Addition of 2% alginate, as in the Alg-Suc sample (**Figure 6.1c**), yielded more spherical and separate particles, possibly due to differences in viscosities and

glass transition temperatures for alginate-containing samples [393]. Apparent differences were also observed between Ca-Alg-Suc (**Figure 6.1e**) and Ca-Alg-Suc-IFC (**Figure 6.1f**) particles. Ca-Alg-Suc-IFC particles were significantly larger granules with lumpy surface characteristics, and these particles do not appear well separated as compared to Ca-Alg-Suc. This suggests that the inversion of feed channels had a significant impact on crosslinking and particle formation. The effects of calcium crosslinking (compare Alg and Ca-Alg or Alg-Suc and Ca-Alg-Suc) did not show significant differences in powder morphology.

Residual moisture contents of spray-dried powders are presented in **Figure 6.1g**. Alg and Ca-Alg powders showed high residual moisture content of $\sim 8\%$, significantly higher than the sucrose-containing formulations, which showed residual moisture of $< 4\%$. Such is likely due to more water solvent present in formulations without sucrose, hence the Alg and Ca-Alg samples were not dried as thoroughly during the spray-drying process. Comparing Ca-Alg-Suc and Ca-Alg-Suc-IFC, Ca-Alg-Suc-IFC showed higher residual moisture. This may be due to some moisture from the calcium chloride crosslinking solution being trapped within the Ca-Alg-Suc-IFC powder, as the crosslinking solution was fed via the inner channel in the Ca-Alg-Suc-IFC sample. Overall, residual moisture content of $< 4\%$ were considered good-quality for spray-dried powders [394], and the Suc, Alg-Suc and Ca-Alg-Suc formulations fulfil this criterion.

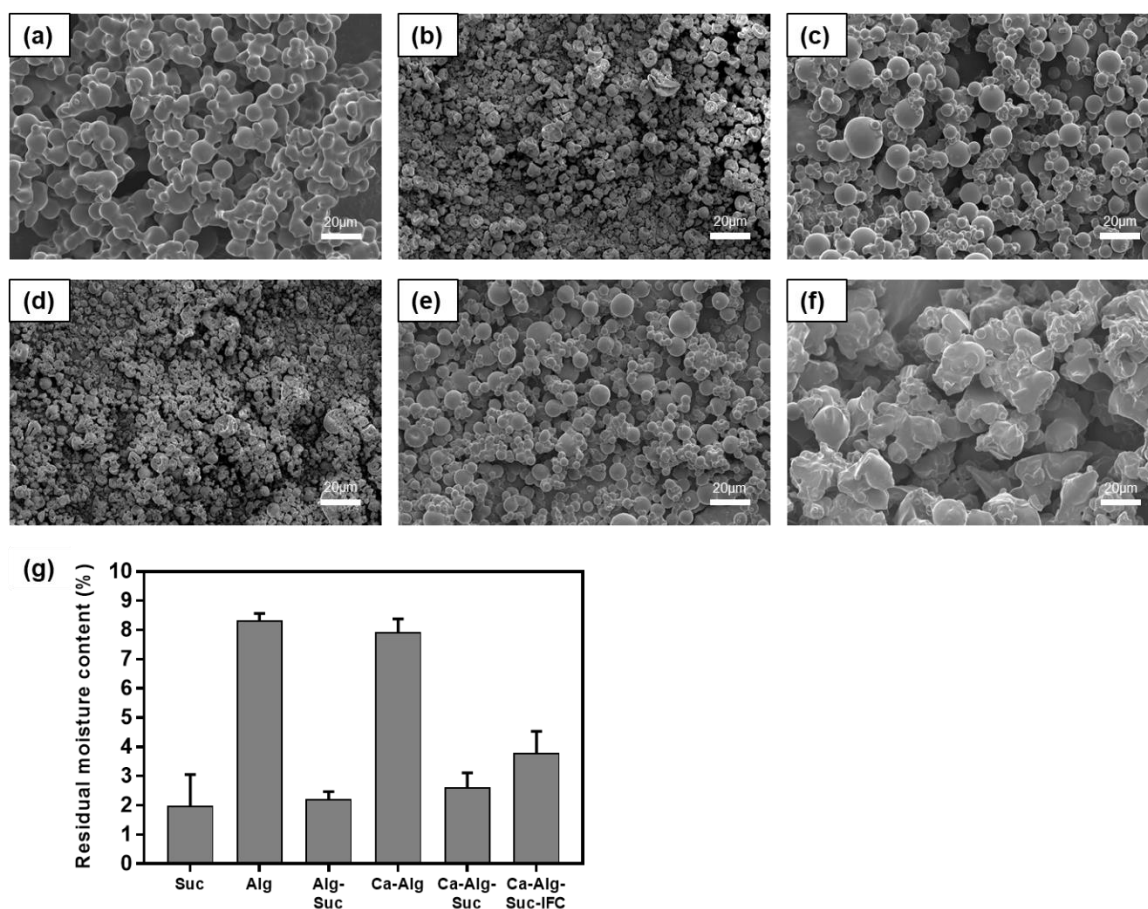


Figure 6.1: Physical properties of spray-dried LGG in various formulations. SEM images of (a) Suc, (b) Alg, (c) Alg-Suc, (d) Ca-Alg, (e) Ca-Alg-Suc, and (f) Ca-Alg-Suc-IFC are included. Residual moisture contents in various formulations are described in (g).

6.3.3. Survivability of LGG after spray-drying

Viable CFU counts of LGG after spray-drying are reported in **Figure 6.2a**. All six formulations yielded $> 10^9$ CFU/g of surviving LGG after spray-drying. The Alg sample yielded highest CFU/g, due to the lower solids content in Alg as compared to sucrose-containing samples, which contributed to higher initial CFU per dry weight in formulations without sucrose. The CFU losses graph (**Figure 6.2b**) provides a better representation of the CFU reduction for various formulations associated with the spray-drying process. Comparing Alg versus Alg-Suc and Ca-Alg versus Ca-Alg-Suc, formulations with sucrose retained significantly higher viability counts of LGG, indicating that sucrose was effective as a protective agent in enhancing LGG survival during the spray-drying process. This is

consistent with prior studies, as sucrose is known to enhance desiccation tolerance of microorganisms by stabilising their cellular membrane and proteins [395], [396]. Comparing crosslinked and un-crosslinked samples (Alg vs Ca-Alg and Alg-Suc vs Ca-Alg-Suc), calcium crosslinking greatly reduced LGG survivability in Alg samples, but did not have a significant impact on LGG survivability in Alg-Suc. A similar phenomenon has been observed in freeze-dried crosslinked alginate, where divalent cations, including Ca^{2+} , demonstrated antagonistic properties in reducing desiccation survivability of probiotics [397]. In the same study [397], sucrose was found to reverse the deleterious effects of divalent cations and restore viability of probiotics in freeze-dried calcium alginate particles. Possibly, a similar mechanism of Ca^{2+} antagonism towards LGG survivability occurred in this spray-drying process, and the addition of sucrose as a protective agent alleviated this effect. Between Ca-Alg-Suc and Ca-Alg-Suc-IFC samples, no significant differences in survivability of LGG were found.

Figure 6.2c shows an epifluorescence image of rehydrated Ca-Alg-Suc with live/dead stained LGG. As seen, Ca-Alg-Suc particles formed insoluble gels upon rehydration in aqueous saline, indicating that crosslinking was effectively achieved during the coaxial spray-drying process. LGG bacteria were seen embedded within crosslinked Ca-Alg-Suc matrices with strong fluorescence in the SYTO 9 channel, indicating good preservation of LGG membrane integrity following spray-drying.

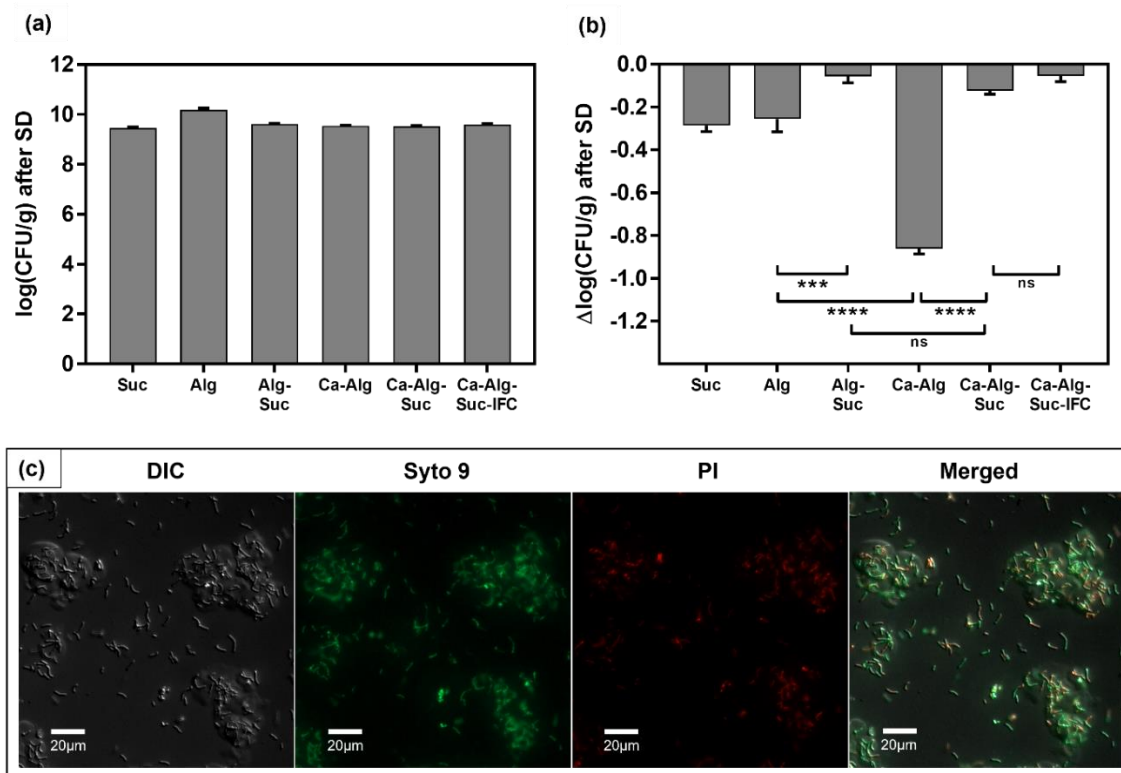


Figure 6.2: Survivability of LGG in various formulations post-spray-drying. (a) Log₁₀(CFU/g) data of LGG in various formulations following spray-drying. (b) Change in log₁₀(CFU/g) counts of LGG in various formulations, as compared to before spray-drying. (c) Epifluorescence microscopy image of live/dead stained LGG bacteria in Ca-Alg-Suc matrix.

6.3.4. Survivability of LGG in SGF

Various spray-dried formulations were exposed to SGF, and the consequent viable LGG counts are presented in **Figure 6.3**. **Figure 6.3c** indicates survivability of LGG in the Suc formulation, i.e., without alginate added, when exposed to SGF pH 2, 2.5 and 3 for 2 h. As seen, LGG in the Suc formulation showed complete susceptibility to pH 2 and pH 2.5 within 1 h of SGF exposure, indicating that sucrose alone did not confer gastroprotective effects on spray-dried LGG. Formulations which incorporated alginate provided higher survivability of LGG. In **Figure 6.3a**, the Alg and Ca-Alg-Suc formulations retained highest viability of LGG after SGF pH 2 exposure, with $\sim 10^9$ CFU/g LGG surviving. From **Figure 6.3b**, the Ca-Alg-Suc matrix was identified as best performing in conferring gastroprotection to LGG, with lowest CFU/g losses of 0.47 log₁₀(CFU/g) following SGF

exposure. Significant LGG survivability differences were identified between the un-crosslinked Alg-Suc and crosslinked Ca-Alg-Suc samples, indicating that the effect of crosslinking was important in enhancing the gastric acid-protective ability of the alginate matrix. Comparing Ca-Alg-Suc and Ca-Alg-Suc-IFC, Ca-Alg-Suc-IFC performed significantly worse in protecting LGG against SGF, possibly due to improper alginate crosslinking. Coaxially spray-dried particles involving multiple feed solutions are known to confer particles with heterogenous core-shell-like inner structures [398]. In the IFC formulation, the alginate-probiotics slurry was fed to the outer channel while the calcium chloride crosslinking agent was fed to the inner channel. This process likely yielded Ca-Alg-Suc-IFC particles which were crosslinked at the core, but un-crosslinked near the surface, resulting in significant viability losses of LGG which were present near the particle surface. In contrast, Ca-Alg-Suc particles were likely to be better crosslinked at its surface, hence forming a calcium alginate surface layer which better protects encapsulated LGG from gastric acid insults.

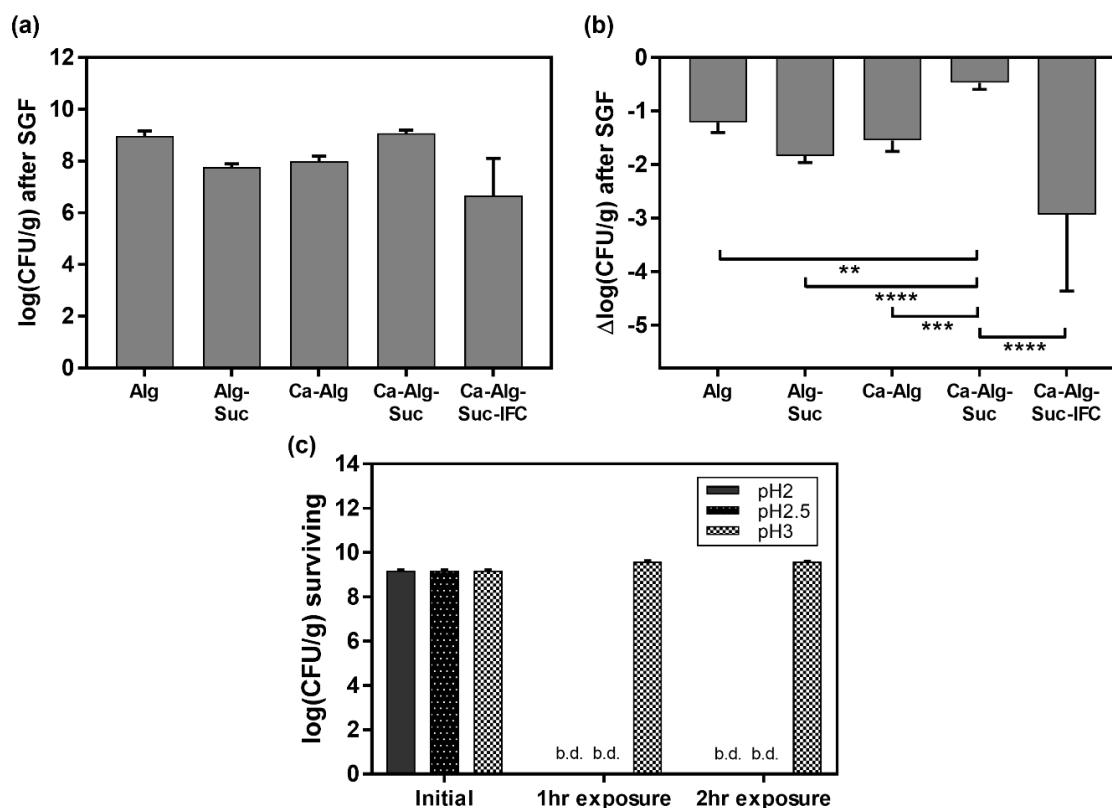


Figure 6.3: Survivability data of LGG in various spray-dried formulations after exposure to SGF. (a) $\text{Log}_{10}(\text{CFU/g})$ data of LGG in various spray-dried formulations following exposure to SGF pH 2. (b) Change in $\text{log}_{10}(\text{CFU/g})$ counts of LGG in various formulations after SGF pH 2 exposure. (c) $\text{Log}_{10}(\text{CFU/g})$ data of LGG in Suc formulation, exposed to SGF of various pH. B.d. indicates that the cell count is below the detection limit of $\sim 1.5 \text{ log}_{10}(\text{CFU/g})$.

The effect of alginate crosslinking on gastroprotection of entrapped probiotics was further tested by exposing the un-crosslinked Alg and crosslinked Ca-Alg-Suc formulations to SGF in varying powder mass to SGF volume ratios. As seen in **Figure 6.4a**, high $\text{log}_{10}(\text{CFU/g})$ counts were obtained for the crosslinked Ca-Alg-Suc formulation, even with decreasing powder mass to SGF volume ratio. In contrast, there was a considerable decline in viable LGG counts for the un-crosslinked Alg formulation, from $9 \text{ log}_{10}(\text{CFU/g})$ to $5.9 \text{ log}_{10}(\text{CFU/g})$, with decreasing powder mass added relative to SGF volume. Likewise in **Figure 6.4b**, changes in the CFU/g reduction of viable LGG in Ca-Alg-Suc powder were minor compared to the significant changes in the CFU/g reduction of viable LGG in Alg powder, for decreasing powder mass ratios. Final pH values of the SGF milieu after incubation with the spray-dried powders were also measured and documented in **Figure 6.4c**. pH values of SGF containing Ca-Alg-Suc powder did not increase significantly from the original pH 2 and were relatively constant with different powder mass to SGF volume ratios. However, for Alg, SGF pH was substantially increased to pH 3.7 upon addition at 50 mg: 5 ml ratio, and a considerable decline in SGF pH values was observed with decreasing powder mass to SGF volume ratio.

In acidic pH, alginate is known to sequester protons and convert to insoluble alginic acid, thereby achieving a pH buffering effect which promotes survivability of encapsulated probiotics [29]. Data from **Figure 6.4** suggest that the un-crosslinked Alg formulation conferred gastroprotection to encapsulated LGG primarily through buffering pH of the entire gastric fluid environment. Accordingly, as the powder mass to SGF volume decreased, the pH buffering capacity of un-crosslinked Alg was hampered, thereby exposing LGG to the detrimental acidic pH, causing significant viability losses. The pH buffering capacity of crosslinked Ca-Alg-Suc was however, independent of the SGF volume, likely due to a localised pH buffering effect achieved within each spray-dried

particle, facilitated by the formation of crosslinked calcium alginate gels surrounding each particle. The crosslinked calcium alginate surface layer has potentially immobilised encapsulated probiotics within the matrix. By the conversion of alginate to alginic acid, excess protons in the vicinity of embedded LGG were sequestered, thereby maintaining a localised pH higher than the external SGF milieu and maintaining high LGG viability.

A proposed mechanism of *in situ* alginate crosslinking by the described coaxial spray-drying technique is hence illustrated in **Figure 6.5a**. The three-fluid coaxial nozzle enables simultaneous flow of the alginate-probiotics solution and the calcium crosslinking agent in the inner and outer feed channels respectively. Crosslinking likely occurred at the tip of the spray-dryer nozzle when both the alginate polymer and calcium chloride crosslinking agent were ejected and in contact. “Egg-box”-like crosslinked calcium alginate structures (**Figure 6.5c**) were rapidly formed at the surface of each atomised alginate-probiotic particle [239], immobilising entrapped probiotics within. This process of crosslinking was likely facilitated by the rapid dehydration of the outer feed channel, which was proximally closer to the heated atomising gas. Calcium chloride in the outer feed channel hence preferentially interacted with alginate from the inner feed channel to rapidly form crosslinked calcium alginate structures, achieving effective crosslinking during the spray-drying process. Upon exposure to acidic SGF (**Figure 6.5b**), individual Ca-Alg-Suc particles rehydrate and swell slightly, but the integrity of each particle was kept intact due to the crosslinked calcium-alginate layer surrounding each particle. Excess protons from the acidic milieu could hence be sequestered by alginate within each particle, providing a localised pH buffering effect which enhances survivability of entrapped probiotics in SGF. Further studies can seek to validate this mechanism by utilizing elemental analysis methods to analyse the calcium content in different spatial regions of the powder particle, or to utilize raman spectroscopy methods to determine the arrangement of cross-linked alginates within each alginate powder particle [399].

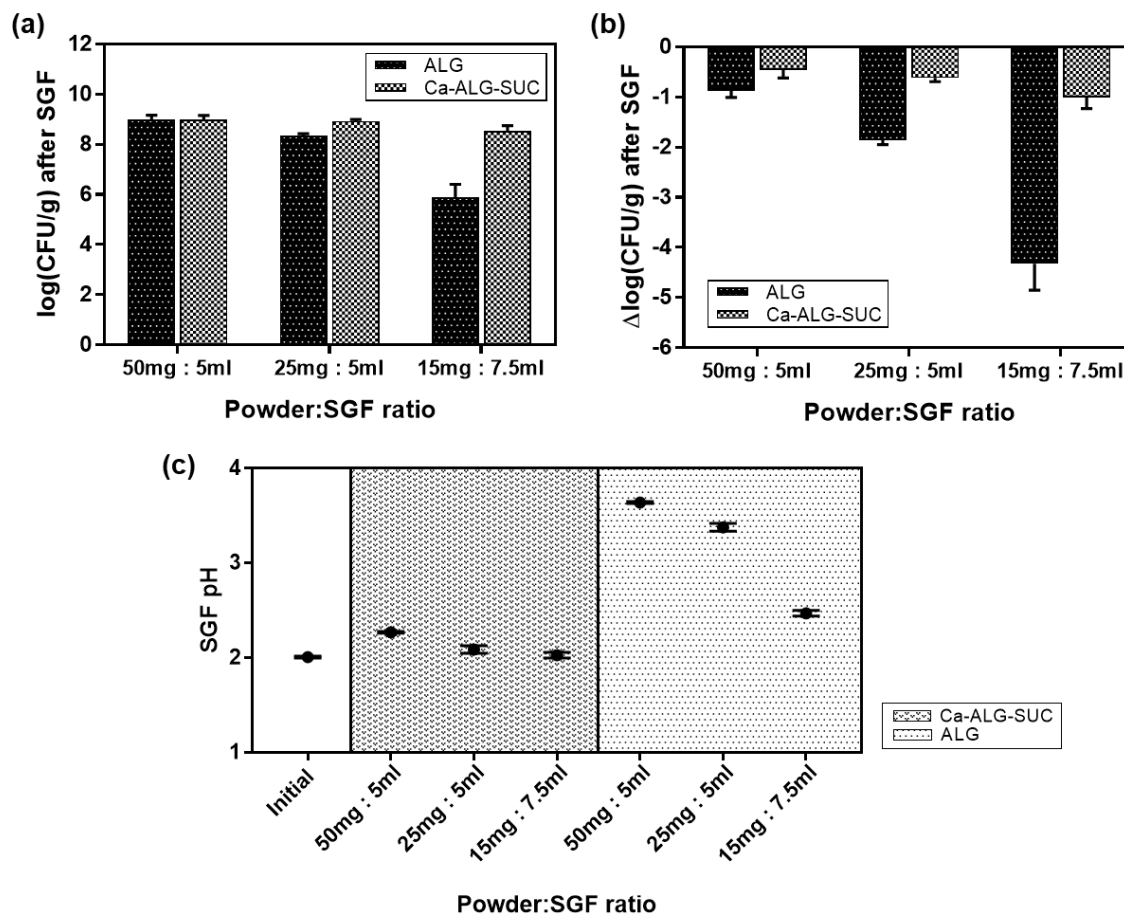


Figure 6.4: Survivability data of LGG in various formulations after exposure to SGF in varying powder:SGF ratios. (a) Log₁₀(CFU/g) data of LGG in un-crosslinked Alg and crosslinked Ca-Alg-Suc formulations exposed to SGF in varying powder:SGF ratios. (b) Change in log₁₀(CFU/g) counts of LGG, as compared to before SGF exposure. (c) pH of SGF media following addition of Ca-Alg-Suc and Alg powder.

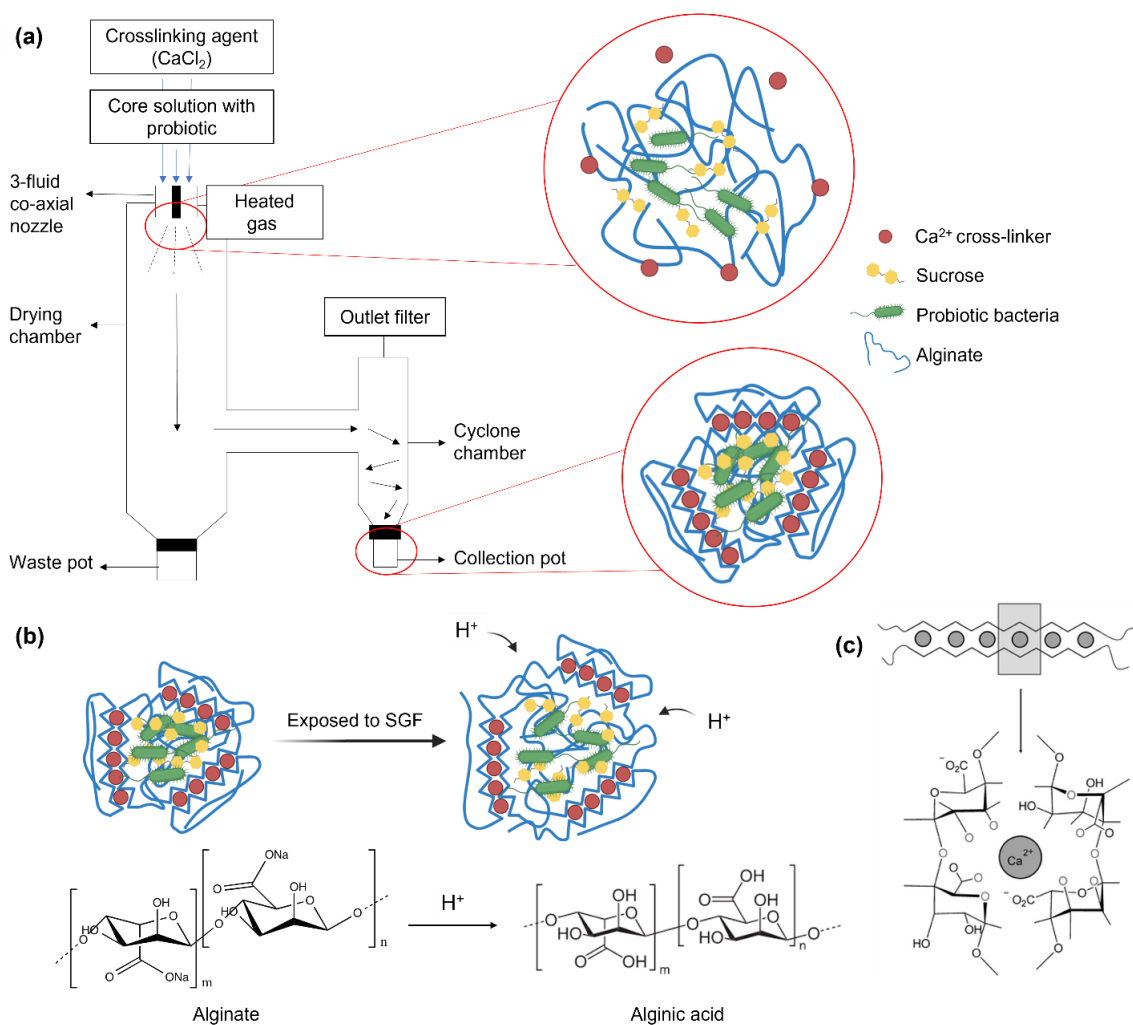


Figure 6.5: Proposed mechanism of *in situ* alginate crosslinking during spray-drying. (a) Schematic diagram of Ca-Alg-Suc particle formation process during coaxial spray-drying. (b) Mechanism of gastroprotection conferred by Ca-Alg-Suc matrix. (c) “Egg-box” chemical structure for calcium alginate [239].

6.3.5. Spray-drying encapsulation of other probiotics

The Ca-Alg-Suc formulation was further adapted for encapsulation of other probiotics, specifically *L. plantarum*, *L. paracasei*, ECN, and kefir probiotics (**Chapter 3**). Viabilities of these probiotics after spray-drying in Suc and Ca-Alg-Suc are shown in **Figure 6.6**. The lactobacilli probiotic strains, *L. plantarum*, *L. paracasei*, and kefir probiotics, attained high post-spray-drying viabilities of $> 10^9$ CFU/g in the Suc and Ca-Alg-Suc formulations, with $< 0.2 \log_{10}(\text{CFU/g})$ losses. This suggests that sucrose was useful in protecting these

lactobacilli probiotics against the harsh conditions involved in spray-drying. For ECN, however, significant viability losses of $>3 \log_{10}(\text{CFU/g})$ were observed after spray-drying. Gram-negative bacteria, like ECN, are generally known to be more susceptible to heat due to the absence of peptidoglycan, which makes their outer membrane more amenable to heat damage [400]. Hence, the proposed spray-drying technique appears to be less useful for Gram-negative probiotics.

Spray-dried *L. plantarum* and *L. paracasei* were also exposed to SGF, and results are presented in **Figure 6.7**. Spray-dried ECN was not studied for its SGF resistance due to poor spray-drying survivability. From **Figures 6.7c** and **6.7d**, *L. plantarum* and *L. paracasei* in Suc formulations showed complete susceptibility to pH 2 within 1 h of SGF exposure. *L. plantarum* and *L. paracasei* in Suc formulations also showed a degree of susceptibility to acidic pH 2.5 and pH 3, with $> 8 \log_{10}(\text{CFU/g})$ reduction in pH 2.5 and $\sim 1 \log_{10}(\text{CFU/g})$ reduction in pH 3 after 2 h exposure. In **Figures 6.7a** and **6.7b**, the Ca-Alg-Suc matrix demonstrated significant improvement in gastroprotection of encapsulated *L. plantarum* and *L. paracasei*. $\sim 10^9$ CFU/g ($0.7 \log_{10}(\text{CFU/g})$ reduction) of *L. plantarum* in Ca-Alg-Suc remained viable following SGF pH 2 exposure, while $\sim 10^8$ CFU/g ($1.9 \log_{10}(\text{CFU/g})$ reduction) of *L. paracasei* remained viable. This finding highlights the suitability of the Ca-Alg-Suc formulation for high throughput spray-drying encapsulation and gastroprotection of other lactobacilli strains.

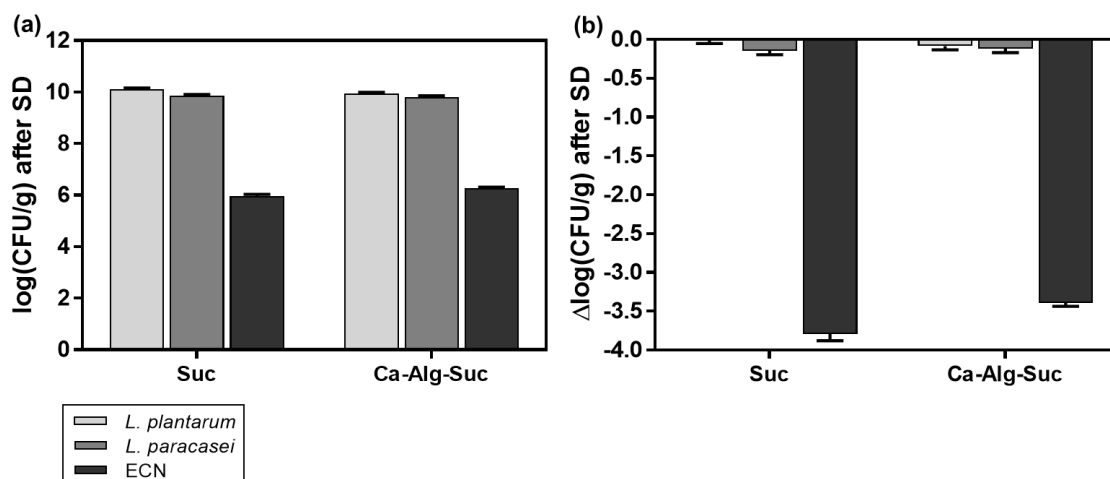


Figure 6.6: Survivability data of *L. plantarum*, *L. paracasei*, and ECN in the crosslinked spray-dried polymeric formulation post-spray-drying. (a) $\log_{10}(\text{CFU/g})$ data of various probiotics in sucrose [Suc] and calcium-alginate-sucrose [Ca-Alg-Suc] formulations following spray-drying. (b) Change in $\log_{10}(\text{CFU/g})$ counts of various probiotics, as compared to before spray-drying.

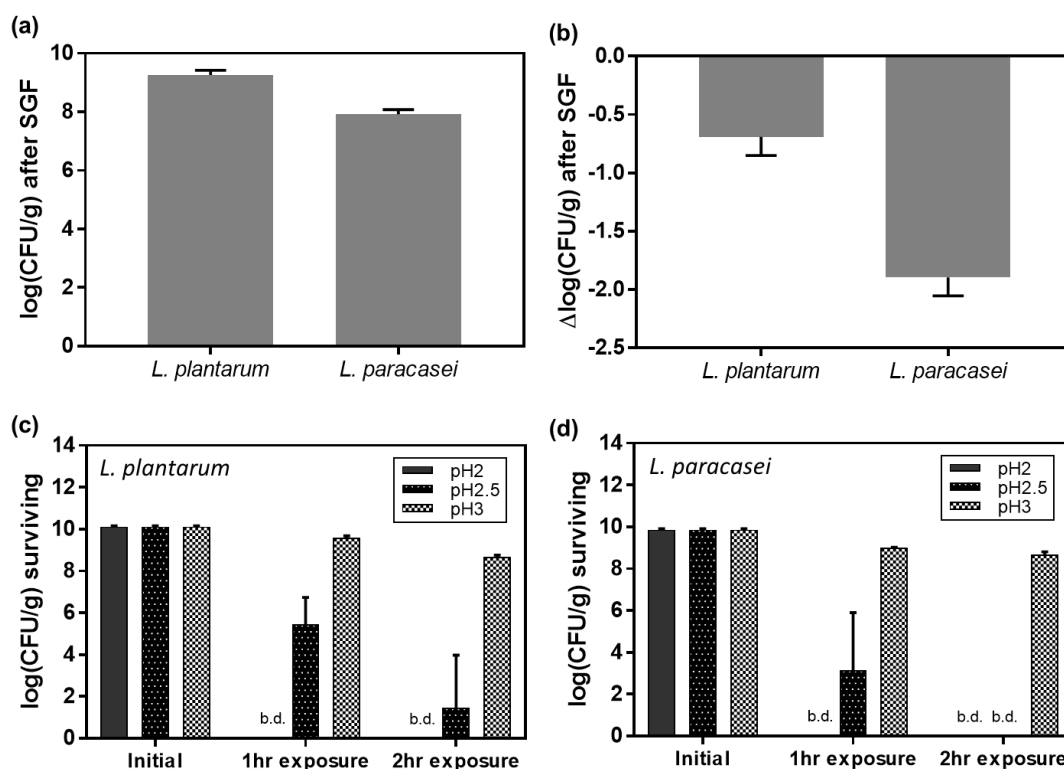


Figure 6.7: Survivability data of spray-dried *L. plantarum* and *L. paracasei* in Suc and Ca-Alg-Suc formulations after exposure to simulated gastric fluid (SGF). (a) $\log_{10}(\text{CFU/g})$ data of various probiotics in the Ca-Alg-Suc formulation following exposure to SGF pH 2. (b) Change in $\log_{10}(\text{CFU/g})$ counts of various probiotics, as compared to before spray-drying. (c) $\log_{10}(\text{CFU/g})$ surviving data for *L. plantarum* at pH 2, pH 2.5, and pH 3. (d) $\log_{10}(\text{CFU/g})$ surviving data for *L. paracasei* at pH 2, pH 2.5, and pH 3.

$\log_{10}(\text{CFU/g})$ counts of various probiotics in Ca-Alg-Suc after SGF pH 2 exposure. (c) $\log_{10}(\text{CFU/g})$ data of *L. plantarum* in Suc formulation, exposed to SGF of various pH. (d) $\log_{10}(\text{CFU/g})$ data of *L. paracasei* in Suc formulation, exposed to SGF of various pH. B.d. indicates that the cell count is below the detection limit of $\sim 1.5 \log_{10}(\text{CFU/g})$.

6.3.6. Trehalose as a protective sugar

The Ca-Alg-Suc formulation had by far been useful in achieving high spray-drying survivability and gastroprotection of lactobacilli probiotic strains. Here, trehalose was explored as an alternative protective sugar to sucrose, to seek greater simulated intestinal fluid (SIF) resistance, and improved shelf-life properties for encapsulated probiotics.

Trehalose, a disaccharide comprising of two glucose monomers, has been commonly utilised to enhance desiccation tolerance of microorganisms in freeze-drying [401] and spray-drying [402] settings. Similar to sucrose, trehalose works by stabilising the cellular membrane and proteins of microbes [395], [396]. However, trehalose is considered advantageous to sucrose due to its higher glass transition temperature (T_g), which helps maintain a glassy, highly viscous, low motility state surrounding microorganisms that can retain microbial viability at room temperature [401], [403]. From Song *et al*'s [403] (**Table 6.2**) and Heljo *et al*'s study [403] (**Figure 6.8**), glass transition temperatures of trehalose and sucrose have been empirically determined to decrease with increasing water concentration, such as in the form of relative vapour pressure or water activity. From Heljo *et al*'s study [403], it was also found that a higher water concentration is required to lower the T_g of trehalose to room temperature (25 °C), as compared to sucrose, indicating that trehalose might yield more room temperature-stable products over shelf life.

Table 6.2: Glass transition temperatures of trehalose and sucrose at various relative vapour pressures. Reproduced with permission from [401].

Relative vapour pressure (%)	Trehalose	Sucrose
0	107.9 ± 1.1	51.2 ± 0.3
11.4	53.8 ± 1.0	31.6 ± 0.7
23.0	40.4 ± 0.1	21.6 ± 0.2
33.2	29.5 ± 0.9	Crystallised
44.1	Crystallised	Crystallised

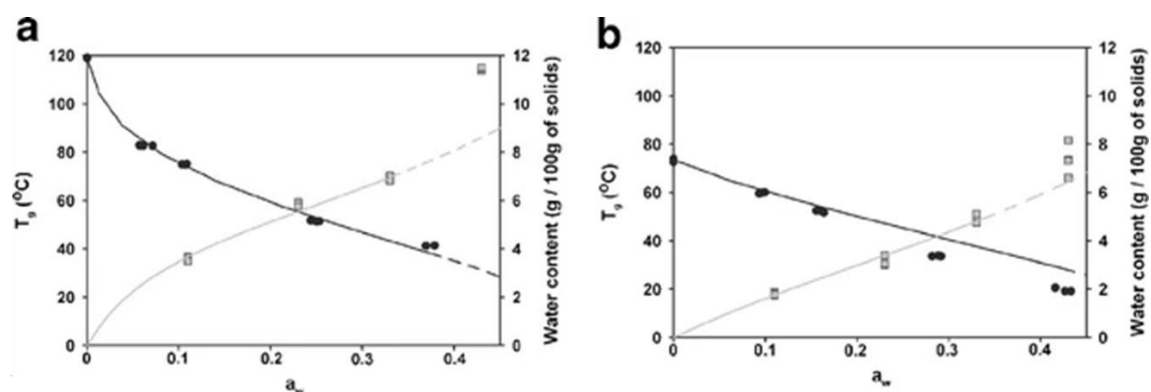


Figure 6.8: Measured and predicted glass transition temperatures (T_g) and water contents as functions of water activity (a_w) for (a) trehalose, and (b) sucrose. The black circles represent measured T_g values, the black lines their Gordon-Taylor models, whereas the grey squares represent measured water contents and the grey lines their Brunauer-Emmett-Teller models. The predicted model lines are dashed after exceeding the a_w range used in model fitting. Reproduced with permission from [403].

The formulations, calcium-alginate-trehalose [Ca-Alg-Tre], which comprises 10% trehalose, and calcium-alginate-sucrose-trehalose [Ca-Alg-Suc-Tre], which comprise 5% sucrose and 5% trehalose, were compared with Ca-Alg-Suc to evaluate post-spray-drying and post-SGF/SIF survival of LGG probiotics (**Figure 6.9**). From **Figure 6.9a**, all three formulations yielded high CFU/g of LGG probiotics after spray-drying, indicating that trehalose was similarly effective as sucrose in protecting LGG during the spray-drying process. For SGF protection, the Ca-Alg-Suc-Tre formulation outperformed Ca-Alg-Suc

and Ca-Alg-Tre, and $9.60 \log_{10}(\text{CFU/g})$ LGG in Ca-Alg-Suc-Tre survived, equivalent to a $\log_{10}(\text{CFU/g})$ reduction of 0.28 (**Figure 6.9b**).

For SIF exposure, Ca-Alg-Tre and Ca-Alg-Suc-Tre significantly increased LGG CFU as compared to the Suc and Ca-Alg-Suc formulations (**Figures 6.9c and d**). This suggests that extracellular trehalose may have an additional protective effect on stabilising bacterial cell membranes, possibly by mitigating the antagonistic effects of bile in emulsifying and disrupting the phospholipids of bacterial cell membranes [200]. The effects of trehalose in enhancing bile tolerance of bacteria has been prior reported in Termont *et al* [404], however, the improved bile tolerance was only observed for intracellularly accumulated trehalose in the tested *Lactococcus lactis* strain, while extracellularly added 7% trehalose did not appear to improve bile tolerance. Between Ca-Alg-Tre and Ca-Alg-Suc-Tre, the reduced trehalose concentration for Ca-Alg-Suc-Tre did not affect protection against SIF for LGG.

Based on these results, Ca-Alg-Suc-Tre was the preferred formulation amongst the three compared due to its better SGF/SIF protection performance (**Figure 6.9**). Ca-Alg-Suc-Tre is also considered advantageous to Ca-Alg-Tre due to a cost reason; trehalose is more expensive than sucrose, priced at ~ US \$1500/kg ($\geq 99\%$ purity, derived from *Saccharomyces cerevisiae*, price from Sigma-Aldrich), whereas sucrose is priced at ~ US \$40/kg ($\geq 99.5\%$ purity, price from Sigma-Aldrich)⁵.

⁵ Note that prices of raw materials for actual manufacturing can be much lower, as economies of scale may be reaped, and there might not be a requirement for high purity sugars.

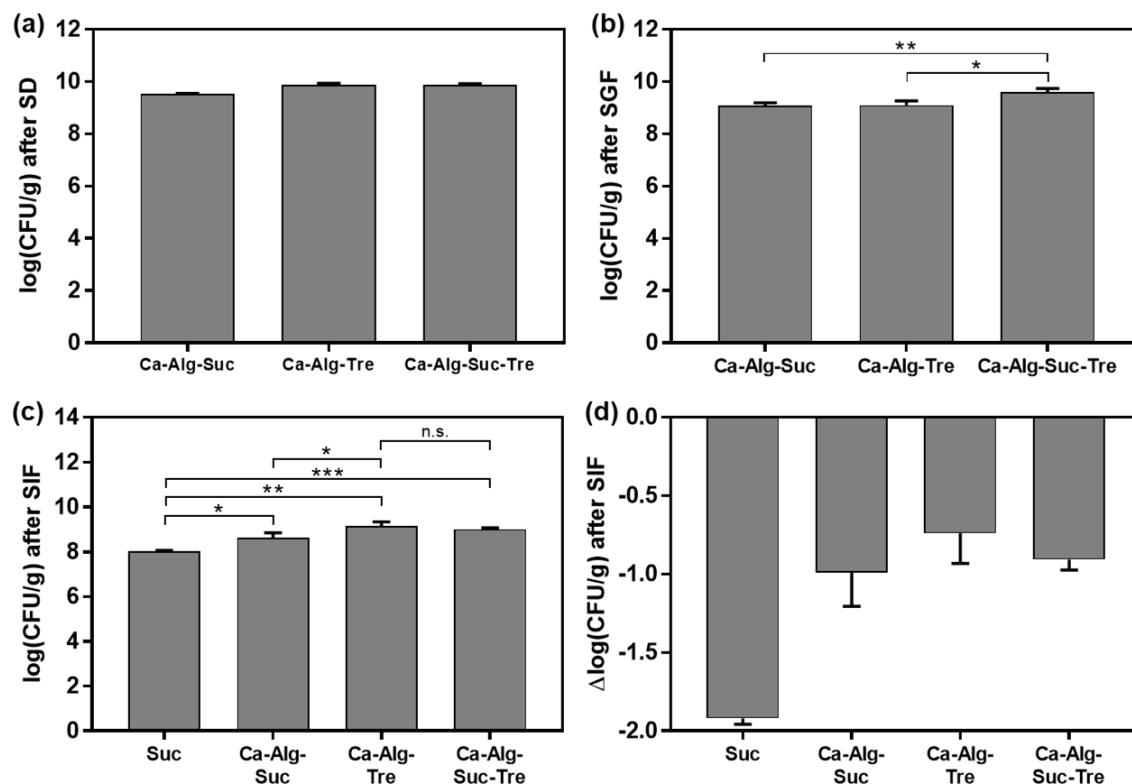


Figure 6.9: Survivability data of spray-dried LGG in Ca-Alg-Suc, Ca-Alg-Tre, and Ca-Alg-Suc-Tre formulations after spray-drying and after exposure to SGF or SIF. (a) $\log_{10}(\text{CFU/g})$ data of LGG in various formulations after spray-drying. (b) $\log_{10}(\text{CFU/g})$ data of LGG in various formulations after SGF exposure. (c) $\log_{10}(\text{CFU/g})$ data of LGG in various formulations after SIF exposure. (d) Change in $\log_{10}(\text{CFU/g})$ counts of LGG in various formulations after SIF exposure.

6.3.7. Calcium lactate as a crosslinking agent

The Ca-Alg-Suc-Tre formulation was compared to the calcium lactate-alginate-sucrose-trehalose [Calac-Alg-Suc-Tre] sample, which utilises calcium lactate, instead of calcium chloride as the crosslinking solution. Calcium lactate is preferable to calcium chloride as the latter has a bitter flavour profile, while the former is known to have a neutral taste. Hence, organoleptic improvements can be achieved by using calcium lactate as the crosslinker. From **Figure 6.10**, the Calac-Alg-Suc-Tre performed similarly well as Ca-Alg-Suc-Tre in achieving good post-spray-drying survivability and SGF/SIF resistance for LGG probiotics, suggesting that calcium lactate was similarly effective in crosslinking alginates by the proposed spray-drying method.

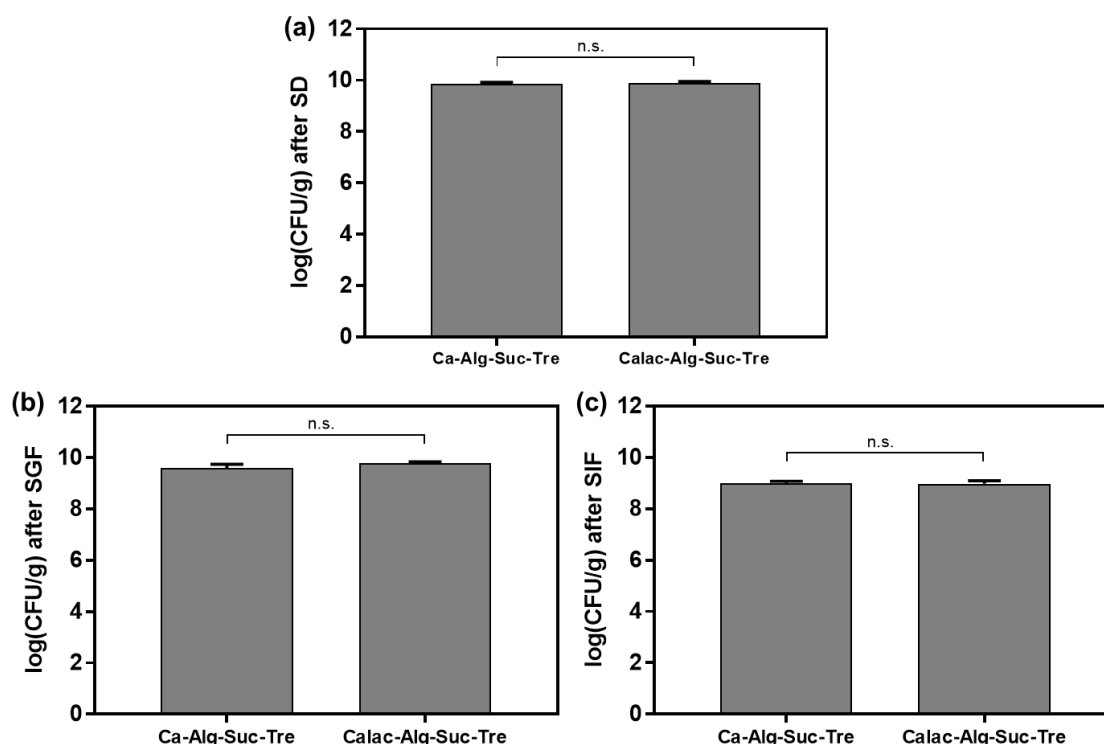


Figure 6.10: Survivability data of spray-dried LGG in Ca-Alg-Suc-Tre and Calac-Alg-Suc-Tre formulations after spray-drying and after exposure to SGF or SIF. (a) $\text{Log}_{10}(\text{CFU/g})$ data of LGG in both formulations after spray-drying. (b) $\text{Log}_{10}(\text{CFU/g})$ data of LGG in both formulations after SGF exposure. (c) $\text{Log}_{10}(\text{CFU/g})$ data of LGG in both formulations after SIF exposure.

6.3.8. Addition of antioxidant agents

The addition of two antioxidant agents (L-cysteine and sodium ascorbate, previously reported in [402], [405]) were explored to seek improvement on room temperature storage stability of encapsulated LGG. The rationale of using antioxidants as additives in desiccation of microorganisms was to mitigate oxidative stresses faced by microorganisms. Oxidative deteriorative reactions, such as the oxidation of membrane lipids, proteins, or nucleic acids, are induced by ambient heat, light, or atmospheric free radicals which are present in the processing and shelf-storage stages of encapsulated probiotics. For lactobacilli probiotics, oxidative stresses also come from ambient oxygen, as many lactobacilli are oxygen sensitive and form reactive oxygen species in aerobic environments

[406]. It was therefore reasoned that the probiotic encapsulation formulation might improve with the incorporation of antioxidants.

From **Figure 6.11**, the formulations with L-cysteine or sodium ascorbate added (Calac-Alg-Suc-Tre-Cys and Calac-Alg-Suc-Tre-Asc) performed similarly as well as the Calac-Alg-Suc-Tre formulation, indicating that the addition of these two antioxidants retained high post-spray-drying survivability and SGF/SIF protection of LGG.

Storage studies were conducted under room temperature (25 °C) or refrigerated (4 °C) conditions for up to eight weeks, and results are presented in **Figure 6.12**. Generally, higher survival of LGG probiotics were observed for samples stored in refrigerated conditions, as compared to room temperature. This is within expectation, as lower temperatures generally lead to higher microorganism survival due to reduced rates of chemical reactions, such as oxidation of lipids or degradation of proteins [380], [381]. From **Figure 6.12a**, the addition of L-cysteine and sodium ascorbate significantly improved room temperature survivability of LGG. Highest CFU was yielded for the Calac-Alg-Suc-Tre-Asc formulation after eight weeks of room temperature storage, with $8.99 \log_{10}(\text{CFU/g})$ remaining viable, equivalent to 0.77 reduction in $\log_{10}(\text{CFU/g})$. This suggests that antioxidants play an important role in maintaining probiotics viability in prolonged ambient temperature storage, and sodium ascorbate is more effective than L-cysteine in achieving this storage stability. For refrigerated storage (**Figure 6.12b**), the Calac-Alg-Suc-Tre, Calac-Alg-Suc-Tre-Cys, and Calac-Alg-Suc-Tre-Asc formulations performed similarly well, with $> 9.4 \log_{10}(\text{CFU/g})$ surviving after eight weeks of storage. These formulations also performed significantly better than the Suc, and Ca-Alg-Suc samples, indicating that the crosslinked alginate matrix and presence of trehalose were important in maintaining refrigerated storage viability of LGG.

The spray-dried samples were also exposed to SGF and SIF after storage to determine if gastrointestinal protective effects were retained after room temperature or refrigerated storage. From **Figure 6.13**, protective effects against SGF and SIF were generally retained for the crosslinked alginate samples (includes Ca-Alg-Suc, Calac-Alg-Suc-Tre, Calac-Alg-

Suc-Tre-Cys, and Calac-Alg-Suc-Tre-Asc). At room temperature storage conditions, the Calac-Alg-Suc-Tre-Asc formulation yielded highest LGG survivability after SGF or SIF, with 8.55 and 7.66 $\log_{10}(\text{CFU/g})$ remaining viable after exposure to respective simulated fluids (**Figures 6.13a and b**). At refrigerated conditions, the crosslinked alginate formulations performed similarly well, with $> 8.7 \log_{10}(\text{CFU/g})$ surviving after eight weeks of storage and SGF exposure, and $> 8.0 \log_{10}(\text{CFU/g})$ surviving after eight weeks of storage and SIF exposure.

In view of all formulations tested, the Calac-Alg-Suc-Tre-Asc formulation performed best in terms of post-spray-drying survivability, organoleptic properties, gastrointestinal protective effects, and room temperature storage stability of probiotics.

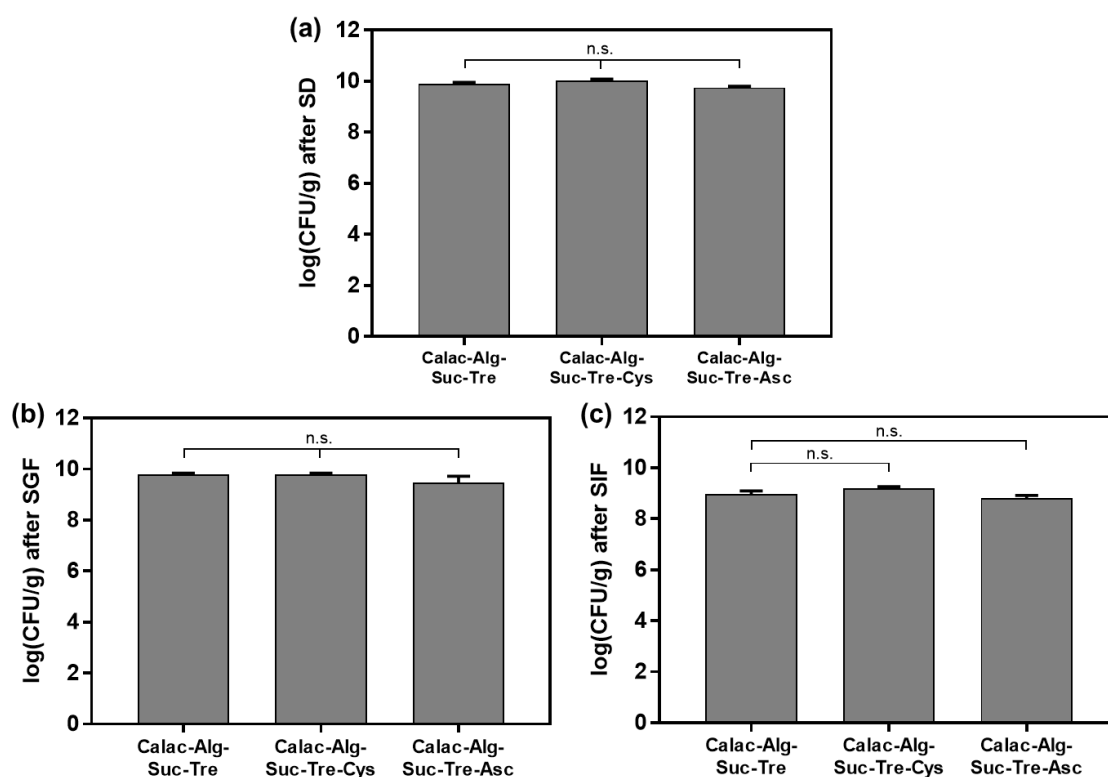


Figure 6.11: Survivability data of spray-dried LGG in Calac-Alg-Suc-Tre, Calac-Alg-Suc-Tre-Cys, and Calac-Alg-Suc-Tre-Asc formulations after spray-drying and after exposure to SGF or SIF. (a) $\log_{10}(\text{CFU/g})$ data of LGG in various formulations after spray-drying. (b) $\log_{10}(\text{CFU/g})$ data of LGG in various formulations after SGF exposure. (c) $\log_{10}(\text{CFU/g})$ data of LGG in various formulations after SIF exposure.

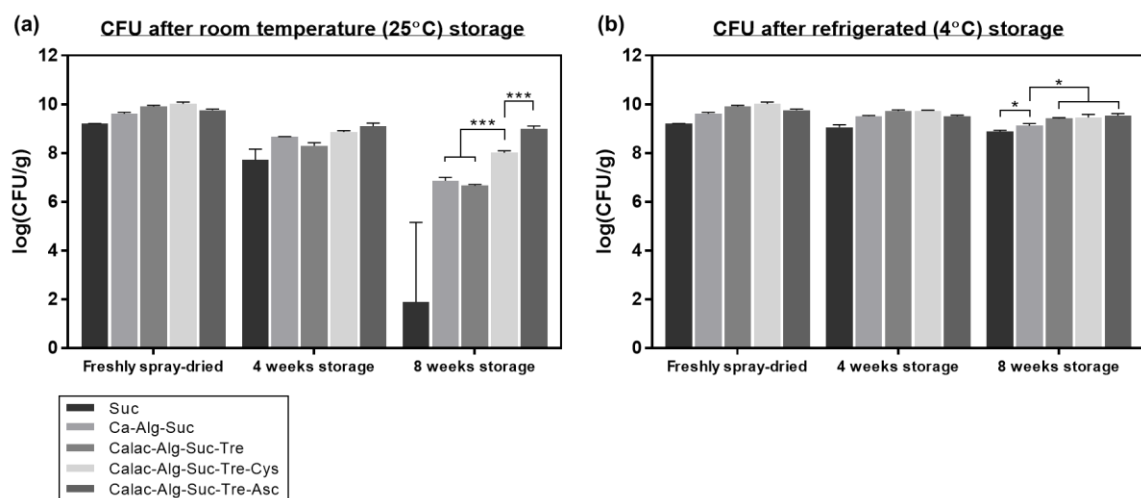


Figure 6.12: Survivability data of Suc, Ca-Alg-Suc, Calac-Alg-Suc-Tre, Calac-Alg-Suc-Tre-Cys, and Calac-Alg-Suc-Tre-Asc formulations after room temperature or refrigerated storage. (a) $\text{Log}_{10}(\text{CFU/g})$ data of LGG in various formulations after four and eight weeks of room temperature storage. (b) $\text{Log}_{10}(\text{CFU/g})$ data of LGG in various formulations after four and eight weeks of refrigerated storage.

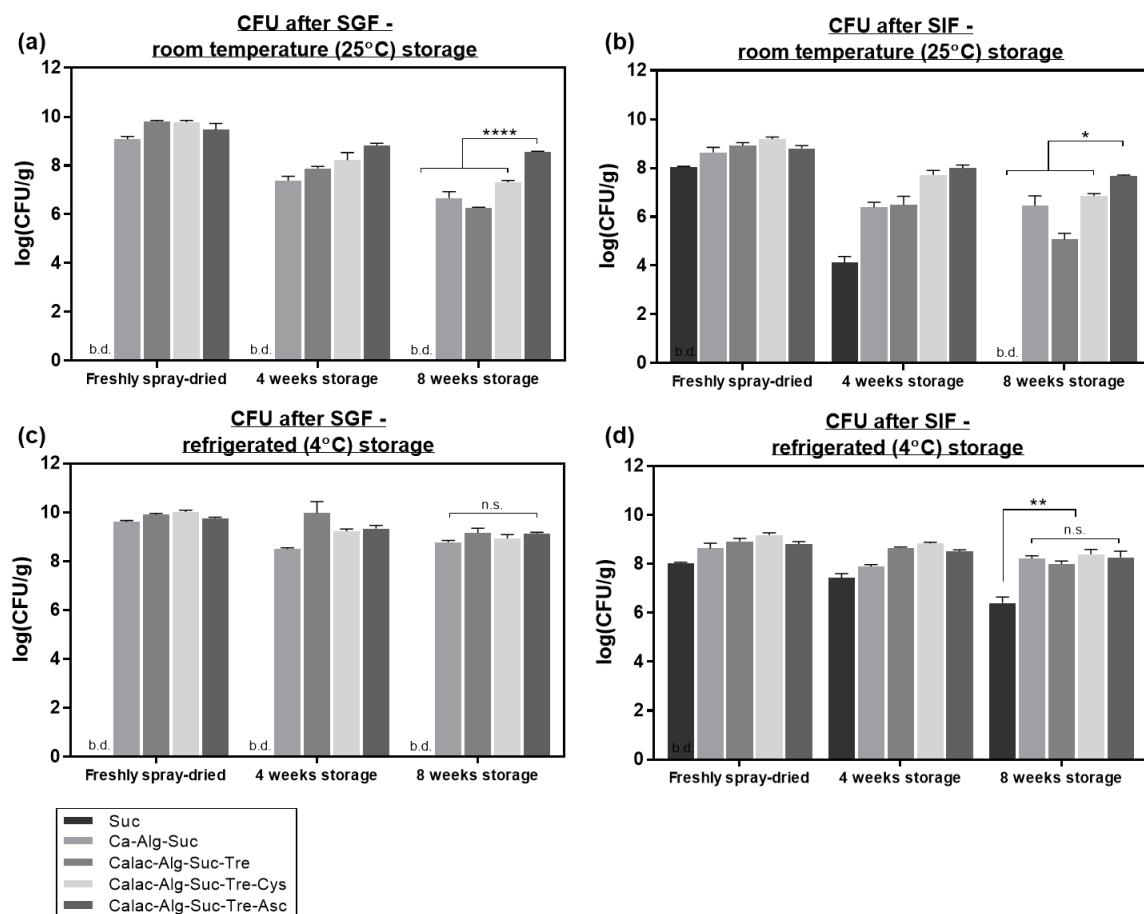


Figure 6.13: Survivability data of spray-dried LGG in Suc, Ca-Alg-Suc, Calac-Alg-Suc-Tre, Calac-Alg-Suc-Tre-Cys, and Calac-Alg-Suc-Tre-Asc formulations after room temperature or refrigerated storage, followed by exposure to SGF or SIF. (a) Log₁₀(CFU/g) data of LGG in various formulations after four and eight weeks of room temperature storage, and SGF exposure. (b) Log₁₀(CFU/g) data of LGG in various formulations after four and eight weeks of room temperature storage, and SIF exposure. (c) Log₁₀(CFU/g) data of LGG in various formulations after four and eight weeks of refrigerated storage, and SGF exposure. (d) Log₁₀(CFU/g) data of LGG in various formulations after four and eight weeks of refrigerated storage, and SIF exposure. B.d. indicates that the cell count is below the detection limit of ~1.5 log₁₀(CFU/g).

6.3.9. Adapting optimised formulation for encapsulating kefir probiotics

In this section, the Calac-Alg-Suc-Tre-Asc formulation was adapted for encapsulating kefir probiotics (**Chapter 3**). A comparison of encapsulated kefir probiotics in Calac-Alg-Suc-Tre-Asc and kefir probiotics in the Suc control set was made in **Figure 6.14**.

Calac-Alg-Suc-Tre-Asc yielded high spray-drying survivability of kefir probiotics with $10.3 \log_{10}(\text{CFU/g})$ viable doses in freshly spray-dried powder (**Figure 6.14a**). Calac-Alg-Suc-Tre-Asc was also effective in protecting kefir probiotics against SGF and SIF, with 9.81 and 9.26 $\log_{10}(\text{CFU/g})$ surviving after exposure to respective simulated fluids (**Figure 6.14a**). Good stability of kefir probiotics in Calac-Alg-Suc-Tre-Asc for up to eight weeks of refrigerated storage was also noted, with 10.1, 9.52 and 9.13 $\log_{10}(\text{CFU/g})$ surviving in the powder, after SGF, and after SIF respectively. Kefir probiotics stored at room temperature also survived, albeit with lower $\log_{10}(\text{CFU/g})$ counts of 9.04, 9.62 and 7.64 respectively in powder, after SGF, and after SIF.

Overall, kefir probiotics were suitable to be encapsulated by the proposed spray-drying technique. Resultant kefir probiotics in the Calac-Alg-Suc-Tre-Asc formulation showed high CFU/g counts and were also imbued with gastrointestinal resistant and shelf-life stable properties.

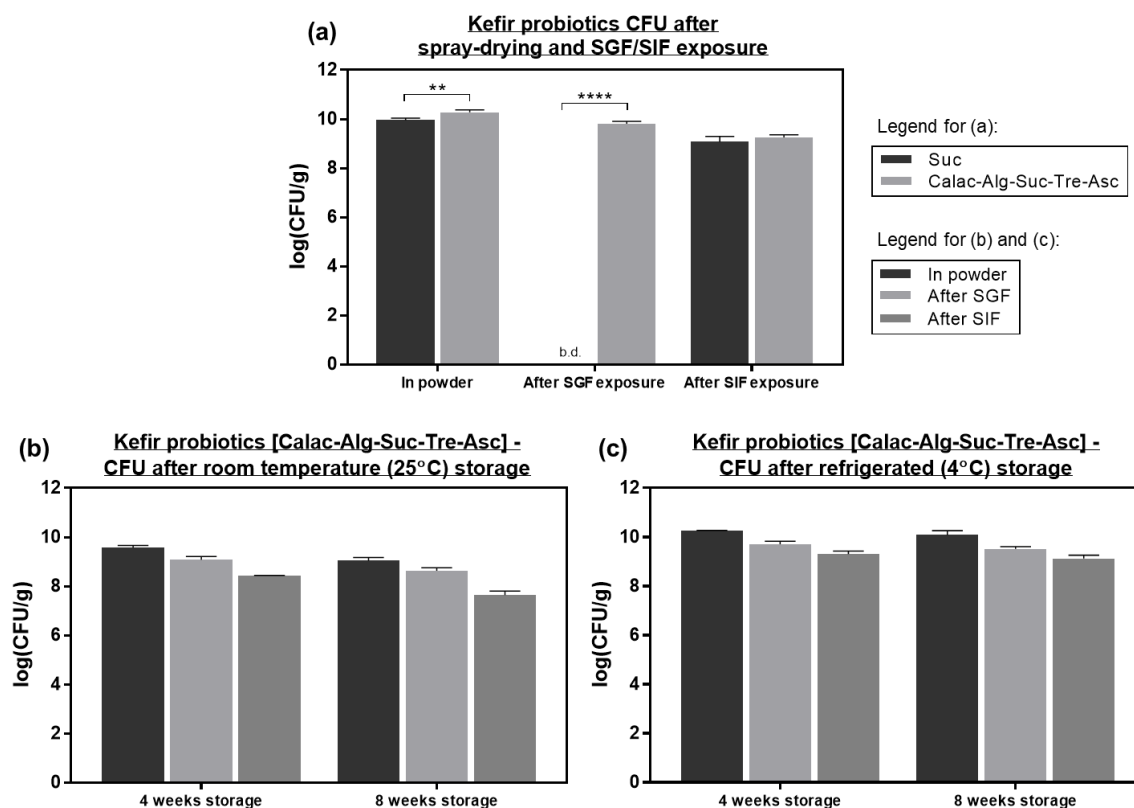


Figure 6.14: Survivability data of spray-dried kefir probiotics in Suc and Calac-Alg-Suc-Tre-Asc formulations after spray-drying, storage, and exposure to SGF or SIF. (a) $\log_{10}(\text{CFU/g})$ data of

freshly spray-dried kefir probiotics in both formulations in powder form, after SGF exposure, and after SIF exposure. (b) $\text{Log}_{10}(\text{CFU/g})$ data of kefir probiotics in Calac-Alg-Suc-Tre-Asc after four and eight weeks of room temperature storage, in powder form, after SGF exposure, and after SIF exposure. (c) $\text{Log}_{10}(\text{CFU/g})$ data of kefir probiotics in Calac-Alg-Suc-Tre-Asc after four and eight weeks of refrigerated storage, in powder form, after SGF exposure, and after SIF exposure. B.d. indicates that the cell count is below the detection limit of $\sim 1.5 \log_{10}(\text{CFU/g})$.

6.4. Conclusion

Encapsulation of probiotics in various formulations was performed using a novel spray-drying technique which utilises coaxial spraying for *in situ* alginate crosslinking. This technique could effectively achieve more than 90% crosslinked alginate in Ca-Alg and Ca-Alg-Suc polymer matrices. Six formulations, namely Suc, Alg, Alg-Suc, Ca-Alg, Ca-Alg-Suc and Ca-Alg-Suc-IFC, were evaluated early in the study to determine if sucrose or crosslinked alginates affected LGG survivability after spray-drying and SGF exposure. Ca-Alg-Suc performed best amongst these six formulations, elucidating the importance of the protective sugar and crosslinked alginate in protecting entrapped probiotics against desiccation and thermal stresses during spray-drying, as well as confer gastroprotection. Enhanced survivability of probiotics in Ca-Alg-Suc was attributed to the formation of crosslinked calcium-alginate surrounding each particle, which immobilises encapsulated probiotics within the matrix. Upon exposure to acidic SGF, excess protons could be sequestered by conversion of alginate to alginic acid, thereby providing a localised pH buffering effect which enhances survivability of entrapped probiotics in SGF. Similar trends in spray-drying and SGF survivals of *L. plantarum* and *L. paracasei* encapsulated in Ca-Alg-Suc were observed, however poor survivability was observed for ECN, indicating that this probiotics encapsulation technique may not be suitable for Gram-negative strains.

In the latter part of the study, other protective agents and crosslinking agents, namely trehalose, calcium lactate, L-cysteine, and sodium ascorbate, were attempted to seek greater SIF resistance, and improved shelf-life properties for encapsulated probiotics. Of various formulations tested, Calac-Alg-Suc-Tre-Asc performed best in terms of post-spray-

drying probiotics survivability, organoleptic properties, gastrointestinal protective effects, and room temperature storage stability of probiotics. The Calac-Alg-Suc-Tre-Asc was further adapted for encapsulation of kefir probiotics, and results showed high CFU/g counts of encapsulated kefir probiotics powder, and kefir probiotics were also imbued with gastrointestinal resistant and shelf-life stable properties.

Overall, this study has yielded an industrially scalable and facile method to produce shelf-stable, gastrointestinal resistant probiotic powders, and fulfils **Objective 5** of this thesis. This novel spray-drying technique can produce dry fine powder particles directly in a single step, which greatly streamlines and simplifies the formulation process. Having a single-step process can also minimise contamination risks and transfer losses, thereby ensuring higher quality and better yield of the resultant probiotic product. Encapsulated dry probiotic powder derived from this technique can be versatilely incorporated into a wide variety of formats, hence has vast commercial applications in the agro-food and pharmaceutical industries. The developed technique has been successfully patented (WIPO PCT International Patent No. WO 2022/177508 A1). Future studies can compare the efficacies of encapsulated and unencapsulated probiotics *in vivo* and in clinical trials, to further ascertain the benefits of this proposed encapsulation technique.

7. Chapter 7

Conclusions and Recommendations

This chapter concludes the main findings of this research work. The research hypotheses and objectives outlined in Chapter 1 and their achievement through this research will be addressed. Major observations and key results from each research chapter are summarised. The scope for further advancement is suggested in the future work section.

7.1. Overall Conclusions

The work for this thesis was inducted with the aim to identify beneficial synbiotics from kefir, and to utilise alginate encapsulation systems to facilitate their delivery to the human intestine. The numerous health benefits of kefir provided an initial motivation for this study, and it was of interest in this research to identify the microbial components of kefir which contribute to its health benefits. This study zoomed in on identifying probiotic strains and prebiotic materials from kefir and explored the feasibility of combining them to formulate a kefir synbiotic product. This thesis is also concerned with developing encapsulation systems to overcome the challenges associated with the oral delivery of probiotics. Two probiotic encapsulation systems were developed in this thesis – a “wet” alginate-extrusion based system, and a “dry” spray-dried encapsulation system. Both systems demonstrated utility in enhancing viability and functionality of probiotics, and facilitated their incorporation in food and beverage matrices, as well as in dietary supplement formulations. This represents a breakthrough in the translational use of food-grade polymeric materials to enhance the efficacy of probiotic formulations. These systems were also successfully adapted for the encapsulation of kefir probiotics, hence contributing towards the development of new consumption formats for consumers to reap the benefits of kefir.

The results attained in this thesis are summarised in detail as follows:

1. This study has successfully isolated and characterised promising probiotic strains from locally sourced milk and water kefir (addresses **Objective 1**). Through a series of *in vitro* screening assays, seven bacterial strains, namely *Lentilactobacillus hilgardii* Kef-w9, *Lactocaseibacillus paracasei* Kef-w14 and Kef-w19, *Liquorilactobacillus satsumensis* Kef-w11 and Kef-w13, *Lactobacillus helveticus* Kef-m4, and *Lentilactobacillus kefiri* Kef-m15, were ultimately identified as potential probiotics, and these were combined to form the “kefir probiotics” formulation. These kefir probiotics exhibited several of the beneficial properties associated with the kefir beverage itself, such as enteric pathogen inhibition effects, antioxidant properties, and potential for cholesterol management through BSH activity. These strains also showed

good safety profile and desirable probiotic characteristics, including good survival in acid and bile environments, non-cytotoxicity and high adhesion to Caco-2 cells, the lack of virulence and AMR genes, and could be consistently produced through batch fermentations. Overall, this study has identified novel and promising probiotic strains from locally sourced kefir which can add diversity to the existing library of probiotic strains and may provide consumers with alternative product formats to attain the health benefits of kefir.

2. Prebiotic polysaccharides were successfully derived from kefir-derived EPS-producing microbes (addresses **Objective 2**). Various bacterial species from water kefir demonstrated EPS-producing properties, of which, EPS from the *L. satsumensis* Kef-w1 strain provided high yields of soluble, LPS-free, α -glucan EPS from Suc-MRS media. The extracted EPS material from *L. satsumensis* Kef-w1 is a branched dextran-like material, consisting of α -1,3/ α -1,4 and α -1,6 glucan bonds. Upon acid hydrolysis, the native EPS from Kef-w1 yielded a shorter chain α -glucan polysaccharide, termed Kef-w1 HP, with DP 50 – 75. Kef-w1 HP exhibited prebiotic qualities, including meeting the selective utilisation criterion of probiotics and non-digestibility by human digestive enzymes. Kef-w1 HP was also found to be utilisable by kefir probiotics, suggesting a potential for these to be applied in combination as kefir synbiotics. Overall, the Kef-w1 HP material is a novel ingredient with prebiotic potential, which may be applied in combination with kefir probiotics to yield a synbiotic formulation or incorporated individually as a functional food additive.
3. Kefir probiotics and kefir prebiotics were combined in a kefir synbiotic formulation and evaluated for their microbiome modulating effects in an *ex vivo* large bowel simulated model (addresses **Objective 3**). Kefir synbiotics demonstrated a propensity to promote *Bacteroidetes* abundance, and generate SCFAs, including acetate, propionate, and butyrate, as by-products. These results suggest that kefir synbiotics could act as functional microbiome-modulating ingredients to confer health benefits on human hosts.

4. An encapsulation system based on alginate extrusion was developed to confer GIT resistance to probiotics and facilitate their addition to carbonated sodas and beers (addresses **Objective 4**). Different alginate formulations, differing in terms of M/G ratio of alginate, whether alginates are sterilised by autoclave or filtration, and the presence of sucrose in the formulation, were examined to determine the optimal formulation for GIT protection of LGG probiotics. Results demonstrated that the Ca-alginate formulations which utilised autoclaved alginates with sucrose (G_ac_suc and M_ac_suc) yielded high survival of encapsulated LGG after SGF exposure. The M_ac_suc formulation was further adapted for encapsulation of probiotics, including LGG, ECN, BL, and kefir probiotics, for addition to various commercial carbonated sodas and beers, including Coke, 7-Up, Tiger Beer, and Guinness. Results showed that the viabilities of all four probiotic candidates were improved in all tested beverages upon alginate encapsulation. The viabilities of all probiotics were also better maintained in the beverages under refrigerated conditions as compared to room temperature. Overall, alginate encapsulation by extrusion can provide beverage manufacturers a novel way to functionalise sodas and beers with alginate-probiotic “bobas”. It is also recommended to store encapsulated probiotics in these beverages under refrigerated conditions to reduce viability losses, and to minimise flavor alterations that could arise due to probiotic metabolism.
5. Finally, a facile, high-throughput spray-drying process for producing dried, alginate-encapsulated probiotics powder with GIT-resistant properties was developed in this thesis (addresses **Objective 5**). This method utilises the principle of *in situ* alginate crosslinking via spray-drying to simultaneously dry, micronise, and encapsulate live microorganisms in calcium-alginate matrices. The optimal formulation, Calac-Alg-Suc-Tre-Asc, yielded high viable counts of LGG probiotics ($>10^9$ CFU/g) after spray-drying and exposure to simulated gastric and intestinal fluids (SGF/SIF). This highlights the ability of this technique to protect entrapped probiotics against desiccation and thermal stresses during spray-drying, as well as confer protection against acidic pH and bile salts. High viability and GIT protection of LGG was also retained after eight weeks of refrigerated and ambient temperature storage. The optimal

formulation also effectively encapsulated kefir probiotics, showing similar results of high post-spray-drying and post-SGF/SIF viabilities. Successful encapsulation of different probiotics via the proposed spray-drying technique highlights the potential of this procedure to be scaled up for commercial applications.

7.2. Future Recommendations

Several studies listed below can aid to further mature the kefir probiotics, kefir prebiotics, and encapsulation systems technologies developed in this thesis.

7.2.1. *In vivo* characterisation of kefir probiotics

This research has so far conducted *in vitro* screening of kefir isolates and has identified seven best-performing strains with promising probiotic properties. Further assessment of these strains would focus on characterising their *in vivo* safety and functionality to facilitate their regulatory approval and use as functional dietary ingredients. Safety is a particularly important aspect for regulatory approval of probiotics. Even though these probiotic strains are derived from kefir, which, as a beverage, has demonstrated a long history of safe consumption, the combination of probiotics in the seven-strain “kefir probiotics” formulation as described in this thesis has not been prior assessed. In addition, *Liquorilactobacillus satsumensis* is not within the QPS list of probiotic organisms designated by EFSA, hence more substantiating evidence will likely be required to ascertain the safety of these strains. To evaluate the safety aspects of the kefir probiotics, animal feeding studies can be conducted to determine if any toxic or detrimental effects were observed on the survival, body weight, food consumption, serum biochemical and haematological indices, allergic responses, inflammatory response, and microbiota composition [407]. Clinical trials would be further required to ascertain the safety of these probiotics in human hosts.

In addition, other functionalities of the kefir probiotics should be further examined. For instance, *ex vivo* or *in vivo* studies can be conducted to detect the persistence or colonisation

of these strains in the GIT [114], [408]. Additional studies can also be conducted to examine the enteric pathogen inhibition, antioxidative, cholesterol management (due to BSH activity), and neurological (due to GABA secretion) effects observed of kefir probiotics. *In vivo* and clinical trials utilising different diseased hosts can be conducted to evaluate if kefir probiotics could treat, alleviate symptoms of, or prevent onset of certain diseases.

The synergy between the individual strains of the multi-strain kefir probiotics blend can also be further studied. Generally, multi-strain probiotics are known to provide a broader range of benefits via more mechanisms of action, however, the synergy between individual strains was not explored in detail within the scope of this thesis. In this work, the ratio of the multi-strain kefir probiotics blend was determined primarily based on the maximum cell density that can be attained for each strain within MRS media. Further optimizations can be done on the ratio of probiotic strains, with more comparative studies of the multi-strain vs individual strains efficacies being evaluated.

7.2.2. Upscaling kefir prebiotic production and further assessment of its safety and functionality

The *L. satsumensis* Kef-w1 HP material has demonstrated usefulness as a potential prebiotic with synbiotic efficacies when combined with kefir probiotics. Further studies can then be done to upscale the production of kefir prebiotics, to improve their commercial applicability. A bioreactor process with optimised fermentation conditions (e.g., stirring, temperature, aerobic/anaerobic, duration of fermentation, Suc-MRS media composition) can be developed to facilitate high-yield batch production of EPS by *L. satsumensis* Kef-w1. Dextranucrase enzymes can also be derived from Kef-w1 to improve purity and efficiency of dextrans from sucrose media [409]. An integrated heat denaturation, ethanol precipitation, and acid hydrolysis process can then be used to form Kef-w1 HP prebiotics. Future work may also explore enzymatic hydrolysis, e.g., using glucanases or dextranases, which has several advantages over acid hydrolysis, including less formation of undesirable by-products, and no need for corrosion resistant processing equipment [410].

In addition, the safety and prebiotic characteristics of Kef-w1 HP should be further ascertained. Similar to probiotics, the safety of prebiotic materials is critical for its regulatory approval as a food ingredient. Purity studies and *in vivo* assessment of Kef-w1 HP should be conducted to evaluate if any undesirable attributes were associated with this material. For prebiotic functionalities, the *in vivo* gut microbiome α -diversity should be assessed following Kef-w1 HP administration, since *ex vivo* studies conducted in this research showed a decreased diversity upon Kef-w1 HP or inulin supplementation. Also, any gas distension effects associated with Kef-w1 HP use could be assessed with *in vitro* or *in vivo* experiments [34].

Another aspect of further work would be to compare between the Kef-w1 EPS and HP with various dextran-like glucans produced by different sources [409]. A comparison of chemical structure can be made between Kef-w1 EPS and commercial dextrans, as well as between the Kef-w1 HP material and commercial dextrans which were hydrolysed to a similar molecular weight. Prebiotic efficacies of other α -glucans with similar structure can also be studied, which can elucidate the structure-prebiotic efficacy relationships of this class of materials. In addition, the yields of Kef-w1 EPS can be compared with commercial production yields to evaluate the techno-economic feasibility of producing the kefir prebiotic material.

7.2.3. Enhancing commercial applicability of encapsulated probiotics in carbonated sodas and beers

Preliminary research conducted in this thesis revealed that alginate encapsulation by extrusion can improve viability of encapsulated probiotics in carbonated sodas and beers. However, more studies need to be conducted to ascertain the commercial feasibility of developing probiotic-sodas and beers through extrusion. One major aspect is to test the organoleptic properties of the alginate-probiotics spheres, e.g., flavour, odour, appearance, mouthfeel, and any evolution of taste profile over storage duration. Mechanical tests, benchmarked against other “boba”, like tapioca, gelatine, konjac, or agar, can be employed

to test the mouthfeel or chewiness of alginate-probiotic spheres. Other aspects of taste profile can be evaluated by an organoleptic and sensory panel.

The second aspect is to test the encapsulated probiotic systems for an extended duration to reflect the longer shelf life required for shelf-stable products. Presently, commercial probiotic drinks have refrigerated shelf lives of around 45 days [203]. Since several of the encapsulated probiotics and beverage systems remained highly viable after 14 days of storage, the test duration can be extended to determine if shelf lives similar to commercial probiotic drinks can be attained. Another possibility would be to store extruded beads separately, before adding them to the beverages. An optimisation of the suspension media containing the extruded beads can be performed to lengthen the shelf-life of beads. Freeze-drying of the extruded alginate-probiotic beads may also be attempted to improve shelf-life stability. These separately stored alginate beads may provide a new consumption format for consumers to functionalise their beverage of choice with beneficial probiotics.

7.2.4. Test other functionalities of the spray-dried probiotics encapsulation system

Finally, further work should be done to expand the commercial potential of the probiotics spray-drying encapsulation technology developed in this thesis. One key aspect would be to scale up production and assess if similar good gastroprotective results and shelf-life stability properties can still be yielded. This research utilized a benchtop spray-dryer and worked with smaller volumes typically not exceeding 50 ml of alginate-probiotic slurry input. Industrial-scale spray-dryers would likely require further optimizations of the developed method to produce encapsulated dried probiotic powder at scale.

It is also of interest to adapt the spray-drying method for the encapsulation of other probiotic genera, such as *Bifidobacterium* and *Streptococcus*, which are also popularly consumed probiotics and known to be susceptible to GIT challenges. Optimisation of the spray-drying technique, e.g., by reducing inlet temperature or utilising other protective agents, can be conducted to facilitate successful encapsulation of these strains. In addition, heat stability is commercially desirable quality which can improve application of

encapsulated probiotics for incorporation into food matrices, e.g., baked goods, milk powder, animal feed. Future studies can evaluate if the spray-drying process, which immobilises probiotics in a crosslinked alginate matrix, might help to improve the heat stability of encapsulated probiotics.

In vivo and clinical studies are also required to assess the effectiveness of encapsulation in enhancing probiotic efficacy. These studies can examine the effects of sequential gastric and intestinal fluids exposure on encapsulated probiotics, as well as the site of probiotics colonisation. The effects of encapsulated probiotics can also be compared with that of non-encapsulated probiotics, to determine if any significant improvements can be observed with the use of encapsulation. Presently, most clinical studies regarding probiotics have been conducted with unencapsulated probiotics, and these studies have not considered encapsulation to preserve the viability of probiotics and to achieve targeted delivery to the human intestines. This could inevitably explain the inconsistencies in the efficacy of probiotics for treatment of various diseases, such as Crohn's disease, metabolic disease, diabetes, psychophysiological diseases, respiratory diseases, and immune system diseases, where the benefit of probiotics appear to vary greatly between patients [64]. Encapsulation of probiotics may therefore be key in evaluating the actual effect of administered dosages on clinical outcomes.

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9. Supplementary information

Table S1: List of all 158 microbial strains isolated from milk and water kefir. 36 strains were selected for well diffusion assay. All kefir-isolates were cultured at 30 °C, static conditions for 3 days in MRS media at the respective aerobic/anaerobic conditions prior to assays.

No.	Kefir isolate species	Strain	Source	Aerobic/ anaerobic (OX/ANA)	Selected for well diffusion assay?
1	<i>Liquorilactobacillus satsumensis</i>	Kef-w1	Water kefir	ANA	Yes
2	<i>Liquorilactobacillus satsumensis</i>	Kef-w2	Water kefir	OX	Yes
3	<i>Liquorilactobacillus satsumensis</i>	Kef-w11	Water kefir	ANA	Yes
4	<i>Liquorilactobacillus satsumensis</i>	Kef-w13	Water kefir	OX	Yes
5	<i>Liquorilactobacillus satsumensis</i>	Kef-w18	Water kefir	OX	Yes
6	<i>Liquorilactobacillus satsumensis</i>	Kef-w22	Water kefir	OX	-
7	<i>Liquorilactobacillus satsumensis</i>	Kef-w23	Water kefir	OX	-
8	<i>Liquorilactobacillus satsumensis</i>	Kef-w24	Water kefir	OX	-
9	<i>Liquorilactobacillus satsumensis</i>	Kef-w25	Water kefir	OX	-
10	<i>Liquorilactobacillus satsumensis</i>	Kef-w26	Water kefir	OX	-
11	<i>Liquorilactobacillus satsumensis</i>	Kef-w27	Water kefir	ANA	-
12	<i>Liquorilactobacillus satsumensis</i>	Kef-w28	Water kefir	ANA	-
13	<i>Liquorilactobacillus satsumensis</i>	Kef-w29	Water kefir	ANA	-
14	<i>Liquorilactobacillus satsumensis</i>	Kef-w30	Water kefir	ANA	-
15	<i>Liquorilactobacillus satsumensis</i>	Kef-w31	Water kefir	ANA	-
16	<i>Liquorilactobacillus satsumensis</i>	Kef-w32	Water kefir	ANA	-
17	<i>Liquorilactobacillus satsumensis</i>	Kef-w33	Water kefir	ANA	-
18	<i>Liquorilactobacillus satsumensis</i>	Kef-w34	Water kefir	ANA	-
19	<i>Liquorilactobacillus satsumensis</i>	Kef-w35	Water kefir	ANA	-
20	<i>Liquorilactobacillus satsumensis</i>	Kef-w36	Water kefir	ANA	-
21	<i>Lactobacillus kefiranofaciens</i>	Kef-m3	Milk kefir	ANA	Yes
22	<i>Lactobacillus kefiranofaciens</i>	Kef-m37	Milk kefir	ANA	-
23	<i>Lactobacillus kefiranofaciens</i>	Kef-m38	Milk kefir	ANA	-
24	<i>Lactobacillus kefiranofaciens</i>	Kef-m39	Milk kefir	ANA	-
25	<i>Lactobacillus helveticus</i>	Kef-m4	Milk kefir	ANA	Yes
26	<i>Acetobacter persici</i>	Kef-w5	Water kefir	OX	Yes
27	<i>Acetobacter persici</i>	Kef-w40	Water kefir	OX	-
28	<i>Acetobacter persici</i>	Kef-w41	Water kefir	OX	-
29	<i>Acetobacter persici</i>	Kef-w42	Water kefir	OX	-
30	<i>Leuconostoc mesenteroides</i>	Kef-m6	Milk kefir	ANA	-
31	<i>Leuconostoc mesenteroides</i>	Kef-m16	Milk kefir	OX	Yes
32	<i>Leuconostoc mesenteroides</i>	Kef-m43	Milk kefir	ANA	-
33	<i>Leuconostoc mesenteroides</i>	Kef-m44	Milk kefir	ANA	-
34	<i>Leuconostoc mesenteroides</i>	Kef-m45	Milk kefir	ANA	Yes
35	<i>Lactocaseibacillus paracasei</i>	Kef-w7	Water kefir	ANA	Yes
36	<i>Lactocaseibacillus paracasei</i>	Kef-w14	Water kefir	OX	Yes

37	<i>Lacticaseibacillus paracasei</i>	Kef-w17	Water kefir	OX	Yes
38	<i>Lacticaseibacillus paracasei</i>	Kef-w19	Water kefir	OX	Yes
39	<i>Lacticaseibacillus paracasei</i>	Kef-w20	Water kefir	ANA	Yes
40	<i>Lacticaseibacillus paracasei</i>	Kef-w46	Water kefir	OX	-
41	<i>Lacticaseibacillus paracasei</i>	Kef-w47	Water kefir	ANA	-
42	<i>Lacticaseibacillus paracasei</i>	Kef-w48	Water kefir	ANA	-
43	<i>Lacticaseibacillus paracasei</i>	Kef-w49	Water kefir	ANA	-
44	<i>Lacticaseibacillus paracasei</i>	Kef-w50	Water kefir	ANA	-
45	<i>Lacticaseibacillus paracasei</i>	Kef-w51	Water kefir	ANA	-
46	<i>Lacticaseibacillus paracasei</i>	Kef-w52	Water kefir	ANA	-
47	<i>Liquorilactobacillus hilgardii</i>	Kef-w8	Water kefir	ANA	Yes
48	<i>Liquorilactobacillus hilgardii</i>	Kef-w9	Water kefir	ANA	Yes
49	<i>Liquorilactobacillus hilgardii</i>	Kef-w10	Water kefir	ANA	Yes
50	<i>Zymomonas mobilis</i>	Kef-w12	Water kefir	OX	-
51	<i>Zymomonas mobilis</i>	Kef-w53	Water kefir	OX	Yes
52	<i>Zymomonas mobilis</i>	Kef-w54	Water kefir	OX	-
53	<i>Zymomonas mobilis</i>	Kef-w55	Water kefir	OX	-
54	<i>Zymomonas mobilis</i>	Kef-w56	Water kefir	OX	-
55	<i>Zymomonas mobilis</i>	Kef-w57	Water kefir	ANA	-
56	<i>Zymomonas mobilis</i>	Kef-w58	Water kefir	ANA	-
57	<i>Zymomonas mobilis</i>	Kef-w59	Water kefir	ANA	-
58	<i>Zymomonas mobilis</i>	Kef-w60	Water kefir	ANA	-
59	<i>Zymomonas mobilis</i>	Kef-w61	Water kefir	ANA	-
60	<i>Lentilactobacillus kefir</i>	Kef-m15	Milk kefir	ANA	Yes
61	<i>Lentilactobacillus kefir</i>	Kef-m62	Milk kefir	ANA	-
62	<i>Liquorilactobacillus nagelii</i>	Kef-w21	Water kefir	ANA	Yes
63	<i>Lactobacillus delbrueckii</i>	Kef-m63	Milk kefir	ANA	Yes
64	<i>Lactococcus lactis</i>	Kef-m64	Milk kefir	OX	Yes
65	<i>Lactococcus lactis</i>	Kef-m65	Milk kefir	OX	-
66	<i>Lactococcus lactis</i>	Kef-m66	Milk kefir	OX	-
67	<i>Lactococcus lactis</i>	Kef-m67	Milk kefir	OX	-
68	<i>Lactococcus lactis</i>	Kef-m68	Milk kefir	OX	Yes
69	<i>Lactococcus lactis</i>	Kef-m69	Milk kefir	OX	-
70	<i>Lactococcus lactis</i>	Kef-m70	Milk kefir	OX	-
71	<i>Lactococcus lactis</i>	Kef-m71	Milk kefir	OX	-
72	<i>Lactococcus lactis</i>	Kef-m72	Milk kefir	OX	-
73	<i>Lactococcus lactis</i>	Kef-m73	Milk kefir	OX	-
74	<i>Lactococcus lactis</i>	Kef-m74	Milk kefir	OX	-
75	<i>Lactococcus lactis</i>	Kef-m75	Milk kefir	OX	-
76	<i>Lactococcus lactis</i>	Kef-m76	Milk kefir	OX	-
77	<i>Lactococcus lactis</i>	Kef-m77	Milk kefir	OX	-
78	<i>Lactococcus lactis</i>	Kef-m78	Milk kefir	OX	-
79	<i>Lactococcus lactis</i>	Kef-m79	Milk kefir	OX	-
80	<i>Lactococcus lactis</i>	Kef-m80	Milk kefir	OX	-
81	<i>Lactococcus lactis</i>	Kef-m81	Milk kefir	OX	-
82	<i>Lactococcus lactis</i>	Kef-m82	Milk kefir	OX	-
83	<i>Lactococcus lactis</i>	Kef-m83	Milk kefir	OX	-
84	<i>Lactococcus lactis</i>	Kef-m84	Milk kefir	OX	-

85	<i>Lactococcus lactis</i>	Kef-m85	Milk kefir	ANA	-
86	<i>Lactococcus lactis</i>	Kef-m86	Milk kefir	ANA	-
87	<i>Lactococcus lactis</i>	Kef-m87	Milk kefir	ANA	-
88	<i>Lactococcus lactis</i>	Kef-m88	Milk kefir	ANA	-
89	<i>Lactococcus lactis</i>	Kef-m89	Milk kefir	ANA	Yes
90	<i>Lactococcus lactis</i>	Kef-m90	Milk kefir	ANA	-
91	<i>Lactococcus lactis</i>	Kef-m91	Milk kefir	ANA	-
92	<i>Lactococcus lactis</i>	Kef-m92	Milk kefir	ANA	-
93	<i>Lactococcus lactis</i>	Kef-m93	Milk kefir	ANA	-
94	<i>Acetobacter fabarum</i>	Kef-m94	Milk kefir	OX	Yes
95	<i>Acetobacter fabarum</i>	Kef-m95	Milk kefir	OX	-
96	<i>Acetobacter fabarum</i>	Kef-m96	Milk kefir	OX	-
97	<i>Acetobacter fabarum</i>	Kef-m97	Milk kefir	OX	-
98	<i>Acetobacter fabarum</i>	Kef-m98	Milk kefir	OX	-
99	<i>Acetobacter fabarum</i>	Kef-m99	Milk kefir	OX	-
100	<i>Acetobacter fabarum</i>	Kef-m100	Milk kefir	OX	-
101	<i>Kluyveromyces marxianus</i>	Kef-m101	Milk kefir	OX	Yes
102	<i>Kluyveromyces marxianus</i>	Kef-m102	Milk kefir	OX	-
103	<i>Kluyveromyces marxianus</i>	Kef-m103	Milk kefir	OX	-
104	<i>Kluyveromyces marxianus</i>	Kef-m104	Milk kefir	OX	-
105	<i>Kluyveromyces marxianus</i>	Kef-m105	Milk kefir	OX	-
106	<i>Kluyveromyces marxianus</i>	Kef-m106	Milk kefir	ANA	-
107	<i>Kluyveromyces marxianus</i>	Kef-m107	Milk kefir	ANA	-
108	<i>Kluyveromyces marxianus</i>	Kef-m108	Milk kefir	ANA	-
109	<i>Acetobacter orientalis</i>	Kef-m109	Milk kefir	OX	Yes
110	<i>Acetobacter orientalis</i>	Kef-m110	Milk kefir	OX	-
111	<i>Acetobacter orientalis</i>	Kef-m111	Milk kefir	OX	-
112	<i>Pichia fermentans</i>	Kef-m112	Milk kefir	OX	Yes
113	<i>Pichia fermentans</i>	Kef-m113	Milk kefir	OX	-
114	<i>Pichia fermentans</i>	Kef-w114	Water kefir	OX	-
115	<i>Saccharomyces cerevisiae</i>	Kef-m115	Milk kefir	OX	Yes
116	<i>Saccharomyces cerevisiae</i>	Kef-w116	Water kefir	OX	-
117	<i>Saccharomyces cerevisiae</i>	Kef-w117	Water kefir	OX	-
118	<i>Saccharomyces cerevisiae</i>	Kef-w118	Water kefir	OX	-
119	<i>Saccharomyces cerevisiae</i>	Kef-w119	Water kefir	OX	-
120	<i>Saccharomyces cerevisiae</i>	Kef-w120	Water kefir	OX	-
121	<i>Saccharomyces cerevisiae</i>	Kef-w121	Water kefir	OX	-
122	<i>Saccharomyces cerevisiae</i>	Kef-w122	Water kefir	OX	-
123	<i>Saccharomyces cerevisiae</i>	Kef-w123	Water kefir	OX	-
124	<i>Saccharomyces cerevisiae</i>	Kef-w124	Water kefir	OX	-
125	<i>Saccharomyces cerevisiae</i>	Kef-w125	Water kefir	OX	-
126	<i>Saccharomyces cerevisiae</i>	Kef-m126	Milk kefir	ANA	-
127	<i>Saccharomyces cerevisiae</i>	Kef-m127	Milk kefir	ANA	-
128	<i>Saccharomyces cerevisiae</i>	Kef-m128	Milk kefir	ANA	-
129	<i>Saccharomyces cerevisiae</i>	Kef-m129	Milk kefir	ANA	-
130	<i>Saccharomyces cerevisiae</i>	Kef-w130	Water kefir	ANA	-
131	<i>Saccharomyces cerevisiae</i>	Kef-w131	Water kefir	ANA	-
132	<i>Saccharomyces cerevisiae</i>	Kef-w132	Water kefir	ANA	-

133	<i>Saccharomyces cerevisiae</i>	Kef-w133	Water kefir	ANA	-
134	<i>Clostridium beijerinckii</i>	Kef-w134	Water kefir	OX	-
135	<i>Clostridium beijerinckii</i>	Kef-m135	Milk kefir	ANA	Yes
136	<i>Clostridium beijerinckii</i>	Kef-m136	Milk kefir	ANA	-
137	<i>Lachancea fermentati</i>	Kef-w137	Water kefir	OX	-
138	<i>Lachancea fermentati</i>	Kef-w138	Water kefir	OX	-
139	<i>Lachancea fermentati</i>	Kef-w139	Water kefir	OX	-
140	<i>Lachancea fermentati</i>	Kef-w140	Water kefir	OX	-
141	<i>Lachancea fermentati</i>	Kef-w141	Water kefir	OX	-
142	<i>Lachancea fermentati</i>	Kef-w142	Water kefir	OX	-
143	<i>Lachancea fermentati</i>	Kef-w143	Water kefir	OX	-
144	<i>Lachancea fermentati</i>	Kef-w144	Water kefir	OX	Yes
145	<i>Lachancea fermentati</i>	Kef-w145	Water kefir	ANA	-
146	<i>Lachancea fermentati</i>	Kef-w146	Water kefir	ANA	-
147	<i>Lachancea fermentati</i>	Kef-w147	Water kefir	ANA	-
148	<i>Candida ethanolica</i>	Kef-w148	Water kefir	OX	Yes
149	<i>Candida ethanolica</i>	Kef-w149	Water kefir	OX	-
150	<i>Candida ethanolica</i>	Kef-w150	Water kefir	OX	-
151	<i>Candida ethanolica</i>	Kef-w151	Water kefir	OX	-
152	<i>Gluconobacter oxydans</i>	Kef-w152	Water kefir	OX	Yes
153	<i>Oenococcus oeni</i>	Kef-w153	Water kefir	ANA	Yes
154	<i>Oenococcus oeni</i>	Kef-w154	Water kefir	ANA	-
155	<i>Oenococcus oeni</i>	Kef-w155	Water kefir	ANA	-
156	<i>Brettanomyces anomalus</i>	Kef-w156	Water kefir	ANA	-
157	<i>Brettanomyces anomalus</i>	Kef-w157	Water kefir	ANA	-
158	<i>Brettanomyces anomalus</i>	Kef-w158	Water kefir	ANA	Yes

Table S2: Growth conditions of microbes used as controls. Static conditions were used for incubation of all following strains.

No.	Species	Strain	Media	Temperature (°C)	OX/ANA	Duration (days)
1	<i>Lactobacillus rhamnosus</i>	GG	MRS	37°C	OX	1
2	<i>Lactobacillus plantarum</i>	WT	MRS	37°C	ANA	2
3	<i>Escherichia coli</i>	K12	LB	37°C	OX	1

Table S3: Growth conditions of pathogens. Static conditions were used for incubation of all following strains.

No.	Species	Strain	Media	Temperature (°C)	OX/ANA	Duration (days)
1	<i>Bacillus cereus</i>	ATCC 11778	Nutrient broth	30	OX	1
2	<i>Salmonella enterica</i>	ATCC-BAA-190	Nutrient broth	37	OX	1
3	<i>Vibrio parahaemolyticus</i>	ATCC 17802	Nutrient broth w/ 3% NaCl	37	OX	1
4	<i>Clostridium perfringens</i>	ATCC 13124	TSB	37	ANA	1
5	<i>Clostridium difficile</i>	ATCC 9689-FZ	TSB	37	ANA	2
6	<i>Klebsiella pneumoniae</i>	KP-1	TSB	37	OX	1
7	<i>Escherichia coli</i> O157:H7	ATCC 43888	TSB	37	OX	1
8	<i>Staphylococcus aureus</i>	USA300	TSB	37	OX	1

Table S4: Antioxidant activity, adhesion, and cytotoxicity to Caco-2 cells of selected kefir isolates. All results were presented as an average of duplicate experiments with standard deviation as indicated.

No.	Kefir isolate species	Strain	Antioxidant activity (AA%)		% adhered to Caco-2 (%)		Cyto-toxicity to Caco-2 (%)	
			Average	Stdev	Average	Stdev	Average	Stdev
1	<i>Liquorilactobacillus satsumensis</i>	Kef-w1	56%	22%	7.59%	0.04%	0.53%	0.92%
2		Kef-w2	51%	9%	1.24%	0.58%	-0.37%	1.05%
3		Kef-w11	32%	27%	10.00%	3.26%	-0.05%	0.30%
4		Kef-w13	58%	25%	3.27%	0.96%	-0.20%	1.12%
5		Kef-w18	62%	19%	8.86%	3.54%	1.21%	0.37%
6	<i>Lactobacillus helveticus</i>	Kef-m4	60%	13%	1.18%	0.31%	0.81%	0.13%
7	<i>Lactocaseibacillus paracasei</i>	Kef-w7	56%	16%	3.98%	1.11%	-0.66%	0.29%
8		Kef-w14	68%	8%	4.85%	1.85%	0.23%	0.30%
9		Kef-w17	63%	20%	3.03%	0.42%	0.07%	0.00%
10		Kef-w19	78%	6%	5.38%	0.22%	0.03%	0.13%
11	<i>Lentilactobacillus hilgardii</i>	Kef-w8	23%	33%	17.65%	5.82%	-2.14%	3.25%
12		Kef-w9	37%	30%	17.22%	10.74%	-3.24%	2.35%
13		Kef-w10	37%	17%	13.33%	6.48%	-1.62%	0.51%
14	<i>Lentilactobacillus kefir</i>	Kef-m15	69%	6%	10.22%	1.54%	0.81%	1.59%
15	<i>Liquorilactobacillus nagelii</i>	Kef-w21	72%	8%	18.08%	2.72%	1.11%	0.10%
16	<i>Lactocaseibacillus rhamnosus</i>	LGG	73%	3%	0.91%	0.20%	0.84%	0.88%

Table S5: Statistical analysis of competitive adhesion/exclusion assay.

No.	Pathogen strain	Probiotic treatment	Average % adhesion	Stdev % adhesion	Dunnett's comparison test	Mean Diff.	95% CI of diff.	Adjusted P-value	Significance level
1	<i>Bacillus cereus</i> ATCC 11778	-	0.41%	0.08%	-	-	-	-	-
2		LGG, competitive adhesion	0.14%	0.09%	Pathogen only VS. LGG, c.a.	0.270%	0.07827 to 0.4617	0.0131	*
3		LGG, competitive exclusion	0.10%	0.01%	Pathogen only VS. LGG, c.e.	0.310%	0.1183 to 0.5017	0.0073	**
4		Kefir probiotics blend, competitive adhesion	0.26%	0.00%	Pathogen only VS. Kefir, c.a.	0.150%	-0.04205 to 0.3414	0.1142	ns
5		Kefir probiotics blend, competitive exclusion	0.12%	0.03%	Pathogen only VS. Kefir, c.e.	0.287%	0.0951 to 0.4786	0.0102	*
6	<i>Salmonella enterica</i> ATCC-BAA-190	-	1.02%	0.24%	-	-	-	-	-
7		LGG, competitive adhesion	0.94%	0.01%	Pathogen only VS. LGG, c.a.	0.083%	-0.3045 to 0.4712	0.8634	ns
8		LGG, competitive exclusion	0.59%	0.03%	Pathogen only VS. LGG, c.e.	0.426%	0.03806 to 0.8138	0.0354	*
9		Kefir probiotics blend, competitive adhesion	1.09%	0.04%	Pathogen only VS. Kefir, c.a.	0.075%	0.4627 to 0.313	0.8984	ns
10		Kefir probiotics blend, competitive exclusion	0.36%	0.06%	Pathogen only VS. Kefir, c.e.	0.656%	0.2685 to 1.044	0.0060	**
11	<i>Escherichia coli</i> O157:H7 ATCC 43888	-	0.13%	0.01%	-	-	-	-	-
12		LGG, competitive adhesion	0.19%	0.02%	Pathogen only VS. LGG, c.a.	0.061%	-0.1241 to 0.0027	0.0584	ns
13		LGG, competitive exclusion	0.08%	0.02%	Pathogen only VS. LGG, c.e.	0.057%	-0.006271 to 0.1206	0.0719	ns
14		Kefir probiotics blend, competitive adhesion	0.21%	0.02%	Pathogen only VS. Kefir, c.a.	0.079%	-0.1424 to -0.01555	0.0217	*
15		Kefir probiotics blend, competitive exclusion	0.17%	0.03%	Pathogen only VS. Kefir, c.e.	0.039%	-0.1023 to 0.02452	0.2222	ns
16	<i>Vibrio parahaemolyticus</i>	-	5.70%	0.42%	-	-	-	-	-
17		LGG, competitive adhesion	8.51%	2.41%	Pathogen only VS. LGG, c.a.	2.808%	-7.041 to 1.424	0.1817	ns

18	ATCC 17802	LGG, competitive exclusion	10.96%	1.05%	Pathogen only VS. LGG, c.e.	5.255%	-9.487 to -1.023	0.0219	*
19		Kefir probiotics blend, competitive adhesion	3.67%	0.47%	Pathogen only VS. Kefir, c.a.	2.035%	-2.197 to 6.267	0.3748	ns
20		Kefir probiotics blend, competitive exclusion	2.97%	0.33%	Pathogen only VS. Kefir, c.e.	2.731%	-1.501 to 6.963	0.1954	ns
21	<i>Klebsiella pneumoniae</i> KP-1	-	0.01%	0.01%	-	-	-	-	-
22		LGG, competitive adhesion	0.00%	0.00%	Pathogen only VS. LGG, c.a.	0.011%	-0.004644 to 0.02688	0.1538	ns
23		LGG, competitive exclusion	0.01%	0.00%	Pathogen only VS. LGG, c.e.	0.008%	-0.007997 to 0.02353	0.3581	ns
24		Kefir probiotics blend, competitive adhesion	0.00%	0.00%	Pathogen only VS. Kefir, c.a.	0.012%	-0.003599 to 0.02793	0.1183	ns
25		Kefir probiotics blend, competitive exclusion	0.01%	0.00%	Pathogen only VS. Kefir, c.e.	0.009%	-0.006652 to 0.02487	0.2559	ns
26	<i>Staphylococcus aureus</i> USA300	-	10.00%	0.51%	-	-	-	-	-
27		LGG, competitive adhesion	8.57%	0.00%	Pathogen only VS. LGG, c.a.	1.429%	-0.2475 to 3.105	0.0865	ns
28		LGG, competitive exclusion	2.48%	0.03%	Pathogen only VS. LGG, c.e.	7.518%	5.842 to 9.194	0.0001	****
29		Kefir probiotics blend, competitive adhesion	3.85%	0.94%	Pathogen only VS. Kefir, c.a.	6.154%	4.478 to 7.83	0.0002	***
30		Kefir probiotics blend, competitive exclusion	3.61%	0.13%	Pathogen only VS. Kefir, c.e.	6.393%	4.717 to 8.069	0.0002	***
31	<i>Clostridium difficile</i> ATCC 9689-FZ	-	0.48%	0.03%	-	-	-	-	-
32		LGG, competitive adhesion	0.12%	0.03%	Pathogen only VS. LGG, c.a.	0.361%	0.2903 to 0.4322	0.0001	****
33		LGG, competitive exclusion	0.03%	0.00%	Pathogen only VS. LGG, c.e.	0.451%	0.3805 to 0.5224	0.0001	****
34		Kefir probiotics blend, competitive adhesion	0.09%	0.02%	Pathogen only VS. Kefir, c.a.	0.391%	0.3205 to 0.4624	0.0001	****
35		Kefir probiotics blend, competitive exclusion	0.09%	0.00%	Pathogen only VS. Kefir, c.e.	0.388%	0.3171 to 0.459	0.0001	****

36	<i>Clostridium perfringens</i> ATCC 13124	-	65.71%	32.32%	-	-	-	-	-
37		LGG, competitive adhesion	30.00%	14.14%	Pathogen only VS. LGG, c.a.	35.714 %	-19.29 to 90.68	0.1920	ns
38		LGG, competitive exclusion	10.14%	2.22%	Pathogen only VS. LGG, c.e.	55.571 %	0.6019 to 110.5	0.0481	*
39		Kefir probiotics blend, competitive adhesion	2.09%	0.00%	Pathogen only VS. Kefir, c.a.	63.619 %	8.65 to 118.6	0.0289	*
40		Kefir probiotics blend, competitive exclusion	0.82%	0.64%	Pathogen only VS. Kefir, c.e.	64.896 %	9.926 to 119.9	0.0268	*

Table S6: Sucrose-supplemented MRS media recipe for EPS production

Sucrose-supplemented MRS component	g/L
Peptone from casein	10
Lab-Lemco powder	8
Yeast Extract	4
Tween 80	1 mL
Di-potassium hydrogen phosphate trihydrate	2.62
Sodium acetate trihydrate	5
Sodium citrate	2.12
Ammonia chloride	1.32
Magnesium sulfate heptahydrate	0.2
Manganese (II) sulfate monohydrate	0.05
Sucrose	80
pH 6.2 ± 0.2 @ 25 °C, adjusted using HCl and NaOH	

Table S7: Carbon-free TS media recipe for culturing pathogenic microbes

Carbon-free TS component	g/L
Peptone from casein	17
Peptone from soya	3
Sodium chloride	5
Dipotassium hydrogen phosphate	2.5
pH 7.3 ± 0.2 @ 25°C	

Table S8: Growth conditions and dilution factors of enteric pathogens for selective substrate utilization assay

Pathogen species	Strain	Media	Incubation Temperature (°C)	Aerobic/ anaerobic (OX/ANA)	Dilution Factor
<i>Bacillus cereus</i>	ATCC 11778	NB	30	OX	10 ⁻⁴
<i>Salmonella enterica</i>	ATCC-BAA-190	NB	37	OX	10 ⁻⁴
<i>Vibrio parahaemolyticus</i>	ATCC 17802	NB	37	OX	10 ⁻⁴
<i>Clostridium perfringens</i>	ATCC 13124	TS	37	ANA	10 ⁻⁴
<i>Clostridium difficile</i>	ATCC 9689-FZ	TS	37	ANA	10 ⁻³
<i>Klebsiella pneumoniae</i>	KP-1	TS	37	OX	10 ⁻⁴
<i>Escherichia coli</i> O157:H7	ATCC 43888	TS	37	OX	10 ⁻⁴
<i>Staphylococcus aureus</i>	USA300	TS	37	OX	10 ⁻⁴

Table S9: Fecal fermentation experiment - Donor details

Donor No.	Donor 1	Donor 2	Donor 3
Age	50	32	69
Gender	Male	Male	Female
Race	Chinese	Chinese	Chinese
Height (cm)	174	176	157
Weight (kg)	72	85	59
Smoking history	Last smoked on 27-Sept-19	None	None
Any illness in the past month?	No	No	No
Average hours of sleep in past week?	7	6-7	8
Hours of sleep previous night	8	7	7
Feeling well on day itself?	Yes	Yes	Yes
GPAQ level	3680	2060	3600
Active/Non-active	Highly active	Moderately active	Highly active
Date of collection	21-Jan-21	1-Feb-21	22-Mar-21
Has he/she been living in Singapore for the past 6 months?	Yes	Yes	Yes
Any alcohol consumption, what types and how much of alcohol consumed per month?	No	No	No
Any antibiotic consumption in the past 3 months?	No	No	No
Any probiotic consumption, what types and how often are the probiotics supplements/foods consumed?	No	Consumes Yakult Ace (1 bottle/day), Meiji yogurt (1 bottle/day), Greek yogurt (4 scoops/day)	Consumes yogurt regularly
Any family history of gastrointestinal problems, e.g. diarrhea, constipation, bloatedness, ulcerative colitis, irritable bowel syndrome, cancer within the gastrointestinal area?	No	No	Bloatedness, loose stool and constipation occurring once a month
Any past diagnosis of gastrointestinal problems within the past 1 month?	No	No	No

Any vaccination in the past 6 months?	No	No	No
Any long-term prescribed medicine, including Traditional Chinese medicine and other alternative medicine?	No	No	No
Any past diagnosis of cancer, diabetes, any neurological disorders, or any psychiatric disorders? If yes, what is the current status of the diagnosed condition?	No	No	Diagnosed with pre-diabetes 5 years ago, but was subsequently cleared
Any dengue fever or zika virus infection in the past year?	No	No	No
Any allergies?	Allergic to naproxen	No	Allergic to penicillin

Table S10: Catalase, lipopolysaccharide (LPS), and EPS-related genes in EPS-producing water kefir bacterial isolates.

No.	Kefir isolate species	Strain	Colony Morphology	Genbank accession number	Catalase	LPS	EPS-related genes	
							Gene_id	Function
1	<i>Liquorilactobacillus satsumensis</i>	Kef-w1	Smooth	JAGPZX00000000	Absent	Absent	KBX33_00115	Polysaccharide biosynthesis
							KBX33_02945	EPS Pel transporter PelG
							KBX33_03315	EPS biosynthesis polyprenyl glycosylphosphotransferase
							KBX33_03345	Polysaccharide biosynthesis C-terminal
							KBX33_06540	Polysaccharide biosynthesis
							KBX33_07255	Polysaccharide pyruvyl transferase
							KBX33_08400	Polysaccharide biosynthesis C-terminal
2	<i>Liquorilactobacillus satsumensis</i>	Kef-w2	Smooth	JAGPZW00000000	Absent	Absent	KBX33_09560	Polysaccharide biosynthesis C-terminal
							KBX49_01940	Polysaccharide pyruvyl transferase
							KBX49_02555	Polysaccharide biosynthesis C-terminal
							KBX49_04300	Polysaccharide biosynthesis
							KBX49_04970	Polysaccharide biosynthesis C-terminal
							KBX49_09700	EPS biosynthesis polyprenyl glycosylphosphotransferase
3	<i>Liquorilactobacillus satsumensis</i>	Kef-w11	Irregular	JAGPZN00000000	Absent	Absent	KBX49_11685	Polysaccharide biosynthesis
							KBX30_02565	EPS Pel transporter PelG
							KBX30_03730	Polysaccharide biosynthesis

							KBX30_05535	Polysaccharide biosynthesis
							KBX30_06680	Polysaccharide biosynthesis C-terminal
							KBX30_07305	Polysaccharide biosynthesis C-terminal
							KBX30_08055	Polysaccharide pyruvyl transferase
4		Kef-w13	Smooth	JAGPZL00000000	Absent	Absent	KBX40_01940	Polysaccharide pyruvyl transferase
							KBX40_02555	Polysaccharide biosynthesis C-terminal
							KBX40_04265	Polysaccharide biosynthesis
							KBX40_05535	Polysaccharide biosynthesis C-terminal
							KBX40_06130	Polysaccharide biosynthesis
							KBX40_09730	EPS biosynthesis polyprenyl glycosylphosphotransferase
5		Kef-w18	Irregular	JAGPZG00000000	Absent	Absent	KBX31_02995	Polysaccharide biosynthesis
							KBX31_04735	Polysaccharide pyruvyl transferase
							KBX31_05265	Polysaccharide biosynthesis
							KBX31_07175	Polysaccharide biosynthesis C-terminal
							KBX31_07515	EPS Pel transporter PelG
							KBX31_09315	Polysaccharide biosynthesis C-terminal
6		Kef-w8	Smooth	JAGPZQ00000000	Absent	Absent	KBX59_06670	Polysaccharide biosynthesis
							KBX59_08435	Polysaccharide biosynthesis
7	<i>Liquorilactobacillus hilgardii</i>	Kef-w9	Smooth	JAGPZP00000000	Absent	Absent	KBX56_05195	Polysaccharide biosynthesis
							KBX56_06975	Polysaccharide biosynthesis
8		Kef-w10	Smooth	JAGPZO00000000	Absent	Absent	KBX58_05300	Polysaccharide biosynthesis
							KBX58_07080	Polysaccharide biosynthesis
9	<i>Liquorilactobacillus nagelii</i>	Kef-w21	Smooth	JAGPZD00000000	Absent	Present	KBX38_02505	Polysaccharide biosynthesis
							KBX38_04595	Polysaccharide pyruvyl transferase
							KBX38_06360	Polysaccharide biosynthesis C-terminal
							KBX38_07565	Polysaccharide biosynthesis
							KBX38_11520	Polysaccharide biosynthesis C-terminal
10	<i>Zymomonas mobilis</i>	Kef-w12	Smooth	JAGPZM00000000	Present	Present	-	-

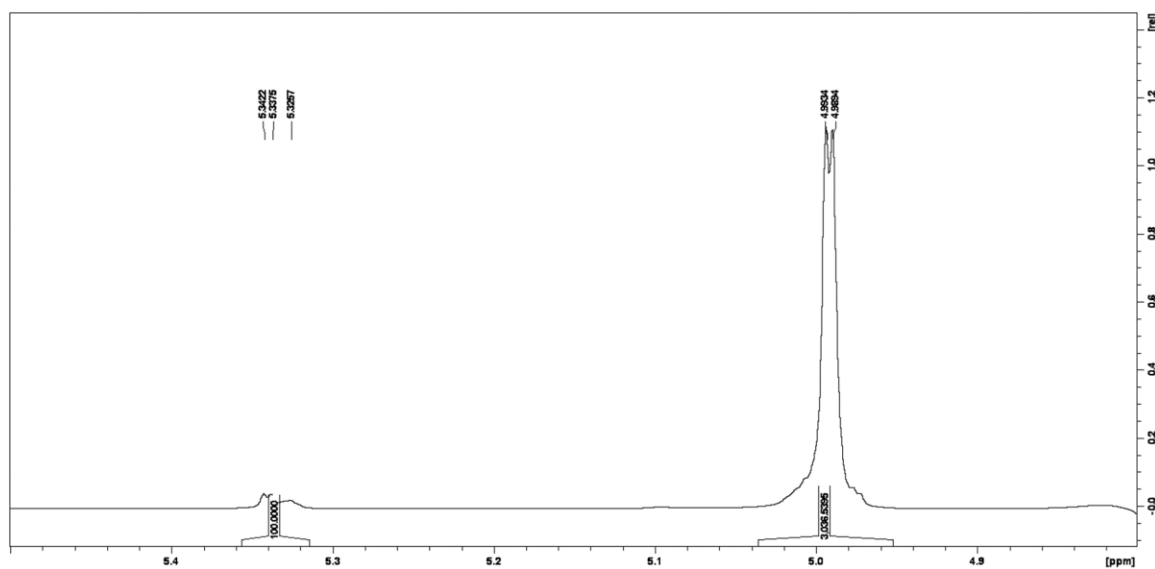


Figure S1: ^1H -NMR spectrum of commercial dextran

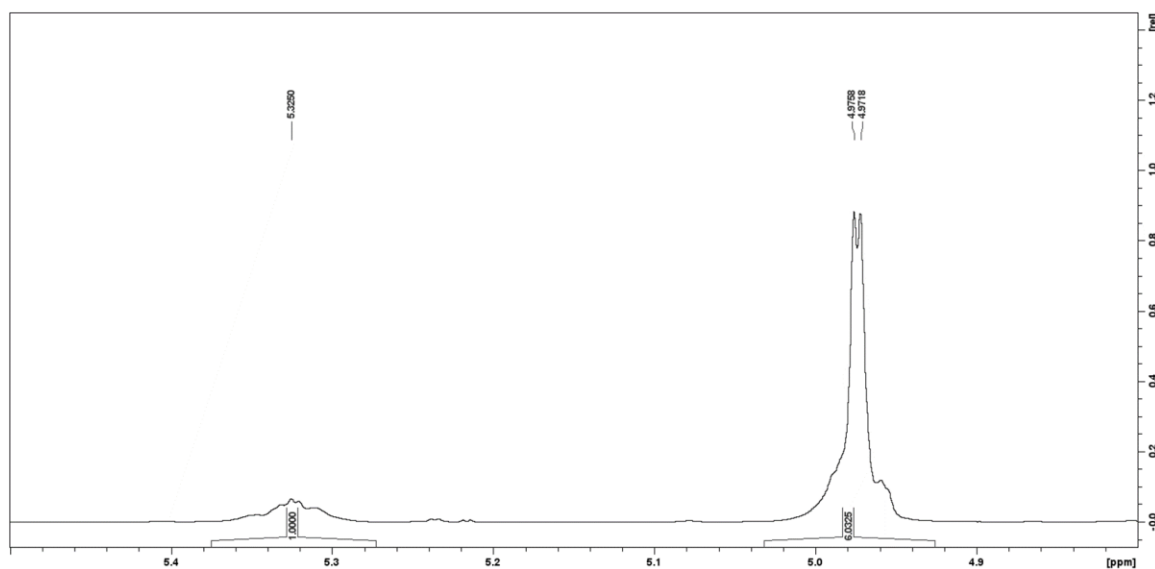


Figure S2: ^1H -NMR spectrum of hydrolysed polysaccharide derived from *L. satsumensis* Kef-w1

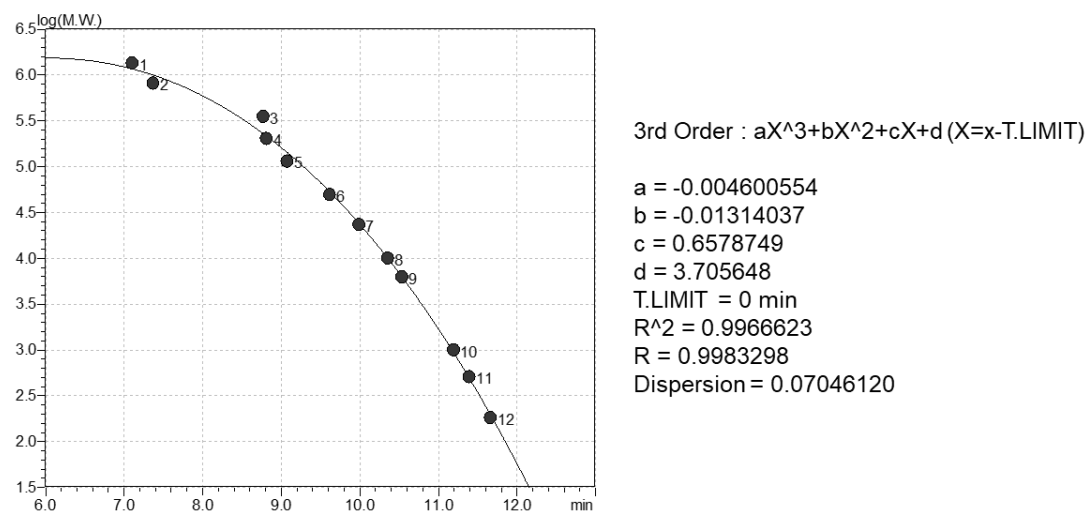


Figure S3: GPC calibration curve

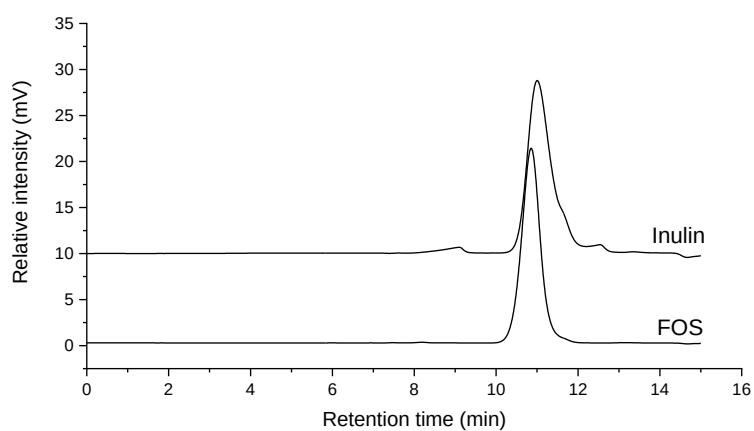


Figure S4: GPC chromatogram of inulin and FOS prebiotics.

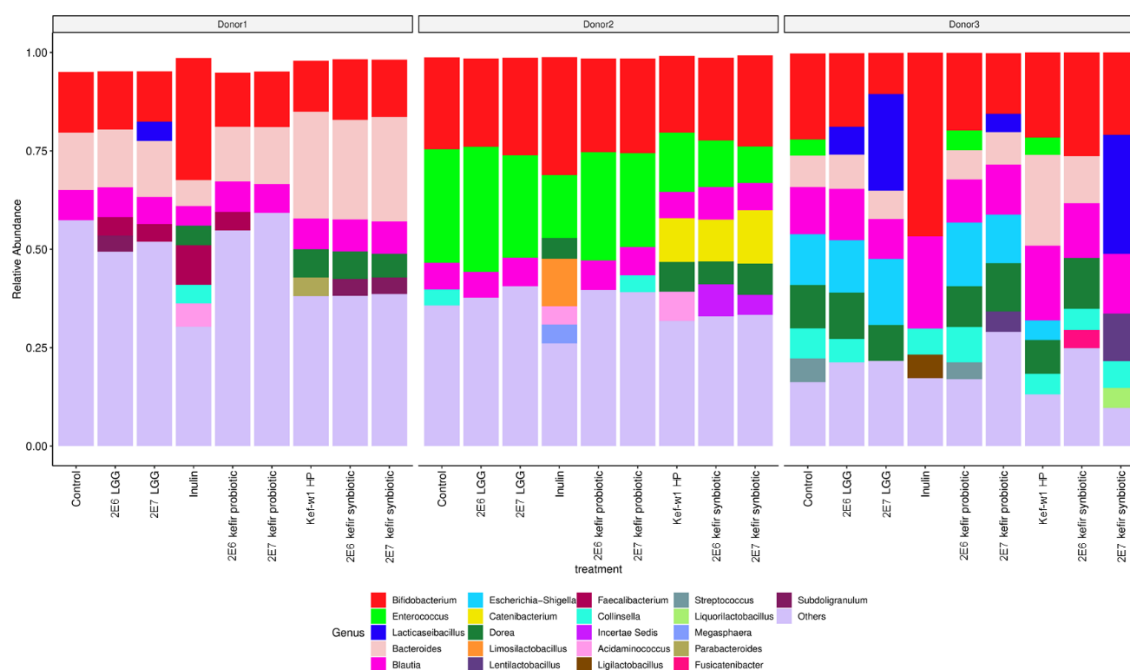


Figure S5: Genera-level community composition of *ex-vivo* microcosms (large bowel simulated model) based on various treatments. Genera with abundance <1% were combined into Others.

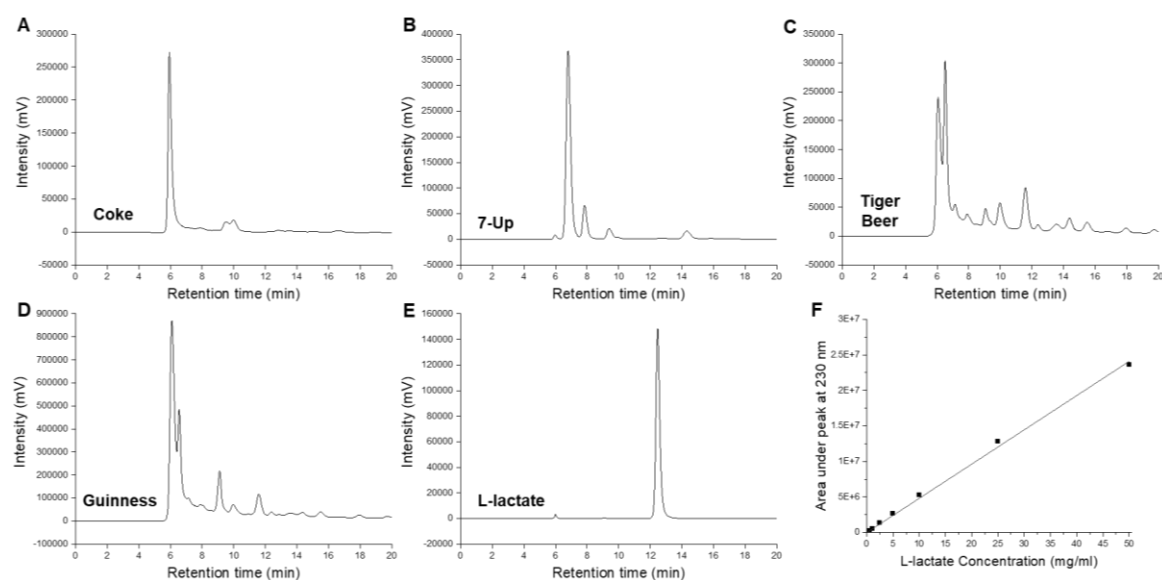


Figure S6: HPLC chromatograms of (a) Coke, (b) 7-Up, (c) Tiger Beer, and (d) Guinness. The L-lactate chromatogram is presented as (e), while the standard curve as (f).

Beverage	Ingredients; pH
	Carbonated water, sucrose, caramel colour, phosphoric acid, flavourings, caffeine, acesulfame potassium, sucralose; pH: 2.26
	Carbonated water, sugar, flavouring, citric acid, malic acid, sodium citrate, potassium sorbate, stevia extract, sodium benzoate, acesulfame potassium, sucralose; pH: 3.20
	Water, malted barley, sugar, hops; pH: 4.44
	Water, malt, barley, hops; pH: 3.71

Figure S7: Nutrition information and ingredient list of four tested beverages, Coke, 7-Up, Tiger Beer and Guinness.