Integration of the functional properties of coconut water kefir into sourdough bread

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Abstract

Sourdough technology is an ancient technology that has been widely described and utilized due to its ability to improve the nutritional quality, texture, sensory properties, flavour and volatile compound composition, and shelf life of the bread. Many studies have reported that sourdough fermented using milk kefir has the ability to produce metabolites such as organic acids, amino acids, exopolysaccharides (EPS) and/or enzymes such as phytases, due to the presence of microorganisms such as lactic acid bacteria (LAB), acetic acid bacteria (AAB) and yeasts. However, few studies have reported on fermentation of sourdough using a coconut water-based medium and kefir grains.

The advantages of using coconut water is its rich nutritional profile with high amounts of glutamic acid, vitamin C, magnesium, vitamin B, arginine, alanine, lysine, calcium, potassium, minerals and antioxidants. These nutrients and sugar content make it an ideal substrate for fermentation.

Kefir itself has many claimed health benefits such as the anticarcinogenic and antimutagenic properties, immunomodulating properties, anti-inflammatory and hypocholesterolemic effects, antihypertensive, antidiabetic, antimicrobial properties and enhanced lactose utilisation. These health effects may be attributed to several components such as the microogorganisms such as LAB, AAB and yeast, exopolysaccharides, organic acids, antioxidants, and bioactive peptides. However, few studies have reported on fermentation of sourdough using a coconut water-based medium and kefir grains.

This thesis aimed at developing a novel sourdough bread with increased functional properties using fermented coconut water kefir culture and pure culture isolates identified from it. The kefir microorganisms ferment the coconut water by consuming the small quantity of sugar available in the substrate.

A study was carried out to understand the fermentation of coconut water kefir supplemented with different sugars (such as glucose and sucrose) at various concentrations. Based on the microbial cell count results, coconut water kefir (CWK) supplemented with 12 g/L of sucrose fermented for 48 h was identified as a suitable material to prepare a sourdough (Chapter 3). Using this CWK culture, a sourdough was prepared and incubated at different temperatures of 4°C, 22°C and 30°C from 0-96h to study the microbial and physico-chemical profile of the sourdough. The sourdough fermentation temperature of 30°C was chosen to incubate the sourdough bread (Chapter 4). The high microbial counts were accompanied by a sharp decline in pH, increase in lactic acid production (D-/L- lactic acid) and increase in TTA, which consequently affected the overall viscosity of the CWK fermented sourdough. The dough volume increased with time when incubated at 30°C temperature. The texture of the CWK fermented sourdough had higher values for hardness, resilience, chewiness, gumminess and springiness after incubation for 48, 72 and 96

h at 30°C. There was significant production of organic acids such as lactic acid (9998.7 ± 18.5 mg/L, 96 h), pyruvic acid (1394.8 ± 2.95 mg/L, 96 h), succinic acid (883.4 ± 2.4 mg/L, 96 h) and malic acid (31.4 ± 8.5 mg/L, 96 h) as well as amino acids such as glutamic acid were produced in notably high quantities.

Five (5) lactic acid bacteria species, 3 acetic acid bacteria species and 5 yeast species were identified and confirmed from kefir, fermented CWK and CWK fermented sourdough. Illumina sequencing was carried out to understand the change in the microbial ecology of a CWK fermented sourdough over time (between 0-96h) (Chapter 5). Each confirmed isolate was assessed for possessing any functional properties such as production of glutamic acid (a flavour enhancing amino acid and a precursor of γ-aminobutyric acid (GABA), phytase enzyme (which reacts with phytate) and EPS production (which acts as a hydrocolloid to improve the overall textural properties of the sourdough bread). Each of these properties was quantified for each isolate (Chapter 6).

The isolates which produced the highest quantities of phytase enzyme (4052.5 ± 171.1 U/ml and 3151.1 ± 383.0 U/ml, respectively) and glutamic acid (260.3 ± 12.1 µmoles/L and 264.89 ± 8.5 µmoles/L, respectively) were identified as *L. fermentum* and *L. plantarum*. These isolates were added in different concentrations (low range of 4.9-5.0 log CFU/ml to the high range of 8.3-9.6 log CFU/ml) to the sourdough to develop a functional sourdough bread. Sensory analyses were carried out for eight experimental sourdough breads but only three sourdough breads were selected based on the outcome of the sensory analysis. Determinations of texture, protein, ash, moisture, carbohydrate, total dietary fibre, total fats, total titratable acidity, and shelf life were carried out on the three finalised sourdough breads. The glycaemic index analysis was also carried out to estimate the impact of the formulated sourdough bread on the blood-glucose level of the consumer. Carboxylic acid analysis and amino acid analysis revealed that significant quantities of carboxylic acids such as lactic acid (828.8 ± 4.8 mg/L) and glutamic acid (187.2 ± 1.5 mg/L) and amino acids such as alanine (654.71 ± 79.24µM/L), proline (310.28 ± 18.93 µM/L) and lysine (156.08 ± 27.11 µM/L) were produced in these sourdough breads which are important flavour components which influence the overall sensory profile of the bread. Production of acids improves the shelf life of the bread due to the lowering of pH and increase in the acidity of the bread, which prevents the growth of spoilage microorganisms (Chapter 7).

In conclusion, kefir grains were able to utilise coconut water supplemented with 12 g/L sucrose as a substrate, which could be used to develop a novel and a functional sourdough. Out of the 13 identified species of LAB, AAB and yeast, two LAB species (*L. plantarum* and *L. fermentum*) which produced the highest quantities of glutamic acid, EPS, and phytase enzyme were incorporated along with the CWK to prepare a sourdough. The importance of phytase enzyme is attributed to its ability to degrade the phytic acid, which in turn leads to increase in the bioavailability of minerals such as calcium, zinc, and iron in its consumers. Glutamic acid, being

the precursor of an important bioactive compound, GABA, is desired in high concentrations in the bread due to the important health properties of GABA to the humans. EPS acts as a hydrocolloid in the bread, which positively influences the textural properties of the final bread product.

The isolates which produced significantly highest quantities of glutamic acid and phytase enzyme were selected for addition into a novel CWK-fermented sourdough bread. Eight such sourdough breads were developed and only three were shortlisted based on the sensory analyses carried out on them. The three short listed breads were analysed for textural properties, PROXIMATE properties, metabolite production such as carboxylic acids and amino acid production and finally, the glycaemic index for each of the three formulated sourdough bread was also determined.

Overall, the integration of functional properties such as of phytase, glutamic acid, EPS (imparting hydrocolloid-like properties) was successfully achieved due to the identification and incorporation of isolates (*Lactobacillus plantarum* and *Lactobacillus fermentum*) into the sourdough. Significant quantity of glutamic acid was detected in the final sourdough bread product and lower GI values for each participant was obtained upon the consumption of the CWK-sourdough bread. Since glutamic acid, phytase, EPS and low GI have been recognised and associated to as health-promoting factors, it could be ascertained that the CWK-sourdough bread is a functional bread product with all of the above-mentioned properties.

The textural and sensory profile of the breads were largely liked by the participants. On the whole, the sensory profile of the CWK fermented sourdough bread was pleasant and was associated with many positive attributes such as sour, porous, moist, light, heterogenous, spongy and buttery.

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Attestation of Authorship

"I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning."

Mansi Jayantikumar Limbad

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List of Abbreviations

Abbreviation	Full form
µl	Micro litres
µMOLES	Micro moles
HQ	2-hydrazinoquinoline
MOPS	3-[N-Morpholino] propanesulfonic acid
CBA	4-Chlorobenzoic acid
AAB	Acetic acid bacteria
AACC	American Association of Cereal Chemists
AAB	acetic acid bacteria agar
API	Analytical Profile Index
ACE	angiotensin-converting enzyme
ANOVA	Analysis of variance
AOAC	Association of Analytical Communities
AUTEC	Auckland University of Technology Ethics Committee
BSE	back-scatter detector
CA	Chalmers agar
CA+S	Chalmers agar in the presence of 5% sucrose and without Calcium carbonate
CA+S+YE	Chalmers agar supplemented with 5% sucrose and 0.5% YE
CA+S-YE	Chalmers agar supplemented with 5% sucrose and no yeast extract
CVA	Canonical Variate Analysis
CFU/ML	Colony-forming units per millilitre
CATA	Check-All-That-Apply
D-	Dextro-rotatory
DNS	Di-nitro Salicylic acid method
DPDS	Dipyridyl disulfide
DNA	Deoxyribonucleic acid
DY	Dough yield
E-DNA	Extracellular DNA
EPS	Exopolysaccharides
EMP	Embden Meyerhof Parnas pathway
EXB	Extraction reagent blanks
G	Gram
GABA	Gamma-Aminobutyric acid
GLU	Glucose
GAD	glutamate decarboxylase
GI	Glycaemic index
H	Hours
HDP	Heavy Duty Platform
HMW	High molecular weight
HSD- TUKEY'S TEST	Honestly significant difference

IUPAC-IUB	International Union of Biochemistry
IPS	Intracellular polysaccharides
KV	Kilo Volts
L	Litre
L-	Leavo Rotatory
LAB	Lactic acid bacteria
LPS	lipopolysaccharides
LC-MS	Liquid Chromatography- Mass Spectrometry
LMW	low molecular weight
M	Metre
ME	Malt extract agar
MCF	methyl chloroformate
MG	Milligram
MIN	Minutes
ML	Milli litre
MM	Milli meter
mM	Milli moles
MSG	Monosodium glutamate
MRS	De Man, Rogosa and Sharpe agar
MANOVA	Multivariate Analysis of Variance
NaOH	Sodium Hydroxide
NCBI-BLAST	National Centre for Biotechnology Information - The Basic Local Alignment Search Tool
OTUs	Operational Taxonomic Units
O.D	Optical Density
OMC	Overly Mature Coconut
Pa.s	Pascal*second
Pa	Pascal
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PCR DGGE	Polymerase Chain Reaction – Deganturing Gradient Gel Electrophoresis
PERMANOVA	Permutational multivariate analysis of variance
POD	Peroxidase
PDM	Polymer dry mass/kg
PPO	Polyphenoloxidase
PC	Principal component
PCA	Principal component analysis
PROXIMATE	Protein, Ash, Moisture, Fats, Carbohydrates, Lipids and Protein
R-STRAIN	Rough surfaced colony
SE	Secondary electrons
SEM	Scanning Electron Microscopy

SCFA	Short chain fatty acids
ASCA	Simultaneous Component Analysis
SIC	Single ion counts
S-STRAIN	Smooth surfaced colony
SD	Standard deviation
SUC	Sucrose
T	Time
TCA	Tricarboxylic acid cycle
TDS	Temporal Dominance of Sensation
TPA	Texture Profile Analysis
TIC	Total Ion Counts
TPP	Triphenylphosphine
TTA	Total titratable acidity
U/ML	UNITS/ML
UPLC	Ultra Performance Liquid Chromatography
USDA	United States Department of Agriculture
V/V	Volume/Volume
W/V	Weight/Volume
W/W	Weight/Weight
YE	Yeast Extract

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As I reach towards the end of my exciting journey as a PhD student in New Zealand, I have not only grown as a researcher but also as an individual as well with life-changing experiences. Getting to the end was not at all easy for me and would not be possible without the guidance and support of all the people near and dear to me, everyone important in their own way.

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“The important thing is to not stop questioning. Curiosity has its own reason for existing”

-Albert Einstein

“All our dreams can come true… if we have the courage to pursue them.”

– Walt Disney

Chapter 1 Thesis introduction and framework

1.1 Thesis introduction

1.1.1 Impact of kefir microorganisms on consumer health

Kefir grains are a cooperative association of microorganisms such as lactic acid bacteria, yeasts and acetic acid bacteria (Arslan, 2015; Pogacic, Sinko, Zamberlin, & Samarzija, 2013). Nalbantoglu et al. (2014) have reported that due to the composition of kefir grains, they are considered as a probiotic resource. Some studies suggest that there are abundant probiotic bacteria present in the gut of kefir consumers, which is correlated to the improvement of health of those individuals (Ahmed et al., 2013; Zheng et al., 2013). Studies by Farnworth (2006) and Rizk et al (2009) have demonstrated that a cell-free extract of kefir enhances the lactose digestibility and has gut relieving properties. Kefir is a potential factor associated with the long life expectancy of the Caucasus population, owing to its many health benefits such as immune-modulation, cholesterol-lowering, anti-allergenic, anti-stress properties, anti-asthmatic, anti-microbial, anti-cancer properties and chemoprevention against colon cancer, aside from its gastrointestinal beneficial effects (Hsu et al., 2018; Khoury et al., 2014; Kim, Chon, Kim, & Seo, 2015; Liu et al., 2006; Miao et al., 2016; Sharifi et al., 2017). Additionally, it has been demonstrated that a mixture of bacteria and yeast, isolated from kefir, is able to prevent diarrhoea and enterocolitis caused by *Clostridium difficile* (Bolla et al., 2013). Besides, kefir showed good efficacy in inhibiting spore formation and aflatoxin B1 production by the fungus *Aspergillus flavus*, which is a toxic compound formed either in the field or during food storage. Therefore, kefir appears as a promising, safe alternative and natural food preservative offering protection against intoxication with aflatoxin B1 (Ismaiel et al., 2011).

1.1.2 Use of non-dairy fermentation media for kefir grains

Apart from using cows' milk, alternative milk substrates such as goats' milk, sheeps' milk, buffalos' milk, camels' milk as well as whey substrates are used to ferment kefir grains (Addeo et al., 2007; Arslan, 2015; Gao and Li, 2016; Magalhaes et al., 2010; Magalhães et al., 2011; Nielsen et al., 2014; Rosa et al., 2017). Due to the positive effect of kefir grains on human health, many non-dairy substances have been tested for their use as fermentation media. Substrates such as soy milk, walnut milk, coconut milk and coconut water, rice and peanut milk have been used (Bau, Garcia & Ida, 2013; Bau, Garcia & Ida, 2015; Bensmira & Jiang, 2012; Cui, Chen, Wang, & Han, 2013; Gao, Zhao, Wu, Brocca & Zhang, 2016; John & Deeseenthum, 2000; Nielsen, Gürakan & Ünlü 2014). Some other sugar-rich substrates such as honey, ginger-beer base, sugar cane juice, molasses, beer wort, cocoa pulp and other fruit and vegetable juices have also been

used (Alzate et al., 2016; Bau et al., 2013; Bau et al., 2015; Corona et al., 2016; Fiorda et al., 2016; Fiorda et al., 2017; Gao and Li, 2016; Nielsen et al., 2014; Puerari et al., 2012; Randazzo et al., 2016; Silva et al., 2009; Zanirati et al., 2015).

1.1.3 Consumer demands for functional sourdough breads

The appreciation of sourdough bread by consumers has increased in recent years, not only due to better knowledge of its nutritional properties, but also because consumers enjoy the stronger flavour of sourdough bread compared with yeasted bread. The flavour of the sourdough bread is enhanced by many factors such as type of cereal flour used, the parameters involved in the preparation of sourdough, the conditions of baking and the metabolism of the fermenting microorganisms. Due to the metabolic activities of these microorganisms, there is enhanced proteolysis in the sourdough which leads to the formation of amino acids. These amino acids are the precursors of aromatic flavour molecules which are the major factor affecting the taste (Katina, Heiniö, Autio & Poutanen, 2006; Pétel, Onno & Prost, 2017).

Compared to sourdough bread, yeast leavened bread has a less strong flavour which most likely occurs due to the slower metabolism in yeast compared that of LAB, hence, lesser production of flavour amino acids (Papadimitriou et al., 2019).

Consumer interest in the healthy aspects of food is continuously growing. The traditional foods are quite often perceived as having nutritional properties that help improve human health and well-being. In the past decade, sourdough fermentation has been associated with the health-promoting properties of bread, and numerous beneficial effects have been reported such as the reduction in phytate content, increased mineral bioavailability, potential anti-oxidant effect, reduction in post-prandial glycaemic response, prolamin hydrolysis and reduction in toxic effect and potential anti-hypertensive effect (Catzeddu, 2019). An increase in iron bioavailability in sourdough bread compared with yeasted bread was demonstrated by Rodriguez-Ramiro et al. (2017) using an intestinal cellular model. The capacity of sourdough microorganisms to hydrolyze gluten proteins has also been utilised to produce gluten-free wheat flour-based bread, which was found not to be harmful to patients with celiac disease (Greco et al., 2011; Rizzello et al., 2014).

1.2 Rationale and Significance of the study

Considering the above, the main aim of this thesis was to study the complete fermentation profile of coconut water kefir and its use as a potential medium as an alternative to milk. The second aim of this thesis was to incorporate the fermented coconut water kefir, to develop a functional sourdough bread. The specific objectives for each chapter were:

i. To study the fermentation profile of the kefir grains to produce a fermented beverage using coconut water as a substrate.
ii. To develop a sourdough using fermented coconut water kefir culture and characterise the sourdough microbially and physico-chemically.
iii. To identify the microbiota in coconut water, kefir, coconut water kefir (CWK) and CWK sourdough using culture-dependent techniques and DNA sequencing methods.
iv. To determine the functional properties of microorganisms identified from kefir, CWK and CWK fermented sourdough.
v. To determine the sensory, physico-chemical profile and glycaemic index of the formulated sourdough bread.

1.3 Structure of thesis

The thesis is a multidisciplinary investigation involving microbiology, food product development and formulation, physico-chemical and biochemical assays, metabolomics, sensory science and advanced statistics.

Chapter one is a general introduction discussing the issues and challenges associated with the limited use of nutrient-rich media (other than milk) for fermenting kefir grains. To the best of my knowledge, this is the first scientific research carried out to understand the fermentation profile of kefir grains using coconut water as the medium. The use of an alternative media to ferment kefir grains, such as fruit and vegetable juices, could provide a healthy and nutritious option for the development of health drinks. General information about the status of kefir-based fermented drinks worldwide has been provided. The impacts of kefir microorganisms on the health of the consumer was discussed and the importance of consuming food developed using probiotic microorganisms such as lactic acid bacteria (LAB) was also highlighted. Finally, the most important challenge, as pointed out by previous studies, is to meet the consumer's demand for developing a sourdough bread with functional properties, high nutrition value, improved flavour, texture and shelf life, low glycaemic index, presence of significant quantities of metabolites such as amino acids and carboxylic acids in the bread.

In Chapter two, a broad literature review was conducted and discussed for kefir grains and the process of kefir grain formation, microbial diversity, the biochemistry of the LAB, health benefits of kefir grains and their application in dairy and non-dairy foods. The use of coconut water as a fermentation medium was highlighted and the gaps in the knowledge about the fermentation of kefir grains with coconut water were acknowledged. This chapter also provided a comprehensive review on topics such as the background of sourdough and types of sourdough along with the importance of functional properties such as phytase activity, production of amino acids, e.g. glutamic acid, exopolysaccharide production and determination of glycaemic index of the final

bread product. This chapter provided the background for this study and proposed practical suggestions and considerations for using fermented coconut water kefir to develop a sourdough with appropriate functional properties.

Chapter three investigated the fermentation profile of coconut water kefir. Microbial profile, amino acid and carboxylic acid production were determined to describe what occurred during fermentation. Scanning electron microscopy was carried out to visualise the kefir grains extracted from kefir-fermented coconut water (CWK). This chapter provides further information about the importance of the fermentation time and the effect of supplemented sugar, such as sucrose and glucose, on the microbial growth during the CWK fermentation.

The next important step was to create a sourdough using the fermented coconut water kefir and study the importance of temperature on the fermentation profile and the physico-chemical properties of the sourdough. This was studied in Chapter four. The organic acid profile, concentrations of D- and L-lactic acid produced, dough leavening, texture and viscosity were determined. Most importantly, the glutamic acid concentration of the dough was also determined.

In Chapter five, the microorganisms were identified using culture-dependent phenotypic identification methods, and by using culture-independent sequencing methods such as Sanger sequencing and Illumina MiSeq sequencing. Five yeast species were confirmed using phenotypic and biochemical identification techniques. Sanger sequencing confirmed the five lactic acid bacteria (LAB) species and three acetic acid bacteria (AAB) species that were identified from kefir, coconut water kefir and coconut water kefir fermented sourdough. Illumina sequencing helped identify the diversity of microorganisms present in coconut water, kefir, fermented CWK and CWK fermented sourdough with time.

The objective of Chapter six was to determine whether the identified LAB, AAB and yeast isolates were capable of possessing any desirable functional properties such as production of phytase enzyme, exopolysaccharides (EPS) and amino acids (such as glutamic acid). Once the species with the highest phytase enzyme activity and capability to produce glutamic acid were identified, they were chosen to prepare a functional sourdough bread. This led to the objective of Chapter seven.

The objective of Chapter seven was to produce a functional sourdough bread using the microorganisms identified with the highest phytase enzyme activity and glutamic acid-producing capability in addition to using fermented CWK as the starter. The glycaemic index of the final bread products was also determined to evaluate the change in the blood glucose level when the bread product(s) were consumed.

1.4 Research outputs from this thesis

Potential peer-reviewed journal publications

Limbad, M. L., Hamid, N., Kantono, K., Pook C., Young, T., Gutierrez-Maddox, N. (2020). Fermentation profile of coconut water kefir. Manuscript in preparation.

Limbad, M. L., Hamid, N., Kantono, K., Pook C., Gutierrez-Maddox, N. (2020). Microbial and physico-chemical characteristics of sourdough prepared with fermented coconut water kefir. Manuscript in preparation.

Limbad, M. L., Hamid, N., Gutierrez-Maddox, N. (2020). Identification of the microbiota in coconut water, kefir, CWK and CWK fermented sourdough using culture-dependent techniques and Illumina-MiSeq sequencing. Manuscript in preparation.

Limbad, M. L., Hamid, N., Gutierrez-Maddox, N. (2020). Functional properties of microorganisms isolated from the formulated sourdough, CWK and kefir. Manuscript in preparation.

Chapter 2 Literature Review

2.1. Kefir

2.1.1 Kefir History

Kefir grains originated from the Caucasus Mountains of the Soviet Union and have been a popular fermented drink for consumption in Russia, Eastern Europe and Middle Eastern countries. It was originally consumed as a fermented milk beverage with kefir grains (Libudzisz & Piatkiewicz, 1990).

The name 'kefir' has emerged from 'keyif', which is a Turkish word meaning 'good feeling' (Kabak & Dobson, 2011). Kefir was produced by the fermentation of milk with kefir grains giving rise to a viscous, acidic, slightly alcoholic and occasionally carbonated probiotic drink (Garrote et al., 2001; Marshall et al., 1984; Yaman et al., 2010). Kefir grains which are irregularly shaped and white or pale white in colour, consist of a combination of various lactic acid bacteria (LAB) and yeast species that are used for developing fermented food products (Beshkova et al., 2002; Simova et al., 2002; Witthuhn et al., 2005). The yeasts and LAB are present in a very robust polysaccharide matrix consisting of galactose and glucose exopolysaccharide (EPS), which is often termed, 'kefiran'. The size of kefir grains varies over a large range of a few millimetres to a few centimetres (Garrrote et al., 2001; Yaman et al., 2010).

With appropriate sub-culturing, kefir grains can remain active for several weeks if grown under precise conditions (Simova et al., 2002). Subculturing can be carried out by adding kefir grains (between 2-10%) to milk (that has been boiled and then cooled down to room temperature of about 25°C) and allowing it ferment for 18-24 h at 25°C (Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011; Otles & Cagindi, 2003; Witthuhn et al., 2005). Acids such as lactic acid, pyruvic acid, acetic acid, hippuric acid, butyric acid, propionic acid, diacetyl, and acetaldehyde are generated during fermentation, that are mainly responsible for imparting the characteristic taste and aroma to kefir (Ahmed et al., 2013; Kesenkas, Dinkcedil, Seccedil, & Gouml, 2011; Kesenkas, Yerlikaya, & Ozer, 2013). Diacetyl is produced by *Streptococcus lactis subsp. diacetlyactis* and some *Leuconostoc spp*. (Cagindi, 2003).

The chemical composition of kefir has been reported in varying concentrations as shown in Table 2.1. Although much of the data are missing, the declared compositions are roughly similar.

Table 2.1. Typical composition of kefir.

Chemical composition of kefir % (w/w)				
	Sarkar, 2007	Wszolek, Tamime, Muir, & Barclay, 2001	Liutkevičius & Šarkinas, 2004	Magalhães, Pereira, Campos, Dragone, & Schwan, 2011
Total solids	Not available	10.6-14.9	Not available	9.62
Crude protein	3	2.9-6.4	4.5	3.61
Carbohydrate	6	3.8-4.7	Not given	Not given
Ash	0.7	0.7-1.1	1.2	Not given
Moisture	89	Not given	86.3	Not given
Fats	0.02	Not given	0.03	2.34

2.1.2 Kefir grain formation

Kefir grains are irregularly shaped, cauliflower blossom-like structures that range in diameter of 3 to 35mm. Scanning electron microscopy of kefir grains suggests that the inner composition of the grains is highly irregular in shape and consists of unstructured material, whereas, the outer biofilm is much more complex consisting of agglomerate of various LAB and yeast species. They also have a very distinctive smell when they increase by about 25% in mass (Magalhães, Pereira, Campos, Dragone, & Schwan, 2011). Recently, the structure of kefir has been studied in further detail, suggesting that the outer layer is composed of lactococci, yeast and lactobacilli (Figure 2.1**)**. In contrast, the inner layer has a higher number of yeast cells compared to the outer layer and has long lactobacilli. The ability of the kefir to auto-aggregate increases with an increase in fermentation time (Figure 2.2) (Wang et al., 2012). Auto aggregation refers to the formation of multicellular clumps between microorganisms of the same strains. This property is related to adhesion to a surface, followed by biofilm formation (Boris, Suarez, & Barbes, 1997). Kefir grains are thin, sheet-like structures, which roll and scroll to form a mature grain-like structure. The smooth side consists of only short lactobacilli while the convoluted side has more yeasts than short lactobacilli. There is a zone of long, curved bacteria within the polysaccharide matrix, which may play a role in forming kefiran or the polysaccharide matrix. Thus, the kefir structure is a product of folding and refolding of the flat sheet-like structures with an increase in the number of microorganisms and polysaccharides as

folding occurs (Marshall et al., 1984). The charges on the microbial cell surface also play a role in auto-aggregation, co-aggregation and microbial adhesion to a surface while contributing to survival in harsh conditions (Xie, Zhou, & Li, 2012). Here, co-aggregation refers to the process of attachment of genetically distinct microorganisms by adhesion mechanism, and influence the formation of complex multi-species biofilms (Rickard, Gilbert, High, Kolenbrander, & Handley, 2003).

As shown in Figure 2.2, the lactic acid bacteria and yeast initially start to auto-aggregate and co-aggregate to form small granules (Figure 2.2A). The aggregation is enhanced as the pH drops. The biofilm producers then adhere to the surface of these small granules due to their cell surface properties and their strong co-aggregation ability, resulting in thin biofilms (Figure 2.2B). Once the biofilm is formed, the co-aggregation of the LAB and yeast in kefir persists to form a three-dimensional microcolony with the granule biofilm (Figure 2.2C). Upon the increase in cell density due to the growth of kefir LAB and yeast, the milk components and the cells that appear in the liquid phase accumulate on the surface of the granule, resulting in the formation of the kefir grains (Figure 2.2D) (Wang et al., 2012).

Figure 2.1: Scanning electron microscopy indicates the microstructure of the kefir grains. (1a) indicates the outer layer of kefir grains which comprises of lactococci, yeast and lactobacilli and (1b) indicates the inner layer of kefir grain with high number of yeast cells and longer lactobacilli (Wang et al., 2012).

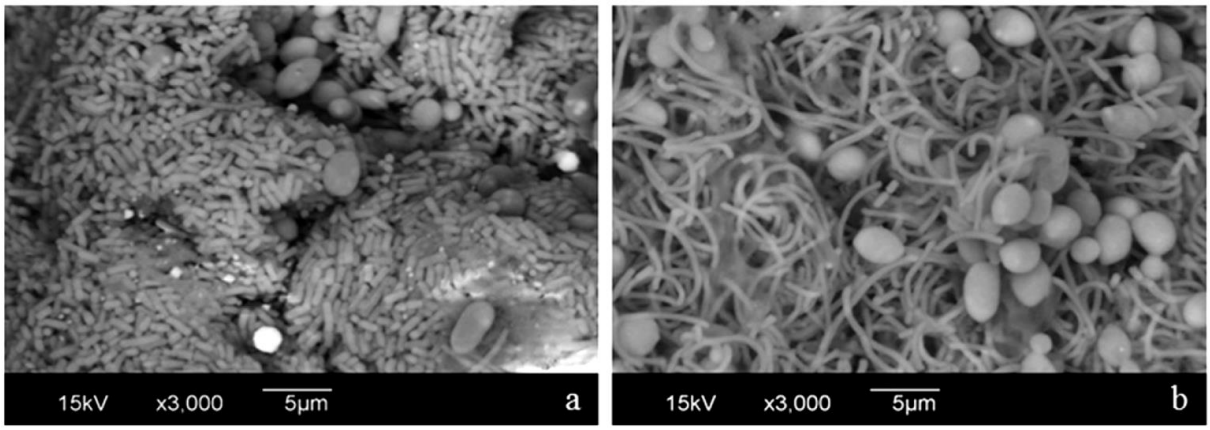
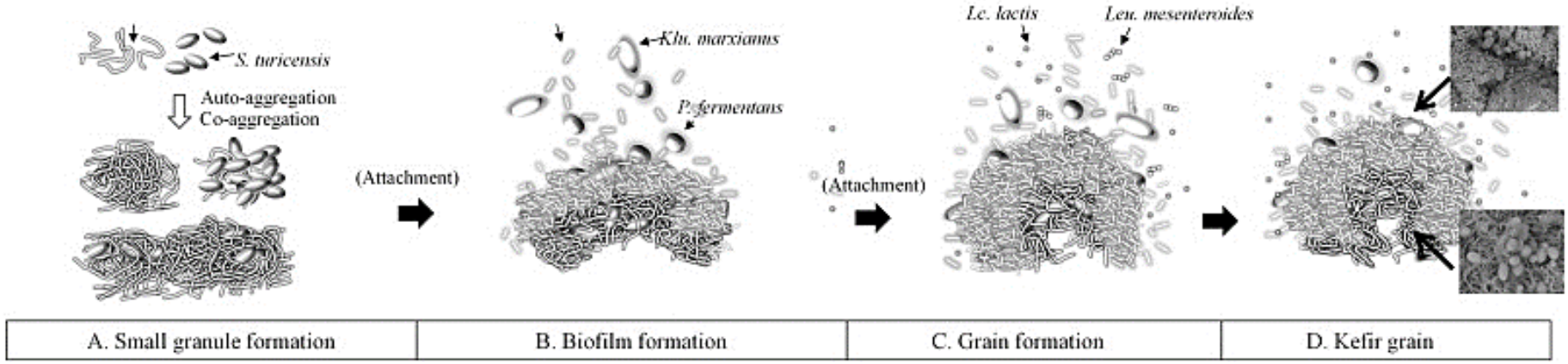


Figure 2.2: A schematic model of kefir grain formation (Wang et al., 2012).



2.1.3 Microbial diversity of kefir grains

Various genera of bacteria and yeasts have been isolated from kefir grains through the years from different countries (Alsayadi, Al Jawfi, Belarbi, & Sabri, 2013; Blaiotta, Di Capua, Romano, Coppola, & Aponte, 2014; Davidovic, Miljkovic, Antonovic, Rajilic-Stojanovic, & Dimitrijevic-Brankovic, 2015; Diosma, Romanin, Rey-Burusco, Londero, & Garrote, 2014; Gulitz et al., 2011; Hsieh et al., 2012; Laureys & De Vuyst, 2014; Magalhaes, Pereira, Dias, & Schwan, 2010; Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013). The carbon and energy sources available for the kefir grains play an important role in determining the concentration of final byproducts and the composition and frequency of the microbial species (Fiorda, de Melo Pereira, Thomaz-Soccol, Rakshit, Pagnoncelli, de Souza Vandenberghe, et al., 2017; Hsieh et al., 2012). Various molecular techniques such as metagenomics and polymerase chain reaction denaturing gradient gel electrophoresis (PCR- DGGE) have been used over the years for determining the microbial species present in kefir grains. This has led to various important findings towards a better understanding of kefir microbial diversity. Microbial species present include lactic acid bacteria (LAB), yeast and acetic acid bacteria (AAB) (da CP Miguel, Cardoso, Magalhães, & Schwan, 2011; Gulitz et al., 2011; Laureys & De Vuyst, 2014; Magalhães et al., 2011; Marsh et al., 2013; Waldherr, Doll, Meißner, & Vogel, 2010). These microorganisms are known to have a symbiotic relationship by sharing their byproducts, growth-stimulating factors and energy sources to survive and propagate (Lopitz-Otsoa, Rementeria, Elguezabal, & Garaizar, 2006).

Various LAB, acetic acid bacteria and yeasts that have been identified by various molecular techniques have been listed below in Table 2.2.

Table 2.2. Summary of the kefir microbiota isolated, along with the techniques used in identifying the isolates.

Technique used to study kefir's microbial profile	Strains/type of isolates	References
Biochemical techniques	*Saccharomyces sp., Kluyveromyces sp., Candida sp., Mycotorula sp., Torulaspora sp., Cryptococcus sp., Pichia sp.* etc.	Kolakowski & Ozimkiewicz, 2012
PCR-based Denaturing Gradient Gel Electrophoresis and species specific PCR	*L. acidophilus, B. bifidum, S. thermophiles, S. thermophiles, L. bulgaricus, Streptococcus spp., S. thermophiles, B. adolescentis, B. longum, B. lactis.*	Theunissen, Britz, Torriani, & Witthuhn, 2005; Leite et al., 2012;
	L. kefiranofaciens subsp. kefirgranum, L. kefiranofaciens subsp. kefiranofaciens, L. kefiri, L. parakefiri, L. parabuchneri, L. amilovorus, L. crispatus and L. buchneri, Leuconostoc mesenteroides, L. mali, L. hordei, Leuconostoc mesenteroides, Enterococcus faecalis, L. lactis, Bifidobacterium psychraerophilum, Zygosaccharomyces fermentati, Saccharomyces cerevisiae, Dekkera bruxellensis, Pichia fermentans	Hsieh, Wang, Chen, Huang, & Chen, 2012
	A. fabarium, A. orientalis, A. lovaniensis, A. fabarium, A. orientalis, A. lovaniensis, Acetobacter aceti, A. rasens	Garofalo et al., 2015; Gulitz et al., 2011; Laureys & De Vuyst, 2014; Magalhaes et al., 2010
DNA sequencing of 16s rRNA region	*L. acidophilus complex, L. amylovorous, L. crispatus, L. galinarium, L. gasseri, and L. jonsonii*	Kullen, Sanozky-Dawes, Crowell, & Klaenhammer, 2000

Pulse Field gel electrophoresis	*Strains of L. acidophilus complex, including L. delbrueckii subspecies (L. delbrueckii subsp. bulgaricus, L. Delbrueckii subsp. delbrueckii, L. delbrueckii subsp. lactis), L. plantarum, L. fermentum, L. rhamnosus, and L. sakei*	Singh, Goswami, Singh, & Heller, 2009
PCR Denaturing Gradient Gel Electrophoresis, 16s rRNA sequencing for yeast	*Kluyveromyces maxianus, Torulaspora delbrueckii, Saccharomyces cerevisiae, Candida kefir, Saccharomyces unisporus, Pichia fermentans, Kazachastania aerobia, Lachanceae meyersii, Yarrowia lipolytica, and Kazachstania unispora*	Leite et al., 2012; Magalhães et al., 2010; Simova et al., 2002; Wang, Chen, Liu, Lin, & Chen, 2008

2.1.4 Biochemistry of LAB

LAB generate adenosine tri-phosphate (ATP) by active degradation of carbohydrates combined to substrate-level phosphorylation. The two key pathways for the metabolism of hexoses are the glycolytic pathway (Embden-Meyerhof pathway) with lactic acid being the main end-product (homofermentative metabolism), and the phosphoketolase pathway, in which other end-products such as acetic acid, propionic acid, CO_2, ethanol and others are formed in addition to lactic acid (heterofermentative metabolism) (Kandler, 1983). Based on the characteristic and exceptional fermentation potential of the LAB and its ability to utilize the substrate, influences its metabolic pathway. The two main groups of LAB based on the end products it generates, are homo- and hetero-fermentative. Hetero-fermentative LABs are further subdivided to facultatively and obligately fermentative species.

As reported by Pessione, (2012), LAB which are able to ferment the hexoses, using Embden–Meyerhof–Parnas (EMP) pathway, and produce lactic acid as the end product, are considered as obligately homofermentative. Some examples of obligately homofermentative LAB are *Pediococcus, Lactococcus, Streptococcus*, and selected *Lactobacillus*. LAB such as *Leuconostoc* and certain *Lactobacillus*, that can produce lactic acid by fermenting the hexoses through EMP pathway, can also metabolise pentoses and sometimes gluconate, since they possess both phosphoketolase and aldolase, and are facultatively heterofermentative (Felis and Dellaglio, 2007).

LAB which can produce significant quantities of end products such as lactic acid, carbon dioxide, acetic acid and/or ethanol by fermenting the hexoses via phosphogluconate pathway, are obligately heterofermentative. Obligately heterofermentative LAB lack fructose-1,6-bisphosphate aldolase, which is a glycolytic enzyme, and therefore cannot degrade the hexoses using EMP pathway (Pessione, 2012).

Figure 2.3: Metabolism of some of the commonly found LAB in food (Luc De Vuyst, Van Kerrebroeck, & Leroy, 2017; Papadimitriou et al., 2019)

Obligate heterofermentative	**Facultative heterofermentative**	**Obligate homofermentative**
L. sanfranciscensis, L. brevis, L. fermentum, L. reuteri, L. pontis, L. rossiae, L. panis L. frumenti Weissella spp., L. zymae, L. siliginis, L. secaliphilus,, L. hammesii, L. acidifarinae, L. namurensis	*L. alimentarius L. paralimentarius L. plantarum, Pediococcus pentosaceus L. plantarum, L. spicheri, L. xiang fangensis*	*L. amylovorus, L. delbrueckii, L. acidophilus, L. johnsonii, L. farciminis, L. crustorum, L. heilongjiangensis, L. mindensis, L. nantensis, L. nodensis*

2.1.5 Health benefits of kefir grains

Microorganisms isolated from kefir have probiotic capabilities in that they have a health-promoting effect on the body. Various health-promoting effects of kefir are enhancement of digestive health, reduction in serum cholesterol, rise in lactose tolerance, improved immune function, control of irritable bowel symptoms, as well as anticarcinogenic properties. Lactobacilli in the kefir have the ability to attach to the epithelial lining in the intestines and to auto-aggregate (Golowczyc et al., 2008). In addition, they are also known for their ability to produce certain bacteriocins and organic acids, which may protect the body from toxins, and have an antimicrobial effect (Sezer & Güven, 2009; Silva, Rodrigues, Xavier Filho, & Lima, 2009). It has been reported that kefir-derived polysaccharide also has an anti-tumor effect, anti-diabetic effect, anti-oxidative effect and plays a role in lowering blood pressure (Ahmed et al., 2011). Various bioactive peptides and proteins which are formed by kefir grains have effect on lymphocyte proliferation, increased absorption of calcium from the intestines, hypertension reduction and immunoglobulin production (Guzel-Seydim, Kok-Tas, Greene, & Seydim, 2011; Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008; Otles & Cagindi, 2003; Parvez, Malik, Ah Kang, & Kim, 2006; Rosa et al., 2017).

Kefir is used for treating atherosclerosis and allergic diseases, cancer and gastrointestinal tract disorders, tuberculosis and has also been associated with longevity in the Caucasus (Cevikbas et al., 1994; Koroleva, 1982; Zourari & Anifantakis, 1988). Hypocholesterolemia, anti-tumoral, immunological and anti-bacterial properties of kefir have been found recently. Kefir is also claimed to have antagonistic effect against *Salmonella kedougou* (Furukawa, Matsuoka, Takahashi, & Yamanaka, 1991; Otles & Cagindi, 2003; Tamai, Yoshimitsu, Watanabe, Kuwabara, & Nagai, 1996; Zacconi, Scolari, Vescovo, & Sarra, 2003).

Blood pressure regulation by kefir is also known, by inhibiting angiotensin-converting enzyme (ACE). ACE is responsible for converting angiotensin I to angiotensin II, a potent vasoconstrictor, that in turn increases the blood pressure (Quirós, Hernández-Ledesma, Ramos, Amigo, & Recio, 2005). Positive effects of kefir are highlighted in Figure 2.4 and Figure 2.5 (Ahmed et al., 2013).

Figure 2.4: Kefir and tumor formation sites in human model (Ahmed et al., 2013)

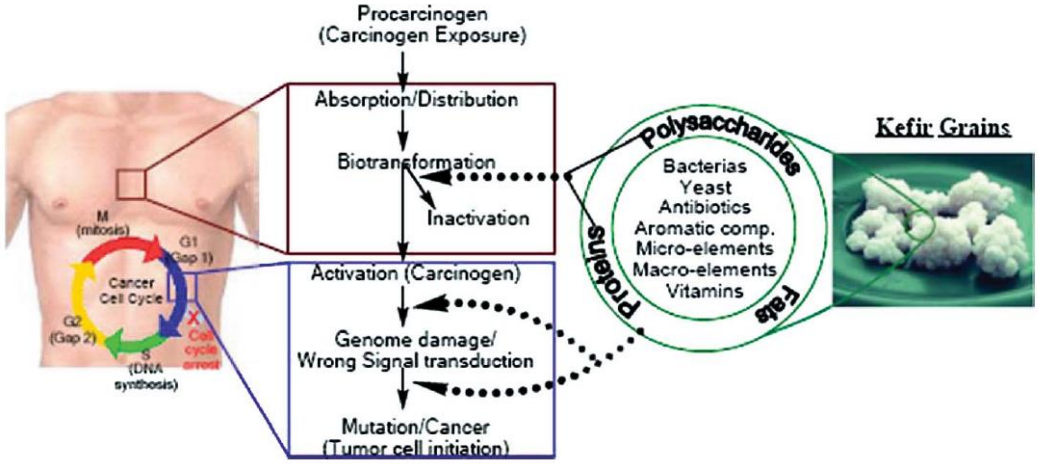
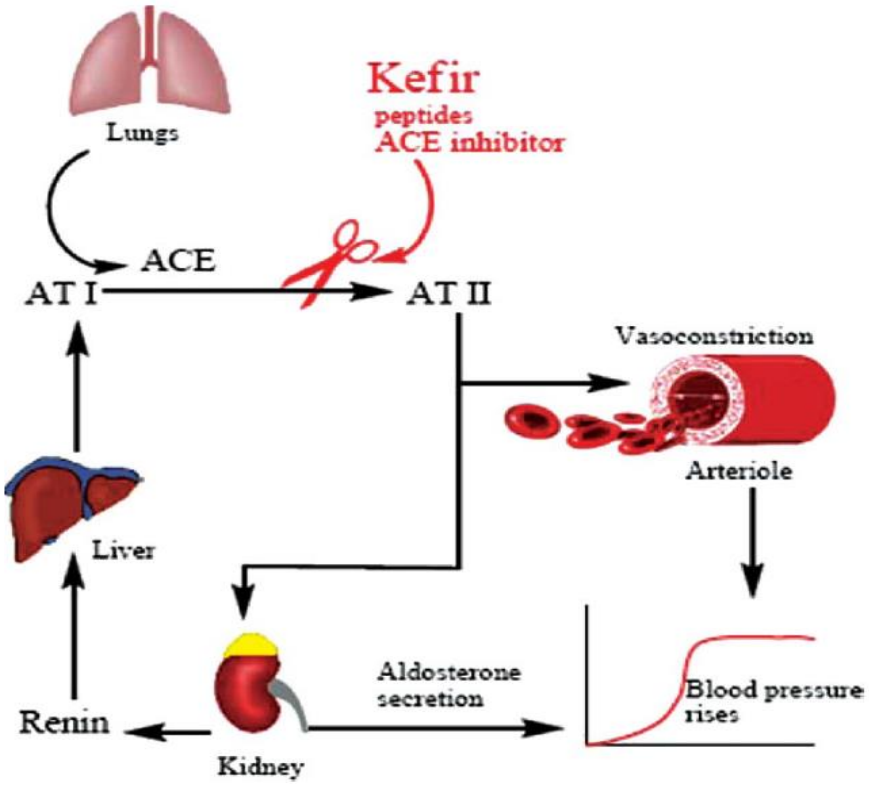


Figure 2.5: Kefir effects on blood pressure regulation (Ahmed et al., 2013).



2.1.6 Dairy and non- dairy kefir foods

The most common fermentation product of kefir is made from milk. A lot of milk-based functional foods are created by using kefir as the inoculum (Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013; Tripathi & Giri, 2014; Khan, 2014). A sample of kefir is added to fresh milk and is allowed to ferment. This fermentation product is then used to inoculate some more fresh milk. The grains are harvested for inoculation purposes. Subsequent kefir batches should be able to produce an infinite amount of kefir under proper environmental conditions (Beshkova, Simova, Simov, Frengova, & Spasov, 2002; Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011; Farnworth, 2006; Otles & Cagindi, 2003; Simova et al., 2002; Witthuhn, Schoeman, & Britz, 2005). Most commonly used sources for milk are cow, sheep and goat milk (Otles & Cagindi, 2003). Kefir is also prepared from commercial starters grown in bovine milk. Kefir starter culture is used in the production of cheese and single cell protein by fermenting cheese whey under aerobic conditions (Filipcev, Šimurina, & Bodroza-Solarov, 2007; Paraskevopoulou et al., 2003).

Addition of milk to kefir grains for fermentation can change the properties of milk such that there is decrease in the concentration of lactose from 4.9% (w/v) to 4.0% (w/v) and the L (+)-lactic acid concentration increases to 0.76% (w/v) from 0.01% (w/v). The acetic acid content increases to 2.73 mg/ml accompanied by a reduction in pH value from pH 6.5-7.0 to pH 4.2 in the first 24 h. The changes in lactic acid concentration, pH and acetic acid production depend mainly on the type of starter culture used, the period of incubation and the medium used to grow the kefir (García Fontán, Martínez, Franco, & Carballo, 2006; Magalhães, Pereira, et al., 2011; Öner, Karahan, & Çakmakçı, 2010). The milk kefir contains 7.6g/L of L-lactic acid and a much lower concentration of 1.1g/L of D-lactic acid, (Frías, Martínez-Villaluenga, & Peñas, 2016; Suriasih, 2000).

Fermentation can also be carried out by adding kefir grains to non-dairy products such as coconut water, soy milk (Liu, Chen, & Lin, 2005), peanut milk (Bensmira & Jiang, 2011), walnut milk (Cui, Chen, Wang, & Han, 2013), rice milk (Otles & Cagindi, 2003) and cocoa-pulp beverage (Puerari, Magalhães, & Schwan, 2012). The present study focuses on coconut water as the fermentation substrate.

2.2. Coconut Water

Coconut (*Cocos nucifera L.*) is one of the important fruit trees in the world. From the various edible parts of the coconut, coconut water is a significant source of nutrition in many tropical and subtropical countries (DebMandal & Mandal, 2011). This liquid endosperm is of cytoplasmic origin which fills the cavity within the coconut (Janick & Paull, 2008). It contains almost all components of the vitamin B group (except for B6 and B12), minerals, proteins,

sugars, amino acids, magnesium, vitamin C, potassium and growth factors such as auxins, cytokinins and gibberellins, which makes it biologically favourable for human nutrition as well as for the growth of microorganisms (Anurag & Rajamohan, 2003; Loki & Rajamohan, 2003; Massey, 2001; Sandhya & Rajamohan, 2006). This isotonic drink is very low in fat content and also contains optimum amounts of RNA phosphorous which play an active role in the transport of amino acids and respiratory metabolism in living cells (Chidambaram, Singaraja, Prasanna, Ganesan, & Sundararajan, 2013). Table 2.3 shows the nutritional composition of young coconut water as reported by USDA (USDA), Arditti (2009) and (Yong, 2009):

Table 2.3: Nutritional composition of young coconut water as reported by the USDA according to Arditti (2009) and (Yong, 2009).

Chemical composition	**Young coconut water**
Energy value	19 kcal
Water	94.99 g/100g
Dry	5.01 g/100g
Ash	0.39 g/100g
Protein	0.72 g/100g
Total lipid, fats	0.2 g/100g
Total dietary fibre	1.1 g/100g
Carbohydrates	3.71 g/100g
Total sugar	2.61 g/100g
Sucrose	9.18 mg/ml
Fructose	5.25 mg/ml
Glucose	7.25 mg/ml
Mannitol	0.8 mg/ml
Sorbitol	15 mg/ml
Myo-inositol	0.01 mg/ml
Scyllo-inositol	0.05 mg/ml
Calcium	24 mg/100g
Magnesium	30 mg/100g
Phosphorus	37 mg/100g
Iron	0.29 mg/100g
Sodium	105 mg/100g
Potassium	312 mg/100g
Manganese	0.142 mg/100g
Zinc	0.1 mg/100g

Chemical composition	Young coconut water
Copper	0.04 mg/100g
Chloride	183 mg/100g
Selenium	0.001 mg/100g
Sulfur	24 mg/100g
Vitamin C, total ascorbic acid	2.4 mg/100g
Thiamin (B1)sx	0.03 mg/100g
Folate, total	0.03 mg/100g
Pyridoxine (B6)	0.032 mg/100g
Folate, food	0.003 mg/100g
Niacin (B3)	0.08 mg/100g
Nicotinic acid (Niacin)	0.64 mg/100g
Riboflavin (B2)	0.057 mg/100g
Pantothenic acid (B5)	0.52 mg/100g
Folate, Dietary Folate Equivalent (DFE)	3 (µg_DFE)
Biotin	0.02 mg/100g
Folic acid	0.003 mg/100g
Alanine	312 µg/ml
γ-Aminobutyric acid	820 µg/ml
β-Alanine	12 µg/ml
Aspartic acid	65 µg/ml
Cystine	0.97-1.17 µg/ml
Arginine	133 µg/ml
Asparagine and glutamine	ca. 60 µg/ml
Glutamic acid	240 µg/ml
Homoserine	5.2 µg/ml
Glycine	13.9 µg/ml
Lysine	150 µg/ml
Leucine	22 µg/ml
Isoleucine	18 µg/ml
Histidine	0.017 g/100g
Methionine	8 µg/ml
Ornithine	22 µg/ml
Phenylalanine	12 µg/ml
Proline	97 µg/ml
Serine	111 µg/ml
Tyrosine	16 µg/ml

Chemical composition	Young coconut water
Tryptophan	39 µg/ml
Threonine	44 µg/ml
Valine	27 µg/ml
Ethanolamine	0.01 µmol/ml
Pyridoline	0.39 mg/ml
Malic acid	34.31 meq/ml
Citric acid	0.37 meq/ml
Shikimic and quinic acids	0.57 meq/ml
Total lipids	0.2 g/100g
Total saturated fatty acids	0.176 g/100g
Total monounsaturated fatty acids	0.008 g/100g
Total polyunsaturated fatty acids	0.002 g/100g
Auxin	0.07 mg/ml
Acid phosphatase	Present (units not given)
Catalase	Present (units not given)
Dehydrogenase	Present (units not given)
Diastase	Present (units not given)
Peroxidase	Present (units not given)
RNA polymerase	Present (units not given)

The electrolyte concentration in coconut water generates an osmotic pressure similar to that of blood which helps in promoting health (Effiong, Ebong, Eyong, Uwah, & Ekong, 2010). Coconut water is also used in callus culture media as an important ingredient (Overbeek, Conklin, & Blakeslee, 1941). Coconut water has a significant impact on anti-ageing, anti-carcinogenic and anti-thrombotic effects due to the presence of a phytohormone (cytokinin) (Kende & Zeevaart, 1997; Rattan & Clark, 1994; Vermeulen et al., 2002). Micronutrients such as vitamins and inorganic ions help in the body's antioxidant system by aiding the removal of oxidizing species (reactive oxygen species) generated in the body due to hypermetabolism (Liu, Lin, Chen, Chen, & Lin, 2005). It is used as a ceremonial gift, traditional medicine and can also be processed into wine and vinegar (Prades, Dornier, Diop, & Pain, 2012). Thus, its use is not limited to being a refreshing drink but is also used as one of the main ingredients in many food items such as bread, ice creams, biscuits and cakes (Chidambaram et al., 2013).

In green coconut water, between pH 5.5 and 6.0, and at 25°C and 35°C, optimum activities of polyphenoloxidase (PPO) and peroxidase (POD) are observed. PPO is an enzyme that is found in chloroplasts, plastids and is also present in the cytoplasm of the ripened senescing plants. It

helps the plant to resist microbial infections and extreme climatic conditions. The ratio of these enzymes (PPO/POD) in coconut water ranges from 0.2 to 16.7 and varies even within similar coconut varieties. This ratio depends on the stage of maturity at harvest, the variety and storage condition of the fruit, the cultivation conditions and on the mode of extraction of the coconut water.

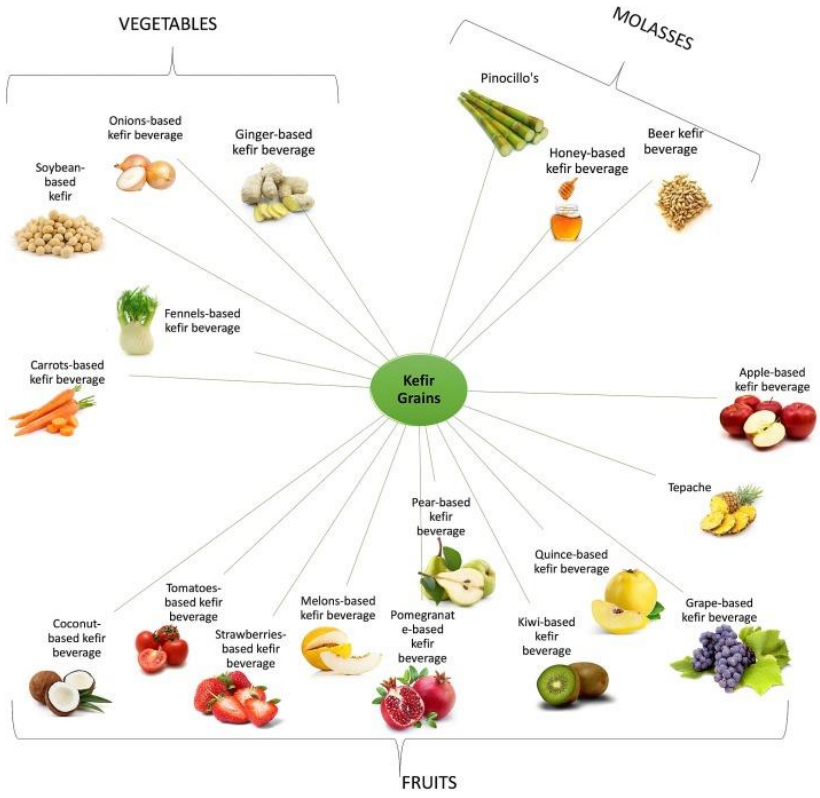
Young coconut water is able to synthesize more than one peptide which has antimicrobial activity and has novel properties and modes of action against human pathogenic bacteria (Mandal et al., 2009). Coconut water also contains (+)-catechin and (-)-epicatechin, which have antimicrobial, anti-cancerous and antioxidant properties (Camargo Prado et al., 2015). In addition, phytohormones such as auxins, cytokinin, kinetin, trans-zeatin, gibberellins, inorganic ions and vitamins are present, which play various roles in delaying ageing, reducing DNA damage by acting as antioxidants, and helping to cure neural diseases such as Alzheimer's. It's use as a potential drug has been suggested to have the ability to reduce the risk of cardiovascular disease and anaemia during pregnancy. Coconut water is often prescribed in cases of indigestion, burning pain during urination, dysuria, gastritis, burning pain of eyes or even expelling of the retained placenta (Prades et al., 2012).

Overly Mature Coconut (OMC) water is lower in the volume of nut water and is slightly salty in taste when compared to fresh young coconut water which is sweet and slightly sour in taste (Jackson, Gordon, Wizzard, McCook, & Rolle, 2004; Terdwongworakul, Chaiyapong, Jarimopas, & Meeklangsaen, 2009). OMC water has a high content of minerals such as sodium and potassium, and contains sugars such as sucrose and some proteins. These properties suggest that OMC water can be developed into a rehydration fluid product. In addition, the maturity of coconut water (due to changes in composition, physiochemical properties, sugar and salt content, and pH) influences the enzyme activity and enzyme inactivation kinetics. Very low thermal resistance is reported for both PPO and POD present in OMC water (Tan, Cheng, Bhat, Rusul, & Easa, 2014).

Different varieties of non-dairy substrates have been inoculated with kefir grains to produce functional probiotic beverages with distinct sensory characteristics (Fontán, Martínez, Franco, & Carballo, 2006). Several fruits (such as strawberries, pear, pomegranate, quinces, grapes and apples), vegetable juices, tea extracts, and honey are a few examples of the medium used for fermentation (Miguel et al., 2011; Grønnevik, Falstad, & Narvhus, 2011). "Tapache" is an example of a fermented drink made with kefir grains, pineapple, brown sugar and cinnamon, which is widely consumed in Mexico. Similarly in southern Italy, a drink called as "Kefir d'uva" is a popular fermented drink made with grape juice and kefir grains (Gaware et al., 2011; Fiorda et al., 2017). Various non-dairy sources for fermenting kefir are illustrated in Figure 2.6,

(Fiorda, Pereira, Soccol, Rakshit, Pagnoncelli, Vandenberghe, et al., 2017). A wide range of fruit juices can be used. There is a potential for using coconut water as a raw material for producing a health drink, using kefir grains. It is prepared by inoculating coconut water with kefir grains, which is then incubated for about 24-48 h to allow appropriate fermentation.

Figure 2.6: Various products used as the medium for fermentation of kefir grains (Fiorda, Pereira, Soccol, Rakshit, Pagnoncelli, Vandenberghe, et al., 2017)



2.3. Coconut Water Kefir

Coconut water kefir is produced by adding kefir grains to the coconut water and allowing it to ferment for 24 to 48 h. There are a few coconut water kefir drinks available commercially in New Zealand, of which, the kefir manufacturing company, Naturally Organic and The Doctor Feel Good are well known. However, no scientific studies have been carried out on coconut water kefir.

The reason for using glucose as a source of sugar for this study is that it is the most readily available source of carbohydrate that can be easily utilised by the kefir microorganisms. Liu et al. (2002) have reported that addition of glucose to kefir grains resulted in higher LAB counts, yeast counts and increased production of lactic acid as a by-product (Liu & Lin, 2000). In addition, sucrose is a readily- available source of carbohydrate, which can be purchased from the supermarkets as “table sugar”. Harta et al., (2004) have reported that increased biomass production for kefir was observed by adding sucrose in the fermentation medium. Sucrose being

a disaccharide, is hydrolysed to produce glucose which can be easily utilised by the microorganisms. It has also been reported that the presence of sucrose supports the rapid sedimentation of kefir grains at the bottom of the container, thus reducing the cost of centrifugal separation (Harta et al., 2004).

2.4. Sourdough

2.4.1 History of Sourdough

Sourdough fermentation is very ancient and is applied worldwide. Since approximately 3000 BC, spontaneous fermentation processes are used in producing sourdough (Brandt & Gänzle, 2006; Cappelle, Guylaine, Gänzle, & Gobbetti, 2013; Catzeddu, 2019; Gobbetti & Gänzle, 2012; Shevchenko et al., 2014). Many food products which use the sourdough fermentation technology are still consumed in form of sweet leavened baked goods which are consumed in North America, the Mediterranean and many European regions (Catzeddu, 2019; W. Hammes & Gänzle, 1998). The mixture of a fraction of flour and water contributes to the formation of the sourdough matrix which is fermented by the microbial community of sourdough consisting of LAB and yeast (Corsetti & Settanni, 2007; Luc De Vuyst & Neysens, 2005; L. De Vuyst, Vrancken, Ravyts, Rimaux, & Weckx, 2009; M. Gobbetti, De Angelis, Corsetti, & Di Cagno, 2005; Marco Gobbetti, Minervini, Pontonio, Di Cagno, & De Angelis, 2016; Hammes & Gänzle, 1998; Minervini, Lattanzi, De Angelis, Celano, & Gobbetti, 2015). In addition to LAB and yeast, acetic acid bacteria are also present in the sourdough (Li, Li, & Bian, 2016; Fabio Minervini, Celano, Lattanzi, De Angelis, & Gobbetti, 2016; Ripari, Cecchi, & Berardi, 2016; Ripari, Gänzle, & Berardi, 2016; Scheirlinck, Van der Meulen, De Vuyst, Vandamme, & Huys, 2009; Scheirlinck et al., 2008).

2.4.2 Sourdough Metabolism

From a microbiological point of view, sourdough is considerably a very specific and stressful ecosystem, with highly variable carbohydrate concentration, variable amounts of acid production (or a characteristic acid environment due to low pH), and limited availability of oxygen (De Vuyst et al., 2014). The microorganisms in sourdough carry out metabolic activities such as acidification (LAB), flavour formation (LAB and yeasts), and leavening (yeasts and hetero-fermentative LAB species) (De Vuyst et al., 2014).

The metabolic activities of LAB reduce the amount of carbohydrate present in the sourdough converting them to organic acids that reduce the pH and acidifies the dough (Zheng, Zhao, Lin, & Gänzle, 2015). The metabolic strategy of certain yeasts during the fermentation is to derive energy from a substrate by using reduced compounds such as ethanol instead of oxygen as the terminal electron acceptor. (Rozpędowska et al., 2011).

Starch is one of the most abundantly available carbohydrates for fermentation in sourdough. It is broken down to maltose, glucose and maltodextrins by endogenous flour enzymes depending on the flour type. Glucose and maltose, therefore are the most abundantly available carbohydrates for sourdough fermentation (Brandt & Gänzle, 2006; Gänzle, 2014).

Wheat proteins mainly consist of proteins such as glutenins and gliadins which are the important storing proteins. Gliadins are alcohol-soluble proteins and glutenins are soluble in dilute acids (Osborne, 1907). Degradation of protein during fermentation is a result of the enzyme action of both cereal and microbial origin (Gänzle et al., 2008). Glutenins are highly polymeric proteins that are divided into high molecular weight (HMW) and low molecular weight (LMW) fractions (Shewry et al., 1986). The gliadins are single units as they contain only intramolecular disulphide bonds. Gliadins are grouped into α-, γ- and ω-type gliadins based on their amino acid compositions.

The final bread quality is influenced by the extent of protein degradation due to the impact it has on the loss of gluten network and its viscoelastic properties. Conversion of protein to amino acids enhances the flavour profile of the sourdough along with the generation of certain bioactive compounds in the sourdough matrix (Gobbetti et al., 2005; Pico, Bernal, & Gómez, 2015; Stromeck, Hu, Chen, & Gänzle, 2011; Thiele, Grassl, & Gänzle, 2004; Vogel et al., 2011). Due to the loss of the amino acid biosynthesis pathways of LAB in the cereal matrix, they are heavily dependent on the amino acids of the cereals (Ganzle et al., 2008; Makarova et al., 2006; Vogel et al., 2011). Additionally, during the sourdough fermentation, yeasts necessitate high amounts of amino acids and therefore, procuring most of the free amino acids (Katina, Poutanen, & Autio, 2006).

Thus, cereal flours consist of carbohydrates, proteins, lipids, phenolic compounds and phytate. Phytate is considered as an anti-nutritional factor for the consumers because it reduces the bioavailability of nutrients (Gänzle, 2014; Rocha, Kalo, & Malcata, 2012). Sourdough fermentation also strongly influences lipid oxidation, which in turn plays an important role in flavour formation (Gänzle, 2014; Vermeulen, Gänzle, & Vogel, 2007). Phenolic compounds including phenolic acids such as ferulic acid, and alkylresorcinols, are considered both an anti-nutritional factor and a health benefit provider (Dykes & Rooney, 2007).

2.4.3 Sourdough characteristics

Sourdough characteristics such as texture, loaf volume, crumb lightness, resilience, softness, flavour are influenced by the microorganisms involved in the fermentation process. They also impact the nutritional quality of the bread in terms of the presence of valuable vitamins, amino acids, dietary fibre and minerals. Texture and colour impart favourable characteristics to sourdough. The shelf life of the sourdough bread in terms of microbial spoilage, water migration,

staling and starch retrogradation also contributes to the overall sourdough characteristics (Arendt, Ryan, & Dal Bello, 2007; Birch, Petersen, & Hansen, 2013; Corsetti & Settanni, 2007).

The unique flavour of sourdough bread is due to the LAB and yeast fermentation. The use of raw materials for fermentation also has a major impact on flavour production (Hansen & Schieberle, 2005). Temperature and water content are factors that influencing the activity of yeast and LAB, and subsequently influence the amounts of the metabolites formed. Generally, LAB and AAB are mostly responsible for causing acidification. Evidence supports that the major proportion of the aroma precursors released by LAB and AAB during sourdough fermentation, are free amino acids (Hansen et al., 1989, Kratochvil and Holas, 1983, Rothe, 1974).

The glycaemic index (GI) for sourdough is low. Novotni et al. (2012) have reported that the addition of 15-22.5% sourdough decreases the GI of the consumers and helps improve overall texture and volume of the sourdough bread. Sourdough can lead to active degradation of starch, which can also decrease the glycaemic reponses. In addition, it has to its positive influence on the overall sensory quality of the fibre-rich, wholegrain and/or gluten free products, mineral bioavailabilty and enhanced accessibility of bioactive compounds (Poutanen, Flander, & Katina, 2009). The digestibility of sourdough bread is better than that of typical bread products, as most of the starch is metabolized influencing the glycaemic index of the final products (De Vuyst et al., 2017; Siepmann, Ripari, Waszczynskyj, & Spier, 2018).

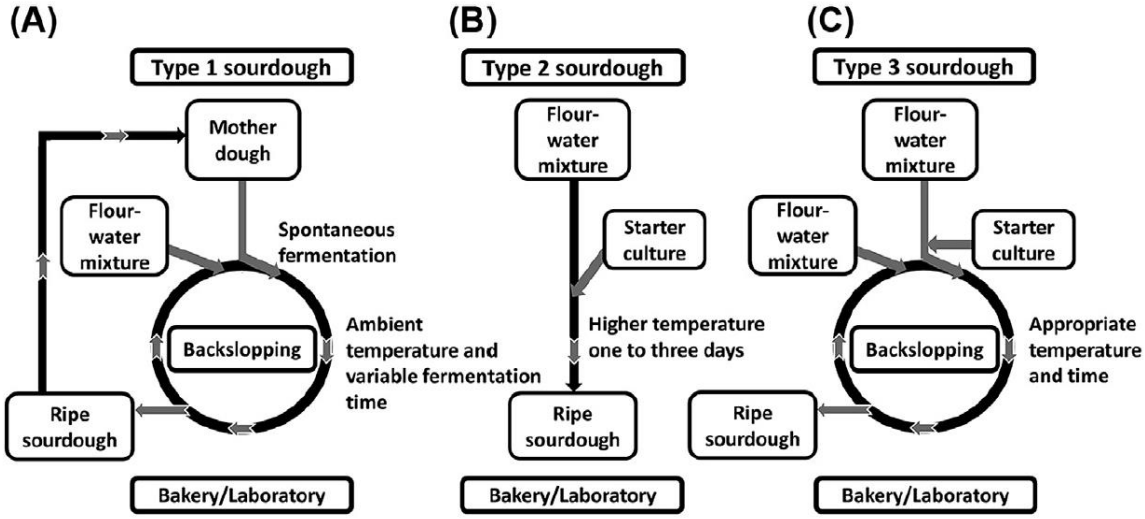
It is also rich in bioactive compounds, vitamins (Arendt et al., 2007; Gänzle, 2014; Gänzle & Vogel, 2003), Ɣ-amino butyrate and phytochemicals (Gänzle et al., 2009; Gobbetti, 1998; Gobbetti, Corsetti, et al., 1994b). It also possesses enhanced anti-fungal activity (Gobbetti, Angelis, Corsetti, & Cagno, 2005) and has high amount of phytase production by LAB which aids in reducing the amount of phytate (anti-nutritional factor) present in the sourdough (Gobbetti, Lavermicocca, Minervini, Angelis, & Corsetti, 2000; Papadimitriou et al., 2019).

2.4.4 Sourdough fermentation types

Sourdough is classified based on the fermentation process and the starter culture used. There are three types of sourdough as shown in Figure 2.7.

Figure 2.7: As described by Papadimitriou et al (2019), the diagram summarises the types of sourdough fermentations, based on the type of inoculum. **A**: Type 1 sourdough produced by spontaneous fermentation, **B:** Type 2 is sourdough fermentation which is initiated by addition of specific type of starter culture to bring about the fermentation, and lastly, **C:** Type 3 is back-

slopped sourdough fermentation process which is also initiated by the addition of specific type of starter culture.



1. **Type 1 sourdough**: This type of sourdough is produced when the fermentation is bought about naturally in the dough, without the addition of any external inoculum or starter cultures (Figure 2.7-A). The first dough produced by this process is called the motherdough. Ideally, about 15% mass/volume of motherdough is added to the subsequent sourdoughs made by adding water to the flour (sub-culturing). An ambient temperature, between 20°C to 30°C is used to carry out fermentation in the doughs (Lhomme, Orain, Courcoux, Onno, & Dousset, 2015; Lhomme et al., 2016; Fabio Minervini, Lattanzi, De Angelis, Di Cagno, & Gobbetti, 2012). Some typical examples of type 1 sourdough fermentation are: San Francisco sourdough, German rye-sourdough and some Italian sourdough, which are firm sourdoughs that contain a mixture of mesophilic LAB and yeast species, thus representing a great diversity of natural starters for the sourdough (Corsetti & Settanni, 2007; De Vuyst et al., 2014). This type of sourdough has been extensively studied to determine the factor which plays an important role in bringing about the diversity in the sourdough. The flour usually contains about 10^4 to 10^7 CFU/g including various LAB and fungi species (Fabio Minervini et al., 2016; Taccari et al., 2016). The characteristic species of LAB found in this type of sourdough are *Lactobacillus fermentum* and *Lactobacillus plantarum* (Weckx et al., 2010).

2. **Type 2 sourdough**:

   It is the type of fermentation obtained by addition of a starter culture to the flour and water mixture (Figure 2.7-B). No back slopping is required for this straight fermentation

process, and the resultant sourdoughs are sold as semi-liquid, dried or heated products (Brandt, 2007; Brandt & Gänzle, 2006; Gaggiano et al., 2007). The starter culture used to prepare this type of sourdough, usually consists of acid-tolerant LAB like *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, or *Lactobacillus sanfranciscensis* (Moroni, Arendt, Morrissey, & Bello, 2010). This is due to the fact that these strains produce acidification at a very fast rate, and are able to generate specific flavour compounds (Brandt, 2007).

3. **Type 3 sourdough**: For the preparation of type 3 sourdoughs, mixed sourdough fermentation process or back slopped starter culture initiation processes can be used (Figure 2.7-C). Traditional back slopping is observed in sourdough initiated with a LAB starter culture (Siragusa et al., 2009). Also, in this type of sourdough, there may exist a competition between spontaneously growing bacteria and yeast versus the microorganisms added through a starter culture. Sometimes, due to stress from the fermentation conditions or when the starter culture is not well adapted to the sourdough ecosystem, the autochthonous LAB species and/or strains may dominate, thus, eliminating the starter culture microorganisms (Lin & Gänzle, 2014; Moroni et al., 2010; Siragusa et al., 2009).

2.4.5 Sourdough LAB and their metabolism

Sourdough LAB are well characterized which mainly consists of obligately and facultative hetero-fermentative and some obligately homo-fermentative LAB species along with Acetobacteraceae. *Lactobacillus sanfranciscensis, Lactobacillus plantarum, Lactobacillus brevis, Pediococcus pentosaceus, Lactobacillus fermentum,* and *Lactobacillus paralimentarius* are some of the species which are frequently isolated from the sourdough. Non sourdough-specific LAB, such as enterococci, lactococci, and streptococci are responsible for acidification of sourdough matrix consisting of flour and water, in particular by activating the flour-native enzymes (Corsetti & Settanni, 2007; De Vuyst & Neysens, 2005; De Vuyst et al., 2014; Gobbetti et al., 2016; Huys, Daniel, & De Vuyst, 2013).

Obligately hetero-fermentative LAB (such as *Lactobacillus fermentum*, *Lactobacillus reuteri*, and *Lactobacillus sanfranciscensis*) are mostly present in the sourdough ecosystem since they are highly adaptive of the carbohydrate metabolism. Evidently, this could be due to their ability to ferment sucrose by sucrose phosphorylase, glucan- and fructansucrase or levansucrase; or maltose by phosphogluconate pathway or by maltose phosphorylase activity (Michael G Gänzle, Vermeulen, & Vogel, 2007; Gobbetti & Gänzle, 2012; Tieking & Gänzle, 2005). Additionally, sourdough LAB possess amino acid conversion mechanisms, which may contribute to microbial growth because of extra ATP formation, and stress response which lead to an environment which

is acidic in nature and has low pH leading to glutamine and glutamate dehydrogenase production, contributing to homeostasis of the intracellular pH at acidic pH.

For homo-fermentative LAB, most carbohydrates are transported by phosphoenolpyruvate-dependent phosphotransferase systems and maltose and sucrose metabolism is generally affected by carbon catabolite repression, whereas for hetero-fermentative LAB, carbohydrates are usually transported by facilitated diffusion and maltose and sucrose metabolism is not exposed to carbon catabolite repression (De Vuyst, Van Kerrebroeck, & Leroy, 2017). Starch degradation by the action of enzymes which may be of microbial origin and maltodextrin uptake may be an adaption mechanism for LAB like *Lactobacillus acidophilus*. Similarly, *Lactococcus lactis* and leuconostocs in sourdough, which are involved in citrate to pyruvate conversion, may produce acetoin/diacetyl, acetate, and/or lactate (Michael G. Gänzle, 2015).

LAB have peptidase enzyme and flour may contain protease enzyme, both of which play a role in nitrogen metabolism and contribute to the formation of (bioactive) peptides as well as amino acids. Flour proteases are activated by reduced pH and by reduction of gluten disulfide bonds, caused through LAB acidification and glutathione reductase activity, respectively. Acidic stress response, redox balancing and flavour formation are all influenced by amino acid production (De Vuyst et al., 2017; Michael G. Gänzle, 2015; Thiele et al., 2004). The production of organic acids, like lactic and acetic acid, ethanol, diacetyl, carbon dioxide, and hydroxy fatty acids, phenyllactic acid, and cyclic dipeptides contribute to antimicrobial and antifungal activity in sourdough (De Vuyst & Vandamme, 1994).

2.4.6 Sourdough yeasts and their metabolism

Generally, only ascomycetous yeasts with fermentative ability are found in sourdough ecosystems (De Vuyst et al., 2016). Some common yeast species isolated from the sourdough are *S. cerevisiae, K. humilis, Torulaspora delbrueckii, Wickerhamomyces anomalus, K. exigua, Pichia kudriavzevii,* and *Candida glabrata* (Legras, Merdinoglu, Cornuet, & Karst, 2007). Glucose, fructose, maltose and sucrose from the flour are fermented via glycolysis pathway with a further breakdown of pyruvate by *S. cerevisiae* into carbon dioxide and ethanol production with the regeneration of NAD^+ (Gobbetti, Corsetti, & Rossi, 1994a; Gobbetti, Corsetti, et al., 1994b). Production of carbon dioxide leads to the dough expansion. Ethanol evaporates very easily during baking, but during fermentation, it could be recycled to produce glycerol and succinate while recycling NAD^+. Glycerol plays an important role as an osmoprotectant and aids in better gas retention ability in the sourdough (thus, influencing sourdough rheology), whereas succinate reduces the pH and influences gluten formation (Aslankoohi, Rezaei, Vervoort, Courtin, & Verstrepen, 2015; Jayaram et al., 2014).

Through invertase activity, the yeast species in the sourdough are able to hydrolyse sucrose and into glucose and fructose consume the available glucose, and provide fructose as well as amino acids to the LAB. The carbohydrates are fermented via glycolysis and subsequent pyruvate breakdown results in formation of carbon dioxide and ethanol. Fructose is then able to act as an alternate electron acceptor for LAB (Vogel, R. F, 2015). Both LAB and yeast in the sourdough adapt to acidic stress, oxidative stress and acetic acid tolerance (Michael G Gänzle et al., 2007; Gobbetti, Corsetti, et al., 1994a).

Apart from the production of carbon dioxide which brings about leavening, yeasts also play a very important role in flavour production during sourdough fermentation (Jayaram et al., 2014). Small quantities of acetic acid and succinic acids are produced by sourdough yeasts, which affect the flavour profile of the leavened dough and also aid in acidification (Jayaram et al., 2013; Jayaram, Cuyvers, et al.,2014; Jayaram, Rezaei, et al., 2014; Rezaei, Jayaram, Verstrepen, & Courtin, 2016). Yeasts significantly add to the antioxidant status of the sourdough by increasing the availability of phenolics (Wang, He, & Chen, 2014), the dephosphorylation of phytate via the action of phytases (Lioger et al., 2007; Moslehi-Jenabian et al., 2010; Reale et al., 2004), and generation of ethylacetate that help in its antifungal actions (Coda, Cassone, et al., 2011; Coda et al., 2013).

2.4.7 Effect of the flour on the sourdough microbiota

Different types of grains are used to produce a sourdough, for example: wheat, rye, cassava, maize, barley, millet, emmer, sorghum, rice, oat, teff, buckwheat, quinoa and amaranth (Abdelghafor, Mustafa, Ibrahim, & Krishnan, 2011; Cappelle et al., 2013; Lhomme et al., 2016; Moroni et al., 2010; Vogelmann, Seitter, Singer, Brandt, & Hertel, 2009). Some legume flours such as: beans, chickpeas, lupine and lentils, and, flour from seeds like: hemp, sunflower and chestnuts are also being explored for the production of sourdough (Bartkiene et al., 2016; Coda, Di Cagno, Edema, Nionelli, & Gobbetti, 2010; Rizzello, Calasso, Campanella, De Angelis, & Gobbetti, 2014; Rizzello, Nionelli, Coda, De Angelis, & Gobbetti, 2010).

Flour quality and its chemical composition determine the presence of elaborate microbial communities and their metabolic impact on the sourdough. Some common LAB species are found universally across all the different types of sourdough fermentations, immaterial of the type of flour uses, are: *L. sanfranciscensis, L. plantarum, L. brevis, P. pentosaceus, L. fermentum* and *L. paralimentarius* (De Vuyst et al., 2014).

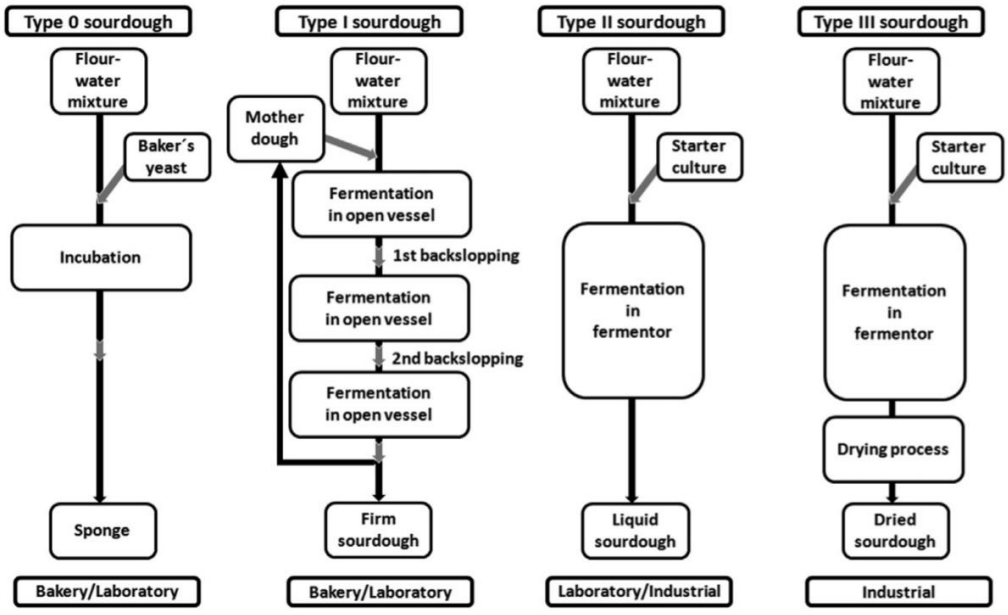
2.4.8 Effect of process parameters on the sourdough microbiota

2.4.8.1 Effect of process technology on the type sourdough

In process technology, there are 4 different types of sourdoughs, which are: type 0, 1, 2, and 3 (Böcker, Stolz, & Hammes, 1995; Brandt, 2007; Brandt & Gänzle, 2006; Corsetti, 2013; Vuyst et al., 2014; Decock & Cappelle, 2005; Hammes et al., 2005; Huys et al., 2013).

Type 0 sourdoughs or pre-doughs are also called as sponge doughs in which baker's yeast is added, which grows very rapidly along with LAB. Traditional or type 1 sourdough are back-slopped daily to maintain active microbes (Corsetti, 2013; Corsetti & Settanni, 2007; Vuyst & Neysens, 2005; Vuyst et al., 2014). The motherdough is used to further inoculate the subsequent sourdough made by adding water and flour without the addition of baker's yeast. Type 2 or semi-solid industrial sourdoughs are made by prolonged fermentation from 2 to 5 days and high water content with only one step propagation (Gänzle et al., 2007; Gobbetti et al., 2005). Type 3 or dried/inactivated sourdoughs are applied as acidifier supplements (non- living) and flavour carriers in the mix and have additional yeast added to them (Hammes et al., 2005; Minervini, Angelis, Cagno, & Gobbetti, 2014). Process parameters influence sourdough fermentation, and help determine its progress and the outcome. These parameters are fermentation time, temperature, leavening, resting, pH, redox potential, water activity, dough yields and refreshment activities, if any (Kerrebroeck, Bastos, Harth, & Vuyst, 2016).

Figure 2.8: Types of Sourdough process based on the technology applied (Vuyst et al., 2017).



2.4.8.2 Temperature of Sourdough Fermentation

The temperature at which the sourdough is fermented, plays a very important role in the microbial ecology of the dough, which in turn influences the metabolite and production kinetics (Katina,

Salmenkallio-Marttila, Partanen, Forssell, & Autio, 2006; Vogelmann et al., 2009). This, in turn, influences the flavour profile of the sourdough in terms of the production of volatile metabolites (Pico et al., 2015). Lower fermentation temperature (between 25-30°C) influences yeasts to form desirable esters in the sourdough whereas, at high temperature, polysaccharide breakdown and lipid oxidation occur at a faster rate (Birch et al., 2013).

Temperatures higher than 30°C shift the balance in the favour of lactic acid production, thus influencing the fermentation quotient of lactic acid: acetic acid ratio within the sourdough system. This leads to higher acidification of the sourdough (Brandt, Hammes, & Gänzle, 2004; Corsetti, 2013). At elevated temperature of more than 35°C, mainly lactic acid is produced within a short duration of sourdough fermentation due to a sharp drop in pH in the system due to the presence of homo-fermentative (and facultative hetero-fermentative) LAB. On the other hand, low fermentation temperature is more suitable for yeast cell growth and metabolism, which is beneficial during leavening since it support formation of carbon dioxide and ethanol (for flavour production) (Häggman & Salovaara, 2008). However, hetero-fermentative LAB species tend to dominate at low temperatures of 27 -28°C and long fermentation periods, producing a mixture of lactic acid, acetic acid, and/or ethanol (Vogel et al., 2011).

Temperature influences the yeast activity during fermentation for type 1 sourdough because at low temperature of 25°C, yeast cells and some LAB utilise glucose and gluco-fructans which makes fructose an available electron acceptor for hetero-fermentative LAB. This helps in the utilisation of mannitol and production of increased amounts of acetic acid for flavour production typical to type 1 sourdough (Corsetti, 2013; Ganzle & Gobbetti, 2013; Gobbetti et al., 2005, 2014). Similarly, for type 2 sourdough, LAB are dominant and therefore, there isn't enough yeast which can metabolise gluco-fructans, therefore making the addition of baker's yeast indispensable during the fermentation end (Corsetti, 2013).

2.4.8.3 Dough Yield and pH of the Sourdough

Dough yield (DY) is the ratio of flour and water in a sourdough system, which determines the sourdough consistency and therefore, making it a visual process parameter during fermentation. DY influences the water activity and the acidity of the doughs (Banu, Vasilean, & Aprodu, 2011; Vogelmann & Hertel, 2011). Low DY of 160 influences the system by effecting flour and making the carbohydrates more available for microorganisms, which in turn influences the activity of endogenous enzymes by lowering the rate of acidification of the dough, as compared to the high DY (DY of 280) sourdough system (Di Cagno et al., 2014).

Low pH (pH less than 4.0) stimulates the growth of LAB which are more acid-tolerant in relation to the high pH (pH exceeding 4.0) which stimulates the selection of Enterococcus, Lactococcus, Leuconostoc, Pediococcus, and Weissella species (Corsetti & Settanni, 2007; Vrancken, De Vuyst, Rimaux, Allemeersch, & Weckx, 2011).

2.4.8.4 Oxygen tension and fermentation time of the Sourdough

Kneading and mixing of sourdough are the means for aerating the sourdough matrix. This oxygen provides for enzymatic, metabolic and microbial activities within the system which is semi-anaerobic, thus influencing the community dynamics, bread texture and flavour formation, and, metabolism and kinetics of the microorganisms. Some microorganisms are capable of growing on the surface of the sourdough where there is more oxygen available, and favours the growth of certain yeast and LAB (Decamps, Joye, De Vos, Courtin, & Delcour, 2016; Viiard, Mihhalevski, Rühka, Paalme, & Sarand, 2013; Vogelmann & Hertel, 2011).

During the process of kneading the dough, the oxygen becomes available for the baker's yeast in the system, which in turn has positive effects on the flavour formation and metabolite production through anaerobic fermentation (Joye, Draganski, Delcour, & Ludescher, 2012). When the baker's yeast is not added, the oxygen is used by lipoxygenases in the flour that catalyse the conversion of lipids to aldehydes and hence affecting the flavour profile of the dough (Decamps et al., 2016; Pico et al., 2015).

Fermentation time of the sourdough directly affects the microbial community and diversity, metabolism and kinetics which is the result of acidification and nutrient consumption due to microbial growth, duration of propagation and frequency of refreshment of the sourdough (Lhomme, Lattanzi, et al., 2015; Vogelmann & Hertel, 2011; Vrancken, Rimaux, et al., 2011). For instance, *L. fermentum* dominates a laboratory wheat sourdough at 30°C with daily backslopping, whereas a mixture of *L. fermentum* and *L. plantarum* prevails at 30°C with backslopping every 2 days (Vrancken, Rimaux, et al., 2011). Similarly, rye sourdoughs propagated at high temperature and a fermentation time of 24 or 48 h select for *L. amylovorus*, *L. crispatus*, *L. frumenti*, *L. johnsonii*, *L. reuteri*, *L. panis*, and *L. pontis*, whereas a rice sourdough propagated by weekly backslopping is characterized by *L. paracasei*, *L. paralimentarius*, and *L. spicheri* (Meroth et al., 2004; Meroth, Walter, et al., 2003; Meuller et al., 2000). Also, bakery rye sourdoughs propagated at high temperature (above 30°C) and for a long fermentation time (at least 10 h) select for *L. amylovorus*, *L. frumenti*, *L. helveticus*, *L. panis*, and/or *L. pontis*, whereas those at ambient temperature (up to 30°C) and short fermentation time (less than 10 h) select for *L. helveticus* (low counts), *L. pontis* (high counts), *L. sanfranciscensis*, and/or *L. zymae* (Viiard et al., 2016).

2.4.9 Sourdough fermented with kefir

Kefir has been applied in regular bread and sourdough bread making, improving the sensorial characteristics, self-life and general properties of breads (Filipcev et al., 2007; Plessas et al., 2012; Plessas et al., 2011). It has been reported that the LAB in the kefir contribute by producing many

flavouring compounds, whereas, the yeast are mainly responsible to bring about the leavening in the dough, during fermentation (Plessas et al., 2005). Literature on creating sourdough breads with kefir is limited, however, may many microorganisms isolated from kefir have been applied to create a sourdough.

2.5. Sourdough microbial ecosystem

Sourdough microbiota essentially consists of LAB and yeast species. In terms of yeasts found in the sourdough ecosystem, only ascomycetous yeasts have been detected due to their fermentation ability compared to the basidiomycete yeast or dimorphic ascomycetes. Some of the common yeasts which have been identified from the sourdough are *S. cerevisiae, Candida colliculosa, Pichia kudriavzevii, Candida humilis, Wickerhamomyces anomalus* and *Saccharomyces exiguous* (Huys, Daniel, & De Vuyst, 2013; Vrancken et al., 2010). When the sourdough is developed in a bakery (which may harbour *T. delbrueckii, W. anomalus, Kazachstania unispora* and *Kazachstania barnettii*) where baker's yeast is used, the stability of the sourdough depends on the cooperation between the LAB, yeasts and baker's yeast (Daniel, Moons, Huret, Vrancken, & De Vuyst, 2011; Vrancken et al., 2010). Compared to the bakery environment, sourdough developed in the laboratory were dominant in *S. cerevisiae, W. anomalus* and *Candida glabrata* species. Heterofermentative LAB such as lactobacilli are the predominant microorganisms present in stable sourdough ecosystems (Huys, Daniel, & De Vuyst, 2013). Approximately 60 lactobacilli species (facultative and obligately heterofermentative and obligately homofermentative) have been isolated from different types of sourdough. The characteristic species for sourdough fermentation process are the obligately heterofermentative lactobacilli which have a very well adapted carbohydrate metabolism (e.g., maltose fermentation ability of *L. sanfranciscensis, L. fermentum* and *L. reuteri*), stress response (e.g., acidic stress response and production of stress proteins) and amino acid assimilation (e.g., the arginine deiminase pathway in *L. fermentum* and *L. reuteri*) (Gänzle, Vermeulen, & Vogel, 2007; Gobbetti, De Angelis, Corsetti, & Di Cagno, 2005; Rollan, Lorca, & de Valdez, 2003). The weissellas that are less predominant in the sourdoughs are obligately heterofermentative, such as *Weissella cibaria* and *Weissella confusa*), pediococci that are facultatively heterofermentative, such as *Pediococcus acidilactici, P. pentosaceus*, and leuconostocs that are obligately heterofermentative such as *Leuc. mesenteroides, Leuconostoc citreum*. The homofermentative lactococci, enterococci, and streptococci are subdominant.

2.6. Kefir Microflora

Many LAB, yeast and AAB species have been isolated and identified in kefir, which makes it a very diverse and complex ecosystem within itself (Rosa et al., 2017). Majority of the microbial biomass consists of the genera *Lactobacillus* and *Lactococcus* that constitutes about 65%–90%, with yeasts comprising the remainder of the biomass (Rosa et al., 2017; Simova et al., 2002).

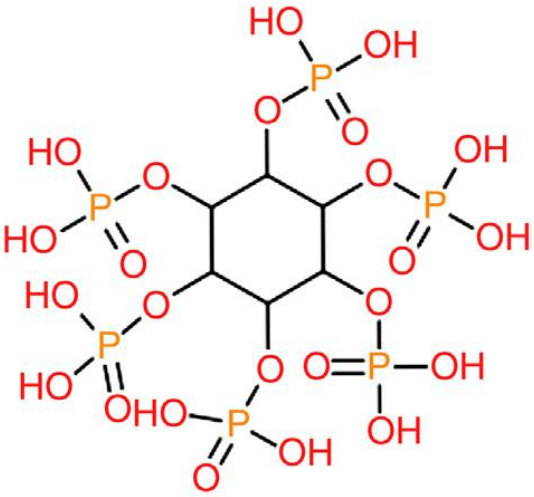
Homofermentative lactococci were previously reported to be the most predominant microorganisms in kefir grains, as reported by Koroleva (1988), however, LAB are now confirmed to be the most dominant microorganisms in kefir grains (Marsh et al., 2013; Dallas et al., 2016; Walsh et al., 2016; Rosa et al., 2017). Some of the widely predominant microorganisms are *Lactobacillus kefiri, L. kefiranofaciens, L. paracasei, L. acidophilus, L. delbrueckii,* and *L. plantarum* while Acetobacter are also integral components of kefir grains (Dertli & Çon, 2017; Gao & Li, 2016; Garofalo et al., 2017; Leite et al., 2012; Yang et al., 2014). A detailed list of all the species identified in kefir and beverages fermented with kefir has been prepared by Van-Wyk (2019), for LAB, AAB and yeast species.

2.7. Phytase

2.7.1 Phytic acid or phytate

Phytic acid is a hexaphosphoric ester of the hexahydric cyclic alcohol meso-inositol (Figure 2.9). Phytic acid, which is also identified as inositol hexakisphosphate (IP6), or as phytate when in salt form, is the principal storage form of phosphorus in several plant tissues. The molecular formula of IP6 is $C_6H_{18}O_{24}P_6$ and molecular mass is 660.04 g/mol. Inositol penta- (IP5), tetra- (IP4) and triphosphate (IP3) are also called phytates (Coban & Demirci, 2017).

Figure 2.9: Chemical structure of phytic acid (Coban & Demirci, 2017).



2.7.2 Phytate- An anti-nutritional factor

2.7.2.1 Mineral bioavailability

Wholemeal foods are a good source of minerals like potassium, calcium, phosphorus, magnesium, zinc and iron. Mg^{2+} reportedly contributes towards health-protective value of wholemeal foods against type 2 diabetes. This mineral bioavailability, however, may be limited due to the presence of about 3-22mg/g of phytate in grains (García-Estepa et al., 1999). Phytate or phytic acid is more concentrated in the aleurone layers of the grain and has a strong chelating ability and forms insoluble complexes with dietary cations, thus, impairing the mineral absorption. Phytate dephosphorylation by phytase enzyme to yield inorganic phosphate and inositol phosphate esters have very little influence on the mineral bioavailability and solubility. Enzymatic phytate degradation depends on parameters like particle size of the flour, acidity, presence of phytase activity, temperature, time of fermentation and water content (Harinder et al., 1998, De Angelis et al., 2003). Yildirim & Arici (2019) have reported high mineral bioavailability for sourdough fermented with *L. plantarum* and *L. brevis* with highest availability for Calcium and magnesium, followed by iron and zinc.

2.7.2.2 Effect of phytate on mineral uptake

Phytate is an anti-nutritional factor since it has a negative impact on the mineral uptake (for minerals like zinc, iron, calcium, magnesium, manganese and copper) in the human diet (Kumar, Sinha, Makkar, & Becker, 2010). This negative impact is caused by the formation of insoluble phytate-mineral complexes, which leads to decreased in mineral availability by not being readily absorbed by the gastrointestinal tract (Konietzny & Greiner, 2003).

Among all the minerals reported above, zinc (Zn^{2+}) bioavailability had the most adverse effect on human beings (Lopez, Leenhardt, Coudray, & Remesy, 2002). Prasad et. al., (1963) had reported the one of the first zinc deficiency in the Egyptian population, who mainly consumed bread and beans. Due to their high consumption of plant-based food, there was a considerable reduction in zinc homeostasis and adsorption in the intestine which resulted in hypogonadism and dwarfism (Oberleas, 1983). Moreover, in humans, the upper part of the digestive tract has reduced microbial population and in addition, the small intestine lacks the phytate-degrading enzymes (Iqbal, Lewis, & Cooper, 1994).

Studies on humans also show that phytate has a very strong inhibitory effect on non-haem iron absorption ($Fe^{+2/+3}$) (Brune, Rossander-Hulthén, Hallberg, Gleerup, & Sandberg, 1992). The microorganisms residing in the colon are capable of dephosphorylating the phytate and consequently releases Ca^{+2} which gets absorbed from the colon (Sandström, Cederblad, Stenquist, & Andersson, 1990).

A list of all the plant based food types and the phytate content has been summaried in Table 2.4, as below.

Table 2.4: Phytate content (mg/g dry matter basis) of all plant-based food types (Greiner & Konietzny, 2006).

Food types	Phytate (mg/g)
Cereals	
Rice (polished, cooked)	1.2–3.7
Rice (unpolished, cooked)	12.7–21.6
Maize bread	4.3–8.2
Unleavened maize bread	12.2–19.3
Wheat bread	3.2–7.3
Unleavened wheat bread	3.2–10.6
Rye bread	1.9–4.3
Sourdough rye bread	0.1–0.3
French bread	0.2–0.4
Flour bread (70% wheat, 30% rye)	0.4–1.1
Flour bread (30% wheat, 70% rye)	0–0.4
Cornflakes	0.4–1.5
Oat flakes	8.4–12.1
Pasta	0.7–9.1
Sorghum	5.9–11.8
Oat porridge	6.9–10.2
Legume-based food	
Green peas (cooked)	1.8–11.5
Soybeans	9.2–16.7
Tofu	8.9–17.8
Lentils (cooked)	2.1–10.1
Peanuts	9.2–19.7
Chickpea (cooked)	2.9–11.7
Cowpea (cooked)	3.9–13.2
Black beans (cooked)	8.5–17.3
White beans (cooked)	9.6–13.9
Kidney beans (cooked	8.3–13.4
Miscellaneous	
Sesame seeds (toasted)	39.3–57.2
Soy protein isolate	2.4–13.1
Soy protein concentrate	11.2–23.4
Buckwheat	9.2–16.2
Amaranth grain	10.6–15.1

2.7.2.3 Effect of phytate on protein digestibility, carbohydrate utilisation and lipid utilisation

Phytate forms a complex with proteins and resists their proteolysis. This process is generally dependant on the pH (Cheryan & Rackis, 1980). A phosphoric group of phytate binds with cationic groups of amino acids to form binary protein-phytate complexes at a pH value lower than the isoelectric point of proteins such as arginine, histidine and lysine (Cosgrove, 1966). These protein-phytate complexes dissolve only at a pH below 3.5, and their interactions are governed

by factors like dietary levels of magnesium and calcium, source and solubility of protein and pH. Therefore the formation of these complexes may affect the protein structures, which may alter its enzymatic abilities, digestibility or solubility (Kemme, Jongbloed, Mroz, Kogut, & Beynen, 1999).

Protein-phytate complexes may be digested by the proteolytic enzyme in vitro (Ravindran, Bryden, & Kornegay, 1995) and even digestive enzymes, such as chymotrypsin, pepsin and trypsin, amylase and lipase are inhibited by phytate (Deshpande & Damodaran, 1989; Inagawa, 1987; Knuckles & Betschart, 1987; Ravindran, 1995; Singh & Krikorian, 1982).

Intake of phytate rich foods in humans, reduces the glycaemic index (blood-glucose response) (Lee et al., 2006) which may be due to the formation of a complex between phytate and carbohydrate. This reduces the solubility of the carbohydrates and thus, its digestibility and absorption of glucose. Phytate may also bind with carbohydrates like starch by the formation of hydrogen bonds or by associating with the proteins bound to the starch (Rickard & Thompson, 1997). Moreover, the reduction in glucose response, i.e., low glycaemic index, as a result of cereal and legume foods consumption may aid diabetics to control blood glucose (Thompson et al., 1987, Yoon et al., 1983). It was also postulated that phytate, by complexing with Ca++ ion, inhibits amylase activity (Selle, Ravindran, Caldwell, & Bryden, 2000). In bean flour, subjected to dephytation, an increment of glycaemic index by 25-30% (42 to 68 GI) in humans had been reported by Thompson et al. (1987). Furthermore, in vitro studies have shown that incubation of human saliva with either wheat or bean starch incorporated with Na phytate reduced the phytilin-mediated hydrolysis of starch (Thompson et al., 1987, Yoon et al., 1983).

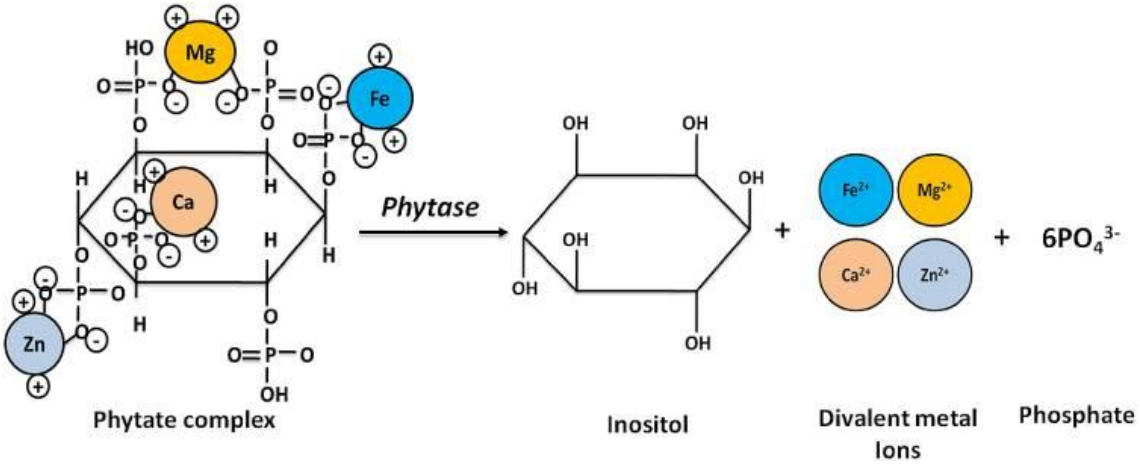
Phytate can form complexes with lipids, derivatives of lipid and with other nutrients known as lipophytins (Vohra & Satanarayana, 2003).

2.8. Classification of phytase

According to the International Union of Biochemistry (IUPAC-IUB), phytase falls under two separate categories depending on the site of hydrolysis. These are 3-phytase, EC 3.1.3.8 or myo-inositol hexakisphosphate 3-phosphohydrolase and 6-phytase, EC 3.1.3.26 or myo-inositol hexakisphosphate 6-phosphohydrolase (Selle, Gill, & Scott, 2007). Based on pH, phytases can be classified into alkaline phytases (optimum activity around pH 8.0) and histidine acid phosphatases (optimum activity at pH 5.0) (Baruah et al., 2007).

Most of the phytate degrading enzyme of microbial origin belong to the acid type (with Bacillus as an exception) (Selle et al., 2007).

Figure 2.10: Schematic representation of phytate hydrolysis in a catalytic reaction (Vashishth, Ram, & Beniwal, 2017).



2.8.1 Microbial phytases

Several phytases from microbial origin are sold as supplements with sales of over $500 million (Zhang, Cao, Hao, & Liu, 2013). Phytase activity for *L. casei* has been reported by Aziz et al. (2015) and phytase activity for *Lactobacillus fructivorans* and *L. sanfranciscensis*has been reported by De Angelis et al. (2003). Cizeikiene et al. (2015) have reported phytase production by various LAB strains like *Pediococcus pentosaceus* KTU05-8 and KTU05-9 at pH 5.5 at the temperature of 30°C, conditions similar to the dough leavening. This study also confirmed increased solubility of calcium ions, zinc ions, iron ions, manganese ions, iron ions and phosphorus ions by 30%. Phytase activity for *Lactobacillus plantarum* and effect of several ions like zinc, manganese, copper, calcium and cobalt on its activity has been studied by Saribuga et al. (2014).

For fungal and yeast phytases, only a few yeast species, such as *S. cerevisiae* and *S. castellii* have been reported as phytase producers (Liu et al., 1998). Yu et al. (2015) have reported phytase production by *Rhodotorula mucilaginosa* JMUY14 strain isolated from the Antarctic and have optimized and characterized its phytase production.

2.9. Phytate degradation during fermentation by phytase enzyme

Phytate degradation occurs due to the presence of phytase enzyme in plants and microorganisms while processing and preparing food items including germinating, cooking, soaking or fermenting them. The process of phytate dephosphorylation varies between plant and microbial species due to the differences in their ability to degrade intrinsic phytate (Egli, Davidsson, Juillerat, Barclay, & Hurrell, 2002). Food preparation or processing does not promise complete phytate hydrolysis

by endogenous phytases, therefore, exogenous phytase are desired since phytate reduction will increase mineral bioavailability, especially for iron (Hurrell, 2003).

Food products like bread have improved by addition of microbial-based phytases or plant protein isolates during fermentation (Fredrikson, Biot, Alminger, Carlsson, & Sandberg, 2001; Haros, Rosell, & Benedito, 2001). Phytic acid content is high for sourdough made with selected LAB strains *L. reuteri* (Sadeghi, Ebrahimi, Mortazavi, & Abedfar, 2019). During fermentation, microorganisms and/or enzymes may be found in raw materials being fermented or in the preparations of the exogenous microbial cultures, to activate the process. Low pH during lactic acid fermentation is due to the metabolism of LAB and AAB producing lactic acid and acetic acid, which are both very favourable for phytase enzyme activity on phytate (Lopez, Gordon, & Fields, 1983). During sourdough fermentation, the acidity of the dough aids in phytate degradation (Bartnik & Florysiak, 1988). Larsson and Sandberg (1991) have reported 96-97% reduction in phytate, when the pH was reduced to 4.6 for 10% sourdough made with oat and rye bran. Complete phytate degradation has been reported for white sorghum and maize gruels by Svanberg, Lorri, & Sandberg (1993) during germination and lactic acid fermentation. Extracellular and intracellular phytate degrading ability by *Rhizopus oligosporus* and *Aspergillus oryzae* has been found in soybean fermented food products like soy sauce, miso, koji and tempeh (Fujita et al., 2003).

2.10. Glutamic acid

Glutamic acid is an important amino acid which is involved in 'umami' taste perception of food and intermediary metabolism (Kondoh, Mallick, & Torii, 2009). It is also involved in enhancing gastric exocrine secretion when consumed with nutrients (Zolotarev, Khropycheva, Uneyama, & Torii, 2009). Glutamic acid is a precursor to other important amino acids like proline and arginine as well as to some bioactive precursors like glutathione and γ-aminobutyric acid (GABA). GABA possesses several well known physiological functions, that is, it is anti-hypertension (Inoue et al., 2003) and anti-diabetic (Hagiwara, Seki, & Ariga, 2004), whereas glutathione plays a role in protecting the mucosa from dietary toxins and peroxide damage (Beyreuther et al., 2007). A study carried out by Tomoe et al. (2009) and by Yamamoto et al. (2009) link the possible usefulness of glutamic acid to enhancing nourishment in the malnourished and elderly individuals.

Among the variety of metabolites produced by LAB, production of glutamic acid by LAB has been attributed to the presence of *gdh* gene (Tanous, Chambellon, Sepulchre, & Yvon, 2005). LAB can be employed in the production of glutamic acid, which can facilitate the production of functional foods rich in GABA. Glutamic acid produced by LAB is mostly L-glutamic acid which is considered to be biologically more active along with the production process being more eco-friendly and safe (Zareian et al., 2012).

Since glutamic acid is the precursor of GABA, the following decarboxylation reaction catalysed by glutamate decarboxylase (GAD) takes place:

$$\mathrm{L-glutamic\ acid} + \mathrm{H^+} \xrightarrow{\text{glutamate decarboxylase}} \mathrm{GABA} + \mathrm{CO_2}$$

The biochemical properties of GAD have been characterised and it has been isolated from various LAB species (Li and Cao, 2010).

2.11. Exopolysaccharide

Exopolysaccharides (EPS) are microbial polysaccharides produces outside the cell either by microorganisms outside the cell wall (Madiedo & Gavilán, 2005; Korakli et al., 2001).

They are also the dietary oligosaccharides which are non-digestible and aid in modulating the activity and composition of the intestinal microflora. This has certain health benefits for humans as it improves the bowel functions, prevents excessive growth of pathogenic bacteria by increased synthesis of short-chain fatty acids and by stimulating intestinal probiotic microorganisms (Ketabi, Dieleman, & Gänzle, 2011). EPS are composed of various polysaccharides, variety of proteins, glycoproteins, and glycolipids, and in some cases, surprising amounts of extracellular DNA (e-DNA) (Flemming, Neu, & Wozniak, 2007).

Depending on the position of the microbial polysaccharide inside the microbial cell, EPS are classified as below- (Bergmaier, 2002; Boels, van Kranenburg, Hugenholtz, Kleerebezem, & De Vos, 2001; Ruas-Madiedo & Reyes-Gavilán, 2005; Kumar, Mody, & Jha, 2007; Yildiz & Karatas, 2018)

1. Polysaccharides constituting the cell wall (structural polysaccharides), such as teichoic acids, lipopolysaccharides (LPS) and peptidoglycans
2. Polysaccharides which provide energy and act as a carbon source for the cell, called cytosolic polysaccharides or
3. EPS that are secreted in the form of biofilm or capsules to the extracellular medium

2.11.1 Purpose of EPS production by microorganisms

Microbial cells produce EPS mainly for protection against abiotic and biotic stress, offers great resistance due to its production, allows its use as a substrate for the growth of microorganism and influences the way microorganism producing EPS interacts with the environment (Czaczyk & Myszka, 2007; Baets, Laing, Francois, & Vandamme, 2002; Limoli, Jones, & Wozniak, 2015; Nwodo, Green, & Okoh, 2012; Ozturk, Aslim, & Suludere, 2010).

2.11.2 LAB and AAB EPS

EPS from LAB can be either homopolysaccharide (four subgroups, Dextrans, β-D-glucans, fructans and others like polygalactan) or heteropolysaccharide. Heteropolysaccharides are often produced by *Lactococcus lactis, Lactococcus. lactis* subsp. *cremoris, Lactobacillus casei, Lactobacillus. Sake* and *Lactobacillus. rhamnosus*) (Mollet, 1996).

Sourdough LAB synthesize a variety of large EPS through enzyme activity of glycosyltransferases. Previous study carried out on EPS suggests that some of the polymers from the fermentation of cereal foods by LAB may be available for food applications and processing through in-situ synthesis (Bounaix et al., 2009; Tieking and Gaenzle, 2005). Synthesis of EPS (glucans and fructans) with prebiotic potential has been reported for sourdough fermentation of sorghum and wheat flours by LAB such as *L. frumenti, L. pontis, L. acidophilus* and *L. reuteri* (Schwab, Mastrangelo, Corsetti, & Gänzle, 2008; Tieking, Kaditzky, Gänzle, & Vogel, 2003; Tieking, Korakli, Ehrmann, Gänzle, & Vogel, 2003). Kralj et al. (2002) have reported that by adding 12% sucrose to the dough during fermentation, *L. reuteri* strain 121 is able for form two types of EPS, a 4,6-disubstituted ά-glucan (reuteran) and a levan. Glucansucrase and fructosyltransferase are the enzymes responsible for the synthesis of above EPS (Van Hijum et al., 2002).

Acetic acid bacteria are Gram-negative obligate aerobes belonging to α-proteobacteria, and have the ability to grow floating in static culture by producing a pellicle in the surface of culture medium. This pellicle is an aggregation of cells in the liquid–air interface in which cells are tightly associated with each other by polysaccharides and other extracellular matrices on the cell surface. The pellicle polysaccharides occur as homopolysaccharide of cellulose or as heteropolysaccharides such as capsular polysaccharide produced by *Acetobacter tropicalis* SKU1100 consisting of glucose, galactose, and rhamnose (Moonmangmee, Toyama et al., 2002).

2.11.3 Yeast EPS

Many yeast species have been reported to produce and secrete EPS and biopolymers (comprised of various mono-, di- or polysachharides or units present in different conformations and structures) (Pavlova, 2014; Rima, Steve, & Ismail, 2012; Van Bogaert, De Maeseneire, & Vandamme, 2009). The biological function of EPS is generally very closely related to the configuration of the yeast cell or in some cases, it may inhibit the growth of surrounding bacterial cells (He, Yang, Yang, & Yu, 2010; S. Li & Shah, 2014; J. Wang, Zhao, Yang, Zhao, & Yang, 2015; J. Zhang et al., 2016). Belz et al. (2019) have reported the EPS production by *Weisella cibaria* incorporated in sourdough fermentation along with a few LAB strains.

2.11.4 Application of EPS in the food industry

Recently, it has been reported that the EPS synthesizing LAB have been employed in making gluten-free food products (Galle, Schwab, Arendt, & Gänzle, 2011; Galle et al., 2012). In-situ formation of dextran showed an increase in the shelf life of the food. Microbial EPS is also a promising alternative to replace the use of hydrocolloids in gluten-free food products (Marco Gobbetti, Rizzello, Di Cagno, & De Angelis, 2014). EPS producing *L. reuteri* E81 has been studied for its effect on sourdough fermentation, and it has been reported to have a positive effect on the overall bread rheology and elasticity (İspirli, Özmen, Yılmaz, Sağdıç, & Dertli, 2020). EPS such as gellan, galactopol, xanthan, pullulan and curdlan are also applied for use in the food industry due to their gas barrier properties and as an edible or a biodegradable membrane/film (Barcelos, Vespermann, Pelissari, & Molina, 2019). LAB producing EPS help in improving the rheological properties such as texture, mouthfeel, stability, shelf life, firmness and mouthfeel of the fermented dairy products (Jaiswal, Sharma, Sanodiya, & Bisen, 2014). Dextran is a type of EPS produced by *L. mesenteroides* and *L. dextranicum* which is used as a food additive, viscosity improving agent and as a stabilizer (Andhare, Chauhan, Dave, & Pathak, 2014; Biliaderis & Izydorczyk, 2006).

2.12. Sourdough bread flavour

The flavour of the sourdough bread is influenced by the enzymatic reactions of LAB and yeasts, by the type of ingredients used for preparing the dough and the temperature at which the bread is baked (Cho & Peterson, 2010). The formation of volatile compounds is achieved within 12 to 24 hours of fermentation which leads to dough acidification and flavour improvement (Hansen & Schieberle, 2005). The fermentation products of microorganisms are aldehydes, acids, esters, ketones and alcohols which are a major route for the formation of volatile compounds in sourdough and bread crumb (Pico, Bernal, & Gómez, 2015). These volatile compounds are responsible for imparting the intense aroma and flavour to the sourdough bread such as alcoholic, fruity, malty and acidic odour (Pétel, Onno, & Prost, 2017; Siepmann, Ripari, Waszczynskyj, & Spier, 2018). The fermentation quotient (molar ratio of acetic acid and lactic acid) which is responsible for taste, is very crucial and depends on the sourdough microbiota involved during the sourdough fermentation (Hansen, 2012; Zhao, Schieber, & Gänzle, 2016). The production of taste-active compounds such as glutamate and glutathione depend on the specific strains present in the sourdough fermentation, and are associated with glutaminase and glutamate decarboxylase activities (Zhao, Schieber, & Gänzle, 2016).

The type of starch and protein present in the flour (Salovaara and Valjakka 1987) also influences the aroma of sourdough, as well as the microbiota present during fermentation (Plessas et al. 2008; Hansen and Schieberle 2005). Makhoul et al. (2015) carried out a study revealing that using a different strain of *S. cerevisiae* has a greater influence on the final profile of aromatic compounds of the bread product, rather than using a different type of flour.

2.13. Sourdough bread innovations

The selection of microorganisms in sourdough fermentation can improve the technological characteristics of bread (Arendt, Ryan, & Dal Bello, 2007; Sadeghi, 2008). Selection of the strains used for fermentation can be based on the EPS producing ability or their ability to synthesis and accumulate bioactive compounds. Such are valuable in the production of functional bread. According to Rizzello et al. (2008), application of microorganisms that have distinct metabolic capability such as gamma-aminobutyric acid (GABA) production (*L. plantarum* and *L. lactis*), gliadins hydrolysis (*L. sanfranciscensis, L. alimentarius, L. brevis* and *Lactobacillus hilgardii*) and peptidase system specificity (*L. sanfranciscensis*) have been used as starter for sourdough fermentation, by using different flour types.

Wholemeal wheat sourdough reportedly had the highest GABA synthesis capability and rye, whole wheat, spelt and kamut sourdough had the highest antioxidant capability (Coda et al., 2012; Rizzello et al., 2008). Coda et al (2008) have also reported that the use of yeast and *L. plantarum* in sourdough fermentation extended the shelf life of the sourdough and improved the taste and structure of the bread produced. Pleassas et al. (2011) have reported the use of kefir for improved sourdough quality, flavour and increased shelf life compared to the conventional sourdough starter culture used with conventional baker's yeast.

Use of legumes, flaxseed and pseudocereals has been reported for the development of wheat sourdough breads. Furthermore, sourdough developed using chia seeds, amaranth and quinoa have been produced in addition to the application of selective LAB starter. LAB isolates such as *L. plantarum*, and *L. rossiae* (for quinoa sourdough), *L. plantarum*, *L. paralimentarius*, and *L. helveticus* (for amaranth sourdough) and *L. plantarum* (for chia sourdough) have been previously applied (Bustos et al., 2017; Jekle et al., 2010; Rizzello et al., 2016). Certain GABA producing strains of LAB such as *L. plantarum* and *Lactococcus lactis* have used with a distinct blend of flours, to produce a sourodugh. Sourdoughs which are spontaneously fermented with wheat sourdough, have reported the application of different flour types such as chick pea flour, bean and lentil flour, and Bambara nut flour (Chinma et al., 2016; Rizzello et al., 2014).

2.14. Proteolysis and acidification of sourdough bread

Enzyme proteinase that is associated with the cell envelope is absent in the sourdough LAB species. Under acidic conditions during sourdough fermentation, cereal-associated proteases assist the generation of amino acids and oligopeptides (De Vuyst, Kerrebroeck, & Leroy, 2017). Furthermore, the solubility of gluten proteins increases due to the reduction in their disulphide bonds by heterofermentative LAB through glutathione reductase. The increased solubility enhances their chances of degradation by proteolytic enzymes (Gänzle, Loponen, & Gobbetti, 2008).

Strain-specific proteolytic activity of LAB may additionally contribute to proteolysis, specifically, the degradation of albumins, globulins, and gliadins during fermentation (Di Cagno et al., 2002; Gänzle, 2014; Gobbetti et al., 2005).

2.15. Glycaemic index (GI)

Novotni et al. (2012) have reported that the addition of 15-22.5% sourdough decreased the GI of the participants and helped improve overall texture, volume and delayed the crumb firming of the sourdough bread. Sourdough can lead to active degradation of starch which can decrease glycaemic reponses. In addition, it has to its positive influence on the overall sensory quality of the fibre-rich, wholegrain and/or gluten free products, mineral bioavailabilty and enhanced accessibility of bioactive compounds (Poutanen, Flander, & Katina, 2009). The digestibility of sourdough bread is better than that of typical bread products, as most of the starch is metabolized influencing the glycaemic index of the final products (De Vuyst et al., 2017; Siepmann, Ripari, Waszczynskyj, & Spier, 2018).

Chapter 3 Fermentation of coconut water kefir

3.1. Introduction

Kefir is made from kefir grains, which are small, white-yellowish irregularly shaped granules with a diameter between 3-35 mm (Tratnik, Božanić, Herceg, & Drgalić, 2006). These grains contain a complex mixture of lactic acid bacteria (LAB), acetic acid bacteria and various yeasts combined with casein and complex sugars in a polysaccharide matrix (Guzel-Seydim, Wyffels, Seydim, & Greene, 2005; Minervini, De Angelis, Di Cagno, & Gobbetti, 2014; Simova et al., 2002).

The microbial diversity in and bioactivities of beverages produced using water kefir grains have been widely studied and shown to contain certain essential bioactive compounds with antioxidant, antimicrobial and anti-inflammatory activities (Fiorda et al., 2017; Rodrigues et al., 2016). The microbiota of kefir has great capability for adaptation into different food substrates which can be utilized to produce new beverages with probiotic function (Corona et al., 2016; Koh at al., 2017; Rodrigues et al., 2016). Previous studies on fermenting vegetable juices using kefir grains have been carried out (Garcia et al., 2020). Therefore, in this chapter, the probiotic activity of the kefir grains will be utilised to produce a fermented beverage using coconut water as a substrate. The fermentation of coconut water with kefir grains will be characterised to develop a starter culture for a laboratory type sourdough. This fermented mixture will eventually be utilised to produce a fermented sourdough and sourdough bread.

Coconut water consumption has increased worldwide. It has important hydrating qualities with enhanced taste profile as well as nutritional and functional health properties such as low-fat content and a good source of electrolytes (Campbell-Falck, Thomas, Falck, Tutuo, & Clem, 2000; DebMandal & Mandal, 2011; Saat, Singh, Sirisinghe, & Nawawi, 2002). Due to all these properties, coconut water was selected as a substrate for fermentation of kefir grains.

Traditionally, to prepare sourdough, the flour is mixed with water and is allowed to ferment naturally. This is an ancient technology which has been used in the Egyptian, Roman and Greek periods (Samuel, 1996). Other types of sourdoughs are prepared by adding a sourdough starter to the flour-water mixture, which is a sourdough pre-ferment maintained for a long duration. The objective of this chapter was to study the fermentation profile of coconut water inoculated with a kefir starter. The microbial cell count in different sugar substrates, the production of lactic acid, other organic acids and amino acids are properties that could determine how a new starter culture for sourdough could be developed using kefir and coconut water.

The reason for using fermented coconut water kefir as a starter for producing a fermented sourdough was based on previous studies which have reported the application of kefir to improve the shelf life by delaying spoilage, improve sensorial qualities and general sourdough characteristics (Filipcev et al., 2007, Plessas et al., 2011). A detailed study on sourdough prepared with fermented CWK will be explained in the next chapter (Chapter 4 of this thesis).

3.2. Materials and Methods

3.2.1 Microbial cultures

Kefir starter (Body Ecology™, New Zealand) was used to ferment coconut water with and without the addition of different sugars (glucose, sucrose and lactose). The fermentation was monitored from 0 to 96 h of incubation at 30°C. The kefir starter contains lactic acid bacteria (LAB), acetic acid bacteria (AAB) and yeast strains.

3.2.2 Coconut water kefir preparation

UFC© Coconut water (manufactured by Universal Food Public Company Limited, Thailand. Purchased at Countdown, Auckland City) was used in this study. Young coconut was purchased from Asian supermarket (Lim Chhour, NZ) and it was punctured in a laminar air hood, to obtain the fresh young coconut water under aseptic conditions. Food-grade sugars such as glucose (Food-grade, obtained from NZ chemical suppliers) and sucrose (Chelsea sugar limited, NZ) were used as additive carbohydrates. Autoclave-able, lidded, plastic containers with 500 ml capacity (Thermo Fisher, NZ) were used for carrying out the fermentation. The sugars were filter-sterilised by preparing a concentrated solution with autoclaved distilled water. The concentrated sugar stocks were prepared for sucrose (0.15% w/v, 0.6% w/v, 1.2% w/v and 11.6% w/v); glucose (0.15% w/v and 1.2% w/v) and lactose (1.35% w/v). The concentrated stock of sugar solution was sterilised using a sterile syringe and sterile filter membrane with 0.2µM pore size (Thermo Fisher NZ). The sterile sugar solution was collected in a sterile bottle. The membrane filtration was performed aseptically in a laminar air-flow hood.

The different variations of sucrose and glucose concentrations used, the amount of coconut water and of kefir are shown in Table 3.3. Different quantities of sucrose and glucose were used to determine the effect of different types of sugars and their different concentrations on the fermentation of coconut water with kefir grains. Kefir grains at the concentration of 1.5 g/L were used for this experimental design and 300 ml of coconut water was used as the fermentation medium. Sucrose and glucose at the concentrations of 6 g/L and 12 g/L were used in various combinations according to the factorial analysis. Factorial analysis is a method in which several permutation strategies are implemented for all the factors utilised for the study by using XLSTAT version 2019.1.2, StatSoft Inc., USA (Anderson & Braak, 2003). The influence of the variables, namely, sugar types- sucrose and glucose, and the concentration of sucrose and glucose on coconut water kefir fermentation was studied employing a full factorial design (Myers, Montgomery, & Anderson-Cook, 2016). Runs were performed at random. Results were analyzed using XLSTAT (version 2019.1.2, StatSoft Inc., USA) software. The statistical significance of the regression coefficients was determined using Student's t-test. The second-order model equation was evaluated using Fischer's test and the proportion of variance explained by the model

obtained was given by the multiple coefficients of determination, R^2. After carrying out the preliminary experiments (refer to Table 3.1, Section 3.3.1) for the resulting thirty permutations, nine such permutations were selected, which are shown in Table 3.3.

3.2.3 Coconut water kefir incubation conditions

The above experimental sets (Table 3.3), were incubated aerobically at 30°C for a period of 96 h in a LabServ incubator (Thermo Fisher Scientific, New Zealand). Samples were taken aseptically every 24 h for monitoring pH, cell counts (CFU/ml) for LAB and yeast and other analysis.

3.2.4 Determination of cell counts

Cell counts were performed using the viable count method to obtain the colony forming unit per ml (CFU/ml) in each sample. Samples were taken from each of the 9 experimental sets at uniform time interval of 24 h (0, 24, 48, 72 and 96 h). Different culture media were used for determining the growth of LAB, yeast and acetic acid bacteria. LAB were grown on De Man, Rogosa and Sharpe agar medium (MRS), yeasts were grown on Malt Extract agar (ME) and acetic acid bacteria were grown on acetic acid bacteria agar (AAB). All the media were supplied by DIFCO and were prepared according to the manufacturer's instructions. The media were autoclaved at 121°C for 15 min at 15psi. All the Petri dishes were bought from Fort Richard Laboratories, Auckland, New Zealand.

A spread plate technique was used for spreading the culture uniformly across the circular agar surface with a sterile glass spreader (Sanders, 2012). Ten-fold dilutions in peptone water were prepared for all samples from each set. A 100µL sample from each dilution was spread on the different agar media. All the spread plates of MRS, ME and AAB were incubated at 30°C in a CO_2 incubator (5% CO_2 concentration) until colonies were visible (72 to 96 h). The spread plates of ME were incubated at 30°C (LabServ incubator, Thermo Fisher Scientific, New Zealand). The colonies were counted after which the colony forming units (CFU/ml) were calculated, where CFU/mL = (no. of colonies x dilution factor)/volume of sample plated.

3.2.5 pH

The pH was determined in accordance with the Association of Analytical Communities (AOAC) methods, where a pH meter (Eutech pH 700 meter, Thermo Fisher Scientific Inc., New Zealand) was used with a glass electrode (Electrode ECFC7252101B, Thermo Fisher Inc., New Zealand). The pH meter electrodes were calibrated against known buffer solutions. The readings were taken when the pH meter was stabilised (Katina, Salmenkallio-Marttila, Partanen, Forssell, & Autio, 2006).

3.2.6 D-/L- Lactic acid content

The concentrations of D- and L-Lactic acids concentrations were determined using Megazyme lactic acid kit K-DLATE01/14 (Megazyme, New Zealand), following the manufacturer's instructions.

3.2.7 Estimation of reducing and non-reducing sugars

The concentration of total reducing sugars was determined using the 3,5-dinitrosalicylic (DNS) acid method (Miller, 1959). The DNS reagent was prepared by dissolving 10.6 grams of 3,5-dinitrosalicylic acid reagent and 19.8 grams of sodium hydroxide (NaOH) into 1416 mL deionised water. Then, 306 grams of potassium sodium tartrate (Rochelle salts), 8.132 grams of melted phenol, and 8.3 grams of sodium metabisulfite were added into the previous solution with stirring (Dutta et al., 2008). A calibration curve was set at 570 nm using standard solutions of glucose, fructose and lactose solutions. Absorbance was determined on a spectrophotometer (Ultrospec 2100 pro UV/visible spectrophotometer).

The concentration of non-reducing sugars, such as sucrose, was estimated using a modified Anthrone method (Finley & Fellers, 1973) which is a colorimetric method involving ethanol-based extraction of sucrose. For the sucrose estimation, 1 ml of CWK sample was boiled with 50 ml of 80% ethanol in 100 ml volumetric flask for 15 min. After cooling extract was diluted to 100 ml with 80% ethanol. To the 1 ml of aliquot added 9 ml of Fehling solution and heated in a boiling water bath for 15 min. Cooled it to room temperature and from which 1 ml of the aliquot was taken and 10 ml of anthrone reagent was rapidly added to it. It was held for 30 min at 40 °C. Simultaneously reagent blank and a standard of 1 ml of 15% w/v of sucrose were also run side by side. The absorbance was read at 610 nm against a reagent blank.

3.2.8 Scanning electron microscopy of coconut water kefir granules

Coconut water kefir granules (extracted after 72 h of incubation in coconut water at 30°C) were filtered using a biological membrane of pore size 0.20 micrometre (µm) and thickness of 150 µm. The granules were then freeze-dried at -80°C using a freeze drier (ALPHA 2-4 LDplus). The freeze-dried coconut water kefir samples were cut transversely into sizes of about 1*1*0.5 cm (length*breadth*height) using a sharp razor blade. This section of the sample was mounted onto a stainless-steel metal using double-sided carbon conductive adhesive tape. A platinum coating was applied in vacuum using the Hitachi E-1045 ion sputter coater for better resolution and to increase the ion conductivity of the lactic acid bacteria and yeast because of their small sizes. This also leads to an increase in electron emission, which helps to capture a stabilised photograph of the granules. A field emission scanning electron micrograph apparatus (SU-70 Schottky, Hitachi,

Japan) at an accelerating voltage of 5 kV. SEM was used to view the microscopic structure of coconut water kefir granules.

3.2.9 Determination of total titratable acidity

Total titratable acidity was determined using the AACC International Method 02-31.01 (AACC, 1999), where 10 ml of coconut water kefir sample was titrated against 0.1 M NaOH, with pH 8.5.

3.2.10 Sample preparation for Liquid Chromatography-Mass Spectrometry (LC-MS)

One ml sample was collected from each of the above sets at 0, 24, 48, 72 and 96 h. The samples were spun down at 1000g for 5min to obtain cell-free supernatants using a GYROZEN centrifuge, model 1580R (Bio-strategy, New Zealand). The supernatant for each sample was collected and was further subjected to derivatisation for LC-MS analysis of the organic acids and amino acids. The samples were stored in -80°C freezer until the derivatisation for LC-MS analysis.

3.2.11 Determination of carboxylic acids using Liquid Chromatography- Mass Spectrometry (LC-MS)

One ml of sample was added to ice-cold 50% MeOH, containing 10 mg/L 4-chlorobenzoic acid (CBA) as an internal standard. The mixture was centrifuged at 10,000xg for 5 min at 4°C (Z216MK, HERMLE Labortechnik GmbH, Germany). The 2-hydrazinoquinoline (HQ) derivatization reaction was conducted by mixing 5 µL of the sample with 100 µL of acetonitrile solution containing 1 mM 2,2′-dipyridyl dipyridyl disulfide (DPDS), 1 mM triphenylphosphine (TPP) and 1 mM HQ. The reaction mixture was incubated at 60 °C for 60 min. The HQ derivatives in the reaction mixture were analyzed using LC-MS .

The carboxylic acids used as standards for this assay were lactic acid, succinic acid, acetic acid, glutamic acid, pyruvic acid and malic acid prepared with 50% methanol, and 20mM sodium hydroxide.

The LC-MS was an Agilent 1260 Series liquid chromatograph comprising a G1311B quaternary pump, G1329B thermostatted autosampler and a G1330B thermostatted column compartment (Agilent Technologies, Santa Clara, USA).

Mobile phase A was 0.1% formic acid in ultrapure water and mobile phase B was 0.1% acetic acid in acetonitrile. The column was a Phenomenex Kinetex EVO C18 column, measuring 2.1x150 mm with 1.7 µm diameter packing material, maintained at 25 °C. The chromatographic gradient was held for 0.1 min at 3% B and then ramped to 6min at 15% B and the to 9 min at 90%

B before decreasing to 3% B at 11 min. The flow rate was 200 µL min-1, the total run time was 16 minutes and the injection volume was 5 µL.

For detection, an Agilent 6420 triple quadrupole mass spectrometer fitted with an Agilent Multimode Ionisation source was operated in positive electrospray mode. Derivatised carboxylic acid peak areas were normalised and quantified by reference to a dilution series of external standards prepared. The external standards prepared were succinic acid, lactic acid, acetic acid, malic acid and pyruvic acid. Data were collected and processed using Agilent MassHunter software.

3.2.12 Amino acids analysis using Liquid Chromatography-Mass Spectrometry (LC-MS)

A 1.4 mL volume of 50% methanol containing 10 mg/L of 2,3,3,3-d4-alanine [d4A] was added to 1 ml samples and mixed in a microcentrifuge tube. The tubes were vortexed to obtain a homogenous mixture. This was followed by centrifugation at 10,000 g for 5min at 4°C (Z216MK, HERMLE Labortechnik GmbH, Germany). A 10 µl volume of extract was used to perform pre-column derivatisation with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate following a method adapted from Salazar et al (2012). The LC-MS was an Agilent 1260 Series liquid chromatograph comprising a G1311B quaternary pump, G1329B thermostatted autosampler and a G1330B thermostatted column compartment (Agilent Technologies, Santa Clara, USA). Mobile phase A was 0.6% formic acid in ultrapure water and mobile phase B was 0.1% formic acid in acetonitrile. The column used was a Phenomenex Kinetex EVO C18 column, measuring 2.1x150 mm with 1.7 µm diameter packing material, maintained at 25 °C. The chromatographic gradient was held for 1 min at 1.5% B and then ramped to 13% B at 8 min, 17% B at 15 min and 80% B at 16 min before returning to 1.5% B at 17.5 min. The flow rate was 300 µL min^{-1}, the total run time was 28 min and the injection volume was 5 µL. For detection, an Agilent 6420 triple quadrupole mass spectrometer fitted with an Agilent Multimode Ionisation source was operated in positive electrospray mode. Optimum Multiple Reaction Monitoring [MRM] transitions were established using Agilent MassHunter Optimiser B06.00 software. Derivatised amino acid peak areas were normalised to the recovery of d4A and quantified by reference to a dilution series of external standards prepared from a commercial amino acid mix (Sigma product A9906, Sigma-Aldrich Pty. Ltd., Sydney, Australia). Data were collected and processed using Agilent MassHunter software.

3.2.13 Statistical analysis

At least five individual replicates of each of the experimental sets were prepared for statistical analysis. All the results are expressed as means of values ± standard deviation for five replicates per sample. One-way and two- way analysis of variance (ANOVA) were applied to determine

level of significance and when the ANOVA was significant, means were separated by pairwise comparison using Tukey's (HSD) test or Fisher (LSD) test at 5% significance level. All statistical analyses were performed using the Statistical Analysis System – XLSTAT-MX version 2012.4.02 (Addinsoft, New York, NY, USA). For analysing the amino acid data for coconut water kefir, web based MetaboAnalyst version 3.0 was used (Chong et al., 2018). All the data was normalized by log-transforming and mean centering. Principal Components Analysis (PCA) was used to identify the effect of time on the change in amino acid profile, based on the underlying structure of the data. ANOVA Simultaneous Component Analysis (ASCA) was performed to identify major patterns with regard to the two factors: glucose and sucrose concentrations and their interaction (Smilde et al., 2005).

Due to a vast number of data generated from the 9 experimental sets with 5 replicates each, the visualisation of data would be exceedingly difficult. Therefore, to visualise the trend in the data, a PCA was used to view the dominant patterns in a data set. Interactive PCA visualisation of a 2D score plot with 95% confidence was used to understand the influence of time on the amino acid concentration. A cluster analysis by using heat map was carried out for better visualisation of the data. For heatmap, each coloured cell on the map corresponds to a concentration value for the amino acids, from a particular experimental set.

Heatmaps were used to identify samples/features in the data that have an unusually high/low value. The distance for heatmaps was measured in Euclidean and the Ward cluster algorithm was used.

3.3. Results and Discussion

3.3.1 Preliminary study

Kefir starter culture (Body Ecology™, New Zealand) was used to ferment CW using different concentrations of glucose, sucrose and lactose. The sugars were added to the fresh coconut water and UFC© treated coconut water. All the experimental sets for the preliminary study were incubated at 30°C (LabServ incubator, ThermoFisher, New Zealand), in triplicates.

Table 3.1: Experimental sets used to carry out the preliminary study.

Experimental sets	Composition
Set 1	300 ml of UFC© coconut water + 1.5g/L of kefir
Set 2	300 ml of Fresh coconut water (young) + 1.5g/L of kefir
Set 3	300 ml of UFC© coconut water + 1.5g Kefir + 1.5g/L of sucrose
Set 4	300 ml of UFC© coconut water + 1.5g Kefir + 6.0g/L sucrose
Set 5	300 ml of UFC© coconut water + 1.5g Kefir + 12.0g/L sucrose
Set 6	300 ml of UFC© coconut water + 1.5g Kefir + 116g/L sucrose
Set 7	300 ml of UFC© coconut water + 1.5g Kefir + 1.5g/L of glucose
Set 8	300 ml of UFC© coconut water + 1.5g Kefir + 6.0g/L glucose
Set 9	300 ml of UFC© coconut water + 1.5g Kefir + 12.0g/L glucose
Set 10	300 ml of UFC© coconut water + 1.5g Kefir + 13.5g/L lactose

Set 6 was supplemented with 116g/L sucrose as a positive control for the preliminary experiment. At the conditions used, 116g/L of sucrose did not completely dissolve in 300 ml of coconut water.

Microbial counts were carried out for all the sets incubated at 30°C at times, T= 0, 24, 48, 72 and 96 h (Table 3.2). From Table 3.2 it can be observed that with time, there was a considerable increase in biomass of LAB, AAB and yeast cells. Significantly high cell concentrations were obtained for all the sets incubated for 96 h time compared to those incubated for 24, 48 and 72 h. Highest significant cell count for LAB, yeast and AAB were obtained for set 5 at 96 h (5.93 ± 0.12 log CFU/ml, 4.03 ± 0.15 log CFU/ml and 3.73 ± 0.12 log CFU/ml respectively, $p<0.05$) and the corresponding f-value was 25.29, 23.68 and 124.81, respectivetly, compared to rest of the time periods and sets.

Triplicate samples were taken from each treatment. The heat-map (Figure 3.1), shows that sets 4, 5, 8, 9 and 10 have the highest log CFU/ml for LAB, yeast and AAB (grown on MRS, ME and acetic acid bacteria agar, respectively) for time, T=48, 72 and 96 h. Similar to the present results, previous studies have demonstrated that by addition of glucose, sucrose or lactose, the concentration of cells increases in the fermentation medium for kefir cell biomass (Golfinopoulos, Papaioannou, Soupioni, & Koutinas, 2009; Harta et al., 2004).

Results from the heat-map further indicate that no significant growth of LAB, yeast and AAB was obtained for sets supplemented with the lowest sugar concentration (sucrose at 1.5 g/L, glucose

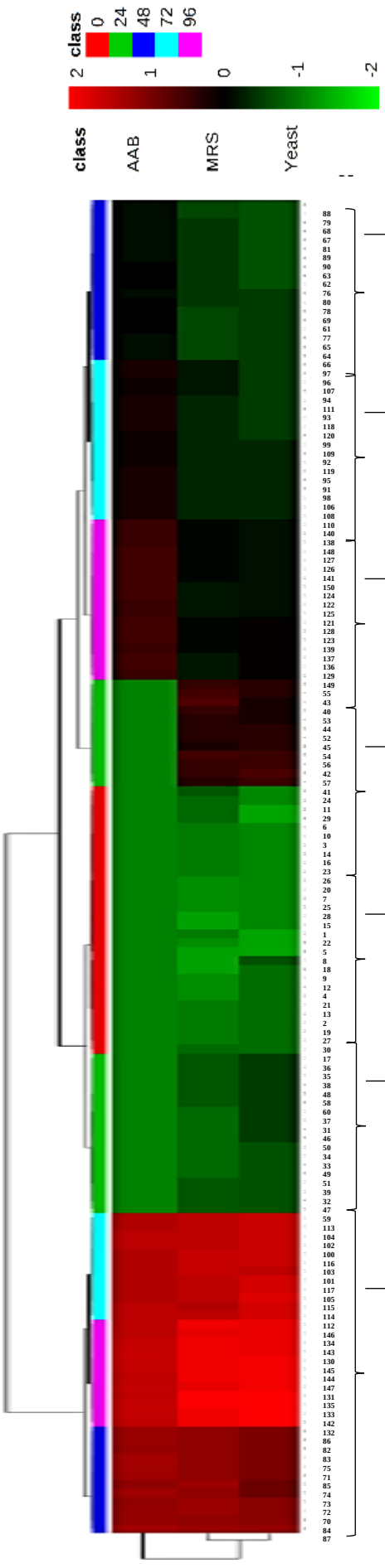
at 1.5 g/L) or those supplemented with lactose (13.5 g/L) (Figure 3.1). The results from the low sugar concentrations are expected. The cell biomass would not increase in the presence of inadequate carbon source for growth. However, very high concentrations of sugar could inhibit the growth of kefir biomass (Harta et al., 2004; Wang & Bi, 2008).

Table 3.4 also shows that there was no significant difference in the microbial counts between those of fresh young coconut water and UFC© treated coconut water (set 2 and 1, respectively).

Since this study is concerned with enhancing sourdough with the functional properties of coconut water kefir, it is very important to obtain a higher number of LAB, AAB and yeast cells for preparing the dough. Sets 4, 5, 8 and 9 had significant growth, hence they were retained for further study. However, since no significant growth was obtained for Sets 3, 6, 7, they were omitted from further study. Set 1 and Set 2 were intended to serve as controls in this study but there was no significant difference between these sets, hence, Set 2 was omitted. Although, considerable cell growth was obtained for Set 10 of CWK supplemented with lactose, it was also omitted since it was designed with the addition of lactose. The lactose component would not be suitable for developing a new coconut water kefir sourdough that can be consumed by lactose-intolerant individuals.

Based on the results from the pilot study, a factorial design was used to determine the influence of 6 and 12 g/L of glucose and sucrose, individually and in combination, on coconut water kefir fermentation (Table 3.3). This type of experimental design using a combination of two sugar types and increase in sugar concentration has been applied in previous studies and had a resultant positive effect on the increase in the overall kefir biomass (Harta et al., 2004; Carmen et al., 2014).

Figure 3.1: Heat-map and hierarchical clustering of microbial counts from coconut water kefir samples supplemented and un-supplemented with varying concentrations of glucose, sucrose and lactose with fresh (young) coconut water and UFC© treated coconut water. The dendrogram represents clusters for LAB, AAB yeast and expresses the relationship with respect to time, T= 0, 24, 48, 72 and 96 h of incubation, based on Pearson's correlation coefficient with average linkage. Each small- sized square in the heat-map expresses normalized counts for LAB, AAB and yeast to the colour range. The red colour indicates higher counts for respective microorganism.



88, 79, 68, 67, 81, 89, 90, 63, 62, 76, 80, 78, 69, 61, 77, 65, 64, 66, 97

96, 107, 94, 111, 93,118, 120, 99, 109, 92, 119, 95, 91, 98 106, 108, 110, 140

138, 148, 127, 126, 141, 150, 124, 122, 125, 121, 128, 123 139, 137, 136, 129, 149, 55, 43

40, 53, 44, 52, 45, 54, 56, 42, 57, 41, 24, 11, 29, 6, 10, 3, 14, 16, 23

26, 20, 7, 25, 28, 15, 1, 22, 5, 8, 18, 9, 12, 4, 21, 13, 2, 19, 27

30, 17, 36, 35, 38, 48, 58, 60, 37, 31, 46, 50, 34, 33, 49, 51, 39, 32, 47

59, 113, 104, 102, 100, 116, 103, 101, 117, 105, 115, 114, 112, 146, 134, 143, 130, 145, 144, 147, 131, 135, 133, 142, 132, 86, 82, 83, 75, 71, 85, 74, 73, 72, 70, 84, 87

For time, T= 0 h: Numbers 1-3 represent set 1, numbers 4-6: represent set 2, numbers 7-9 represent set 3, numbers 10-12 represent set 4, numbers 13-15 represent set 5, numbers 16-18 represent set 6, numbers 19-21 represent set 7, numbers 22-24 represent set 8, numbers 25-27 represent set 9 and numbers 28-30 represent set 10.

For time, T= 24 h: Numbers 31-33 represent set 1, numbers 34 to 36 represent set 2, numbers 37-39 represent set 3, numbers 40-42 represent set 4, numbers 43-45 represent set 5, numbers 46-48 represent set 6, numbers 49-51 represent set 7, numbers 52-54 represent set 8, numbers 55-57 represents set 9 and numbers 58-60 represent set 10.

For time, T= 48 h: Numbers 61-63 represent set 1, numbers 64 to 66 represent set 2, numbers 67-69 represents set 3, numbers 70-72 represent set 4, numbers 73-75 represent set 5, numbers 76-78 represent set 6, numbers 79-81 represent set 7, numbers 82-84 represent set 8, numbers 85-87 represents set 9 and numbers 88-90 represent set 10.

For time, T=72 h: Numbers 91-93 represent set 1, numbers 94 to 96 represent set 2, numbers 97-99 represents set 3, numbers 100-102 represent set 4, numbers 103-105 represent set 5, numbers 106-108 represent set 6, numbers 109-111 represent set 7, numbers 112-114 represent set 8, numbers 115-117 represents set 9 and numbers 118-120 represent set 10.

For time, T= 96 h: Numbers 121-123 represent set 1, numbers 124 to 126 represent set 2, numbers 127-129 represents set 3, numbers 130-132 represent set 4, numbers 133-135 represent set 5, numbers 136-138 represent set 6, numbers 139-141 represent set 7, numbers 142-144 represent set 8, numbers 145-147 represents set 9 and numbers 148-150 represent set 10.

Table 3.2: ANOVA table for LAB, AAB and yeast counts for all the experimental sets (Table 2.4) for time, T= 0, 24, 48, 72 and 96 h.

	f.value	**p.value**	**-log$_{10}$(p)**	**FDR**	**Fisher's LSD**
AAB	124.81	6.30E-46	45.2	1.89E-45	48 - 0; 72 - 0; 96 - 0; 48 - 24; 72 - 24; 96 - 24; 96 - 48
LAB	25.295	6.63E-16	15.178	9.95E-16	24 - 0; 48 - 0; 72 - 0; 96 - 0; 48 - 24; 72 - 24; 96 - 24; 96 - 48
Yeast	23.682	4.38E-15	14.359	4.38E-15	24 - 0; 48 - 0; 72 - 0; 96 - 0; 72 - 24; 96 - 24; 96 - 48

3.3.2 Microbial profile of kefir fermentation

Coconut water (CW) fermentations for all the factorial sets generated from the preliminary study are shown in Table 3.3. The fermentations were monitored from 0 h to 96 h of incubation at 30°C, and were analysed for bacterial and yeast growth.

Table 3.3: The composition of nine experimental sets obtained from factorial design.

Experimental sets	Coconut water (ml)	Kefir grains (g/L)	Added glucose (g/L)	Added sucrose (g/L)
Set 1	300	1.5	0	0
Set 2	300	1.5	0	6
Set 3	300	1.5	0	12
Set 4	300	1.5	6	0
Set 5	300	1.5	6	6
Set 6	300	1.5	6	12
Set 7	300	1.5	12	0
Set 8	300	1.5	12	6
Set 9	300	1.5	12	12

3.3.2.1. Lactic Acid Bacteria (LAB)

The concentrations of LAB during the fermentation are shown in Table 3.4. In all the sets inclusive of the un-supplemented CW and supplemented CW with different combinations of glucose and sucrose, LAB produced significant cell biomass concentration from the time of inoculation. CW supplemented with sucrose at 12 g/L (Set 3) produced the highest LAB cell concentration, 5.72 ± 0.04 log CFU/ml ($p \leq 0.05$) after 96 h. The second-highest concentration, 5.64 ± 0.02 log CFU/ml ($p \leq 0.05$) was obtained from Set 9, CW to which both glucose (12 g/L) and sucrose (12 g/L) in the ratio of 1:1, were added. The least cell biomass production (5.02 log CFU/ml, $p \leq 0.05$) occurred in CW which was not supplemented with either glucose or sucrose (control set). A bar graph for the growth of LAB is shown in Figure 3.2.

The two highest LAB concentrations were obtained from sucrose-supplemented CW (Sets 3 and 9). This was expected since sucrose consists of glucose and fructose each of which serves as a fermentable source of energy for growth of the LAB. The LAB are known to utilise a wide range of sugars to obtain energy by fermentative metabolism. Glucose, fructose, galactose and mannose, sucrose and lactose are fermented by almost all species of the LAB (Endo and Dicks, 2014; Harta et al., 2004; Zajšek, Goršek, & Kolar, 2013).

The presence of a comparable cell biomass concentration of LAB after 96 h of fermentation in sucrose and glucose (both 12 g/L) supplemented CW (Set 9) in relation to that in CW containing

sucrose (12 g/L), Set 3, indicates that the sugar content in CW provides energy for the growth of the bacteria. Although the statistical tool employed revealed a significant difference between growth in Set 9 and in Set 3, the absolute difference in the cell concentration was less than one order of magnitude. This was true with all the experimental sets. The results suggest that there might be some kind of sugar (substrate) saturation of the enzymes involved in the metabolism of the sugar (Harta et al., 2004; Wang & Bi, 2008). This could also explain the lack of significant difference between Set 2 (CW containing 6 g/L sucrose) and Set 5 (CW containing 6 g/L each of glucose and sucrose).

In addition to substrate saturation effect, the results suggest that there might be catabolite repression occurring between glucose and sucrose. The acid products of fermentation could also inhibit the growth of LAB as supported by 2D score plot for the increase in organic acids during coconut water kefir fermentation (Figure 3.9) (Grønnevik et al., 2011; Irigoyen, Arana, Castiella, Torre, & Ibanez, 2005).

Furthermore, the cell numbers produced in all experimental sets (approximately 5 log CFU/ml) are low compared to those of the LAB from other substrates such as fructose and lactose in other studies (Cheirsilp, Suksawang, Yeesang, & Boonsawang, 2018; Harta et al., 2004; Zajšek, Goršek, & Kolar, 2013). This suggests that despite the presence of more than one type of sugar at high concentration, there is some other factor that is limiting the growth of LAB in CW. LAB are known to be fastidious microorganisms which require more than two growth factors such as vitamins and amino acids, and could be also be affected by the nitrogen concentration (Dailin et al., 2016; Ghasemlou, Khodaiyan, Jahanbin, Gharibzahedi, & Taheri, 2012; Maeda, Zhu, Suzuki, Suzuki, & Kitamura, 2004; Taniguchi, Nomura, Itaya, & Tanaka, 2001; Yokoi & Watanabe, 1992). Wang and Bi (2008) studied the influence of various carbohydrates on the growth of kefir microorganisms in MRSL (De Man-Rogosa-Sharpe containing Lactose) medium, which is formulated to enrich the growth of LAB. They concluded that increased growth of kefir microorganisms and high kefir biomass was produced by the addition of sucrose, lactose, glucose, maltose and fructose. The addition of maltose resulted in the highest kefir biomass production. Hence, there is good evidence that CW is lacking in one or more essential nutrients for LAB growth (Wang & Bi, 2008).

The F-values obtained for the variables affecting the growth show that the length of cultivation time had the most significant effect (357.99, $p \leq 0.01$). This is a known fact with any cell growth that given all the factors for the growth, there would be an increase in growth as time increases until the stationary phase is reached.

The second significant factor that affects growth of the LAB in CW is the presence of glucose on its own. This effect may be due to the property of glucose as the simplest monosaccharide that can be metabolized by most living cells including the LAB. Although either glucose or sucrose

could individually affect growth of the LAB, when the two sugars are both added to CW, the effect on the increase in the LAB growth, F= 7.668*** ($p \leq 0.001$) had a lower impact than that of glucose alone (F=73.907 ** ($p \leq 0.01$). As discussed earlier, this may be explained by catabolite repression, also known as the glucose effect, where glucose represses the utilization of sucrose. Furthermore, the production of acids from the fermentation of sugars could have inhibited cell reproduction.

3.3.2.2 Yeast

The concentrations of yeast during the fermentation are shown in Table 3.5. From the time of inoculation of coconut water with kefir, non-supplemented and supplemented with glucose and/or sucrose, significant yeast cell biomass was produced. A bar graph for the growth of yeast is shown in Figure 3.3.

In the coconut water fermented with kefir and 12g/L of sucrose (Set 3), the highest significant yeast cell concentration of 4.98 ± 0.03 log CFU/ml was achieved after 96 h of fermentation ($p \leq 0.05$). The second highest significant yeast cell concentration was obtained for Set 9 supplemented with 12 g/L of glucose and 12 g/L of sucrose with 4.87 ± 0.02 log CFU/ml ($p \leq 0.05$). The control set of coconut water fermented with kefir (Set 1, with no addition of sugars) had the least yeast cell concentration of 4.04 ± 0.02 log CFU/ml after 96 h of incubation at 30°C. It can be noted that the highest yeast cell concentrations were obtained for Set 3 and Set 9, which were both supplemented with 12 g/L of sucrose.

As with LAB cell concentration which had high values for set 3 and set 9 (Table 3.4), the yeast cell concentrations (Table 3.5) for both set 3 and set 9 also had the highest concentrations ($p \leq 0.05$). Similar results have been reported by Laureys and De Vuyst (2014) for the growth of yeast cells in water kefir (fermented sugar solutions) supplemented with sucrose. In set 3, yeast cells were able to utilise sucrose as the source of carbon that provides an environment supporting the growth of LAB (Martínez-Torres, Gutiérrez-Ambrocio, Heredia-del-Orbe, Villa-Tanaca, & Hernández-Rodríguez, 2017; Neve & Heller, 2002; Zajšej & Goršek, 2011). In natural environments such as where ripe or spoiled fruit are, the LAB exist with yeast that are able to metabolise sucrose to produce glucose and fructose in the environment. These monosaccharides become available for the LAB to utilize (Almeida, Rachid, & Schwan, 2007; Giraffa, 2004; Magalhaes et al., 2010).

Utilisation of sucrose by yeast cells is also supported by studies carried out on yeast cells of water kefir, which are able to convert sucrose into the monosaccharides glucose and fructose through the enzyme invertase producing glucose and sucrose as free metabolites (Sikander, 2007).

A similar pattern, where yeast cell concentrations were lower than LAB cell concentration has been reported earlier (Garrote, Abraham, & De Antoni, 2001).

The F-values obtained for the variables affecting yeast growth show that the length of fermentation (time) and sucrose concentration had the most significant effect (6728.24 and 861.38, $p \leq 0.001$ respectively). This is expected with any cell growth, that given all the factors for growth, there would be an increase in growth as time increases until the stationary phase is reached. The high F- value indicates that the high sucrose concentration of 12g/L had a significant impact resulting in good growth of yeast cells.

3.3.2.3 Acetic Acid Bacteria (AAB)

Growth of AAB was observed only after incubation for 48 h on Acetic Acid Bacteria Agar (Table 3.6). Acetic acid bacteria are known to grow better in the presence of lactic acid produced during fermentation (Gulitz et al., 2011; Martínez-Torres, Gutiérrez-Ambrocio, Heredia-del-Orbe, Villa-Tanaca, & Hernández-Rodríguez, 2017). A bar graph for the growth of AAB is shown in Figure 3.4.

The highest growth of AAB was observed in Set 3 supplemented with sucrose at 12 g/L and in set 9 (supplemented with both glucose and sucrose at 12g/L each) with similar values of 2.3 ± 0.01 log CFU/ml. There was significant growth of AAB at the later stages of fermentation in the presence of sucrose, however, this cannot be supported by any previous studies due to the limited literature available on the effect of sucrose on the growth of acetic acid bacteria.

Figure 3.2: Change in the counts of LAB for kefir fermented coconut water kefir sets over 96 h of fermentation. Upper case letters A, B, C, D, E, F: Different letters across fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same treatment set differ significantly (Fisher's least significant difference, $p < 0.05$). Lower case letters a,b,c: Different letters within the same time interval for different sets differ significantly (Fisher's least significant difference, $p < 0.05$).

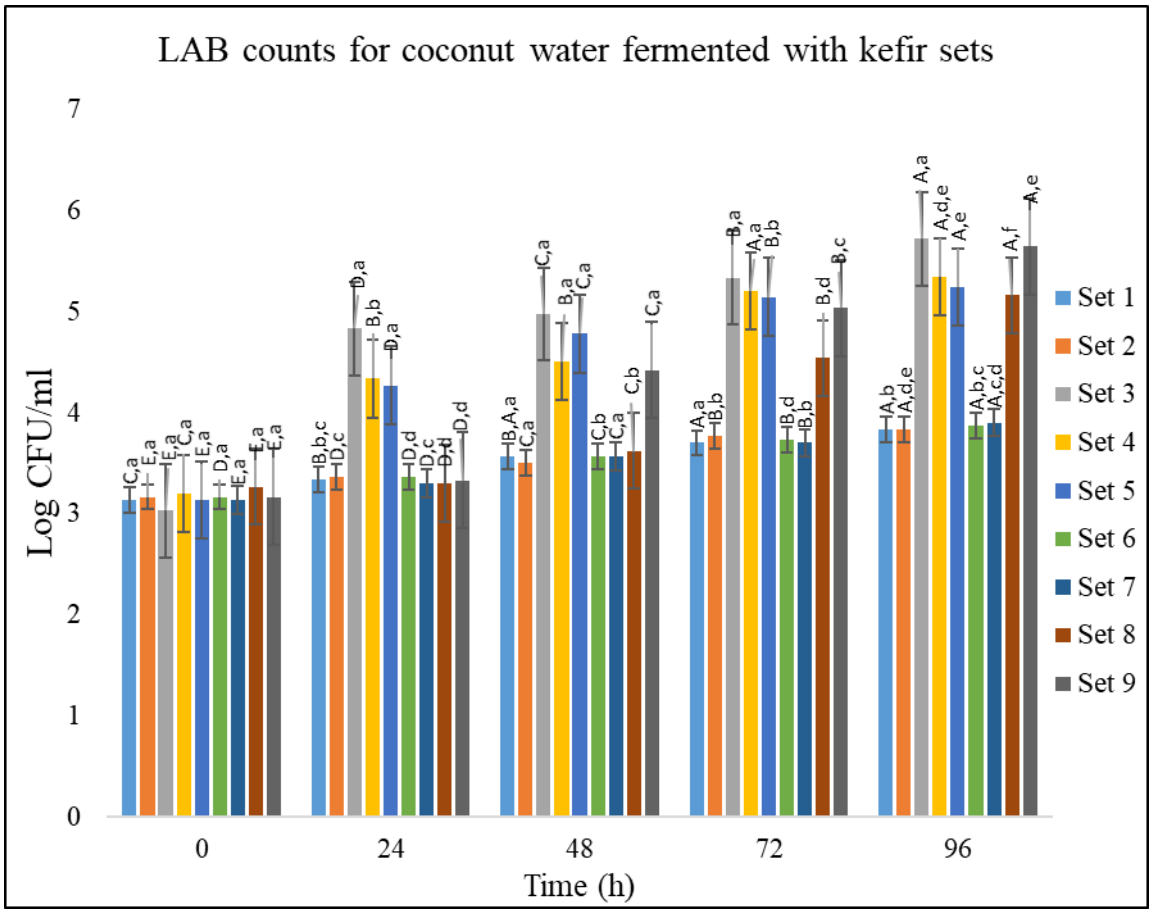


Figure 3.3: Change in the counts of yeast for kefir fermented coconut water kefir sets over 96h of fermentation. Upper case letters A, B, C, D, E, F: Different letters across fermentation times- 0h, 24h, 48h, 72h and 96 h for the same set treatment differ significantly (Fisher's least significant difference, $p < 0.05$). Lower case letters a,b,c: Different letters within the same time interval for different sets differ significantly (Fisher's least significant difference, $p < 0.05$).

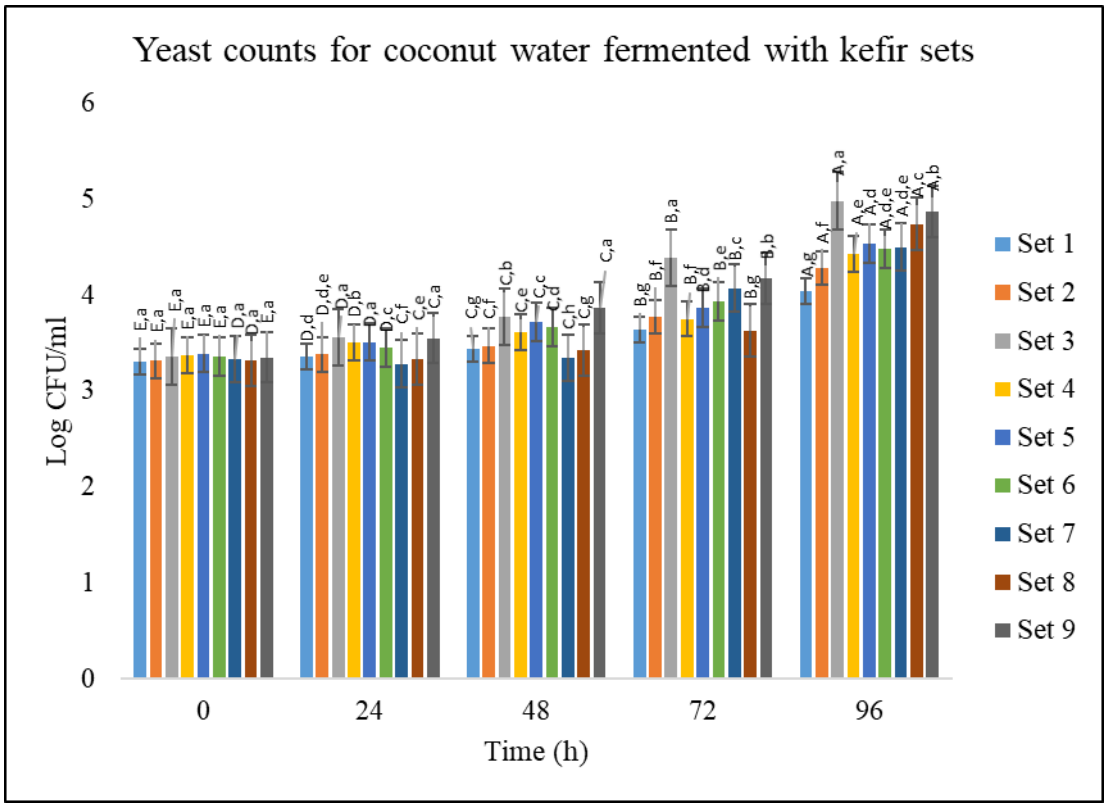
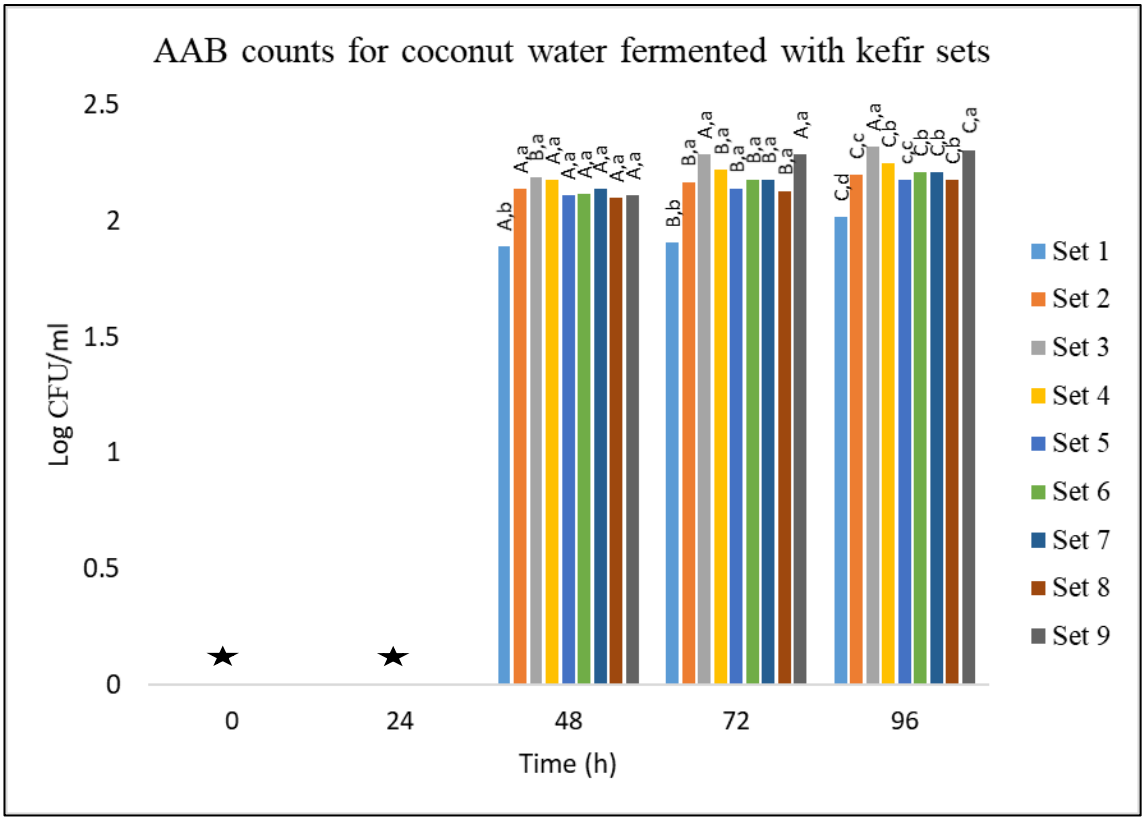


Figure 3.4: Change in the counts of AAB for kefir fermented coconut water kefir sets over 96h of fermentation. Upper case letters A, B, C, D, E, F: Different letters across fermentation times- 0h, 24h, 48h, 72h and 96 h for the same set treatment differ significantly (Fisher's least significant difference, $p < 0.05$). Lower case letter a,b,c: Different letters within the same time interval for different sets differ significantly (Fisher's least significant difference, $p < 0.05$).



" ★ " - For incubation time 0 h and 24 h, no growth was detected for acetic acid bacteria.

Table 3.4: The cell concentration of Lactic acid bacteria (log CFU/ml) isolated from coconut water kefir fermented at 30°C with 5% CO2. Each value is the mean of triplicate readings. A, B, C, D, E, F= Different letters within the same row (different fermentation times- 0h, 24h, 48h, 72h and 96h for the same set (treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c= Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Sets	Added Glucose (g/L)	Added Sucrose (g/L)	Log CFU/ml for LAB					F value (ANOVA model)						
			0 h	24 h	48 h	72 h	96 h	Suc (g/L)	Glu (g/L)	Time	Suc (g/L)* Glu (g/L)	Suc (g/L)* Time	Glu (g/L) *Time	Suc (g/L)* Glu (g/L)* Time
Set 1	0	0	$3.34 \pm 0.02^{C,a}$	$4.34 \pm 0.3^{B,b,c}$	$4.35 \pm 1.16^{B,A,a}$	$5.08 \pm 0.04^{A,a}$	$5.1 \pm 0.03^{A,b}$	5.60 ***	73.90***	357.99***	64.65***	7.76***	3.36**	7.66***
Set 2	0	6	$3.37 \pm 0.01^{E,a}$	$4.29 \pm 0.06^{D,c}$	$4.84 \pm 0.02^{C,a}$	$5.17 \pm 0.04^{B,b}$	$5.28 \pm 0.08^{A,d,e}$							
Set 3	0	12	$3.34 \pm 0.02^{E,a}$	$4.84 \pm 0.02^{D,a}$	$4.95 \pm 0.1^{C,a}$	$5.33 \pm 0.07^{B,a}$	$5.72 \pm 0.04^{A,a}$							
Set 4	6	0	$3.34 \pm 0.11^{C,a}$	$4.54 \pm 0.24^{B,b}$	$4.55 \pm 0.08^{B,a}$	$5.32 \pm 0.1^{A,a}$	$5.33 \pm 0.1^{A,d,e}$							
Set 5	6	6	$3.35 \pm 0.02^{E,a}$	$4.78 \pm 0.03^{D,a}$	$4.94 \pm 0.05^{C,a}$	$5.14 \pm 0.03^{B,b}$	$5.24 \pm 0.03^{A,e}$							
Set 6	6	12	$3.38 \pm 0.01^{D,a}$	$3.42 \pm 0.07^{D,d}$	$3.7 \pm 0.02^{C,b}$	$4.48 \pm 0.07^{B,d}$	$5.45 \pm 0.07^{A,b,c}$							
Set 7	12	0	$3.34 \pm 0.21^{E,a}$	$4.28 \pm 0.06^{D,c}$	$4.53 \pm 0.08^{C,a}$	$5.14 \pm 0.02^{B,b}$	$5.35 \pm 0.04^{A,c,d}$							
Set 8	12	6	$3.33 \pm 0.03^{E,a}$	$3.4 \pm 0.07^{D,d}$	$3.62 \pm 0.03^{C,b}$	$4.54 \pm 0.06^{B,d}$	$5.16 \pm 0.07^{A,f}$							
Set 9	12	12	$3.33 \pm 0.07^{E,a}$	$3.53 \pm 0.07^{D,d}$	$4.42 \pm 0.11^{C,a}$	$5.04 \pm 0.03^{B,c}$	$5.64 \pm 0.02^{A,e}$							

Table 3.5: The cell concentration of yeast (log CFU/ml) isolated from coconut water kefir fermented at 27±3°C temperature on Malt extract agar. Each value is the mean of triplicate readings. A, B, C, D, E, F= Different letters within the same row (different fermentation times- 0h, 24h, 48h, 72h and 96h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a,b,c= Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Sets	Added Glucose (g/L)	Log CFU/ml for yeast						F value (ANOVA model)						
		Added Sucrose (g/L)	0 h	24 h	48 h	72 h	96 h	Suc (g/L)	Glu (g/L)	Time	Suc (g/L) *Glu (g/L)	Suc (g/L)* Time	Glu (g/L)* Time	Suc (g/L)* Glu (g/L)* Time
Set 1	0	0	$3.30 \pm 0.02^{E,a}$	$3.38 \pm 0.02^{D,d}$	$3.44 \pm 0.02^{C,g}$	$3.64 \pm 0.02^{B,g}$	$4.04 \pm 0.02^{A,g}$	861.38***	7.66**	6728.24***	272.18***	67.33***	71.98***	47.07***
Set 2	0	6	$3.31 \pm 0.02^{E,a}$	$3.36 \pm 0.02^{D,d,e}$	$3.47 \pm 0.02^{C,f}$	$3.77 \pm 0.02^{B,f}$	$4.28 \pm 0.02^{A,f}$							
Set 3	0	12	$3.36 \pm 0.03^{E,a}$	$3.56 \pm 0.03^{D,a}$	$3.77 \pm 0.01^{C,b}$	$4.39 \pm 0.02^{B,a}$	$4.98 \pm 0.03^{A,a}$							
Set 4	6	0	$3.37 \pm 0.02^{E,a}$	$3.5 \pm 0.02^{D,b}$	$3.61 \pm 0.02^{C,e}$	$3.75 \pm 0.02^{B,f}$	$4.43 \pm 0.02^{A,e}$							
Set 5	6	6	$3.39 \pm 0.02^{E,a}$	$3.58 \pm 0.02^{D,a}$	$3.72 \pm 0.02^{C,c}$	$3.87 \pm 0.03^{B,e}$	$4.53 \pm 0.02^{A,d}$							
Set 6	6	12	$3.36 \pm 0.03^{E,a}$	$3.45 \pm 0.03^{D,c}$	$3.67 \pm 0.02^{C,d}$	$3.93 \pm 0.02^{B,d}$	$4.48 \pm 0.04^{A,d,e}$							
Set 7	12	0	$3.33 \pm 0.03^{D,a}$	$3.28 \pm 0.02^{C,f}$	$3.34 \pm 0.02^{C,h}$	$4.07 \pm 0.02^{B,c}$	$4.5 \pm 0.11^{A,d,e}$							
Set 8	12	6	$3.32 \pm 0.01^{D,a}$	$3.33 \pm 0.03^{C,e}$	$3.42 \pm 0.01^{C,g}$	$3.63 \pm 0.02^{B,g}$	$4.74 \pm 0.11^{A,c}$							
Set 9	12	12	$3.35 \pm 0.01^{E,a}$	$3.56 \pm 0.04^{D,a}$	$3.86 \pm 0.02^{C,a}$	$4.17 \pm 0.01^{B,b}$	$4.87 \pm 0.02^{A,b}$							

Table 3.6: The cell concentration of Acetic acid bacteria (Log CFU/ml) isolated from coconut water kefir fermented at 30°C on Acetic acid bacteria agar. Each value is the mean of triplicate readings. A, B, C, D, E, F= Different letters within the same row (different fermentation times- 0h, 24h, 48h, 72h and 96h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a,b,c= Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Sets	Added Glucose (g/L)	Added Sucrose (g/L)	Log CFU/ml for Acetic acid bacteria				F value (ANOVA model)						
			0h and 24h – No growth	48 h	72 h	96 h	Suc (g/L)	Glu (g/L)	Time	Suc (g/L)*Glu (g/L)	Suc (g/L)* Time	Glu (g/L)* Time	Suc (g/L)*Glu (g/L)*Time
Set 1	0	0	0	$1.89 \pm 0^{A,b}$	$1.91 \pm 0.01^{B,b}$	$2.02 \pm 0.01^{C,d}$	1.37	4.56***	3.73***	0.95	1.37	0.70	1.35
Set 2	0	6	0	$2.14 \pm 0^{A,a}$	$2.17 \pm 0.01^{B,a}$	$2.2 \pm 0.01^{C,c}$							
Set 3	0	12	0	$2.19 \pm 0.53^{B,a}$	$2.29 \pm 0.01^{A,a}$	$2.32 \pm 0.01^{A,a}$							
Set 4	6	0	0	$2.18 \pm 0^{A,a}$	$2.22 \pm 0.01^{B,a}$	$2.25 \pm 0.01^{C,b}$							
Set 5	6	6	0	$2.11 \pm 0.01^{A,a}$	$2.14 \pm 0.01^{B,a}$	$2.18 \pm 0^{C,c}$							
Set 6	6	12	0	$2.12 \pm 0.01^{A,a}$	$2.18 \pm 0.01^{B,a}$	$2.21 \pm 0^{C,b}$							
Set 7	12	0	0	$2.14 \pm 0^{A,a}$	$2.18 \pm 0.01^{B,a}$	$2.21 \pm 0.01^{C,b}$							
Set 8	12	6	0	$2.1 \pm 0.01^{A,a}$	$2.13 \pm 0.01^{B,a}$	$2.18 \pm 0.01^{C,c}$							
Set 9	12	12	0	$2.11 \pm 0.01^{A,a}$	$2.29 \pm 0.58^{A,a}$	$2.31 \pm 0.01^{A,a}$							

3.3.3 Determination of Residual Glucose and Sucrose concentrations and Total Titratable acidity at the end of fermentation.

Reducing sugar (glucose) concentration in the experimental sets during fermentation was determined using the di-nitro salicylic acid method (DNS) whereas the non-reducing sugar (sucrose) concentration was determined using a modified Anthrone method. Coconut water was used as the blank for estimating glucose and sucrose concentration. All the experiments were done in triplicates. Error bars for the graphs represent the standard deviation in the data.

As shown in Table 3.7 and Table 3.8, the concentrations of residual glucose and sucrose were respectively determined at regular time intervals during the fermentation. The utilisation of sugars is indicated by the decreasing concentration over time for glucose and sucrose. Figure 3.5 and Figure 3.6 show a decreasing pattern for the sugar concentrations with fermentation time in all the sets. A significant increase in glucose concentration ($p < 0.05$) was observed after 24 h and 48 h in Sets 5, 6, 8 and 9 (Figure 3.5) that accompanied an increase in growth of the kefir microorganisms. This could be due to the metabolism of sucrose by yeast, yielding glucose and fructose, which is demonstrated in Figure 3.6 where there was a significant reduction in sucrose concentration after 24 h of fermentation. There was a significantly steep reduction in residual sucrose concentration after 96 h of CWK fermentation accompanied by a slight significant reduction in residual glucose concentration.

There was a general trend with the added glucose and sucrose metabolism in CWK. As shown in Table 3.7 and Table 3.8, Set 7 (supplemented with 12g/L of glucose) and Set 4 (supplemented with 6g/L of glucose) demonstrated very high glucose utilisation values of 70% and 74%, respectively. The high glucose utilisation for these sets indicate that the added glucose was the main source of carbon available to the LAB and yeast. Set 2 (supplemented with 6g/L of sucrose) and Set 8 (supplemented with 12g/L of glucose and 6g/L of sucrose) showed high sucrose utilisation of 79 and 81%, respectively.

Overall, there was a trend in glucose utilisation that was observed in the data. For sets 2 and 3 (supplemented with 6 and 12 g/L of sucrose respectively), there was an increase in the residual glucose concentration for 24 h and then the concentration gradually declined upto 96 h. For sets 4 and 6 (supplemented with 6 and 12 g/L glucose respectively), there was a gradual decline over 96 h fermentation time. For all the sets supplemented with a combination of both glucose and sucrose (6 and 12 g/L), the residual glucose concentration increased upto 24 h and then gradually declined until 96 h fermentation time. The overall trend for sucrose utilisation is that the concentration of residual sucrose gradually decreases for all the sets, with the fermentation time.

In general, sucrose was utilised more than glucose. The utilisation of sugars is reflected in the increase in cell biomass for LAB, yeast and AAB, which metabolised the available glucose and/or sucrose. Such observed decrease in sucrose concentrations in kefir fermented coconut water in this study agrees with the utilisation of sucrose by water kefir microorganisms reported by Laureys & De Vuyst (2014). Similar reductions in the levels of sucrose and glucose have been observed in other fermentations as in sourdough (Gobbetti, Corsetti, & Rossi, 1994; Thiele, Gänzle, & Vogel, 2003). Significant reduction of sucrose concentration in kimchi has also been reported by Kang et al (2019), with fermentation. Study of sucrose hydrolysis has also been carried out in kimchi, which supports this study. Microorganisms such as LAB and yeast secrete dextransucrase (invertase) enzyme that hydrolyses sucrose to produce D-glucose and D-fructose. The D-fructose produced in this way, is taken up as a carbon and energy source and the D-glucose is used for biosynthesis of dextran via polymerization (Hahn, Woo, Park, & Lee, 1997; Kim, Min, Kim, & Han, 2001).

During the fermentation, the sugars glucose and sucrose were being metabolised to produce acids, which correlated with the drop in the pH (Table 3.12). and an increase in the overall acidity of the medium. This acidity measured as the total titratable acidity (TTA) in Table 3.9 increased significantly with time ($p<0.05$). Set 3 (supplemented with 12g/L of sucrose) showed the significantly highest amount of TTA (13.65 ± 0.04, $p<0.05$) at 96 h. Second highest TTA was observed with Set 8 (supplemented with glucose at 12g/L and sucrose at 6g/L), with a TTA of 12.97 ± 0.02, $p<0.05$. Set 7 (supplemented with glucose at 12g/L) produced the third highest TTA of 12.86 ± 0.02, $p<0.05$. The lowest TTA at 11.54 ± 0.01A ($p<0.05$) was observed with Set 2 (supplemented with sucrose at 6g/L).

A big increase in total titratable acidity (up to 1% at the end of fermentation) was previously reported for milk kefir grains by Chen et al (2009) that was accompanied with a decrease in pH and an increase in lactic acid and other organic acids production (Chen, Wang, Chen, Liu, & Chen, 2009). The transformation of sugars to lactic acid and other organic acids by the LAB is an important step during fermentation that influences the pH and acidity of fermented products such as kimchi (Jeong et al., 2011; Kang, Jung, & Seo, 2015).

Figure 3.5: Change in residual glucose concentration for all the sets with time. A,B,C,D,E,F= Different letters across fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment differ significantly using Fisher's least significant difference ($p < 0.05$). a,b,c: Different letters within the same time interval for different sets differ significantly using Fisher's least significant difference ($p < 0.05$).

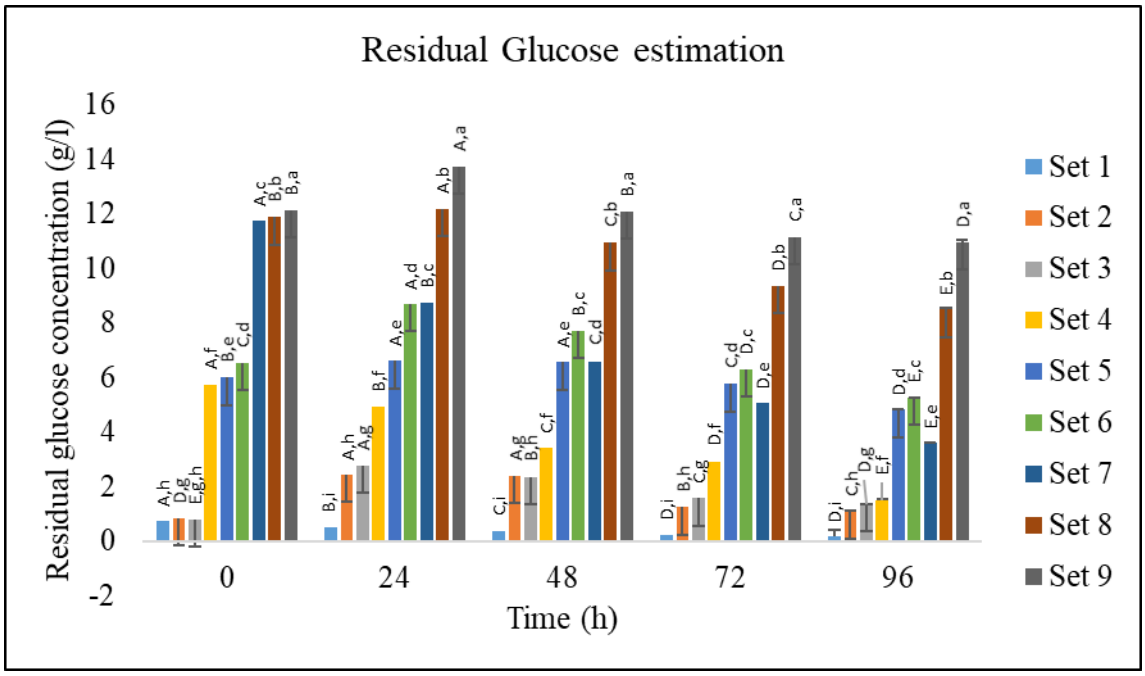


Figure 3.6: Change in residual sucrose concentration for all the sets with time. A,B,C,D,E,F= Different letters across fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment differ significantly using Fisher's least significant difference ($p < 0.05$). a,b,c: Different letters within the same time interval for different sets differ significantly using Fisher's least significant difference ($p < 0.05$).

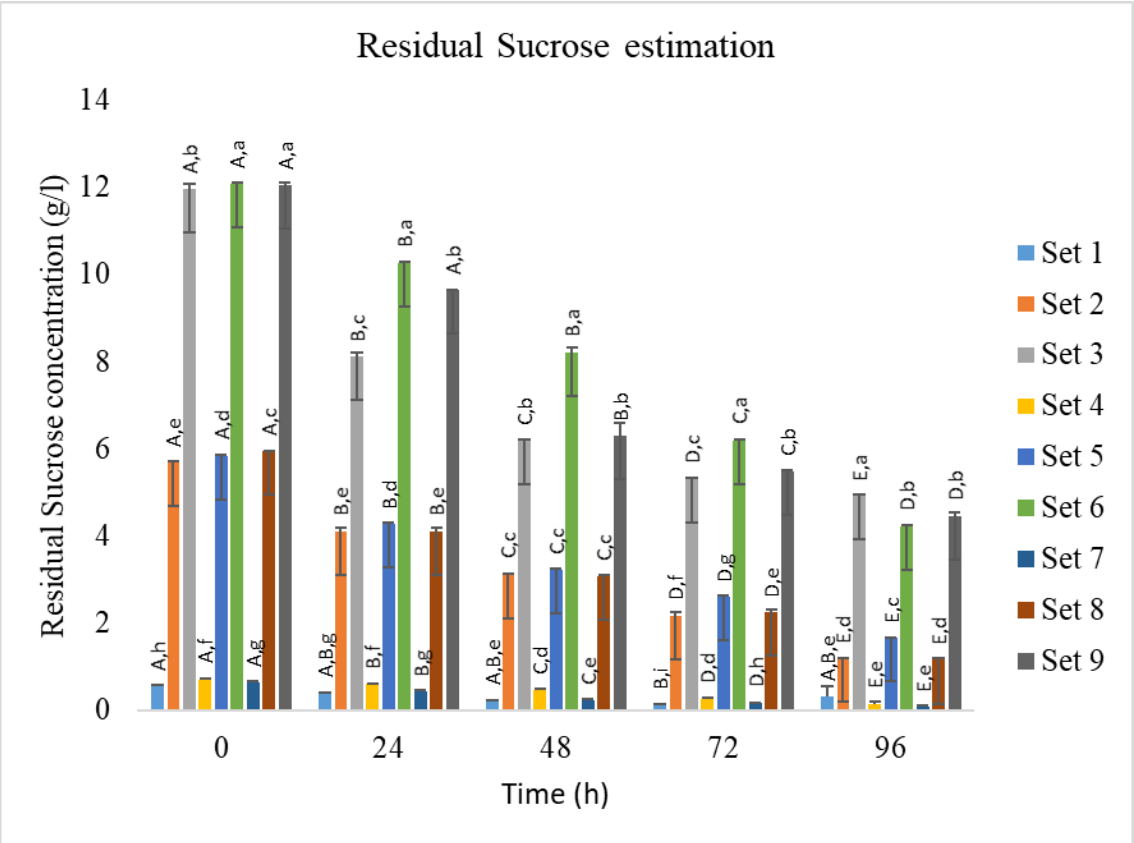


Table 3.7: The estimation of glucose for the different treatments of coconut water kefir fermented at 30°C with 5% CO_2 concentration. All the values for each experimental set represent the mean of triplicate readings. **A, B, C, D, E, F**= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). **a, b, c, d**: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Sets	Added Glucose (g/L)	Added Sucrose (g/L)	Glucose concentration (g/L)					F value (ANOVA model)						
			0 h	24 h	48 h	72 h	96 h	Suc (g/L)	Glu (g/L)	Time	Suc (g/L)* Glu (g/L)	Suc (g/L)* Time	Glu (g/L)* Time	Suc (g/L)* Glu (g/L)* Time
Set 1	0	0	0.73 ± 0.11A,h	0.52 ± 0.1B,i	0.37 ± 0.2C,i	0.22 ± 0.14D,i	0.15 ± 0.02D,i	5847.42***	56912.02***	5598.43***	681.80***	346.22***	206.21***	44.01***
Set 2	0	6	0.83 ± 0.11D,g	2.43 ± 0.12A,h	2.37 ± 0.11A,g	1.23 ± 0.07B,h	1.08 ± 0.2C,h							
Set 3	0	12	0.79 ± 0.07E,g,h	2.75 ± 0.04A,g	2.35 ± 0.21B,h	1.56 ± 0.04C,g	1.34 ± 0.06D,g							
Set 4	6	0	5.72 ± 0.04A,f	4.94 ± 0.21B,f	3.41 ± 0.11C,f	2.92 ± 0.15D,f	1.48 ± 0.18E,f							
Set 5	6	6	5.99 ± 0.16B,e	6.6 ± 0.08A,e	6.55 ± 0.16A,e	5.75 ± 0.12C,d	4.81 ± 0.13D,d							
Set 6	6	12	6.54 ± 0.18C,d	8.68 ± 0.14A,d	7.69 ± 0.12B,c	6.3 ± 0.19D,c	5.25 ± 0.13E,c							
Set 7	12	0	11.73 ± 0.14A,c	8.71 ± 0.11B,c	6.59 ± 0.07C,d	5.04 ± 0.18D,e	3.58 ± 0.07E,e							
Set 8	12	6	11.87 ± 0.15B,b	12.18 ± 0.11A,b	10.93 ± 0.12C,b	9.35 ± 0.22D,b	8.48 ± 0.13E,b							
Set 9	12	12	12.14 ± 0.12B,a	13.73 ± 0.05A,a	12.09 ± 0.04B,a	11.13 ± 0.07C,a	10.94 ± 0.07D,a							

Table 3.8: The estimation of sucrose for the different treatments of coconut water kefir fermented at 30°C with 5% CO_2 concentration. All the values for each experimental set represent the mean of triplicate readings.

A, B, C, D, E, F= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). **a, b, c, d**: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). * **p < 0.05.** ** **p < 0.01.** *** **p < 0.001.**

Sets	Added Glucose (g/L)	Added Sucrose (g/L)	Sucrose concentration (g/L)					F value (ANOVA model)						
			0 h	24 h	48 h	72 h	96 h	Suc (g/L)	Glu (g/L)	Time	Suc (g/L)* Glu (g/L)	Suc (g/L)* Time	Glu (g/L)* Time	Suc (g/L)* Glu (g/L)* Time
Set 1	0	0	0.55 ± 0.04A,h	0.39 ± 0.06A,B,g	0.22 ± 0.11A,B,e	0.13 ± 0.08B,i	0.31 ± 0.07A,B,e	312.76***	85369.1***	10120.4***	89.280***	71.107***	2506.51**	64.128***
Set 2	0	6	5.68 ± 0.11A,e	4.1 ± 0.04B,e	3.12 ± 0.06C,c	2.17 ± 0.09D,f	1.19 ± 0.11E,d							
Set 3	0	12	11.96 ± 0.04A,b	8.13 ± 0.11B,c	6.19 ± 0.12C,b	5.32 ± 0.13D,c	4.92 ± 0.13E,a							
Set 4	6	0	0.71 ± 0.13A,f	0.61 ± 0.09B,f	0.47 ± 0.08C,d	0.26 ± 0.12D,g	0.15 ± 0.04E,e							
Set 5	6	6	5.84 ± 0.11A,d	4.29 ± 0.1B,d	3.23 ± 0.12C,c	2.61 ± 0.03D,d	1.66 ± 0.11E,c							
Set 6	6	12	12.08 ± 0.05A,a	10.27 ± 0.1B,a	8.22 ± 0.09B,a	6.19 ± 0.02C,a	4.23 ± 0.09D,b							
Set 7	12	0	0.64 ± 0.1A,g	0.43 ± 0.01B,g	0.22 ± 0.12C,e	0.17 ± 0.11D,h	0.08 ± 0.12E,e							
Set 8	12	6	5.95 ± 0.13A,c	4.1 ± 0.07B,e	3.07 ± 0.02C,c	2.25 ± 0.02D,e	1.15 ± 0.14E,d							
Set 9	12	12	12.05 ± 0.02A,a	9.64 ± 0.1A,b	6.3 ± 0.08B,b	5.49 ± 0.08C,b	4.46 ± 0.15D,b							

Table 3.9: The estimation of total titratable acidity for the different treatments of coconut water kefir fermented at 30°C with 5% CO_2 concentration. All the values for each experimental set represent the mean of triplicate readings. A, B, C, D, E, F= Different letters within the same row (different fermentation times- 0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c, d: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Sets	Added Glucose (g/L)	Added Sucrose (g/L)	Total Titratable acidity (0.1 M NaOH)					F value (ANOVA model)						
			0 h	24 h	48 h	72 h	96 h	Suc (g/L)	Glu (g/L)	Time	Suc (g/L)* Glu (g/L)	Suc (g/L)* Time	Glu (g/L)* Time	Suc (g/L)* Glu (g/L)* Time
Set 1	0	0	$5.21 \pm 0.02^{E,e}$	$6.50 \pm 0.04^{D,f}$	$8.85 \pm 0.04^{C,f}$	$10.26 \pm 0.05^{B,e}$	$11.71 \pm 0.02^{A,g}$	0.95	1.01	1.14	0.99	0.99	1.0	1.0
Set 2	0	6	$5.29 \pm 0.02^{E,d,e}$	$6.89 \pm 0.01^{D,c}$	$9.04 \pm 0.05^{C,d}$	$10.09 \pm 0.04^{B,f}$	$11.54 \pm 0.01^{A,h}$							
Set 3	0	12	$5.42 \pm 0.04^{E,c,d}$	$6.32 \pm 0.04^{D,g}$	$8.85 \pm 0.02^{C,f}$	$9.160 \pm 0.56^{B,e}$	$13.65 \pm 0.04^{A,a}$							
Set 4	6	0	$5.40 \pm 0.05^{E,c,d}$	$6.57 \pm 0.04^{D,e}$	$8.93 \pm 0.02^{C,e}$	$10.60 \pm 0.06^{B,d}$	$12.44 \pm 0.04^{A,f}$							
Set 5	6	6	$5.33 \pm 0.01^{E,c,d,e}$	$6.68 \pm 0.08^{D,d}$	$8.76 \pm 0.03^{C,g}$	$10.63 \pm 0.04^{B,d}$	$12.47 \pm 0.03^{A,f}$							
Set 6	6	12	$5.66 \pm 0.03^{E,a,b}$	$7.20 \pm 0.02^{D,a}$	$9.48 \pm 0.07^{C,b}$	$11.07 \pm 0.04^{B,a}$	$12.56 \pm 0.04^{A,e}$							
Set 7	12	0	$5.41 \pm 0.04^{E,c,d}$	$6.56 \pm 0.02^{D,e,f}$	$9.29 \pm 0.03^{C,c}$	$10.96 \pm 0.05^{B,b,c}$	$12.86 \pm 0.02^{A,c}$							
Set 8	12	6	$5.76 \pm 0.30^{E,a}$	$7.13 \pm 0.02^{D,b}$	$9.58 \pm 0.06^{C,a}$	$11.01 \pm 0.03^{B,a,b}$	$12.97 \pm 0.02^{A,b}$							
Set 9	12	12	$5.48 \pm 0.06^{E,b,c}$	$6.92 \pm 0.03^{D,c}$	$8.9 \pm 0.05^{C,e,f}$	$10.89 \pm 0.03^{B,c}$	$12.69 \pm 0.03^{A,d}$							

3.3.4 Determination of D-/L- Lactic acid content and pH

D- lactic acid, L- lactic acid and pH were measured to determine the products of CWK fermentation. The LAB and yeast growing during the fermentation of coconut water influenced the lactic acid concentration of the medium through carbohydrate metabolism. Production of L- and D- lactic acid during the fermentation influences the overall quality of the product. The presence of D-lactic is undesirable as it forms non-metabolisable constituents which may accumulate in the blood (Olieman & Vries, 1988). To determine the D-/L- lactic acid content in the coconut water kefir sourdough, Megazyme lactic acid kit K-DLATE01/14 was used (Figure 3.7 and Figure 3.8).

Figure 3.7: L- lactic acid concentration in the coconut water kefir samples for all the different treatments incubated for 0 h, 24 h, 48h, 72h and 96 h at 30°C. Error bars represent standard deviations. All the D- and L- lactic acid values are significant with $p<0.05$. A, B, C, D, E, F= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$).

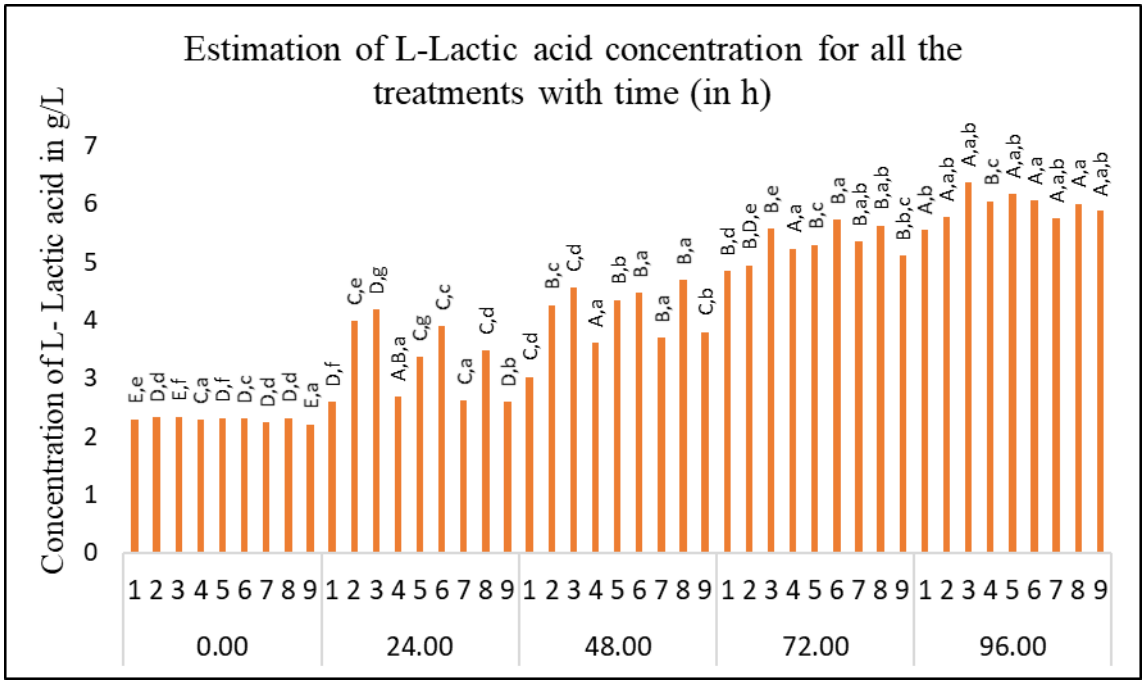
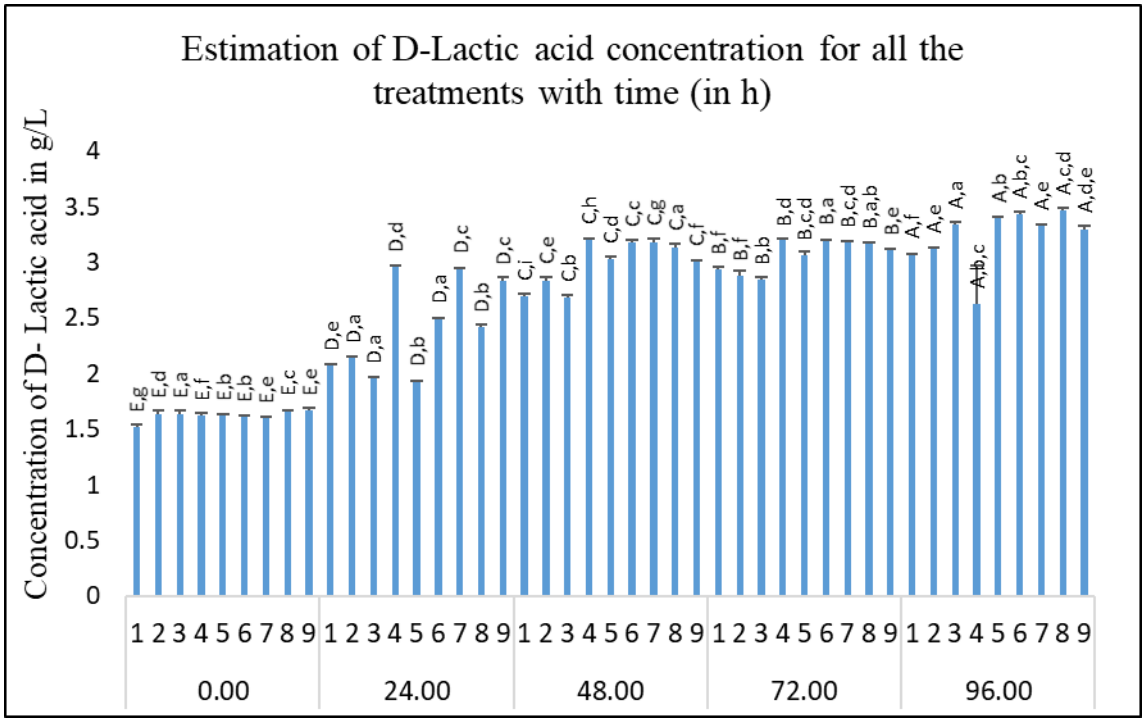


Figure 3.8: D- lactic acid concentration in the coconut water kefir samples for all the different treatments incubated for 0 h, 24 h, 48h, 72h and 96 h at 30°C. Error bars represent standard deviations. All the D- and L- lactic acid values are significant with $p<0.05$. A, B, C, D, E, F= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$).



The concentration of L-lactic acid was almost double that of the concentration of D-lactic acid as observed in Figure 3.7 and Figure 3.8, which is supported by Table 3.10 and Table 3.11. The increase in lactic acid can be explained by the utilisation of sugars during fermentation of coconut water with kefir. This agrees with the study carried out by Liu and Lin (2000), on milk kefir grains, which has reported an increase in overall lactic acid content of milk kefir grains with time.

The highest amount of L-lactic acid (Table 3.11) was produced in Set 3 (with 12g/L of sucrose) at 96 h (6.38 ± 0.06 g/L, $p<0.05$). This was followed by Set 4 (6.05 ± 0.13) and Set 1 (5.56 ± 0.1A), $p<0.05$.

The highest concentration of D- lactic acid (Table 3.10) was produced in Set 8 (3.46 ± 0.05). The next highest concentrations were obtained with Set 6 (3.43 ± 0.03), Set 3 (3.34 ± 0.04) and Set 9 (3.30 ± 0.05), $p<0.05$.

A significant increase in the concentrations of D-/L-lactic acid was observed over time for all sets ($p<0.05$), which correlate to the metabolism of the supplemented sugars glucose and sucrose as discussed above (Figure 3.5 and Figure 3.6).

There is some physiological importance in terms of type of lactic acid that is produced in the fermented milk kefir (Farnworth, 2008). Two stereoisomers, L- and D- isomers are formed in kefir grains by the action of homo-fermentative and hetero-fermentative microorganisms. In the gastrointestinal tract, both D and L-Lactic acid isomers are completely absorbed, however, their proportions for the production of glucose and glycogen differ hugely. L-lactic acid is completely converted to glycogen at a rapid metabolic rate, whereas, D- lactic acid is metabolised at a much slower rate and is also excreted out of the body with urine (Uniacke-Lowe, 2011). It has been reported that kefir grains contain mainly L- lactic acid but it varies greatly with the medium in which the kefir grains are grown. Yoghurt was found to contain 40 – 60 g/L of L- lactic acid and 40 – 55g/L of D- lactic acid. At the end of milk kefir fermentation, the concentration of L-lactic acid was approximately 7.6g/L compared to the much lower concentration of D-lactic acid, which was 1.1g/L (Frías, Martínez-Villaluenga, & Peñas, 2016; Farnworth and Mainville, 2008; Suriasih, 2000). These results are comparable to the results obtained in this study, where a higher overall concentration for L-lactic acid has been obtained compared to D- lactic acid concentration.

Table 3.10: The concentration of D-Lactic acid estimated for the different treatments of coconut water kefir fermented at 30°C with 5% CO_2 concentration. All the values for each experimental set represent the mean of triplicate readings. A, B, C, D, E, F= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Sets	Added Glucose (g/L)	Added Sucrose (g/L)	Concentration of D-Lactic acid in g/L					F value (ANOVA model)						
			0 h	24 h	48 h	72 h	96 h	Suc (g/L)	Glu (g/L)	Time	Suc (g/L)* Glu (g/L)	Suc (g/L)* Time	Glu (g/L) *Time	Suc (g/L)* Glu (g/L)* Time
Set 1	0	0	$1.52 \pm 0.05^{E,e}$	$2.07 \pm 0.02^{D,f}$	$2.69 \pm 0.04^{C,d}$	$2.94 \pm 0.04^{B,d}$	$3.07 \pm 0.01^{A,c}$	202.23 ***	25.16**	787.29 ***	17.14**	14.21**	29.60***	16.87**
Set 2	0	6	$1.64 \pm 0.05^{D,d}$	$2.14 \pm 0.03^{C,e}$	$2.84 \pm 0.05^{B,c}$	$2.88 \pm 0.07^{B,d,e}$	$3.12 \pm 0.02^{A,b}$							
Set 3	0	12	$1.64 \pm 0.04^{E,f}$	$1.96 \pm 0.01^{D,g}$	$2.69 \pm 0.03^{C,d}$	$2.84 \pm 0.05^{B,e}$	$3.34 \pm 0.04^{A,a,b}$							
Set 4	6	0	$1.62 \pm 0.04^{C,a}$	$2.96 \pm 0.03^{A,B,a}$	$3.20 \pm 0.02^{A,a}$	$3.20 \pm 0.02^{A,a}$	$3.23 \pm 0.59^{A,a,b}$							
Set 5	6	6	$1.62 \pm 0.04^{D,f}$	$1.93 \pm 0.02^{C,g}$	$3.03 \pm 0.04^{B,b}$	$3.06 \pm 0.07^{B,c}$	$3.40 \pm 0.02^{A,a,b}$							
Set 6	6	12	$1.61 \pm 0.02^{D,c}$	$2.49 \pm 0.02^{C,c}$	$3.18 \pm 0.03^{B,a}$	$3.19 \pm 0.02^{B,a}$	$3.43 \pm 0.03^{A,a}$							
Set 7	12	0	$1.60 \pm 0.02^{D,b}$	$2.94 \pm 0.02^{C,a}$	$3.18 \pm 0.06^{B,a}$	$3.18 \pm 0.02^{B,a,b}$	$3.32 \pm 0.03^{A,a,b}$							
Set 8	12	6	$1.66 \pm 0.01^{D,d}$	$2.42 \pm 0.04^{C,d}$	$3.14 \pm 0.05^{B,a}$	$3.16 \pm 0.03^{B,a,b}$	$3.46 \pm 0.05^{A,a}$							
Set 9	12	12	$1.67 \pm 0.03^{E,a}$	$2.84 \pm 0.06^{D,b}$	$3.00 \pm 0.03^{C,b}$	$3.11 \pm 0.03^{B,b,c}$	$3.30 \pm 0.05^{A,a,b}$							

Table 3.11: The concentration of L-Lactic acid estimated for the different treatments of coconut water kefir fermented at 30°C with 5% CO_2 concentration. All the values for each experimental set represent the mean of triplicate readings. A, B, C, D, E, F= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Sets	Added Glucose (g/L)	Added Sucrose (g/L)	Concentration of L-lactic acid in g/L					F value (ANOVA model)						
			0 h	24 h	48 h	72 h	96 h	Suc (g/L)	Glu (g/L)	Time	Suc (g/L)*Glu (g/L)	Suc (g/L)* Time	Glu (g/L)* Time	Suc (g/L)*Glu (g/L)*Time
Set 1	0	0	$2.29 \pm 0.02^{E,g}$	$2.59 \pm 0.06^{D,e}$	$3.03 \pm 0.05^{C,i}$	$4.85 \pm 0.03^{B,f}$	$5.56 \pm 0.1^{A,f}$	34.70***	773.68***	6070.6***	220.96***	7.37***	87.13***	22.38***
Set 2	0	6	$2.33 \pm 0.01^{E,d}$	$3.98 \pm 0.01^{D,a}$	$4.25 \pm 0.04^{C,e}$	$4.94 \pm 0.03^{B,f}$	$5.77 \pm 0.05^{A,e}$							
Set 3	0	12	$2.34 \pm 0.04^{E,a}$	$4.18 \pm 0.02^{D,a}$	$4.55 \pm 0.06^{C,b}$	$5.57 \pm 0.05^{B,b}$	$6.38 \pm 0.06^{A,a}$							
Set 4	6	0	$2.29 \pm 0.02^{E,f}$	$2.68 \pm 0.03^{D,d}$	$3.61 \pm 0.04^{C,h}$	$5.23 \pm 0.09^{B,d}$	$6.05 \pm 0.13^{A,b,c}$							
Set 5	6	6	$2.32 \pm 0.03^{E,b}$	$3.37 \pm 0.6^{D,b}$	$4.35 \pm 0.03^{C,d}$	$5.29 \pm 0.08^{B,c,d}$	$6.18 \pm 0.05^{A,b}$							
Set 6	6	12	$2.31 \pm 0.05^{E,c}$	$3.89 \pm 0.02^{D,a}$	$4.47 \pm 0.05^{C,c}$	$5.73 \pm 0.06^{B,a}$	$6.06 \pm 0.03^{A,b,c}$							
Set 7	12	0	$2.25 \pm 0.07^{E,e}$	$2.62 \pm 0.06^{D,c}$	$3.69 \pm 0.04^{C,g}$	$5.35 \pm 0.11^{B,c,d}$	$5.75 \pm 0.12^{A,e}$							
Set 8	12	6	$2.32 \pm 0.06^{E,c}$	$3.47 \pm 0.05^{D,b}$	$4.69 \pm 0.02^{C,a}$	$5.63 \pm 0.06^{B,a,b}$	$6 \pm 0.04^{A,c,d}$							
Set 9	12	12	$2.21 \pm 0.04^{E,e}$	$2.59 \pm 0.05^{D,c}$	$3.79 \pm 0.03^{C,f}$	$5.11 \pm 0.07^{B,e}$	$5.88 \pm 0.05^{A,d,e}$							

Table 3.12: The change in pH estimated for the different treatments of coconut water kefir fermented at 30°C with 5% CO_2 concentration. All the values for each experimental set represent the mean of triplicate readings. A, B, C, D, E, F= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Sets	Added Glucose (g/L)	Added Sucrose (g/L)	pH					F value (ANOVA model)						
			0 h	24 h	48 h	72 h	96 h	Suc (g/L)	Glu (g/L)	Time	Suc (g/L)*Glu (g/L)	Suc (g/L)* Time	Glu (g/L)*Time	Suc (g/L)*Glu (g/L)*Time
Set 1	0	0	$5.55 \pm 0.01^{A,g}$	$5.35 \pm 0.01^{B,b}$	$4.29 \pm 0.01^{C,d}$	$4.13 \pm 0.01^{D,b}$	$3.81 \pm 0.05^{E,a}$	6703.6***	33508.76***	129832.67***	6764.92***	885.76***	4007.05***	744.99***
Set 2	0	6	$5.62 \pm 0.01^{A,e}$	$5.19 \pm 0.01^{B,c}$	$4.35 \pm 0.01^{C,b}$	$4.15 \pm 0.01^{D,a}$	$3.77 \pm 0.06^{E,a}$							
Set 3	0	12	$5.88 \pm 0.02^{A,a}$	$3.84 \pm 0.01^{B,g}$	$3.02 \pm 0.01^{C,h}$	$2.86 \pm 0.02^{D,i}$	$2.82 \pm 0.01^{E,e}$							
Set 4	6	0	$5.61 \pm 0.01^{A,e,f}$	$4.75 \pm 0.01^{B,d}$	$4.33 \pm 0.01^{C,c}$	$3.95 \pm 0.01^{D,c}$	$3.79 \pm 0.01^{E,a}$							
Set 5	6	6	$5.76 \pm 0.01^{A,d}$	$3.81 \pm 0.01^{B,h}$	$3.02 \pm 0.01^{C,h}$	$2.92 \pm 0.01^{D,h}$	$2.84 \pm 0.01^{E,e}$							
Set 6	6	12	$5.86 \pm 0.01^{A,a}$	$3.84 \pm 0.01^{B,g}$	$3.05 \pm 0.01^{C,g}$	$2.96 \pm 0.01^{D,g}$	$2.85 \pm 0.01^{E,e}$							
Set 7	12	0	$5.6 \pm 0.01^{A,f}$	$5.37 \pm 0.01^{B,a}$	$4.37 \pm 0.01^{C,a}$	$3.87 \pm 0.01^{D,d}$	$3.55 \pm 0.01^{E,b}$							
Set 8	12	6	$5.81 \pm 0.01^{A,c}$	$3.98 \pm 0.01^{B,f}$	$3.21 \pm 0.01^{C,f}$	$3 \pm 0.02^{D,f}$	$2.92 \pm 0.01^{E,d}$							
Set 9	12	12	$5.83 \pm 0.01^{A,b}$	$4.23 \pm 0.02^{B,e}$	$3.4 \pm 0.02^{C,e}$	$3.12 \pm 0.01^{D,e}$	$3.03 \pm 0.01^{E,c}$							

As shown in Table 3.12, a significantly sharp decline in the pH was observed during fermentation in Set 3 between 0 and 48 h, with the final pH of 2.82. This was the lowest final pH observed in comparison to all the other sets after 96 h ($p<0.05$). Set 5 also had a significantly low pH of 2.84 ± 0.01, $p<0.05$ at 96 h. The lowest pH for Set 3 and Set 5 correlated to a high TTA (Table 3.9). The highest pH values at 96 h were observed in Set 1 (3.81 ± 0.05) and Set 2 (3.77 ± 0.06), which correlated to the lowest TTA (Table 3.9).

Interestingly, the F values for glucose, sucrose, time, sucrose*glucose, glucose*time, sucrose*time and sucrose*glucose*time were all highly significant for D-Lactic acid, L-Lactic acid and pH, with $p<0.001$. This means that all the factors individually and in combination, had a significant influence in the pH decrease.

Studies have been carried out on milk kefir with or without supplementing soy, and pH has been known to steadily decrease for a fermentation carried out for up to 21 days (Chen et al., 2009; Gul, Mortas, Atalar, Dervisoglu, & Kahyaoglu, 2015). A similar study on milk kefir was carried out in which fresh milk was supplied every day for a total of 28 cycles. It was found that the pH continued to steadily decrease to pH 3.4 (Chen et al., 2009).

In the present study using coconut water kefir grains, the pH values agree with the previous fermentation studies carried out on milk where there was a steady drop in pH after 96 h without any replenishment of the medium during fermentation. According to Odet (1995), the pH of milk kefir varied between 4.2 and 4.6 Lower pH values were observed by Garrote et al. (2001) that was attributed to metabolic activity of the kefir grains.

3.3.5 Carboxylic acids detected using LCMS chromatographic method

Organic acid analysis was carried out on kefir- fermented coconut water to determine changes in the concentration of these acids - malic acid, lactic acid, acetic acid, pyruvic acid and succinic acid. The concentrations of these acids in this section are expressed in mg/L. Principal component analysis (PCA) was carried out to assess the variation in these acids for all nine sets at different concentrations of glucose and sucrose during fermentation for 96 h. Samples were taken at time, T= 0, 24, 48, 72 and 96 h.

The union of principal components with manifest variables, measured by loading (association between principal component scores and manifest variables), can also be studied qualitatively in order to interpret a principal component. If all the variables in a component are positively correlated with each other, all the loadings will be positive. Negative correlations among variables and negative loadings are considered non-relevant in PCA. In the interpretation of PCA, a negative loading simply means that a specified characteristic is deficient in a latent variable associated with the given principal component.

The PCA- 2D score plots (Figure 3.9) distinguished the higher concentrations of carboxylic acids produced after longer fermentation times (24h to 96 h) from those at the start of fermentation (0 h). PC1 and PC2 demonstrated 75.2% and 12.3% of the total variation, respectively. The scores for the different kefir fermented coconut water at different times (T=0, 24, 48, 72 and 96 h) are clearly shown. Five groups were clearly separated according to fermentation time (T=0, 24, 48, 72 and 96 h) indicating distinct changes in acids during fermentation. Samples at time, T=0 had negative scores along PC1. With increasing fermentation time (T= 24, 48, 72 and 96 h), sample scores became increasingly positive which meant that the acid concentrations were increasing.

The PCA biplot in Figure 3.10 shows both the scores and loadings plot for carboxylic acids in all the kefir fermented coconut water fermented at 0, 24, 48, 72 and 96 h. T=0 h. The control, (T=0h) had significantly high negative scores that corresponded to high negative loadings for malic acid. This finding was supported by ANOVA results shown in Table 3.19 and Appendix 1, which indicated that malic acid concentration was significantly high for set 3, while set 9 had significantly low malic acid ($p<0.5$). The fact that malic acid was only found at the start time of fermentation (T=0 h) indicated that malolactic fermentation may be occurring during coconut water kefir fermentation. Malic acid is known to be converted to lactic acid (Liu, 2002; Vegara et al., 2014). With increasing fermentation time, most of the sets at time, T= 24, 48 and 72 h had significantly high positive scores, which corresponded to the high positive loadings of lactic acid, acetic acid, pyruvic acid. This finding was supported by ANOVA results shown in Table 3.13, Table 3.14, Table 3.15, Table 3.16, Table 3.17, Table 3.18 and Appendix 1 that showed significantly higher concentrations of lactic acid, acetic acid and pyruvic acid in fermented

samples with time. This suggests that malic acid was slowly being converted to lactic acid (Liu, 2002; Vegara et al., 2014).

Successful conversion of malic acid to lactic acid, known as malolactic fermentation, has been reported in wine using *L. casei* and *Lactococcus oenos* (Agouridis, Bekatorou, Nigam, & Kanellaki, 2005; Plessas et al., 2007). Malic acid has also been produced in fermented food and beverages such as kimchi and wine which involve both lactic acid bacteria and yeast (Linko, Javanainen, & Linko, 1997; Volschenk, Van Vuuren, & Viljoen-Bloom, 2006; Yang & Chang, 2010). Lactic acid was identified as the primary non-volatile organic acid that increased gradually throughout fermentation. Lactic acid could also be produced by further fermentation of malic acid during the malolactic fermentation, consistent with a previous report by Vegara et al., (2014).

Guzel-Seydim et al. (2000) have reported that there is was increase in lactic acid production during milk kefir fermentation (Guzel-Seydim, Seydim, & Greene, 2000). It has been observed that kefir microorganisms were able to significantly increase the lactic acid concentration along with other acids present in the fermentation medium ($p<0.05$). Acetic acid also increased with fermentation in this study. Interestingly, the acetic acid concentration in milk kefir grains (fermented in both cow and buffalo milk) has been found to be present in high quantities at the end of fermentation (Erkaya & Şengul, 2011).

In this study on coconut water kefir fermentation, pyruvic acid was generated in significant amounts due to the presence of various strains of lactic acid bacteria. Pyruvic acid can then be reduced to lactic acid. Thus, pyruvate is a critical intermediate in the homo- and hetero-fermentative pathways. Different strains of lactic acid bacteria breakdown pyruvate differently. *L. brevis* degrades pyruvate to lactate and acetate (thus increasing both lactic acid and acetic acid concentrations in the medium (Liu, 2003).

Another group separation in PC1 at T=96 h showed samples with the highest positive scores and high positive loadings that correspond to succinic acid (Figure 3.10). This observation was supported by ANOVA results, where succinic acid was significantly the highest at 96 h fermented samples ($p<0.05$) (Table 3.15). Weckx et al., (2010) reported a correlation between the presence of *L. plantarum* in the fermentation medium with succinic acid production.

Figure 3.9: PCA-2D score plots of PC1 and PC2 for the carboxylic acids found in the different sets of coconut water kefir with time (T=0, 24, 48, 72 and 96 h). The samples are colour-coded as below:

Legends- : T= 0 h; : T= 24 h ; : T=48 h; : T=72 h; : T= 96 h

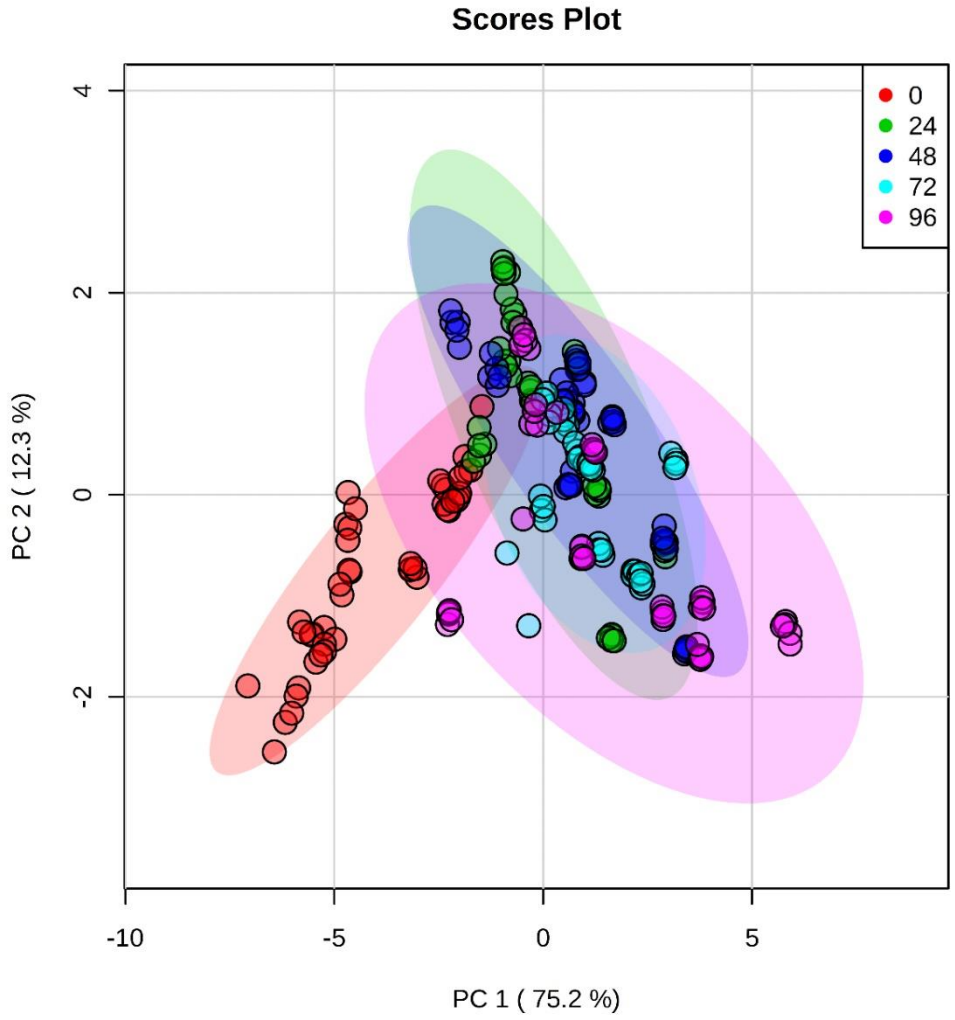
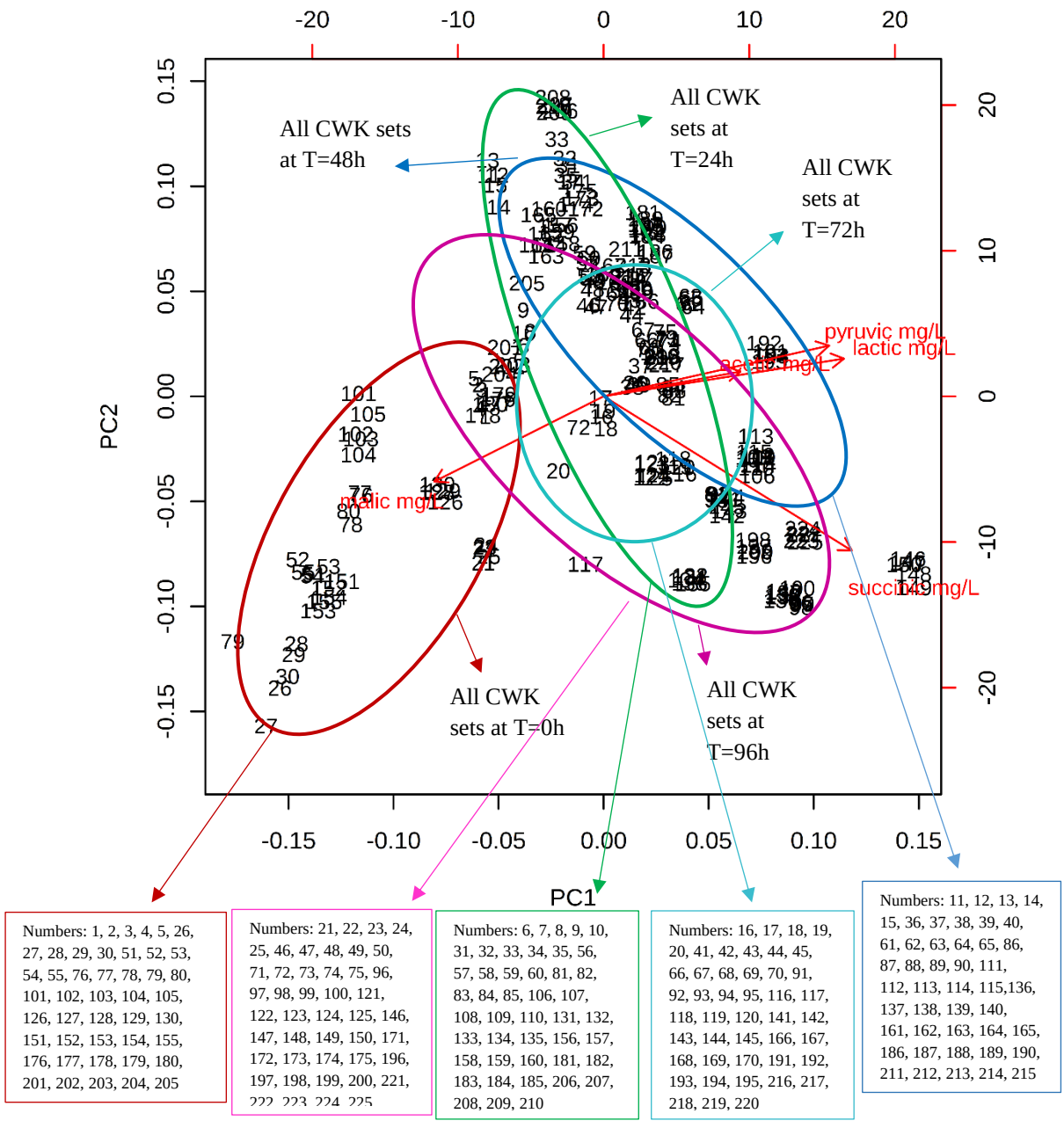


Figure 3.10: Principal components analysis (PCA) biplot for carboxylic acids in all the coconut water kefir sets at different times (T=0, 24, 48, 72 and 96 h). The correlation loadings are presented as numbers and refer to carboxylic acid concentration present in each set sample at a particular time.



The coconut water kefir samples are numbered as below: Numbers 1-25: belong to Set 1; Numbers 1-5 belong to T=0 h, numbers 6-10 belong to T=24 h, numbers 11-15 belong to T=48 h, numbers 16-20 belong to T=72 h and numbers 21-25 belong to T=96 h. Numbers 26-50: belong to Set 2; Numbers 26-30 belong to T=0 h, numbers 31-35 belong to T=24 h, numbers 36-40 belong to T=48 h, numbers 41-45 belong to T=72 h and numbers 46-50 belong to T=96 h. Numbers 51-75: belong to Set 3; Numbers 51-55 belong to T=0 h, numbers 56-60 belong to T=24 h, numbers 61-65 belong to T=48 h, numbers 66-70 belong to T=72 h and numbers 71-75 belong to T=96 h. Numbers 76-100: belong to Set 4; Numbers 76-80 belong to T=0 h, numbers 81-85 belong to T=24 h, numbers 86-90 belong to T=48 h, numbers 91-95 belong to T=72 h and numbers 96-100 belong to T=96 h. Numbers 101-125: belong to Set 5; Numbers 101-105 belong to T=0 h, numbers 106-110 belong to T=24 h, numbers 111-115 belong to T=48 h, numbers 116-120 belong to T=72 h and numbers 121-125 belong to T=96 h. Numbers 126-150: belong to Set 6; Numbers 126-130 belong to T=0 h, numbers 131-135 belong to T=24 h, numbers 136-140 belong to T=48 h, numbers 141-145 belong to T=72 h and numbers 146-150 belong to T=96 h. Numbers 151-175: belong to Set 7; Numbers 151-155 belong to T=0 h, numbers 156-160 belong to T=24 h, numbers 161-165 belong to T=48 h, numbers 166-170 belong to T=72 h and numbers 171-175 belong to T=96 h. Numbers 176-200: belong to Set 8; Numbers 176-180 belong to T=0 h, numbers 181-185 belong to T=24 h, numbers 186-190 belong to T=48 h, numbers 191-195 belong to T=72 h and numbers 196-200 belong to T=96 h. Numbers 221-245: belong to Set 9; Numbers 221-225 belong to T=0 h, numbers 226-230 belong to T=24 h, numbers 231-235 belong to T=48 h, numbers 236-240 belong to T=72 h and numbers 141-145 belong to T=96 h.

Figure 3.11: Heatmap and hierarchical clustering of carboxylic acids profiles from kefir fermented coconut water samples. Dendrogram on the left, represents the sample clusters based on Pearson's correlation coefficient with average linkage. Each carboxylic acid in the heat-map expresses normalised acid content respected to the colour range, red and green colour indicates higher and lower concentrations of the corresponding acid.

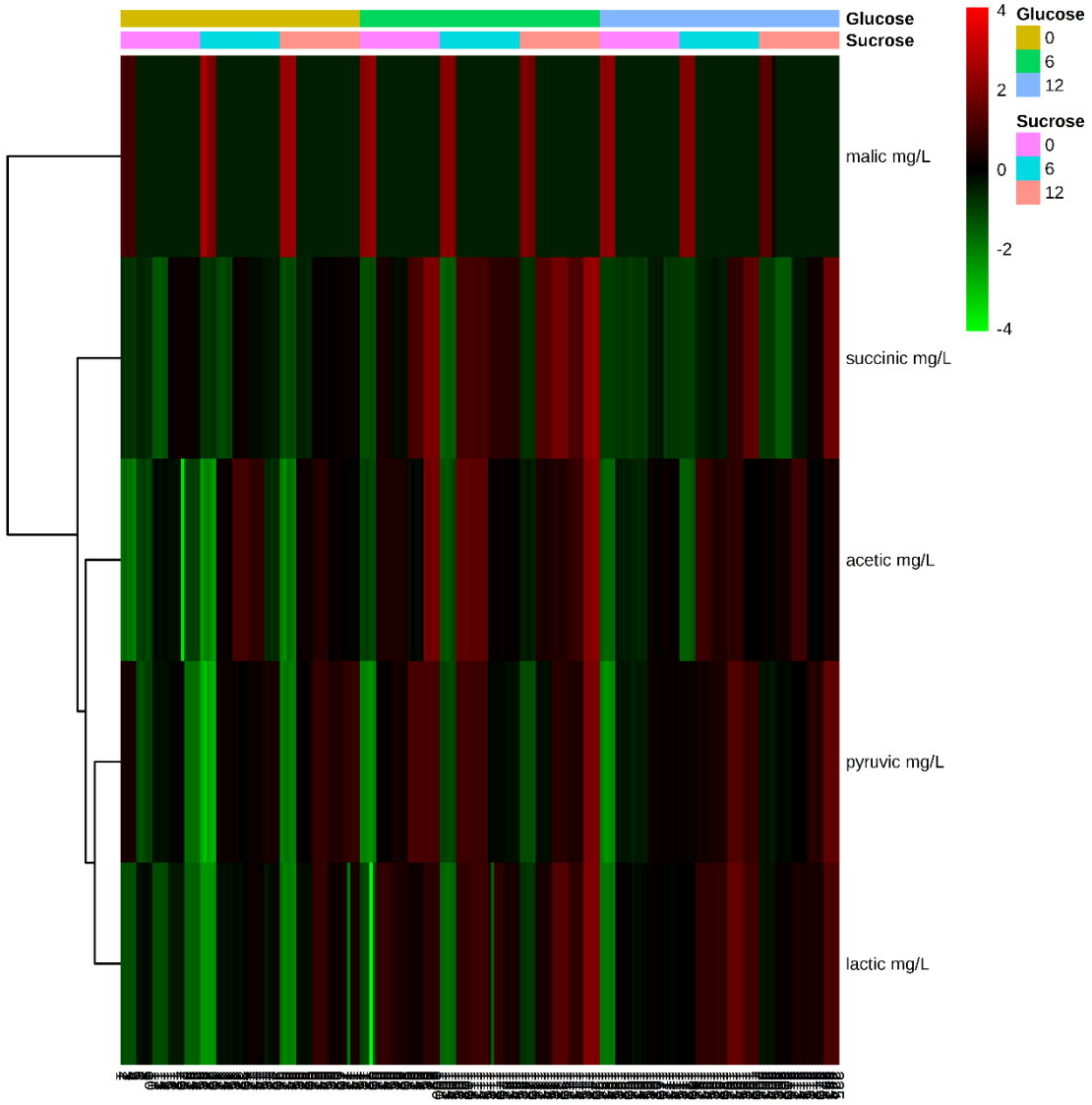


Table 3.13: Two- way ANOVA table for carboxylic acid profile for the effect of glucose and sucrose addition during the coconut water kefir fermentation from time, T=0, 24, 48, 72 and 96 h).

Carboxylic acids (mg/L)	Glucose (F.val)	Glucose (raw.p)	Glucose (adj.p)	Sucrose (F.val)	Sucrose (raw.p)	Sucrose (adj.p)	Interaction (F.val)	Interaction (raw.p)	Interaction (adj.p)
Succinic acid	36.4	2.38E-14	1.19E-13	7.7145	0.000581	0.000953	3.4064	0.010012	0.025031
Lactic acid	24.491	2.59E-10	6.48E-10	13.567	2.82E-06	1.41E-05	3.7488	0.005696	0.025031
Acetic acid	20.326	8.16E-09	1.36E-08	10.676	3.79E-05	9.47E-05	1.199	0.31223	0.39029
Pyruvic acid	5.7587	0.003659	0.004574	7.423	0.000762	0.000953	2.7012	0.031557	0.052595

Results of the heat map (Figure 3.11) and those in Table 3.13 demonstrate that lactic acid, acetic acid, pyruvic acid and succinic acids were present in high relative abundance for sets 3, 4, 6, 8 and 9 (shown in red). Of these sets, sets 3, 6 and 9 were supplemented with 12g/L of sucrose and sets 4 and 8 were supplemented with 6 g/L of glucose and 6 g/L of sucrose respectively. Hence, high concentrations of acids were obtained in sets with added sucrose.

All the above acids are desirable for good acidification property of kefir microorganisms, LAB and acetic acid bacteria. The dendrogram showed a correlation between the carboxylic acids with respect to the concentration of added glucose and sucrose. Lactic acid and pyruvic acid are closely related, which are both very closely related to acetic acid. Acetic, pyruvic and lactic acid are all related to succinic acid, finally the distant relationship between malic acid and the rest of the acids is shown. A study carried out by Li, Xin, Na, & Jun (2019) on Tibetian kefir fermented beverage production have reported the presence of a considerable number of carboxylic acids which were short and medium-chain (C4:0-C12:0). Dongmo, Sacher, Kollmannsberger, & Becker (2017) have reported that ethyl esters could play a role of precursors for the production of carboxylic acids during fermentation. Additionally, they have also stated that acetic acid was the representative carboxylic acid giving mild vinegar flavour to the beverage.

Previous studies carried out by Cho et al. (2006) and Xiong et al. (2014) showed that sucrose could be better utilized by LAB than fructose or glucose, consistent with their rapid growth by consuming sucrose during the early stage of fermentation. It has also been reported in the same study that sucrose was utilized throughout sauerkraut fermentation (Cho et al., 2006; Xiong, Li, Guan, Peng, & Xie, 2014). A similar study to support sucrose utilisation during fermentation was carried out by Siragusa et al. (2014), where sucrose was gradually degraded into glucose and fructose for lactic acid production. These studies support the finding in the present study obtained in Sets 4, 5, 6, 8 and 9, all of which had added sucrose, and produced high lactic acid concentration.

The concentration of lactic acid obtained using LC-MS is comparable to the total concentrations of D- and L-lactic acid obtained using the enzyme detection kit (Section 3.3.4). The total concentration of D- lactic acid and L- lactic acid produced for set 3 (supplemented with 12 g/L of sucrose) after 96 h of CWK sourdough fermentation was significantly the highest of all sets, with a value of 9.72 ± 0.1 g/L, ($p<0.05$). This value is comparable to the (total) lactic acid detected using LC-MS after 96h fermentation of CWK sourdough at 9.9 ± 0.01 g/L, ($p<0.05$), after unit conversion. Similarly, all the other data for D-/L- lactic acid detected using the enzyme detection kit are comparable to the total lactic acid concentration detected in sourdough using LC-MS. For these comparisons, the concentrations detected by LC-MS expressed as mg/L were converted to g/L.

Table 3.14: Lactic acid production monitored at different time intervals in kefir fermented coconut water. a, b, c, d…, mean values of mg/L for lactic acid, with different superscripts within the same row (different time interval) differ significantly ($p < 0.05$). A, B, C mean values of mg/L for lactic acid, with different superscripts within the same column (different treatment of samples) differ significantly ($p < 0.05$). Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Sets	Added Glucose (g/L)	Added Sucrose (g/L)	Lactic acid in mg/L					F value (ANOVA model)						
			0 h	24 ours	48 h	72 h	96 h	Suc (g/L)	Glu (g/L)	Time	Suc (g/L) *Glu (g/L)	Suc (g/L)* Time	Glu (g/L)* Time	Suc (g/L)*Glu (g/L)* Time
Set 1	0	0	827.3 ± 14.8^{E,a,b,c}	2362.8 ± 14.01^{D,c,d}	5880.6 ± 13.79^{C,c}	6043.6 ± 82.67^{B,b}	8618.2 ± 21.0^{A,b,c}	13.9 ***	14.6***	1623.2***	24.1 ***	2.1*	2.3*	6.4***
Set 2	0	6	866.3 ± 6.5^{E,a,b,c}	2346.9 ± 16.2^{D,c,d}	5615.3 ± 84.26^{C,d}	6492.1 ± 16.26^{B,b}	7716.6 ± 7.1^{A,c,d,e}							
Set 3	0	12	870.3 ± 41.7^{E,a,b,c}	3414.8 ± 36.6^{D,a}	6858.2 ± 77.88^{C,a}	7645.2 ± 14.3^{B,a}	9998.7 ± 18.5^{A,a}							
Set 4	6	0	732.6 ± 37.1^{D,b,c,d}	2463.2 ± 16.4^{C,c,d}	5643.4 ± 65.3^{B,a}	6743.7 ± 11.7^{B,a,b}	8816. ± 59.6^{A,b,c}							
Set 5	6	6	746.8 ± 32.3^{D,c,d}	2257.6 ± 11.8^{C,d}	5541.6 ± 35.7^{B,d}	6831.6 ± 20.9^{A,B,a,b}	8205.3 ± 57.9^{A,b,c,d}							
Set 6	6	12	796.7 ± 11.0^{E,a,b,c,d}	2315.9 ± 78.8^{D,c,d}	5381.3 ± 43.1^{C,d}	6211.2 ± 48.8^{B,b}	7432.4 ± 57.2^{A,d,e}							
Set 7	12	0	728.8 ± 15.2^{E,d}	2980.2 ± 63.6^{D,b}	4520.8 ± 128.2^{C,e}	6209.2 ± 25.5^{B,b}	6763.8 ± 13.7^{A,e}							
Set 8	12	6	722.7 ± 12.4^{D,d}	2049.35 ± 86.4^{C,e}	6281.1 ± 84.1^{B,b}	6166.1 ± 47.4^{B,b}	8146.1 ± 75.6^{A,b,c,d}							
Set 9	12	12	788.4 ± 59.66^{E,a,b,c,d}	3072.3 ± 13.6^{D,b}	5444.5 ± 28.4^{C,d}	6325.7 ± 55.6^{B,b}	9058.1 ± 21.3^{A,a,b}							

Table 3.15: Succinic acid production monitored at different time intervals in kefir fermented coconut water. a, b, c, d…,represent the mean values of mg/L of Succinic acid, with different superscripts within the same row (different time interval) differ significantly ($p < 0.05$). A, B, C mean values of mg/L for Succinic acid, with different superscripts within the same column (different treatment of samples) differ significantly ($p < 0.05$). Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Sets	Added Glucose (g/L)	Added Sucrose (g/L)	Succinic acid in mg/L					F value (ANOVA model)						
			0 h	24 h	48 h	72 h	96 h	Suc (g/L)	Glu (g/L)	Time	Suc (g/L)* Glu (g/L)	Suc (g/L)*Time	Glu (g/L)* Time	Suc (g/L)* Glu (g/L)* Time
Set 1	0	0	$27.8 \pm 2.6^{E,a}$	$33.9 \pm 2.3^{D,d}$	$74.1 \pm 1.9^{C,d}$	$184.0 \pm 3.6^{B,f}$	$783.1 \pm 3^{A,b}$	1273.2***	4786.9***	2712.4***	807.0***	459.2***	770.5***	417.1***
Set 2	0	6	$26.3 \pm 1.4^{E,b}$	$39.3 \pm 2.1^{D,d}$	$85.4 \pm 2.7^{C,b}$	$194.7 \pm 3.5^{B,e}$	$645.1 \pm 3.4^{A,f}$							
Set 3	0	12	$27.3 \pm 1.4^{E,a,b}$	$56.2 \pm 2.3^{D,a}$	$97.6 \pm 2.2^{C,a}$	$296.8 \pm 2.9^{B,e}$	$883.4 \pm 2.4A^{,a}$							
Set 4	6	0	$16.3 \pm 0.7^{E,d}$	$46.1 \pm 3.6^{D,b}$	$85.1 \pm 2.5^{C,b}$	$250.7 \pm 3.1^{B,b,c}$	$669.9 \pm 1.5^{A,e,f}$							
Set 5	6	6	$22.2 \pm 1.2^{E,c}$	$46.7 \pm 2.4^{D,b}$	$82.2 \pm 4.9^{C,b,c}$	$260.5 \pm 3.4^{B,a}$	$643.1 \pm 5.9^{A,f}$							
Set 6	6	12	$27.2 \pm 1.7^{C,a,b}$	$35.2 \pm 3.9^{C,d}$	$73.2 \pm 4.4^{C,d}$	$263.3 \pm 2.3^{B,a}$	$749.8 \pm 3.1^{A,b,c}$							
Set 7	12	0	$22.3 \pm 0.1^{E,c}$	$34.4 \pm 2.2^{D,d}$	$74.5 \pm 2.2^{C,d}$	$244.8 \pm 4^{B,c}$	$724.4 \pm 1.7^{A,c,d}$							
Set 8	12	6	$23.2 \pm 0.3^{E,c}$	$40.1 \pm 2.0^{D,c}$	$64.3 \pm 4.6^{C,e}$	$256.7 \pm 4.3^{B,a,b}$	$686.9 \pm 2.9^{A,d,e}$							
Set 9	12	12	$23.3 \pm 0.9^{E,c}$	$33.1 \pm 0.5^{D,d}$	$75.5 \pm 2.9^{C,c,d}$	$219.4 \pm 3.5^{B,d}$	$690.8 \pm 4.8^{A,d,e}$							

Table 3.16: Acetic acid production monitored at different time intervals in kefir fermented coconut water. a, b, c, d…,n mean values of mg/L for Acetic acid, with different superscripts within the same row (different time interval) differ significantly ($p < 0.05$). A, B, C mean values of mg/L for Acetic acid, with different superscripts within the same column (different treatment of samples) differ significantly ($p < 0.05$). Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$).

Sets	Added Glucose (g/L)	Added Sucrose (g/L)	Acetic acid in mg/L					F value (ANOVA model)						
			0 h	24 h	48 h	72 h	96 h	Suc (g/L)	Glu (g/L)	Time	Suc (g/L)*Glu (g/L)	Suc (g/L)* Time	Glu (g/L)* Time	Suc (g/L)*Glu (g/L)*Time
Set 1	0	0	$26.3 \pm 3.1^{D,e,f}$	$115.0 \pm 3.1^{C,b}$	$197.9 \pm 3.5^{B,d}$	$213.4 \pm 29.8^{B,e}$	$244.5 \pm 2.3^{A,e}$	66.9***	42.1***	9346.5***	41.3***	4.1*	8.7***	14.3***
Set 2	0	6	$29.3 \pm 2.7^{E,g}$	$95.4 \pm 4.6^{D,f}$	$213.5 \pm 5.1^{C,c}$	$240.8 \pm 4.1^{B,a,b}$	$285.8 \pm 3.5^{A,b}$							
Set 3	0	12	$43.1 \pm 3.5^{E,a}$	$124.6 \pm 3.4^{D,a}$	$233.6 \pm 6.9^{C,a}$	$252.9 \pm 4.3^{B,a,b}$	$294.9 \pm 2.4^{A,a}$							
Set 4	6	0	$25.4 \pm 2.6^{E,f,g}$	$97.8 \pm 2.8^{D,e,f}$	$215.6 \pm 4.1^{C,b,c}$	$230.2 \pm 4.5^{B,b,c,d}$	$286.8 \pm 5.5^{A,a,b}$							
Set 5	6	6	$37.6 \pm 4.0^{D,b,c}$	$127.4 \pm 3.1^{C,a}$	$219.5 \pm 5.5^{B,b,c}$	$224.3 \pm 3.2^{B,c,d,e}$	$290.2 \pm 2.1^{A,a,b}$							
Set 6	6	12	$33.77 \pm 3.3^{E,c,d}$	$113.5 \pm 2.8^{D,b,c}$	$219.7 \pm 4.9^{C,b,c}$	$239.1 \pm 8.3^{B,a,b}$	$265.8 \pm 71.4^{A,c}$							
Set 7	12	0	$30.4 \pm 2.7^{E,d,e}$	$105.2 \pm 2.1^{D,d}$	$198.1 \pm 2.1^{C,d}$	$218.4 \pm 6.4^{B,d,e}$	$254.4 \pm 3.9^{A,d}$							
Set 8	12	6	$32.8 \pm 1.9^{E,c,d}$	$107.6 \pm 3.8^{D,c,d}$	$219.7 \pm 5.74^{C,b,c}$	$233.7 \pm 6.27^{B,b,c,d}$	$285.1 \pm 2.1^{A,c,d}$							
Set 9	12	12	$38.9 \pm 7.6^{E,a,b}$	$103.5 \pm 3.1^{D,d,e}$	$192.9 \pm 4.88^{C,d}$	$223.3 \pm 1.9^{B,c,d,e}$	$261.6 \pm 6.1^{A,c,d}$							

Table 3.17 Pyruvic acid production monitored at different time intervals in kefir fermented coconut water. a, b, c, d…,n mean values of mg/L area for Pyruvic acid, with different superscripts within the same row (different time interval) differ significantly ($p < 0.05$). A, B, C mean values of mg/L for Pyruvic acid, with different superscripts within the same column (different treatment of samples) differ significantly ($p < 0.05$). Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). ∗ $p < 0.05$. ∗∗ $p < 0.01$. ∗∗∗ $p < 0.001$.difference ($p < 0.05$). ∗ $p < 0.05$. ∗∗ $p < 0.01$. ∗∗∗ $p < 0.001$).

Sets	Added Glucose (g/L)	Added Sucrose (g/L)	Pyruvic acid in mg/L					F value (ANOVA model)						
			0 h	24 h	48 h	72 h	96 h	Suc (g/L)	Glu (g/L)	Time	Suc (g/L)* Glu (g/L)	Suc (g/L)* Time	Glu (g/L)* Time	Suc (g/L)*Glu (g/L)*Time
Set 1	0	0	$665.8 \pm 2.1^{E,a}$	$758.3 \pm 2.1^{D,b,c}$	$865.2 \pm 3.6^{C,a,b}$	$940.1 \pm 4.1^{B,b,c}$	$1284.7 \pm 2.9^{A,b}$	4.5*	110.6***	3391.2***	77.3***	51.5***	28.7***	21.6***
Set 2	0	6	$630.2 \pm 5.2^{E,b}$	$780.4 \pm 2.57^{D,a,b}$	$860.2 \pm 8.8^{C,a,b}$	$965.6 \pm 9.7^{B,a,b}$	$1289.7 \pm 5.5^{A,b}$							
Set 3	0	12	$668.5 \pm 7.3^{E,a}$	$799.6 \pm 3.9^{D,a}$	$886.5 \pm 8.8^{C,a,b}$	$976.4 \pm 1.3^{B,a,b}$	$1394.8 \pm 2.95^{A,a}$							
Set 4	6	0	$557.2 \pm 6.3^{E,d,e}$	$787.2 \pm 2.9^{D,a}$	$836.6 \pm 4.6^{C,b,c}$	$967.6 \pm 3.7^{B,a,b}$	$1252.1 \pm 4.7^{A,b}$							
Set 5	6	6	$638.3 \pm 8.5^{E,a,b}$	$742.2 \pm 8.6^{D,c}$	$840.3 \pm 6.3^{C,b,c}$	$929.5 \pm 4.2^{B,c}$	$1378.5 \pm 8.9^{A,a}$							
Set 6	6	12	$542.9 \pm 5.6^{D,e}$	$783.7 \pm 8.9^{D,a,b}$	$864.3 \pm 8.3^{C,a,b}$	$913.9 \pm 2.8^{B,e}$	$1235.8 \pm 2.9^{A,b}$							
Set 7	12	0	$594.2 \pm 9.3^{E,c}$	$741.3 \pm 2.7^{D,c}$	$804.6 \pm 6.7^{C,c}$	$956.4 \pm 2.7^{B,a,b,c}$	$1249 \pm 4.9^{A,b}$							
Set 8	12	6	$510.4 \pm 5.5^{E,f}$	$610.1 \pm 9.9^{D,e}$	$720.5 \pm 8.7^{C,d}$	$958.3 \pm 5.4^{B,a,b}$	$1242.6 \pm 5.3^{A,b}$							
Set 9	12	12	$585.4 \pm 2.6^{D,c,d}$	$712.4 \pm 6.9^{C,d}$	$862.3 \pm 7.9^{B,a,b}$	$861.5 \pm 6.7^{B,d}$	$1360.2 \pm 8.8^{A,a}$							

Table 3.18: Malic acid production monitored at different time intervals in kefir fermented coconut water. a, b, c, d…, n mean values of mg/L for Malic acid, with different superscripts within the same row (different time interval) differ significantly ($p < 0.05$). A, B, C mean values of mg/L for Malic acid, with different superscripts within the same column (different treatment of samples) differ significantly ($p < 0.05$). Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$).

Sets	Added Glucose (g/L)	Added Sucrose (g/L)	Malic acid concentration in mg/L at Time=0 h	F value (ANOVA model)						
				Suc (g/L)	Glu (g/L)	Time	Suc (g/L)*Glu (g/L)	Suc (g/L)*Time	Glu (g/L)*Time	Suc (g/L)*Glu (g/L)*Time
Set 1	0	0	29.6 ± 7.5^{a}	13.3***	22.6***	1971.7***	7.8***	13.3***	22.6***	7.8***
Set 2	0	6	$27.5 \pm 5.3^{a,b}$							
Set 3	0	12	31.4 ± 8.5^{a}							
Set 4	6	0	$27.6 \pm 6.1^{a,b}$							
Set 5	6	6	$24.4 \pm 5.6^{b,c}$							
Set 6	6	12	19.9 ± 6.8^{c}							
Set 7	12	0	$26.7 \pm 6.5^{a,b}$							
Set 8	12	6	$22.9 \pm 7.7b^{,c}$							
Set 9	12	12	11.6 ± 6.7^{d}							

3.3.6 Amino acids composition in kefir fermented coconut water

Amino acid analysis was carried out using the LCMS chromatographic method to determine changes in amino acid composition in the kefir fermented coconut water. All the 9 experimental sets of coconut water kefir (Table 3.3) were analysed at times, T=0, 24, 48, 72 and 96 h.

The PCA-2D score plot shown in Figure 3.12, described 75.9% and 7.7% of the total variation in Principal component 1 (PC1) and Principal component 2 (PC2) on the x and y-axis respectively. The scores for the different kefir fermented coconut water at different times (T=0, 24, 48, 72 and 96 h) are clearly shown. The generated PCA model had a high fitting (R2=0.84) and high-predicting quality (Q2=0.82). Five groups were clearly separated according to fermentation time (T=0, 24, 48, 72 and 96 h) indicating distinct changes in amino acids during fermentation. Samples at time 0 had negative scores along PC1. With increasing fermentation time (T= 24, 48, 72 and 96 h), sample scores became increasingly positive.

The PCA biplot in Figure 3.13, generated for all the 18 identified amino acids shows both the scores and loadings plot for amino acids in all the kefir fermented coconut water fermented at 0, 24, 48, 72 and 96 h. The T=0 h (control) samples had significantly high negative scores that corresponded to high negative loadings for tyrosine, valine, leucine, isoleucine, methionine and phenylalanine. This finding was supported by ANOVA results in Appendix 4 and Appendix 5, that showed significantly higher values for tyrosine, valine, leucine, isoleucine, methionine and phenylalanine for, T= 0 h for all the sets.

With increasing fermentation, most of the sets at time, T= 24, 48 and 72 h had significantly high positive scores, which corresponded to the high positive loadings of glutamic acid. All the fermented samples at time 24, 48, 72 and 96 h had positive scores, which corresponded to high positive loadings of glutamic acid. These observations are supported by results summarised in the ANOVA table, Appendix 4 and Appendix 5 that showed significantly higher values for glutamic acid, particularly in samples at T= 24, 48 and 72 h. The presence of glutamic acid in these fermentations is desirable in the current study as this amino acid could enhance the flavour profile of the sourdough bread being developed in this study.

The 95% confidence ellipse can be seen in Figure 3.12. The ellipses clearly define statistically significant class separation. The ellipsoid for 96 h samples was wide vertically with variance that can be explained along PC2. The ellipses for T=48 and 72 h overlap tightly and corresponded to high positive glutamic acid loading along PC1 as seen in the PCA-biplot in Figure 3.13.

Glutamate dehydrogenase activity in LAB, which leads to the production of glutamic acid, is a very important criterion for selecting the strains utilised for producing fermented foods like cheese. The production of glutamate from α-ketoglutarate by LAB produces flavour compound which is associated with the formation of very desirable aroma in fermented foods (Tanous, Kieronczyk, Helinck, Chambellon, & Yvon, 2002).

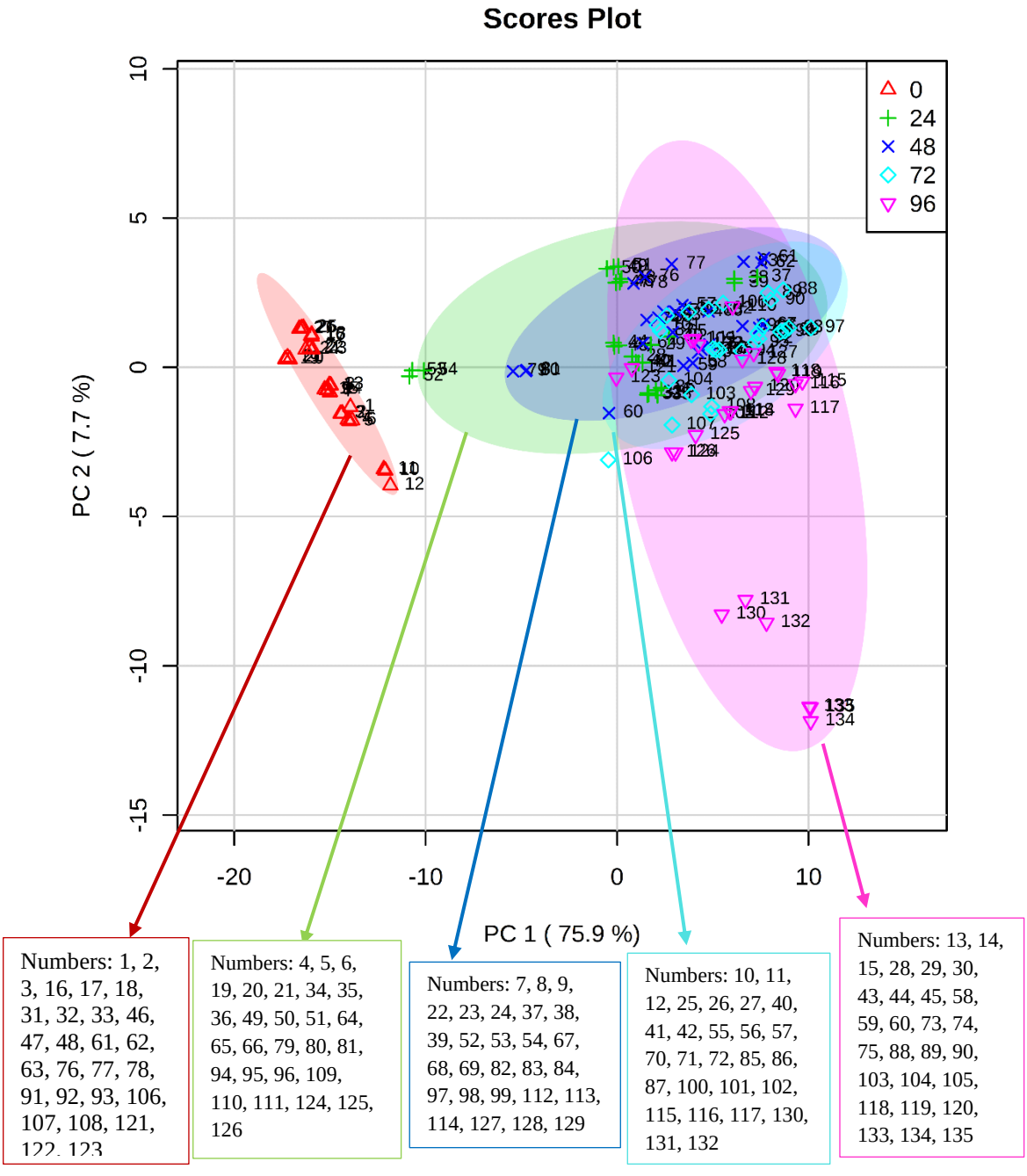
Kondoh et al (2009) have reported that glutamic acid is an important multi-functional amino acid which is also involved in intermediary metabolism and excitatory neurotransmission (Kondoh, Mallick, & Torii, 2009). Glutamate, the anionic form of glutamic acid, can be further converted into glutamine or gamma-amino butyric acid (GABA). During metabolism, when sugars are converted into pyruvic acid through the glycolytic pathway, α-ketoglutaric acid (intermediate product of TCA cycle) is converted to glutamate. Employing LAB, on the other hand, the potential to produce glutamic acid can facilitate the production of functional foods rich in bioactive molecules such as GABA. GABA possesses several well-known physiological functions such as anti-hypertension (Inoue et al., 2003) and anti-diabetic (Hagiwara, Seki, & Ariga, 2004). Glutamic acid or monosodium glutamate (MSG) is used as a flavour enhancer in foods (Zareian et al., 2012). Primarily, this AA is used in food for its umami flavour (Jyothi, Sasikiran, Nambisan, & Balagopalan, 2005). Furthermore, a number of studies have shown the possible usefulness of glutamic acid in enhancing nourishment in the elderly and in patients with poor nutrition (Tomoe et al., 2009; Yamamoto, Tomoe, Toyama, Kawai, & Uneyama, 2009). The vital benefit of LAB produced glutamic acid is that the amino acid produced using this approach is biologically active and the production process is considered to be reliable and environmental-friendly (Zareian et al., 2012).

Several studies carried out in sourdough show that glutamine deamidation by LAB during wheat sourdough fermentation could be the reason for the increase in glutamate levels that could positively impact the flavour of the bread (Gobbetti, Simonetti, et al., 1994).

Glutamic acid can be formed from proteolytic activity resulting in the deamination of glutamine. Glutamic acid can also be formed from transamination of other amino acids as the preferred amino group acceptor such as α-ketoglutarate, which is converted to glutamic acid in this way (Fernandez & Zuniga, 2006; Smit, Smit, & Engels, 2005). Increased production of glutamic acid with incubation time has been reported for kefir grains fermentation of cow and buffalo milk (Gul et al., 2015).

Figure 3.12: The PCA 2D Score plot derived for 18 amino acids for kefir fermented coconut water sets at times, T=0, 24, 48, 72 and 96 h. The score plot shows clear differentiation between fermented samples with time. The correlation loadings are presented as numbers and refer to amino acid concentration present in each set sample at a particular time

Legends- △ : T= 0 h; + : T= 24 h ; × : T=48 h; ◇ : T=72 h; ▽ : T= 96 h



Variables are numbered as shown below: Numbers 1-15: belong to Set 1, numbers 16-30: belong to Set 2, numbers 31-45: belong to Set 3, numbers 46-60: belong to Set 4, numbers 61-75: belong to Set 5, numbers 76-90: belong to Set 6, numbers 91-105: belong to Set 7, numbers 106-120: belong to Set 8, numbers 121-135: belong to Set 9.

Figure 3.13: PCA Biplot for all the kefir fermented coconut water sets at time, T=0, 24, 48, 72 and 96 h. The variables are numbered as shown below:

Numbers 1-15: belong to Set 1, numbers 16-30: belong to Set 2, numbers 31-45: belong to Set 3, numbers 46-60: belong to Set 4, numbers 61-75: belong to Set 5, numbers 76-90: belong to Set 6, numbers 91-105: belong to Set 7, numbers 106-120: belong to Set 8, numbers 121-135: belong to Set 9.

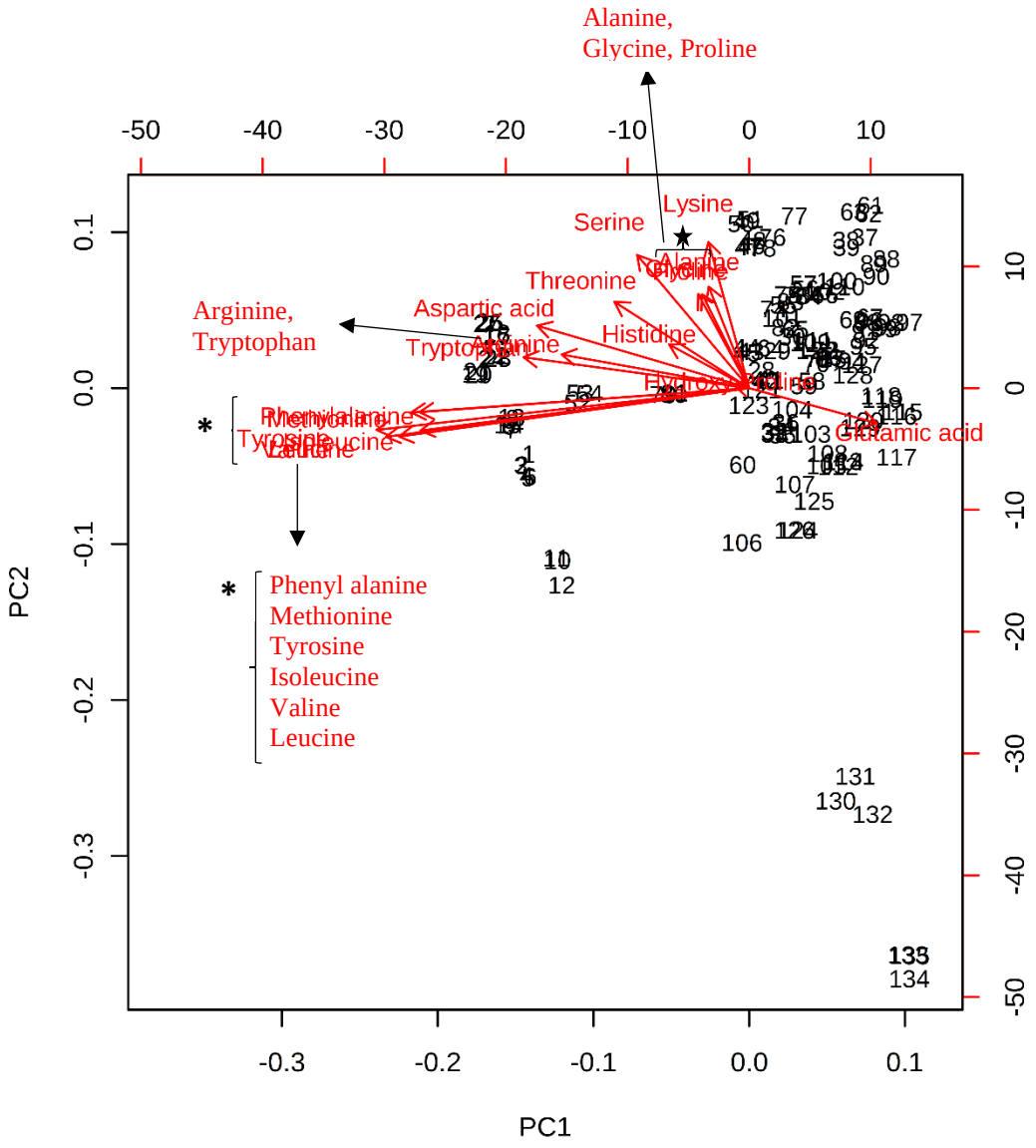
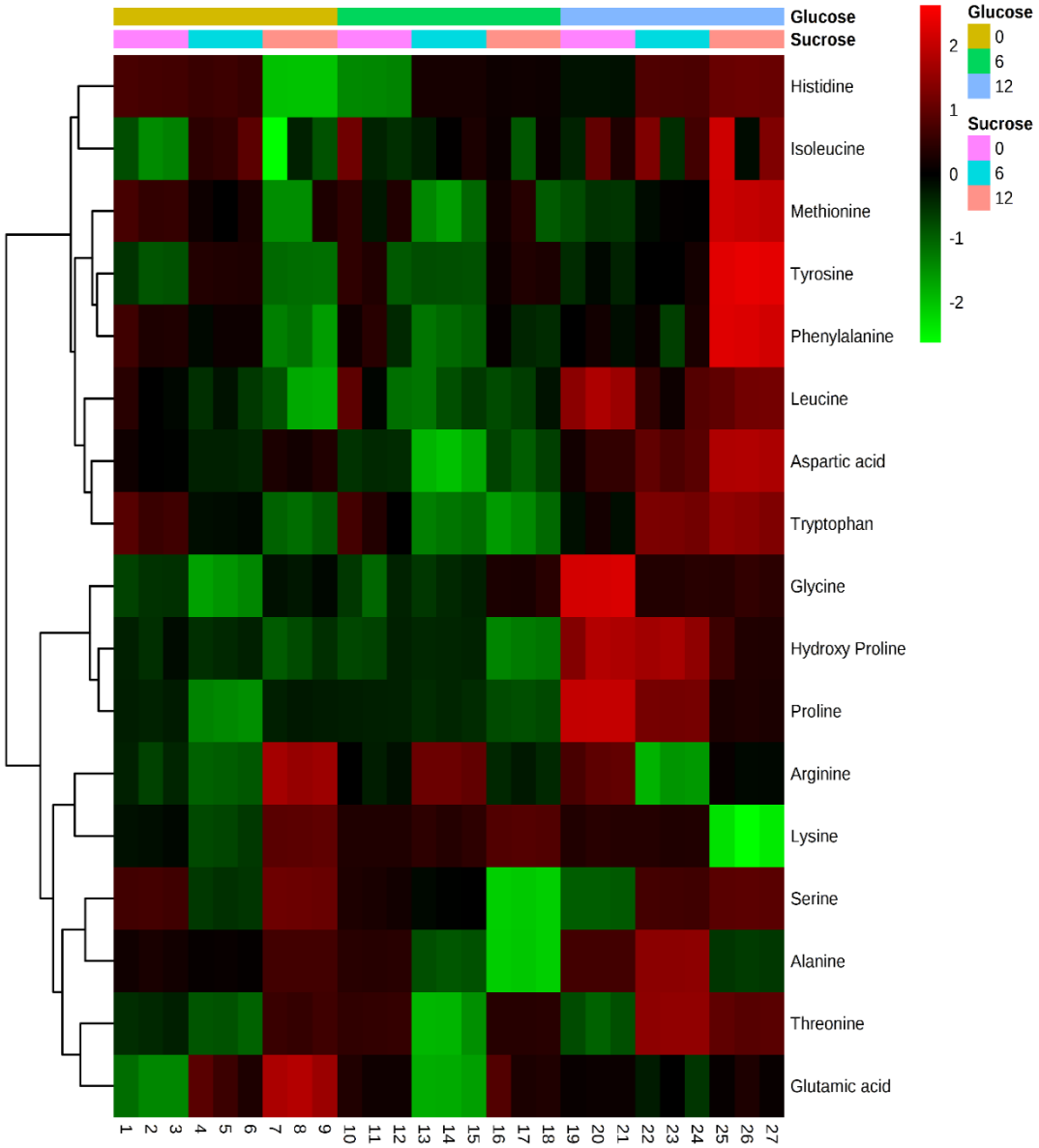


Figure 3.14: Heat-map and hierarchical clustering of amino acid profile from kefir fermented coconut water sample sets with added glucose and sucrose at time, T= 48 h. Dendrogram represents amino acid clusters based on Pearson's correlation coefficient with average linkage. Each square in the heat-map expresses normalized amino acid content respected to the colour range. The red colour indicates higher content of the corresponding compound. The sample numbers are shown as below:

For time, T= 48 h: Numbers 1-3 represent set 1, numbers 4-6: represent set 2,…, numbers 25-27 represent set 9.



In order to further understand the effect of glucose and sucrose addition in kefir fermented coconut water on amino acid profiles, the heatmap for 48-h fermentation was generated as seen in Figure 3.14. In Set 3 (corresponding to numbers 7, 8 and 9) at T= 48 h glutamic acid was present in high relative abundance (shown in red). The concentration of glutamic acid was between 21.21 ± 0.3 - 41.25 ± 1.06 µM/L. This set also had a significantly high concentration of glutamic acid compared to the rest of the sets as supported by ANOVA results (Appendix 5). Set 3 had 12 g/L sucrose, added to coconut water kefir.

A similar study was carried out on the water kefir microflora by Neve and Heller in 2002, who reported that yeasts in kefir are responsible for the breakdown of sucrose into glucose and fructose units (Neve & Heller, 2002). All the changes in the amino acid profile can be related to the differences in the number of microorganisms that thrive in the different sugar medium during the coconut water kefir fermentation.

Gronnevik et al. (2011) reported that the presence of amino acids in milk kefir may be due to the combined effects of the release of amino acids during the course of fermentation, assimilation of peptides and proteolytic activity. It was reported that change in glutamic acid may have been due to decarboxylation to γ -aminobutyric acid (Gronnevik, Falstad, & Narvhus, 2012).

3.3.7 Analysis of kefir granules using scanning electron microscopy (SEM)

Samples of coconut water kefir were completely dehydrated and frozen at -80°C and freeze-dried using Alpha 2-4 LD plus. The freeze-dried samples were then coated with platinum using the Hitachi E-1045 ion sputter coater and were observed using the Hitachi TM3030 plus SEM, 15KV, Secondary electrons (SE) and back-scatter detector (BSE). Figure 3.15, Figure 3.16 and Figure 3.17 show the SEM images of coconut water kefir.

Figure 3.15: The surface appearance of the coconut water kefir microflora (magnification of upto x 4000).

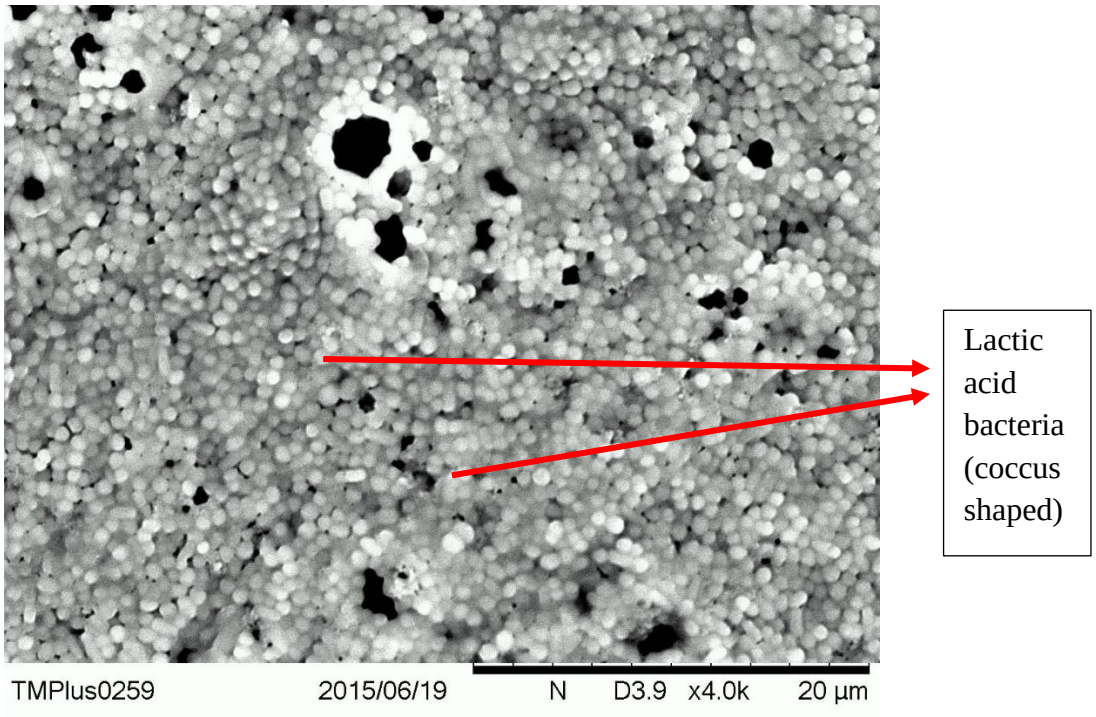


Figure 3.16: The surface appearance of the cross-section of the coconut water kefir microflora (magnification of upto x 10,000).

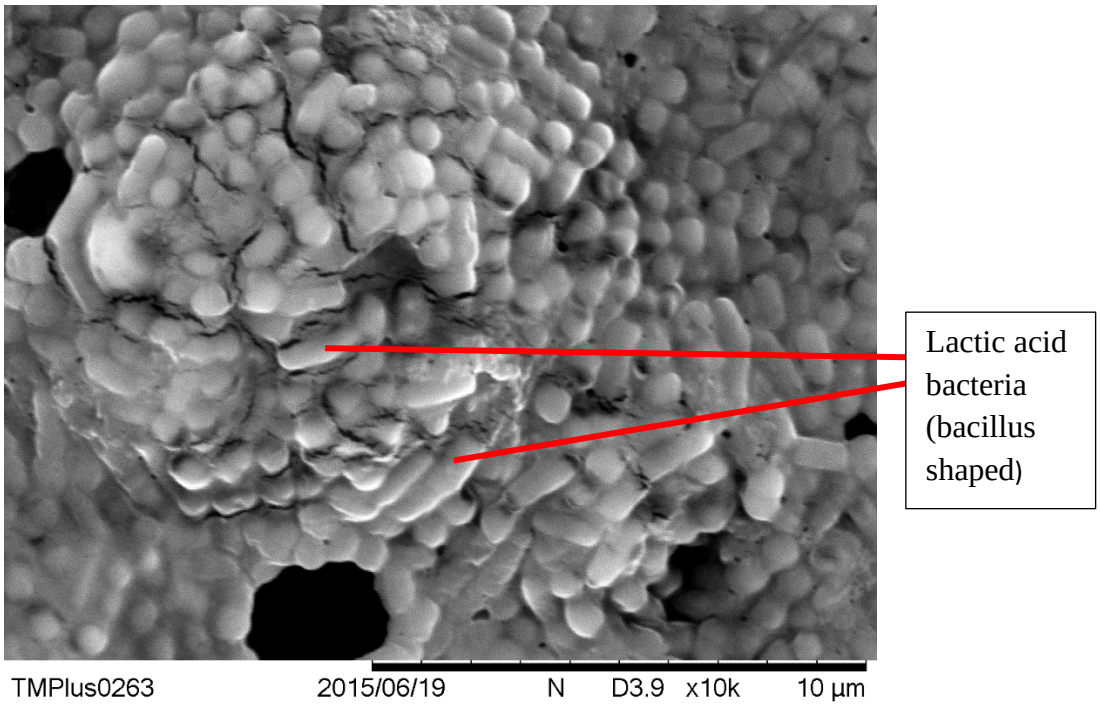
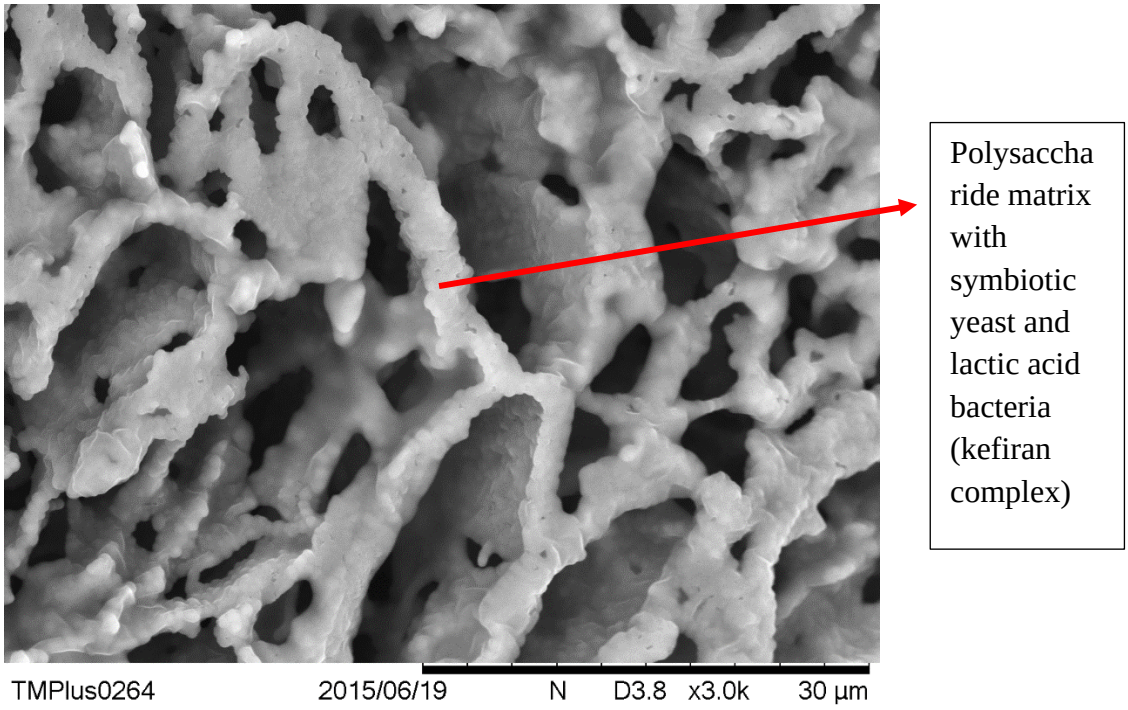


Figure 3.17: SEM of kefir network, in coconut water kefir, on the polysaccharide scaffold (magnification of upto x 3000).



The frozen coconut water kefir samples and dehydrated coconut water kefir showed a smooth surface. A thin cross-section obtained from both the samples appeared to have a rough surface, when observed under SEM. Magnification of up to 45x produced a good image of kefir grain surface (Figure 3.17). Under higher magnification, coccus-shaped and bacillus-shaped bacteria were visible (Figure 3.15 and Figure 3.16). These were most likely the LAB. The SEM results could not distinguish between the lactobacilli and the acetic acid bacteria.

A study carried out by Rea et al. (1996) and Neve and Heller (2002) reported that lactococci were the dominating bacteria on the kefir surface. They also reported that cocci were dominantly present in the water kefir samples along with some short and long rods (Neve & Heller, 2002; Rea et al., 1996). In the present study, the coconut water kefir cross-section showed a relatively higher density of bacterial cells than those of yeast cells.

The previous studies carried out on the structure of milk kefir, support the SEM results observed in this study, that is, a greater proportion of lactic acid bacteria were found to be present compared to yeast (Chin-Wen, Hsiao-Ling, & Liu, 1999; Guzel-Seydim, Wyffels, Seydim, & Greene, 2005; Rea et al., 1996).

3.4 Summary

To summarise, the microbial count for coconut water fermented with kefir and supplemented with 12 g/L sucrose (set 3) had the highest cell count for LAB, yeast and AAB. For the utilisation of glucose and sucrose by kefir microorganisms during coconut water fermentation, sucrose was utilised more than was glucose. There was increased acidity at the end of 96 h fermentation period of the coconut water kefir grains, due to which there was a reduction in pH value and significant production of carboxylic acids such as D-/L-lactic acid, acetic acid and pyruvic acid. Eighteen amino acids were identified, which were produced during fermentation, of which glutamic acid was present in higher quantities. There was increased production of L- lactic acid which was almost twice that of D- lactic acid. This, in turn, led to a decrease in the pH of CWK to pH 2.82 ± 0.01, after 96 h of incubation. Overall, there was higher production of carboxylic acids such as lactic acid, acetic acid and pyruvic acid in all the CWK fermented sets which were incubated for 48, 72 and 96 h compared to 0 and 24 h. Interestingly, malic acid was only detected at the start of CWK fermentation (T=0 h), which could be due to the malolactic fermentation occurring during coconut water kefir fermentation or its conversion to lactic acid. From all the 18 identified amino acids in fermented CWK, glutamic acid was produced in higher quantities compared to the rest of the amino acids after longer incubation times (T=48, 72 and 96 h). Set 3 supplemented with sucrose only and incubated for 48 h had the highest production of glutamic acid during the CWK fermentation. Scanning electron microscopy revealed that bacillus and coccus-shaped microorganisms (most probably LAB) were present in higher density in the CWK cross-section, compared to yeast. The SEM results could not distinguish between the lactobacilli and the acetic acid bacteria. Overall, since set 3 that was incubated for 48 h had significantly the highest cell counts (supplemented with 12 g/L of sucrose), it was selected to produce sourdough in further experiments.

Chapter 4 Microbial and physico-chemical characteristics of sourdough prepared with fermented coconut water kefir

4.1. Introduction

Sourdough is a fermented mixture of flour and water. The fermentation is the result of the growth of lactic acid bacteria (LAB), acetic acid bacteria (AAB) and yeast (Gobbetti, Minervini, Pontonio, Di Cagno, & De Angelis, 2016; Minervini, Celano, Lattanzi, De-Angelis, & Gobbetti, 2016). The fermented dough acts as the leavening agent in sourdough bread. This is in contrast to other breads that are leavened by baker's yeast.

The differences between sourdough bread and baker's yeast-leavened bread are well recognized and have been documented by many authors (Laatikainen et al., 2017). Sourdough fermentation affects the properties of both the dough and the resulting bread. It extends shelf life through the inhibition of spoilage fungi and bacteria, improves the bread's flavour, enhances nutritional properties, increases loaf volume, and delays staling (Arendt et al., 2007; Björck & Elmståhl, 2003; Katina, Heiniö, Autio, & Poutanen, 2006; Katina, Sauri, Alakomi, & Mattila-Sandholm, 2002; Ryan, Dal Bello, & Arendt, 2008). Also in sourdough, cell counts of LAB are higher than those of yeast by 1 log unit, compared to the other breads (Gobbetti, 1998; Ottogalli, Galli, & Foschino, 1996).

The microbial activity at different temperatures affects the pH and total acidity of the sourdough in terms of production of D- and L- lactic acid and carboxylic acids. Increasing acidity, however, leads finally also to a systematic degradation of gluten proteins (Thiele, Gänzle, & Vogel, 2003) and thus results in softer, less elastic dough with inferior gas holding properties, if fermentation is permitted extensively to obtain intensive acidification (Clarke et al., 2003). Accordingly, the acidity level of sourdough and subsequent bread dough must be carefully controlled, as recognized by Clarke et al. (2003).

In this study, sourdough leavening, texture, and viscosity have also been analysed to determine the effect of temperature on the quality of the sourdough being produced. The increase in leavening is related to the increase in the metabolic activities of the sourdough microorganisms and production of acids. There could be weakening of gluten structure in sourdough (Thiele et al. 2004), which could be due to the solubility of pentosans and the formation of exopolysaccharides (EPS), which play a crucial role in determining dough texture. The retention of gas by the dough matrix could affect the texture and viscosity of the resultant sourdough fermented over time for 96 h at 22°C and 30°C.

There are several factors that affect the sourdough fermentation such as the type of sourdough produced based on the process technology applied. The different types of sourdough (types 0, 1, 2 and 3) are distinguished based on whether a starter culture is added to the flour-water mixture, whether back slopping is carried out or not, on the time of incubation and lastly, on the final output – that is, if the dough is a sponge, a firm dough, a liquid sourdough or a dried sourdough (Brandt, 2007; Brandt & Ganzle, 2006; Corsetti, 2013; De Vuyst et al., 2014; Decock & Cappelle, 2005; Hammes et al., 2005; Huys et al., 2013). The second very important factor which affects the sourdough fermentation is the temperature at which it is fermented (Vrancken, Rimaux, et al., 2011). This directly affects the volatile profile of the bread produced (Katina et al., 2006; Pico et al., 2015), the fermentation quotient (ratio of lactic acid: acetic acid – since higher temperature causes a shift towards higher lactic acid production, thus enhancing the dough acidification) (Brandt et al., 2004; Corsetti, 2013) and it also affects the microbial community dynamic and associated metabolite production kinetics (facultative heterofermentative or homofermentative) (Katina et al., 2006; Vogelmann & Hertel, 2011; Vrancken, Rimaux, et al., 2011). The third factor which affects the sourdough is the ratio between flour and water used (dough yield and water activity) and the pH of the dough (dough acidification) (Vogelmann & Hertel, 2011). Water activity and osmotic strength of the sourdough along with the salt and/or sugar influence the growth of LAB and yeast in the dough (Minervini et al., 2014; Paramithiotis et al., 2010). Elevated osmotic pressure or low values of water activity (also because of a low DY) has more influence on LAB than yeasts (Venturi et al., 2012). Additionally, factors such as redox potential, oxygen tension and stirring of the sourdough also influence the dough (Decamps, Joye, De Vos, Courtin, & Delcour, 2016). Oxygen gets introduced to the dough matrix while mixing the dough, and which gets consumed due to microbial and enzymatic activities during the resting time of the sourdough. Redox potential could exert significant stress on the LAB species, depending on whether homo- and/or hetero-fermentative are present, which directly impacts the volatile profile of the dough due to the microbial and enzymatic activity in the sourdough (Capuani et al., 2014; Vermeulen, Kretzer, Machalitza, Vogel, & Ganzle, 2006).

Thus, the objective of this chapter was to understand the microbial and physio-chemical properties of the sourdough prepared with a fermented mixture of coconut water kefir (48 h fermented mixture of coconut water kefir supplemented with 12 g/L of sucrose (Set 3, Table 3.3, Chapter 1). This sourdough was categorised as a laboratory type 2 sourdough since the sourdough was prepared in a laboratory with the addition of starter culture/inoculum. The sourdough was incubated at different temperatures (4°C, 22°C and 30°C) to understand the effect of temperature on the fermentation of sourdough.

The physico-chemical changes of this sourdough were studied over time. Determination of microbial counts, acidification in terms of production of carboxylic acids and lowering of pH, determination of the concentration of D-/L- lactic acid, dough leavening, colour, texture, rheology and total titratable acidity was also carried out to understand the fermentation of the sourdough with time.

To understand the fermentation in the sourdough, microbial cell counts were determined over the period of 96 h using MRS, ME and AAB agar plates, for all the three temperatures. The importance of determining the microbial cell counts is to understand the effect of temperature on the microbial cells in the sourdough. Some of the microorganisms would be able to grow well at 22°C and the others may grow well at 30°C. The influence of temperature on sourdough microorganisms has been studied previously by Meroth, Walter, Hertel, Brandt, & Hammes (2003), who reported the influence of the dominating microbial species in sourdough fermentation on the taste and sensory perception of the final sourdough bread. The effects of sourdough fermentation on bread quality result from the metabolic properties of the sourdough microflora, the activity of enzymes of the flour, and, indirectly, the effects of acidification on the solubility of flour constituents and enzyme activities (Thiele, Gänzle, & Vogel, 2002).

4.2. Materials and Methods

4.2.1 Preparation of sourdough using fermented coconut water kefir

Starter cultures: Body Ecology (Body Ecology™, New Zealand) kefir powder at 1.5 g was used to ferment 300 ml of coconut water (UFC© Coconut water, manufactured by Universal Food Public Company Limited, Thailand was purchased at Countdown, Auckland City). This was supplemented with different concentrations of glucose and sucrose. The coconut water kefir cultures (CWK) were incubated at 30°C for 48 h in a LabServ incubator (Thermo Fisher Scientific, New Zealand) until high counts of microorganisms were obtained as observed in Chapter 3, Section 3.3.2.

Sourdough preparation:

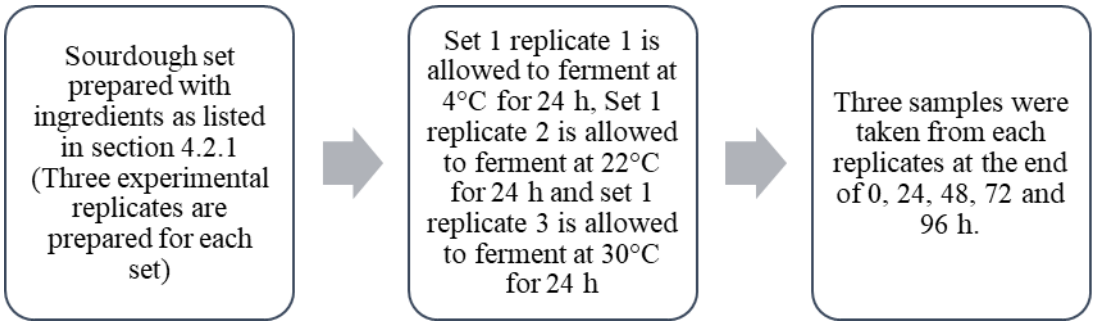
Different formulations of experimental sourdough were produced with varying sugars and sugar concentrations as shown in Table 4.1. Three hundred (300) ml of 48 h fermented CWK was mixed with 600 g of high-grade white flour (Home brand, Countdown, Auckland), 3.0 g salt (Countdown, Auckland) and 1.5 ml of canola oil (Countdown, Auckland).

Table 4.1: Formulations of sourdough sets.

Sourdough sets	Coconut water (ml)	Kefir grains (g/L)	Glucose (g/L)	Sucrose (g/L)	Flour (g)	Salt (g)	Oil (ml)
Set 1	300	1.5	0	0	600	3	1.5
Set 2	300	1.5	0	6	600	3	1.5
Set 3	300	1.5	0	12	600	3	1.5
Set 4	300	1.5	6	0	600	3	1.5
Set 5	300	1.5	6	6	600	3	1.5
Set 6	300	1.5	6	12	600	3	1.5
Set 7	300	1.5	12	0	600	3	1.5
Set 8	300	1.5	12	6	600	3	1.5
Set 9	300	1.5	12	12	600	3	1.5

The flour was placed into a stainless steel mixing bowl (5L capacity). Salt was then added into the flour and mixed for 1 min at speed 1 using an electronic mixer (Kenwood, New Zealand). Then, kefir-fermented coconut water was added into the flour mixture by mixing at a speed 1 for 5 min until a homogenous dough was obtained.

All the individual sets of dough prepared from their respective CWK fermentum were labelled accordingly (Set 1 to Set 9). These sets were prepared in triplicates and were then allowed to ferment at 4°C, 22°C and 30°C between 0-96 h. Samples were taken from each dough set every 24 h until the end of 96 h of fermentation.



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4.2.2 Sourdough incubation conditions

Sourdoughs were prepared using the recipe as shown in Table 4.1 and were each incubated for 24 h at different temperatures, viz., 4°C, 22°C and 30°C in LabServ incubators (Thermo Fisher Scientific, New Zealand).

4.2.3 Determination of microbial cell counts

Cell counts were performed for all the samples on De Man, Rogosa and Sharpe medium (MRS), Malt Extract (ME) and acetic acid bacteria agar (Difco, New Zealand). All the media were prepared as per the manufacturer's instructions and were autoclaved at 121°C for 15 min. One g of dough was diluted in 9 ml of peptone water and was mixed using a rotor to mix the contents uniformly. One ml of this mixture (1gm dough: 9 ml peptone water) was then transferred to a fresh tube containing 9 ml peptone water to make another 10 fold dilution. Dilutions up to 10^{-4} were made and were spread plated on each of the agar media. A spread plate technique was carried out by spreading a 100 µL of the culture uniformly across the agar surface, using a sterile glass spreader (Sanders, 2012). All the spread plates on the different media were incubated at 30°C in a 5% CO2 incubator (LabServ, Thermo Fisher, New Zealand) until the colonies were visible after 72 to 96h. The colonies were counted after which the colony forming units (CFU/ml) were calculated, where CFU/mL = (no. of colonies x dilution factor)/volume of sample plated.

4.2.4 Measurement of pH

To measure the pH of dough samples, 10 g of sourdough sample was added to 100 ml distilled water (Katina et al., 2006). This mixture was then blended. The reading for each dough mixture was taken using a digital pH meter (Eutech pH 700 meter, Thermo Fisher Scientific Inc, New Zealand) with a glass electrode (Electrode ECFC7252101B, Thermo Fisher Scientific Inc, New Zealand). Before measurement, the pH meter was calibrated with buffers (Thermo Fisher Scientific Inc, New Zealand) at pH 4.0 and 7.0. The pH measurement was performed in triplicate for each sample (at 0, 24, 48, 72 and 96 h).

4.2.5 Texture analysis of formulated sourdough

Texture Profile Analysis (TPA) was performed to determine sourdough texture in terms of hardness, springiness, cohesiveness, chewiness, gumminess and resilience. Each sourdough sample was cut using a knife to obtain a cube (2cm x 2cm x 2cm). Analysis was performed using a Stable Micro Systems TA.XTplus Texture Analyser equipped with a Film Support Rig (HDP/FSR) on a Heavy Duty Platform (HDP/90) with a 5 mm stainless steel probe (P/55) and a 5 kg load cell. The texture analyser was set to measure force in the compression mode with a pre-test speed of 2.0 mm/s, a test speed of 1.0 mm/s, and a post-test speed of 10.0 mm/s time 30s; load cell: 50 Kg; trigger force: auto-10g. Target mode was set to distance set at 5 mm. Data acquisition rate was set at 500 pps. Significant differences between texture readings of the different samples were tested using Two-way ANOVA on XLSTATS with Tukey's post hoc comparison tests, where $p < 0.05$ ($\alpha = 0.05$) indicates significant differences between means.

Table 4.2: Textural parameters used for TPA and their definition (Abdelghafor et al., 2011)

Parameters	Definition
Hardness	The force needed to compress the objects using a given amount. The peak force during the first compression cycle, expressed in 1b or N
Springiness	The elasticity of materials that can be recovered when the compressive force is removed. The time duration between the end of the first bite and the start of the second bite. It is calculated by the time duration of second compression by the time duration of first compression
Cohesiveness	The strength of the internal bonds in the sample. It is defined as the ratio of the positive force area during the second compression to that during the first compression.
Gumminess	The energy needed to break down a semi-solid food ready for swallowing. It is calculated by hardness multipled by cohesiveness.
Chewiness	The energy needed to chew a solid food into a state for swallowing. It is calculated by gumminess multiplied by springiness.
Resilience	The ability required to return to the original position after being compressed. It is calculated by the software (TA.XTplus, NZ)

4.2.6 Determination of D-/L- lactic acid content

The concentrations of D and L-lactic acids were determined using Megazyme lactic acid kit K-DLATE01/14 (Megazyme, New Zealand), following the manufacturer's instructions.

4.2.7 Measurement of leavening

Sourdough weighing 20g (equivalent to 20ml) was prepared in a 100ml volumetric cylinder. They were placed in a proofing chamber with 60% humidity for a period of 96 h at 22°C and 30°C. Dough rise was measured regularly 24 h interval for a total time period of 96 h. Maximum leavening rate (ml/h) at 22°C and 30°C, was calculated from the highest volume reached during the incubation period, divided by the time at which that volume was first observed (Caballero et al., 1995).

4.2.8 Determination of dough colour

A Hunter lab (45/0, Colourflex) chroma meter was used to measure the colour of the sourdough and samples and the analysis was done according to Whale, Singh, Behboudian, Janes, and Dhaliwal (2008) (Whale, Singh, Behboudian, Janes, & Dhaliwal, 2008). The dough was cut in a small wedge and was placed in a Petri dish, which was covered and the lens of the Chroma meter placed on top. The colour was measured from 3 different positions. Before measuring, the Chroma meter was calibrated with a white tile and checked for recalibration in between measurements. Colour values were recorded as L* (0 = black, 100 = white), a* (-a* = greenness and +a* = redness), and b* (-b* = blueness, +b* = yellowness). The parameter, cylindrical coordinate C* (chroma) was calculated using the below formula (Minolta, 1994). The samples were prepared in triplicate for the analysis.

$$C^* = (a^2 + b^2)\ ½$$

4.2.9 Analysis of dough viscosity

A Brookfield RST Rheometer, version 1.5.4, 2015 was used for all the dough rheological measurements. The probe VT-40-20, S/N 3600137 was inserted inside the sample for rheology measurements. The dough viscosity was measured as Pascal*seconds (Pa.s), shear stress in Pascals (Pa) and shear rate as $second^{-1}$ (1/s). The sample was placed in a cylinder and was enclosed using a lid, with enough space only to allow the probe to enter the sample. Tests were performed at a constant temperature of 30°C. A rest period of 10 min was allowed to enable the sample to recover from the stresses induced by preparation (Brookfield, 1996). The samples were prepared in triplicates (Moore, Bello, & Arendt, 2008).

4.2.10 Determination of total titratable acidity

Total titratable acidity (TTA) of the dough was determined by the AACC International Method 02-31.01 (AACC, 1999). A 10g sample of coconut water kefir sourdough samples was dissolved in 100 ml deionised water and then titrated with 0.1M NaOH (pH 8.5).

4.2.11 Determination of carboxylic acids by Liquid Chromatography-Mass Spectrometry (LC-MS)

The dough samples from each set incubated at 30°C were taken in triplicate at times, T=0, 24, 48, 72 and 96 h. In order to extract the organic acids present in the dough samples, a 50 mg sample of dough was mixed with 1ml of autoclaved deionised water in a stomacher bag. The mixture was homogenised in a Stomacher 400 Circulator (Thermofisher, Australia) to obtain a liquid slurry of the dough.

The method for determination of carboxylic acids in CWK fermented sourdough using LCMS was the same as that in Chapter 3 for carboxylic acid analysis (section 3.2.11).

4.2.12 Determination of fermentation quotient

The fermentation quotient was calculated as a molar ratio between lactic acid and acetic acid (Galli et al., 2019). The determination of lactic and acetic acid from the dough was carried out using the LC-MS method as mentioned in section 4.2.11, materials and method section in this Chapter.

4.2.13 Statistical analysis

At least five individual replicates of each of the sourdough sets were prepared for statistical analysis. All the results are expressed by means of values ± standard deviation of three separate determinations. One-way and two- way analysis of variance (ANOVA) were applied to determine the level of significance and when the ANOVA was significant, means were separated by pairwise comparison using Tukey's (HSD) test or Fisher (LSD) test at 5% significance level. All statistical analyses were performed using the Statistical Analysis System – XLSTAT-MX version 2012.4.02 (Addinsoft, New York, NY, USA).

Canonical Variate Analysis (CVA) was performed on all the data using XLSTAT (version 2018.5) (Addinsoft, USA). CVA minimizes residual variability and maximizes the distances between samples (Delarue & Sieffermann, 2004). Additionally, Multivariate Analysis of Variance (MANOVA) tests were used to determine if significant differences existed between the factors (α =.05).

CVA analysis was used for this study as it marks each experimental set and highlights it on the graph rather than just taking the mean of all the sourdough sets. CVA was also used as it computes factorial axis describing the effect of each product/experimental data set. CVA axes maximize the distances between product means while minimizing the residual variability. This analysis is equivalent to a PCA on mean product scores, with a specific descriptor weighting: the inverse of the residual covariance matrix (Hand & Taylor, 1987; Jobson, 2012; Monrozier & Danzart, 2001; Tomassone, Danzart, Daudin, & Masson, 1988).

4.3. Results and Discussion

4.3.1 Cell counts from the coconut water kefir sourdough

Coconut water kefir (with and without the addition of varying concentrations of glucose and sucrose) was used to ferment the dough to produce a sourdough, which was incubated at two different temperatures, 22°C and 30°C. Microbial counts were carried out for all coconut water kefir fermented dough sets (incubated at both 22°C and 30°C) at times, T= 0, 24, 48, 72 and 96 h. All the samples were evaluated in triplicates.

Appendix 7 shows the cell counts of LAB, yeast and AAB on MRS, ME and AAB agar, respectively. The cell counts were an average of three replicate plates for each type of agar. The cell counts for LAB varied from 3.07 ± 0.06 (at 0 h, 30°C) - 13.82 ± 0.92 (at 96 h, 30°C) log CFU/ml.

The log CFU/ml for yeasts obtained was 2.75 ± 0.05 (at 0 h, 4°C) - 11.38 ± 0.07 (at 96 h, 30°C). The counts for AAB ranged between 1 ± 0.03 (at 48 h, 4°C) - 3.22 ± 0.08 (at 96 h, 30°C). Overall, it can be observed that set 3 supplemented with sucrose at 12g/L had significantly highest cell count at 96 h at 30°C for LAB, yeasts and AAB with the values of 13.82 ± 0.92, 11.38 ± 0.07 and 3.22 ± 0.08, respectively ($p<0.05$). Overall, all the sets incubated at 30°C produced higher cell counts than those incubated at 22°C. These results are supported by CVA analysis (Figure 4.1).

Therefore, CVA analysis was used to analyse the results of cell counts of each experimental set of CWK fermented sourdough at the different temperature (22°C and 30°C) and different time points (between 0 – 96 h) rather than just the mean cell counts of all the sourdough set(s).

CVA was used to summarize the results of cell counts for LAB, yeast and AAB after incubation for 0, 24, 48, 72 and 96 h at 22°C and 30°C. Figure 4.1 describes the canonical variates where sample discrimination was explained by 99.93% (sum of both the factors, F1 (dimension one – explained 97.02%) and F2 (dimension 2 – explained 2.91%)). Hotelling-Lawley Multivariate Analysis of Variance (MANOVA) showed significant differences $F(267,502) = 903.04$, $p < 0.0001$) for both time and temperature. Factor 1 (F1) explained 97.02%, of the variance, separating the coconut kefir fermented dough sets in terms of time (0 h to 96 h) and temperature (22°C and 30°C) of incubation. All the sets for time 0 and 24 h of incubation had high negative scores and with increase in incubation time along F1 had increasingly positive scores for samples incubated at 48, 72 and 96 h. In addition, samples incubated at 30°C had more positive scores for all corresponding sets at 48, 72 and 96 h at 22°C.

Overall from Figure 4.1, at a given time point, there was more cell growth in all the sets incubated at 30°C compared to all the sets incubated at 22°C after the same incubation period. It can be observed that all the sets incubated at 48, 72 and 96 h at 30°C have significantly higher cell counts compared to those in all the sets incubated at 22°C. Significantly more cells were produced only after 48h incubation and not at 0 or 24h. There were lower cell counts at 22°C compared to those at 30°C.

CVA loadings plot of cell counts for LAB on MRS, yeasts on ME and AAB on AAB agar and the Hotelling-Lawley MANOVA test showed significant cell count differences $F(267,502) = 903.04$, $p < 0.0001$) based on the type of growth (LAB, yeast or AAB). These loadings on the CVA loading plot show both incubation time and incubation temperature affects the cell count of LAB, yeast and AAB.

Interestingly, from Figure 4.2**,** all the sets incubated at 30°C had correspondingly higher loading along F1. The higher loadings corresponded to higher cell concentrations of LAB, AAB and yeast. All the sets incubated at 30°C showed high cell counts compared to 22°C for times 48, 72 and 96 h.

As expected with shorter incubation times (24h), little growth of LAB, yeast and AAB was observed for all the dough sets incubated at both 22°C and 30°C. With time, greater growth was observed for all the sets incubated at 30°C compared to all the sets incubated at 22°C.

Figure 4.1: Canonical Variate Analysis bi-plot of cell counts from dough fermented with coconut water kefir for 0, 24, 48, 72 and 96 h incubated at 22°C and 30°C. Hotelling-Lawley MANOVA test showed significant cell count differences based on the time and temperature of fermentation. This bi-plot represents the centroid variables of each environment which show correlation between the two factors on the factor axes.

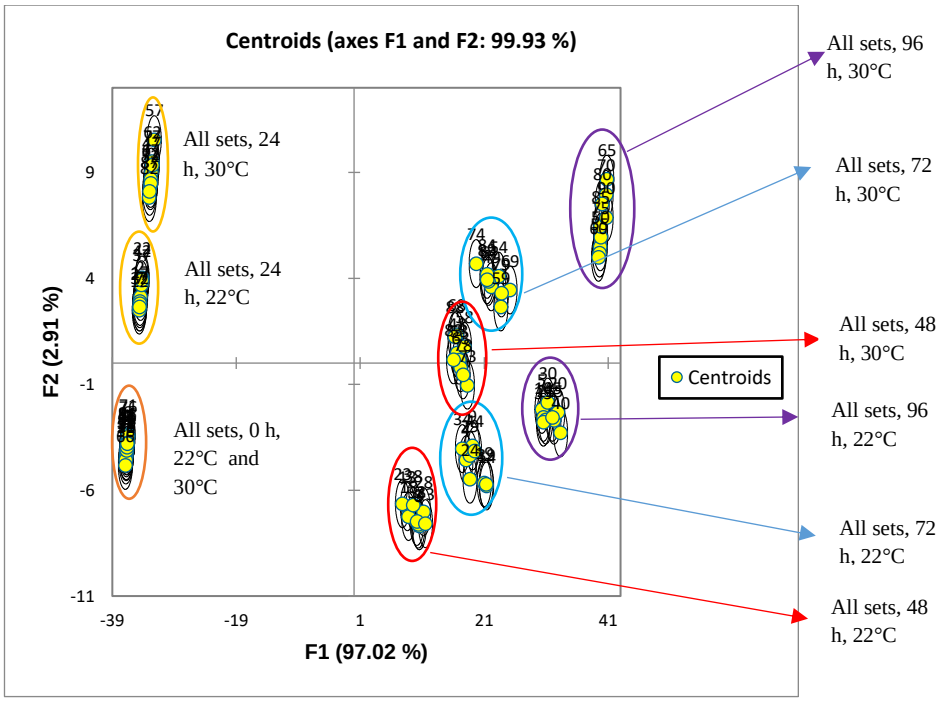
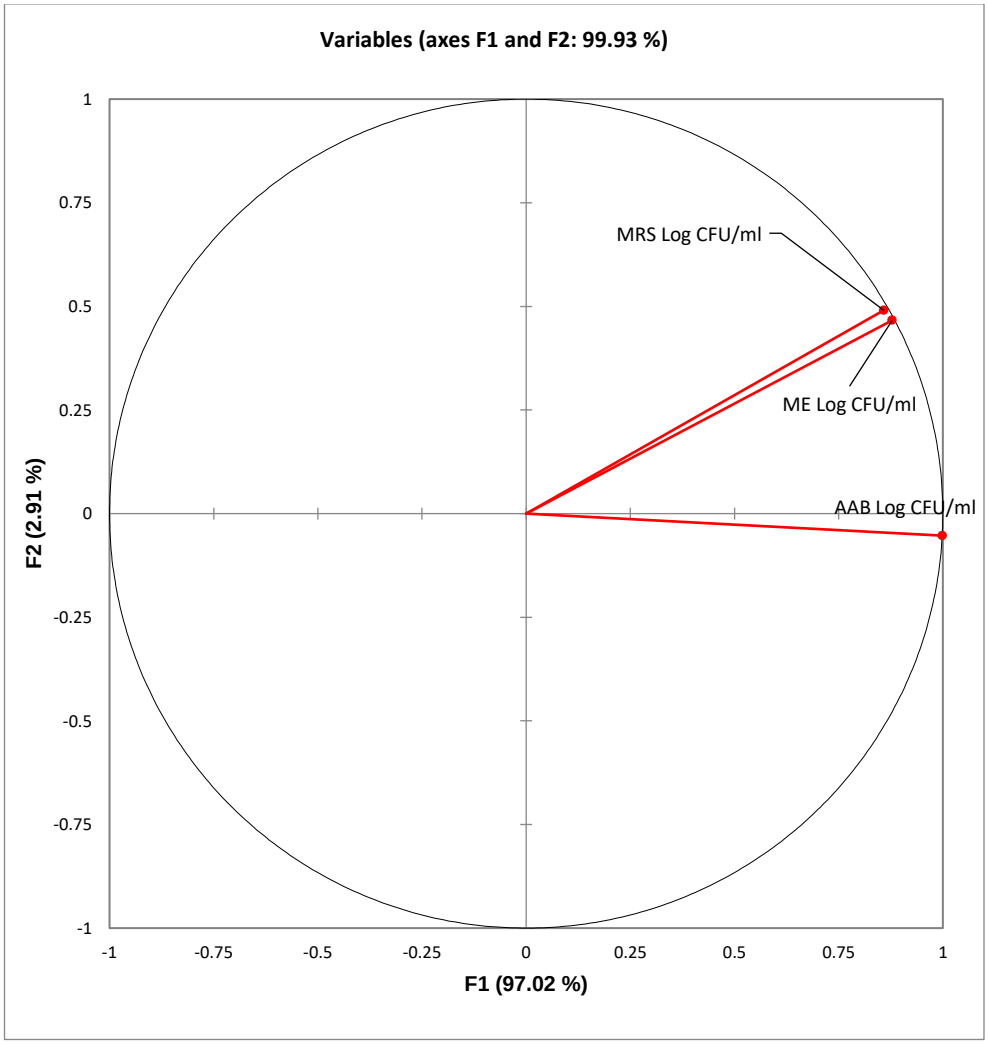


Figure 4.2: Canonical Variate Analysis loadings plot of cell counts for LAB on MRS, yeasts on ME and AAB on AAB agar. Hotelling-Lawley MANOVA test showed significant cell count differences F (267,502) = 903.04, p < 0.0001) based on the growth of LAB, yeast and AAB. This loadings plot shows the variables of each LAB, yeast and AAB cell concentration which are correlated with the two factors on the factor axes.



Prior studies carried out by Meroth, Walter, Hertel, Brandt, & Hammes (2003) described the importance of temperature on the growth of yeast and LAB in sourdough at different temperatures such as 25°C, 30°C and 40°C. The use of genotypic (randomly amplified polymorphic DNA [RAPD] and specific PCR) identification techniques have revealed that the dominating strains of LAB and yeast differed, suggesting that within those species, adaptations to different temperatures exist. This can support the results obtained for this study at 22°C and 30°C. In addition, growth of AAB and yeast was reported to be supported at a lower temperature range of 26°C to 28°C, whereas LAB grew best between 30°C and 35°C

(Francieli Begnini Siepmann, Sousa de Almeida, Waszczynskyj, & Spier, 2019). These results support our finding that significantly higher growth at 30°C ($p<0.05$), based on the ANOVA results (Appendix 7). As reported by Gänzle, Ehmann, & Hammes, (1998) and Vogelmann & Hertel, (2011), LAB dominated in sourdough fermented for a long incubation time at a temperature of 30°C, which support the above results. A mixture of acetic acid and lactic acid and/or ethanol was produced. Certain yeast species like *S. cerevisiae* and *C. humilis* grew faster at 30°C. Additionally, Meroth, Walter, Hertel, Brandt, & Hammes, (2003) reported that type 2 sourdough microorganisms grew the best at 30°C which helped stabilise the presence of certain LAB species like *Lactobacillus crispatus, L. fermentum*, and *L. pontis.*

Lattanzi et al. (2013) reported the cell count for LAB in sourdough as 9 log CFU/g and 8 log CFU/g for yeast, whereas, Rocha and Malcata (2012) reported 10.11 ± 0.05 CFU/g of LAB and 8.84 ± 0.88 CFU/g of yeast in sourdough, which corresponds to the results obtained in this study obtained for LAB.

The cell counts obtained for LAB (13.82 ± 0.92 log CFU/ml significantly highest for set 3 at 96 h) and yeast (11.38 ± 0.07 log CFU/ml significantly highest for set 3 at 96 h) for sourdough in this study were higher than that reported in the literature due to a few reasons such as the origin of the flour could make a difference (Ercolini et al., 2013, Weckx et al., 2010). The concentration and type of nutrients present in the flour, such as carbohydrates, may affect the cell densities of sourdough (Minervini et al., 2012). The role of the type of flour in the process leading to the formation of the microbial structure of the sourdough deserves further studies. Also, the external factors like changes in temperature could impact the cell counts of LAB (Vogelmann and Hertel, 2011, Vrancken et al., 2011).

Overall, the cell density for LAB, yeasts and acetic acid bacteria in the fermented sourdough which were 13.82 ± 0.92, 11.38 ± 0.07 and 3.22 ± 0.08 log CFU/ml respectively, are higher than those obtained for fermented coconut water kefir (results as shown in Chapter 3, Table 3.4, Table 3.5 and Table 3.6). The cell counts from fermented CWK after 96 h were 5.72 ± 0.04 for LAB, 4.98 ± 0.03 for yeasts and 2.32 ± 0.01 for AAB. Since the temperature of fermentation was the same for CWK and for sourdough, the higher cell densities in sourdough might be best attributed to higher concentrations of nutrients available in the formulated sourdoughs than those in coconut water.

4.3.2 Determination of pH, D- lactic acid, L-lactic acid and total titratable acidity in coconut water kefir sourdough

Canonical variate analysis was used to summarize the pH, D- lactic acid, L-Lactic acid and total titratable acidity (TTA) of the coconut water kefir fermented sourdough at 0, 24, 48, 72 and 96 h incubation at 22°C and 30°C.

Figure 4.3 describes the canonical variates where sample discrimination was explained by 99.53%. Hotelling-Lawley Multivariate Analysis of Variance (MANOVA) showed significant differences $F(356, 653) = 302.152$, $p < 0.001$) for both time and temperature of the CWK sourdough fermentation. Factor 1 (F1) separates the coconut kefir fermented dough sets in terms of time and temperature of incubation for pH, D-lactic acid, L-lactic acid and TTA data, explaining 98.76% of the variance along the x-axis.

Overall, a pattern can be identified with all the sets at 96, 72 and 48 h incubation at 30°C having a higher positive score along F1. This result corresponds to higher positive loadings (cell counts) in Figure 4.2. Sourdoughs prepared at 22°C for 0 and 24 h demonstrated high pH values (between 5.71 ± 0.08 and 5.31 ± 0.08 respectively, $p<0.05$), as shown by negative loadings along F1.

According to Figure 4.3, higher acid concentrations, lower pH values and high TTA concentrations were observed at 30°C.

Higher concentrations of D-lactic acid, L-lactic acid and TTA were obtained after 48 h (2.08 ± 0.04, 6.65 ± 0.06 and 8.9 ± 0.06 g/L respectively, $p<0.05$), 72 h (2.61 ± 0.01, 7.56 ± 0.36 and 10.55 ± 0.11g/L respectively, $p<0.05$) and 96 h (3.71 ± 0.07, 10.69 ± 0.36 and 12.81 ± 0.15 g/L respectively, $p<0.05$) in all the sets as shown by the high positive scores and high positive loadings along F1. Interestingly, all the sets incubated at 30°C have higher values than all the sets incubated at 22°C for the same length of incubation. In Figure 4.3, it can be observed that the increase at 30°C was higher for D-lactic acid, L-lactic acid and TTA compared to that at 22°C after 24 h.

Figure 4.3: Canonical Variate Analysis bi-plot for pH, D- lactic acid, L-lactic acid and total titratable acidity from dough fermented with coconut water kefir incubated for 0, 24, 48, 72 and 96 h at 22°C and 30°C. Hotelling-Lawley MANOVA test showed significant differences among the sets, based on the time and temperature of fermentation. This bi-plot represents the centroid variables of each environment which are correlated with the two factors on the factor axes.

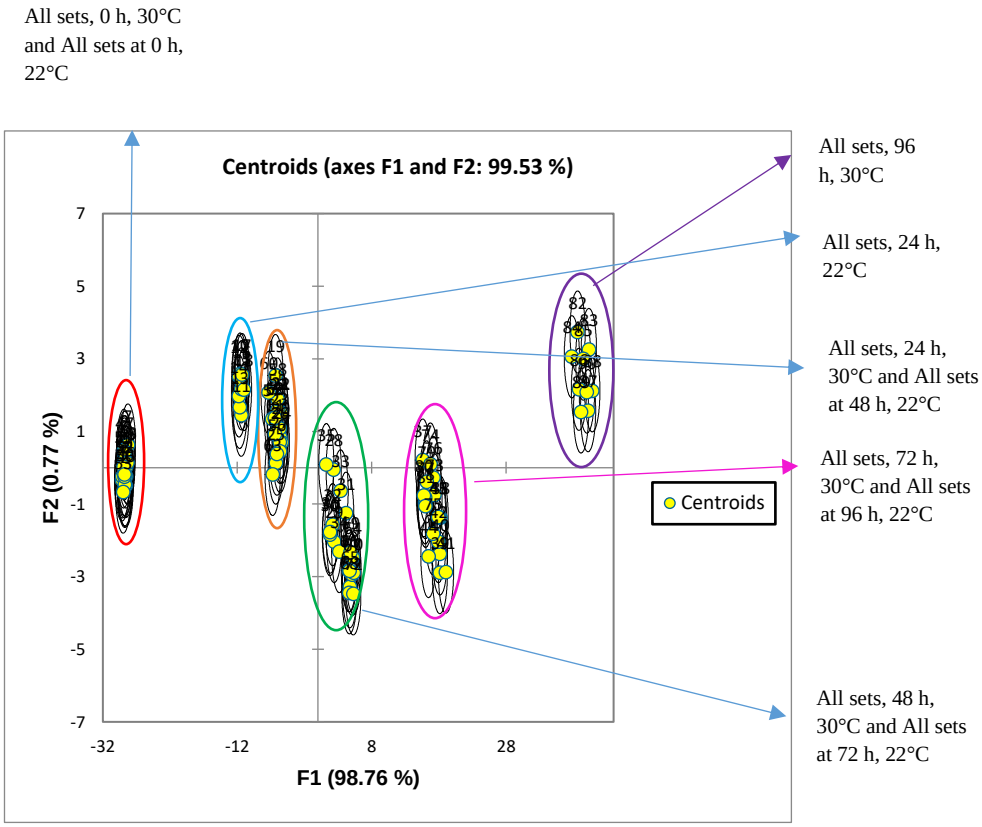
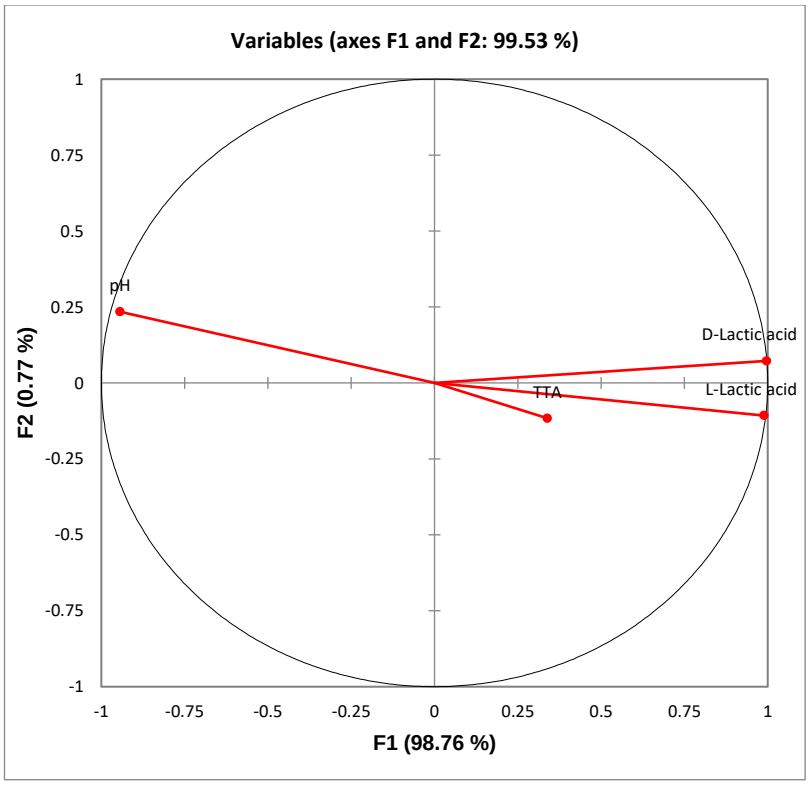


Figure 4.4: Canonical Variate Analysis loadings plot for pH, D- Lactic acid, L-Lactic acid and total titratable acidity. Hotelling-Lawley MANOVA test showed significant differences F (356, 653) = 302.152, $p < 0.001$). This loadings plot shows all the above variables which are correlated with the two factors on the factor axes.



The results of this study are consistent with several other studies, where the parameters which influence the dough acidification such as pH, TTA and D-/L-Lactic acid, have been assessed during fermentation. Low-pH values are suitable for the growth of acid-tolerant lactobacilli, whereas higher values select for species of *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Weissella*. The low pH values in the dough meant that there has been acidification of the dough due to acetic acid and lactic acid production by acid-tolerant LAB species. Therefore, high D-/L-lactic acid content is found in the dough at the end of the fermentation period resulting in low pH and high TTA values. (Aponte et al., 2014; Corsetti & Settanni, 2007; Minervini, Di Cagno, et al., 2012; Robert, Gabriel, & Fontagné-Faucher, 2009; Vrancken, Rimaux, Weckx, Leroy, & De Vuyst, 2011; Zotta, Piraino, Parente, Salzano, & Ricciardi, 2008).

4.3.3 Determination of leavening and colour in coconut water kefir sourdough

Figure 4.5 describes the canonical variates where sample discrimination was explained by 99.66% of the variation. Hotelling-Lawley Multivariate Analysis of Variance –MANOVA showed significant differences $F(356, 653) = 2941.71$, $p < 0.001$) for the effect of both time and temperature on the CWK sourdough fermentation. Factor 1 (F1 on the x-axis) separates the coconut kefir fermented dough sets in terms of time and temperature of incubation for leavening and colour, explaining 99.06% of the variance along the x-axis. This means that both, the dough rise (leavening) and colour were affected by the time and temperature of incubation.

All sets incubated at 96 h have the highest positive score (high values for colour and leavening) along F1 for incubation at 30°C, whereas all the sets at 0 and 24 h have high negative scores (low values for colour and leavening) along F1 for 30°C and 22°C.

The positive scores from the loadings plot (Figure 4.6) for the sets at 48, 72 and 96 h incubated at 22°C and 30°C correspond to the positive loadings for leavening, colour (factor 'l' and factor 'b'), which means that higher leavening and colour values were obtained for all the sets incubated at 30°C for 48, 72 and 96 h. There was a general increase in leavening and colour as shown along F1. At 30°C, there are high positive loadings for both leavening and colour variables compared to those at 22°C accompanying an increase fermentation time. This was observed at 24, 48, 72 and 96 h. This means that the leavening and colour had significantly higher values at 30°C compared to all the sets incubated at 22°C for all 24, 48, 72 and 96 h. Furthermore, the highest positive loading corresponding to the higher colour and leavening values was obtained from all the sets incubated at 30°C after 96 h of fermentation.

Interestingly, an overlap can be observed in Figure 4.5, where all the CWK sourdough sets at 48 h at 30°C were 24 h faster than all the sets at 22°C. The increase at 30°C at all incubation times was higher for leavening and colour compared to those at 22°C after 24 h. The results of these findings are consistent with the literature on sourdough leavening and were due to the fermentation activity of the microorganisms in the bread. Leavening of the sourdough is a result of carbon dioxide production by fermentation activities of LAB, yeast and AAB (Neumann, Stephan, & Brümmer, 2006). Leavening is associated with the production of carbon dioxide in the dough is stabilised in the aqueous dough, which leads to expansion of dough volume or mass. Upon saturation, the gas is (entrapped in the form of bubbles) within the dough gradually dilate and expand the dough. The leavened dough becomes foam-like, consisting of a semi-solid aqueous phase where gas bubbles are distributed (Dobraszczyk, Campbell, & Gan, 2001; Gan, Ellis, & Schofield, 1995; MacRitchie, 1976).

All the samples incubated at 30°C after 24 h have high positive loadings for colour and leavening. In the scope of instrumental analysis, the greenness/redness of the surface correlates with a* value, while the white/black correlates to l* value and blueness/yellowness relates with b* value. Therefore, significant higher loadings from the loadings plot for surface whiteness and yellowness (l* and b*) can be associated with the sourdough during leavening which provided good surface brightness. All the sets incubated at 0 and 24 h, and all the sets incubated at 22°C at 48 and 72 h have a darker dough surface, with l* values between 41.6 ± 0.4 - 44.8 ± 0.8.

The change in colour could be associated with the high leavening capacity of the dough, which may be due to the increase in the cell size causing a variation in the pattern of light reflection (Tlapale-Valdivia et al., 2010). As for colour, the changes in these parameters (L*, a* and b*) are the best indicators of changes in the appearance of the sourdough during fermentation. For most studies on breads, l* and b* had negligible change in colour with dough fermentation, which is different from the results obtained in this study. The results obtained for this study for high l* and b* values are consistent with the findings for similar surface brightness that have been reported for wheat dough bread affected by *L. brevis* and dry sourdough preferment (Komlenić et al., 2010). Lamsal & Faubion, (2009) have reported that the colour of the dough depends on the type of flour used and the enzymes present in the flour. In this study, a fine grade wheat flour was used, which may have contributed to the high whiteness and yellowness of the dough surface.

Figure 4.5: Canonical Variate Analysis bi-plot for leavening and colour from dough fermented with coconut water kefir at 0, 24, 48, 72 and 96 h incubated at 22°C and 30°C. Hotelling-Lawley MANOVA test showed significant differences among the sets, based on the time and temperature of fermentation. This bi-plot represents the centroid variables of each environment which are correlated with the two factors on the factor axes.

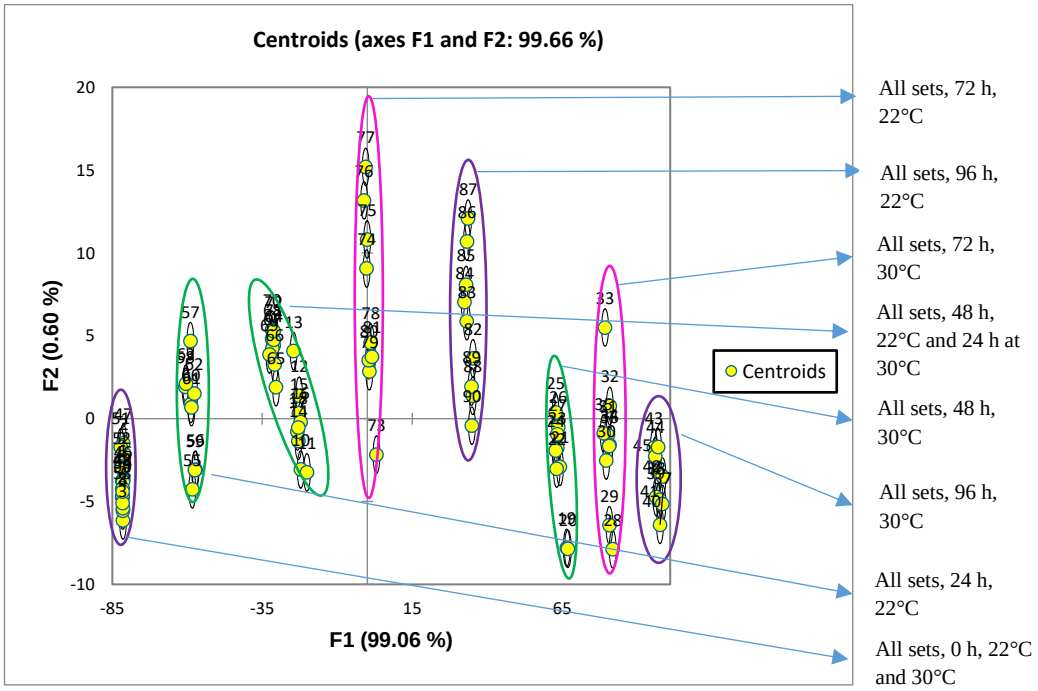
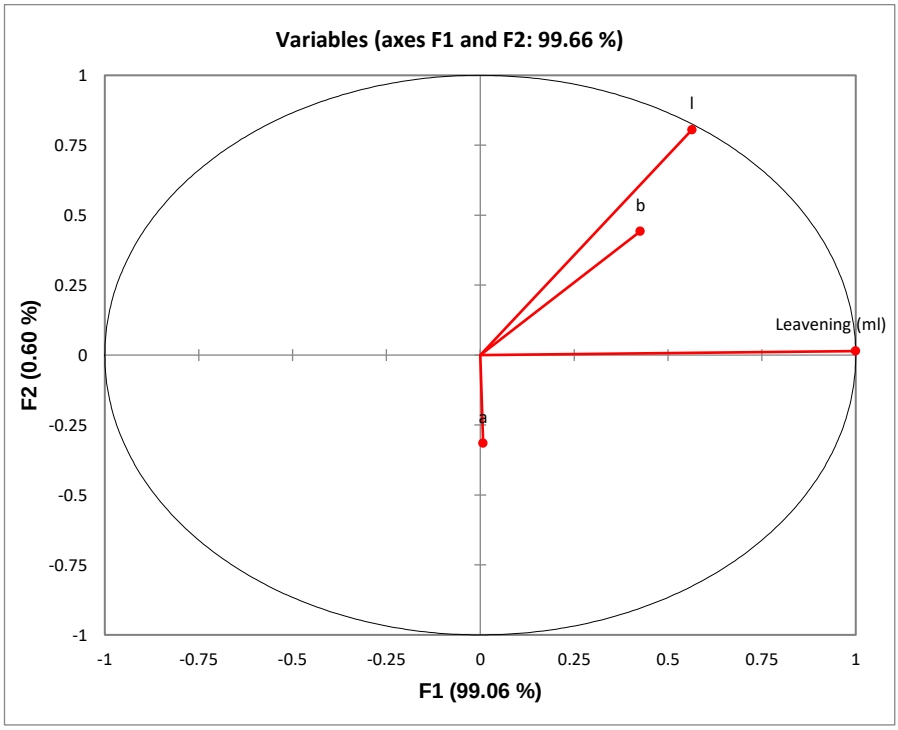


Figure 4.6: Canonical Variate Analysis loadings plot for colour and leavening for CWK fermented sourdough at 22°C and 30°C. Hotelling-Lawley MANOVA test showed significant differences ($F(356, 653) = 2941.71$, $p < 0.001$). This loadings plot shows all the above variables (leavening and colour variables: l*, a* and b*) which are correlated with the two factors (F1 and F2) on the factor axes.



4.3.4 Analysis of dough texture and viscosity

A two-way ANOVA analysis was carried out to analyse the change in dough viscosity and texture with time (0, 24, 48, 72 and 96 h) for all the CWK fermented dough sets (Table 3.3). Viscosity of the CWK fermented sourdoughs ranged from 605.52 pa.s to 1217.73 pa.s over 96 h of fermentation (Table 4.3). With increase in time, there was a significant gradual increase in the viscosity for all the dough sets incubated at both 22°C and 30°C ($p<0.05$). All the coconut water fermented sourdough sets had significantly high viscosity at the end of 96 h (1217.73 ± 1.16 pa.s), compared to the control dough sets (606.67 ± 6.82 pa.s). In addition, dough sets incubated at 30°C (1217.73 ± 1.16 pa.s) had significantly higher viscosity compared to sets incubated at 22°C (804.4 ± 4.4 pa.s).

The increase in dough viscosity may be due to increased hydration of flour macromolecules (or starch) by CWK and the production of carbon dioxide by the metabolic activities of the sourdough

microorganisms (Najafi, Pourfarzad, Zahedi, Ahmadian-Kouchaksaraie, & Khodaparast, 2016). As reported by Corsetti et al., (1998), fermented sourdough showed the highest viscosity when gas production is highest. These gas bubbles interrupt the elastic dough network and lead to a highly viscous dough.

Production of lactic acid and acetic acid by LAB could lead to increase in viscosity of the dough, with a consequent reduction in pH. Acidity increases the solubility of gluten particles and leads to their swelling which consequently leads to the exposure the hydrophobic section due to rise in intamolecular forces which causes the protein deployment. Proteolysis of a substantially large protein molecule generates a stable and less elastic mixture with higher extensibility. The rheological properties of the final dough depend on the amount of sourdough used to bring about leavening and on the expansion of gluten during fermentation (Clarke et al. 2004; Corsetti et al. 2000; Hoseney 1994; Takeda et al. 2001). Limited proteolysis allows greater retention of CO_2, precisely influencing the softness of the final product (Thiele et al. 2002).

Wehrle and Arendt, (1998) reported that the dough viscosity is affected by acidification and pH levels, which could explain the increase in the viscosity in this study. As reported by Wrigley and Bietz, (1988), solubility of some protein fractions are affected due to the low pH levels. Enzymatic breakdown is highly dependent on the changes in the pH of the surrounding medium. For example, depending on the variety of wheat and its status of germination, the carbohydrate degrading enzymes such as amylase, pentosanase and cellulase have a pH optima between 3.6-5.6 (Fox and Mulvihill 1982). Acidification could be used to inhibit the α-amylase activity. Depending on the substrate, the flour proteinases and peptidases are active at pH levels ranging between 4.0 – 9.0. This could explain the mechanism of change in the viscosity of the dough during fermentation in this study. Due to the low pH and high acidity, the activity of certain enzymes and protein which act on the flour, may have been inhibited, leading to increase in the viscosity of the dough.

The results for pH and TTA in this study are in accordance with the results explained in Section 4.3.2 (Appendix 8), which positively correlated with the results for this study for viscosity, as explained above. Results showed that there was a significant decrease in pH by almost 1.5 log unit (after 96 h of fermentation) for all the coconut water kefir fermented dough sets incubated at 22°C. A decrease of almost 2 log units for all the dough sets incubated at 30°C was observed. Significantly low pH was obtained for all the dough sets after 96 h of fermentation for both 22°C and 30°C. The lowest pH value was obtained for set 6 (3.52 ± 0.24) followed by set 5 (3.64 ± 0.3). Total titratable acidity was significantly the highest at the end of 96 h for all the coconut water kefir fermented doughs at both 22°C and 30°C. The significantly highest TTA was obtained from set 6 at 96 h (12.81 ± 0.1), followed by set 5 (12.6 ± 0.21) at 96 h. These results correspond

to a pH drop and acidification of the sourdough which in turn affect the CWK fermented sourdough's viscosity.

Texture was characterised based on hardness, resilience, cohesiveness, gumminess, springiness and chewiness. The PCA biplot score plot shown in Figure 4.7, described 73.49% and 12.97% of the total variation in Principal component 1 (F1) and Principal component 2 (F2) respectively. This separates all the different texture characteristics along x-axis, explain that all the sets incubated at 72 and 96 h have the highest values for all the texture characteristics like resilience, springiness, gumminess, chewiness, cohesion and hardness.

The scores for all the sets of sourdough fermented with coconut water kefir at different times (T=0, 24, 48, 72 and 96 h) at both 30°C and 22°C are clearly shown. All the fermented sourdough sets incubated at 30°C for 48 and 96 h had high positive loadings for cohesiveness (along F1), compared to the control sourdough sets incubated at 30°C (T= 0 h), which had very high negative loadings along F1. This is supported by the ANOVA results from Table 4.6, it can be observed that dough cohesiveness was significantly the high for all sets incubated for 24 h at 22°C. Dough cohesiveness was significantly the highest for sets 1 (control set) and 4 (supplemented with 6g/L of glucose) after 48 h of incubation, whereas for set 2, maximum cohesiveness was achieved at 72 h of incubation ($p<0.05$). Dough cohesiveness was significantly the highest for set 5 (supplemented with 6 g/L of glucose and sucrose) and set 9 supplemented with 12g/L of glucose and sucrose) incubated at 30°C at 96 h with the values of 0.71 ± 0.01 and 0.59 ± 0.26 respectively ($p<0.05$). For incubation at 22°C at 96 h, cohesiveness was significantly the highest in set 8 (supplemented with 12 g/L of glucose and 6 g/L of sucrose) and set 2 (supplemented with 6 g/L of sucrose) with 0.66 ± 0.1 and 0.55 ± 0.09 respectively ($p<0.05$).

Dough cohesiveness is an important predictive property for bread quality (Mnif, Besbes, Ellouze-Ghorbel, Ellouze-Chaabouni, & Ghribi, 2013). A study carried out on wheat dough functionality by Davidou et al. (1996) reported that doughs which are more cohesive, result in a higher specific volume and softer breads.

From the PCA plot, it can be observed that all the CWK fermented sourdough sets incubated at 30°C for 72 h had high positive loadings along F1 for hardness, resilience, chewiness, gumminess and springiness. These results are supported by the ANOVA results as shown in Table 4.4, Table 4.5, Table 4.6, Table 4.7, Table 4.8 and Table 4.9, which are discussed in detail below. Table 4.4 shows changes in dough hardness (N) with time, for all the dough sets incubated at 30°C and 22°C. For all dough sets, there was a gradual increase in hardness at both temperatures. Maximum hardness was observed for all dough sets at the end of 72 h of fermentation at 30°C, and at the end of 96 h of fermentation for all the sets incubated at 22°C. The highest significant value for

hardness of 20.34 ± 0.24 was obtained for set 1. This was followed by set 2 (20.21 ± 0.28) at 30°C after 72 h of incubation ($p<0.05$).

At 22°C, sets 6 and 2 had the highest hardness values of 20.85 ± 0.28 and 20.78 ± 0.34 respectively after 72 h of incubation ($p<0.05$). For all the sets incubated at 30°C, hardness values were significantly higher than all sets incubated at 22°C ($p<0.05$).

Maximum dough resilience for all the sets was obtained after 48 h for both 30°C and 22°C. The values were significantly higher than those at the start of dough fermentation (0 h) (Table 4.5). Additionally, with incubation at 22°C, sets 2 and 5 had the highest resilience values respectively of 11.18 ± 1.55 and 11.17 ± 1.14 at 48 h incubation time ($p<0.05$).

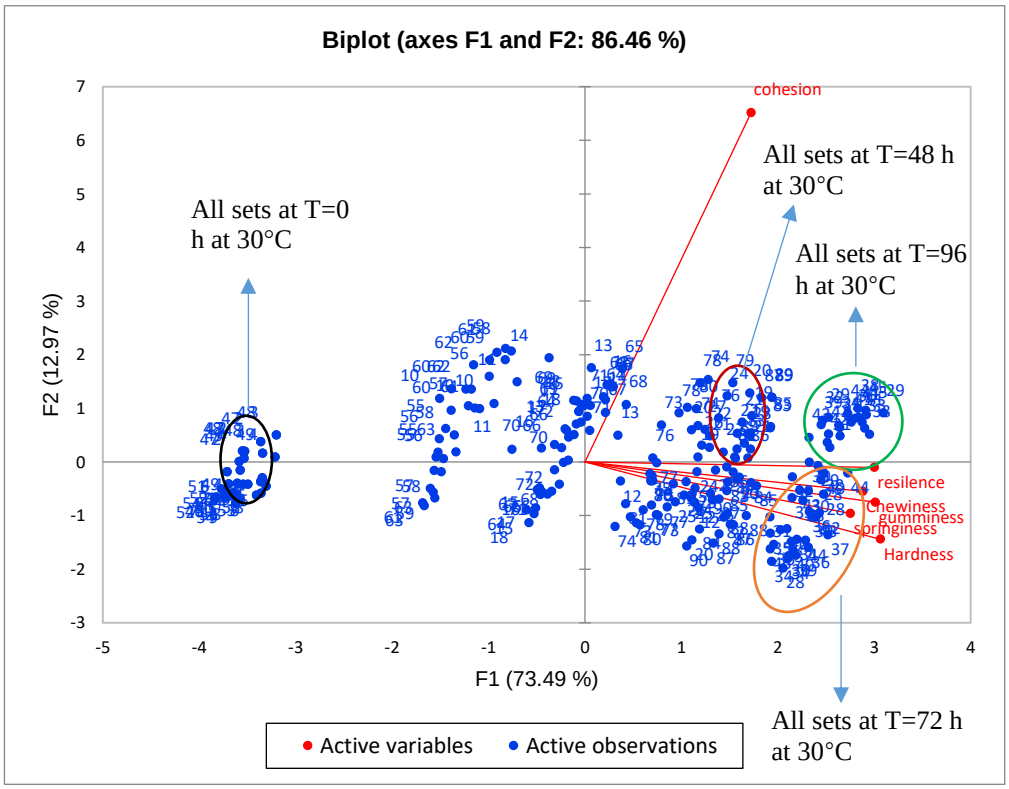
Dough gumminess was significantly the highest at 48 h for all sets incubated at 30°C (Table 4.7). On the other hand, gumminess was significantly the highest for all the sets at 24 h, incubated at 22°C. There was no significant increase in dough gumminess after 24 h, up until 96 h of incubation for all sets incubated at 22°C. For all the sets incubated at 30°C, there was no significant difference between incubation times of 48, 72 and 96 h. Dough gumminess was significantly the highest in sets 1, 2 and 8 incubated at 30°C at 96 h with the values of 7.85 ± 0.1, 7.78 ± 0.25 and 7.71 ± 0.34 respectively ($p<0.05$). For incubation at 22°C after 96 h of incubation, gumminess was significantly the highest set 4 and set 7 with 5.78 ± 0.01 and 5.75 ± 0.02 respectively ($p<0.05$).

For all the sets incubated at 22°C and 30°C, springiness (mm) was significantly the highest after 48 h of incubation, $p<0.05$ (Table 4.8). For all sets, there was no significant difference between the incubation times of 48, 72 and 96 h for both temperatures of 22°C and 30°C. Dough springiness was significantly the highest in sets 4 and 9 incubated at 30°C for 96 h with the values of 42.95 ± 0.08 and 42.83 ± 0.23 respectively ($p<0.05$). For incubation at 22°C after 96 h of incubation, springiness was significantly the highest in set 3 and set 5 with 35.65 ± 0.51 and 35.64 ± 0.4 respectively ($p<0.05$).

Chewiness (N × mm) was significantly the highest for all the dough sets incubated for 72 h at 22°C and 30°C, except for sets 3, 5 and 7 (Table 4.9). For sets 5 and 7, chewiness was significantly the highest at 48 h for incubation at 30°C and at 72 h for 22°C, respectively. For set 3, chewiness was significantly the highest at 48 h of incubation at 22°C and 72 h of incubation at 30°C. Dough chewiness was significantly the highest in sets 4 and 6 incubated at 30°C at 96 h with the values of 6.31 ± 0.22 and 6.29 ± 0.21 respectively ($p<0.05$). For incubation at 22°C after 96 h of incubation, chewiness was significantly the highest set 2 and set 6 with 7.7 ± 0.19 and 7.5 ± 0.39 respectively ($p<0.05$).

The texture analysis results are supported by Armero & Collar (1997), who reported that hardness was highly correlated with gumminess and chewiness in wheat doughs. Hardness in fact has the highest weighting in the determination of gumminess (gumminess = cohesiveness * hardness) and chewiness (chewiness = gumminess * springiness, and springiness). Fermented dough springiness is correlated with cohesiveness and hardness. Hence the softer and more cohesive the dough is, better the recovery after compression. The authors also mentioned that the high hardness value of fermented sour doughs can be due to lower pH and higher TTA with increase in time, which supports the results in this study as shown in Figure 4.3. Fermented dough resilience and gumminess correlate positively with hardness. Harder doughs recovered their shape more quickly, thus characterising high elasticity in the dough.

Figure 4.7: Principal components analysis (PCA) bi-plot for all the coconut water kefir fermented dough sets at 30°C and 22°C sampled at T=0, 24, 48, 72 and 96 h. The correlation loadings represents the individual texture parameters along with viscosity (pa.s).



Samples numbers 1-9 belong to set 1, set 2, ..set 9 respectively, incubated at 30°C at time, T=0 h.
Numbers 10-18 belong to set 1, set 2, ..set 9 respectively, incubated at 30°C at time, T=24 h.
Numbers 19-27 belong to set 1, set 2, ..set 9 respectively, incubated at 30°C at time, T=48 h.
Numbers 28-36 belong to set 1, set 2, ..set 9 respectively, incubated at 30°C at time, T=72 h.
Numbers 37-45 belong to set 1, set 2, ..set 9 respectively, incubated at 30°C at time, T=96 h.
Numbers 46-54 belong to set 1, set 2, ..set 9 respectively, incubated at 22°C at time, T=0 h.
Numbers 55-63 belong to set 1, set 2, ..set 9 respectively, incubated at 22°C at time, T=24 h.
Numbers 64-72 belong to set 1, set 2, ..set 9 respectively, incubated at 22°C at time, T=48 h.
Numbers 73-81 belong to set 1, set 2, ..set 9 respectively, incubated at 22°C at time, T=72 h.
Numbers 82-90 belong to set 1, set 2, ..set 9 respectively, incubated at 22°C at time, T=96 h.

Table 4.3: Viscosity analysis of the coconut water kefir fermented sourdough. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Set 1						**Set 2**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	612.07 ± 2.57E,a	681.6 ± 11.82D,a	781.53 ± 2.71C,a	844.67 ± 2.24B,a	1215.7 ± 2.1A,a	608.13 ± 3.54E,a	679.93 ± 5.73D,a	786.53 ± 10.75C,a	846.63 ± 4.28B,a	1215.4 ± 2.78A,a
22°C	611.87 ± 2.13E,a	661.89 ± 1.82D,b	702.02 ± 0.56C,b	732.07 ± 0.99B,b	770.61 ± 1.59A,b	610.34 ± 2.13E,a	664.32 ± 0.31D,b	701.95 ± 1.55C,b	731.95 ± 1B,b	771.13 ± 1.58A,b
Set 3						**Set 4**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	606.53 ± 1.46E,a	685.17 ± 4.67D,a	800.07 ± 14.26C,a	844.93 ± 0.42B,a	1214.97 ± 4.27A,a	612.3 ± 2.41E,a	677.83 ± 4.31D,a	773.9 ± 15.27C,a	845.67 ± 1.8B,a	1214.07 ± 2.49A,a
22°C	607.5 ± 5.64E,a	691.94 ± 1.15D,b	763.83 ± 4.9C,b	731.06 ± 2.3B,b	794.15 ± 5.01A,b	612.15 ± 2.47E,a	662.61 ± 1.44D,b	701.07 ± 2.5C,b	730.82 ± 1.57B,b	773.39 ± 0.52A,b
Set 5						**Set 6**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	610.3 ± 3.27E,a	682.4 ± 6.05D,a	775.47 ± 24.64C,a	844.63 ± 1.72B,a	1214.33 ± 4.09A,a	608.97 ± 5.75E,a	687.37 ± 5.58D,a	772.8 ± 3.29C,a	846.2 ± 1.47B,a	1214.43 ± 3.46A,a
22°C	609.97 ± 6.48E,a	662.33 ± 1.25D,b	701.11 ± 1.14C,b	730.52 ± 1.31B,b	770.67 ± 1.72A,b	605.69 ± 5.58E,a	661.38 ± 1.32D,b	702.64 ± 1.21C,b	731.9 ± 1.52B,b	770.43 ± 0.76A,b
Set 7						**Set 8**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	606.67 ± 6.82E,a	682.07 ± 9.54D,a	797.07 ± 3.25C,a	845.6 ± 4.91B,a	1213.7 ± 5.05A,a	606.5 ± 3.24E,a	687.9 ± 2.86D,a	775.93 ± 8.56C,a	845.6 ± 2.17B,a	1215.5 ± 3.52A,a
22°C	605.36 ± 4.68E,a	662.64 ± 1.74D,b	701.46 ± 1.88C,b	731.93 ± 1.39B,b	771.36 ± 1.33A,b	605.52 ± 0.99E,a	661.67 ± 2.68D,b	701.25 ± 1.75C,b	731.26 ± 1.18B,b	770.59 ± 1.45A,b
Set 9						**F Value**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**Time**	**Temperature**	**Time*Temprature**		
30°C	609.9 ± 5.91E,a	711.57 ± 4.6D,a	783.7 ± 15.11C,a	845.6 ± 4.52B,a	1217.73 ± 1.16A,a					
22°C	605.98 ± 4.58E,a	703.21 ± 0.75D,b	754.39 ± 4.83C,b	732.59 ± 0.58B,b	804.4 ± 4.4A,b	41097.03***	38203.20***	15264.26***		

Table 4.4**:** Texture profile analysis of the coconut water kefir fermented sourdough for dough hardness. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Set 1						**Set 2**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	12.26 ± 0.55C,a	12.19 ± 0.13C,a	18.1 ± 0.16B,a	20.34 ± 0.24A,a	20.3 ± 0.38A,a	12.26 ± 0.55D,a	15.82 ± 0.19C,a	17.95 ± 0.19B,a	20.21 ± 0.28A,a	20.78 ± 0.34A,a
22°C	12.26 ± 0.55E,a	13.5 ± 0.46D,a	14.98 ± 0.36C,b	16.9 ± 0.45B,b	19.36 ± 0.13A,b	11.52 ± 0.18C,a	13.89 ± 0.33B,b	14.75 ± 0.26B,b	17.1 ± 0.3A,b	18.81 ± 0.14A,b
Set 3						**Set 4**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	11.52 ± 0.18D,a	15.72 ± 0.16C,a	18.01 ± 0.06B,a	20.1 ± 0.15A,a	20.15 ± 0.66A,a	12.11 ± 0.2C,a	13.18 ± 0.22C,a	17.81 ± 0.37B,a	20.1 ± 0.52A,a	20.28 ± 0.2A,a
22°C	12.11 ± 0.2D,a	14.03 ± 0.55C,a	15.19 ± 0.27B,C,b	16.71 ± 0.25B,b	18.96 ± 0.4A,b	11.93 ± 0.15E,a	13.15 ± 0.33D,a	15.51 ± 0.44C,b	17.13 ± 0.6B,b	19.32 ± 0.4A,a
Set 5						**Set 6**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	12.93 ± 0.15C,a	13.32 ± 0.1C,a	18.15 ± 0.4B,a	20.17 ± 0.44A,a	20.21 ± 0.34A,a	12.36 ± 0.62D,a	15.85 ± 0.24C,a	18.17 ± 0.23B,a	19.96 ± 0.21A,a	20.85 ± 0.28A,a
22°C	12.36 ± 0.62D,a	13.32 ± 0.39D,a	15.3 ± 0.39C,b	17.2 ± 0.23B,b	19.25 ± 0.3A,a	12.02 ± 0.52D,a	13.68 ± 0.09C,D,b	14.43 ± 0.15C,b	16.86 ± 0.2B,b	18.59 ± 0.37A,b
Set 7						**Set 8**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	12.02 ± 0.52D,a	15.64 ± 0.14C,a	17.8 ± 0.32B,a	19.87 ± 0.58A,a	20.17 ± 0.31A,a	12.13 ± 0.14D,a	15.89 ± 0.23C,a	17.78 ± 0.45B,a	20.04 ± 0.19A,a	20.11 ± 0.05A,a
22°C	12.13 ± 0.14D,a	13.92 ± 0.42D,b	15.11 ± 0.38C,b	17.19 ± 0.32B,b	19.56 ± 0.53A,a	12.43 ± 0.28C,a	14.02 ± 0.26B,a	14.65 ± 0.32B,b	17.36 ± 0.46A,b	18.94 ± 0.08A,b
Set 9						**F Value**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**Time**	**Temperature**	**Time*Temprature**		
30°C	12.43 ± 0.28D,a	15.68 ± 0.12C,a	18.46 ± 0.09B,a	20.2 ± 0.39A,a	20.22 ± 0.26A,a	1000.62***	339.65***	53.02***		
22°C	11.94 ± 0.17D,a	14.43 ± 0.38C,a	14.58 ± 0.3C,b	16.98 ± 0.4B,b	18.73 ± 0.53A,b					

Table 4.5: Texture profile analysis of the coconut water kefir fermented sourdough for dough resilience. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Set 1						**Set 2**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	6.27 ± 2.57C,a	9.84 ± 11.82B,a	11.06 ± 2.71A,a	11.62 ± 2.24A,a	12.01 ± 2.1A,a	6.44 ± 3.54D,a	9.78 ± 5.73B,a	10.55 ± 10.75A,B,a	12.16 ± 4.28A,a	12.66 ± 2.78A,a
22°C	6.27 ± 2.13D,a	9.99 ± 1.82C,a	10.96 ± 0.56B,C,a	11.93 ± 0.99A,B,a	12.5 ± 1.59A,a	6.44 ± 2.13D,a	9.94 ± 0.31B,a	11.18 ± 1.55A,B,a	12.06 ± 1A,a	12.62 ± 1.58A,a
Set 3						**Set 4**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	6.13 ± 1.46D,a	9.85 ± 4.67B,a	11.44 ± 14.26A,B,a	12.32 ± 0.42A,a	12.11 ± 4.27A,a	6.21 ± 2.41D,a	9.8 ± 4.31B,a	10.93 ± 15.27A,B,a	11.87 ± 1.8A,a	12.04 ± 2.49A,a
22°C	6.13 ± 5.64D,a	9.98 ± 1.15B,a	10.91 ± 4.9A,B,a	11.58 ± 2.3A,a	13 ± 5.01A,a	6.21 ± 2.47D,a	10.18 ± 1.44B,a	10.99 ± 2.5A,B,a	12.07 ± 1.57A,a	13.01 ± 0.52A,a
Set 5						**Set 6**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	6.44 ± 3.27D,a	9.9 ± 6.05B,a	10.96 ± 24.64A,B,a	11.89 ± 1.72A,a	12.04 ± 4.09A,a	6.01 ± 5.75D,a	9.7 ± 5.58B,a	10.92 ± 3.29A,B,a	11.91 ± 1.47A,a	12.08 ± 3.46A,a
22°C	6.44 ± 6.48D,a	10.08 ± 1.25B,a	11.17 ± 1.14A,B,a	12.3 ± 1.31A,a	12.77 ± 1.72A,a	6.01 ± 5.58D,a	9.55 ± 1.32B,a	10.5 ± 1.21A,B,a	11.72 ± 1.52A,a	13.28 ± 0.76A,a
Set 7						**Set 8**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	5.74 ± 6.82D,a	9.83 ± 9.54B,a	10.72 ± 3.25A,B,a	12.1 ± 4.91A,a	12.73 ± 5.05A,a	6.66 ± 3.24D,a	9.71 ± 2.86B,a	10.86 ± 8.56A,B,a	11.85 ± 2.17A,a	12.34 ± 3.52A,a
22°C	5.74 ± 4.68D,a	9.98 ± 1.74B,a	10.62 ± 1.88A,B,a	12.05 ± 1.39A,a	12.91 ± 1.33A,a	6.66 ± 0.99D,a	9.51 ± 2.68B,a	10.89 ± 1.75A,B,a	11.93 ± 1.18A,a	12.67 ± 1.45A,a
Set 9						**F Value**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**Time**	**Temperature**	**Time*Temperature**		
30°C	6.48 ± 5.91D,a	9.73 ± 4.6B,a	10.48 ± 15.11A,B,a	12.15 ± 4.52A,a	12.96 ± 1.16A,a	2489.07	21.01	16.38		
22°C	6.48 ± 4.58D,a	9.82 ± 0.75B,a	10.92 ± 4.83A,B,a	11.87 ± 0.58A,a	12.87 ± 4.4A,a					

Figure 4.8: Bar graph for texture profile analysis of the coconut water kefir fermented sourdough for dough cohesiveness. A, B, C, D, E,= Capital alphabets differ significantly using Fisher's least significant difference ($p < 0.05$), which are used for different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment. a, b, c: Alphabets used for different treatments for the same time interval, differ significantly using Fisher's least significant difference ($p < 0.05$).

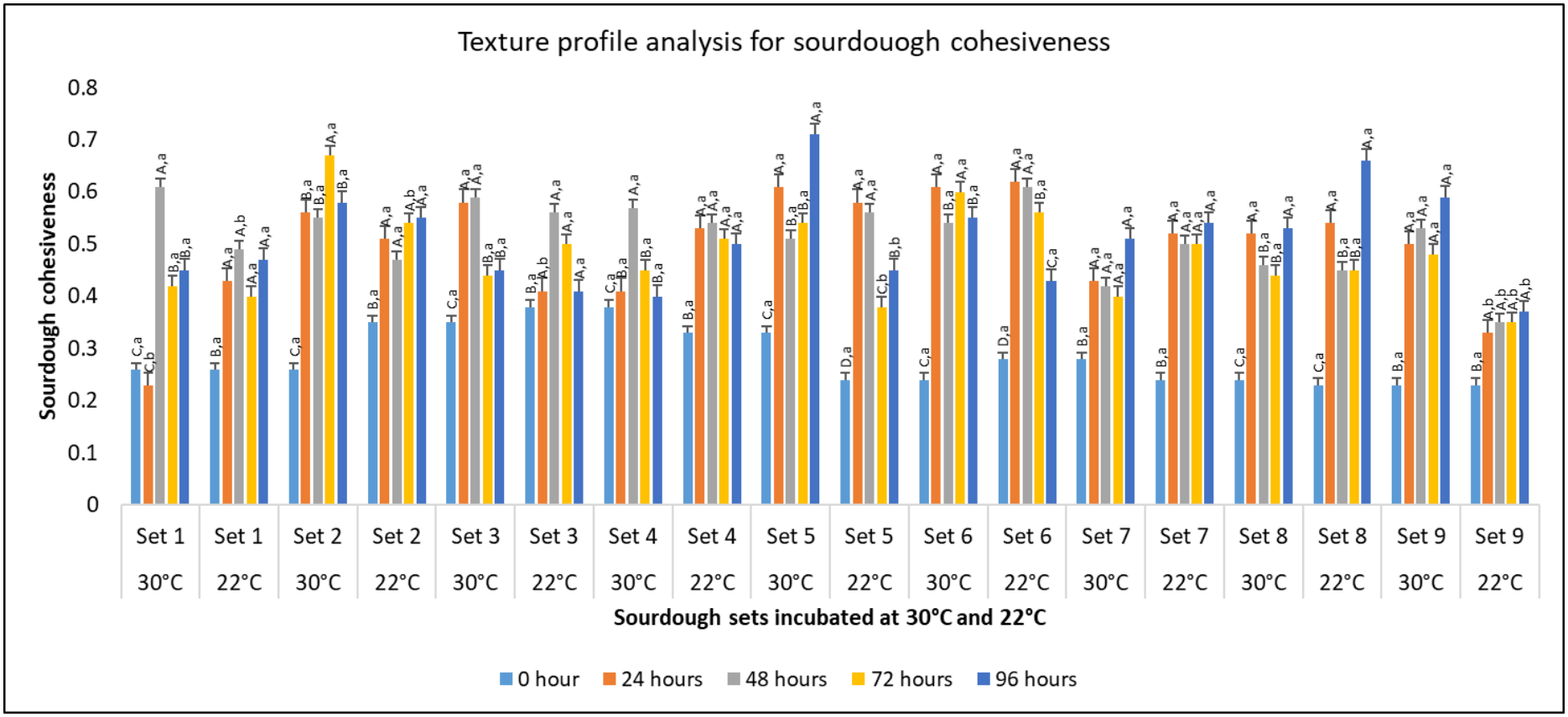


Table 4.6**:** Texture profile analysis of the coconut water kefir fermented sourdough for dough cohesiveness. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Set 1						Set 2				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
30°C	0.26 ± 0.04C,a	0.23 ± 0.03C,b	0.61 ± 0.08A,a	0.42 ± 0.15B,a	0.45 ± 0.19B,a	0.26 ± 0.04C,a	0.56 ± 0.01B,a	0.55 ± 0.23B,a	0.67 ± 0.09A,a	0.58 ± 0.15B,a
22°C	0.26 ± 0.04B,a	0.43 ± 0.05A,a	0.49 ± 0.19A,b	0.4 ± 0.16A,a	0.47 ± 0.1A,a	0.35 ± 0.03B,a	0.51 ± 0.14A,a	0.47 ± 0.23A,a	0.54 ± 0.24A,b	0.55 ± 0.09A,a
Set 3						Set 4				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
30°C	0.35 ± 0.03C,a	0.58 ± 0.1A,a	0.59 ± 0.05A,a	0.44 ± 0.14B,a	0.45 ± 0.21B,a	0.38 ± 0.04C,a	0.41 ± 0.02B,a	0.57 ± 0.06A,a	0.45 ± 0.15B,a	0.4 ± 0.12B,a
22°C	0.38 ± 0.04B,a	0.41 ± 0.13A,b	0.56 ± 0.11A,a	0.5 ± 0.13A,a	0.41 ± 0.1A,a	0.33 ± 0.03B,a	0.53 ± 0.19A,a	0.54 ± 0.17A,a	0.51 ± 0.11A,a	0.5 ± 0.11A,a
Set 5						Set 6				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
30°C	0.33 ± 0.03C,a	0.61 ± 0.1A,a	0.51 ± 0.09B,a	0.54 ± 0.14B,a	0.71 ± 0.01A,a	0.24 ± 0.02C,a	0.61 ± 0.14A,a	0.54 ± 0.16B,a	0.6 ± 0.13A,a	0.55 ± 0.16B,a
22°C	0.24 ± 0.02D,a	0.58 ± 0.26A,a	0.56 ± 0.17A,a	0.38 ± 0.08C,b	0.45 ± 0.11B,b	0.28 ± 0.02D,a	0.62 ± 0.08A,a	0.61 ± 0.02A,a	0.56 ± 0.21B,a	0.43 ± 0.24C,a
Set 7						Set 8				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
30°C	0.28 ± 0.02B,a	0.43 ± 0.18A,a	0.42 ± 0.03A,a	0.4 ± 0.25A,a	0.51 ± 0.12A,a	0.24 ± 0.03C,a	0.52 ± 0.18A,a	0.46 ± 0.09B,a	0.44 ± 0.21B,a	0.53 ± 0.2A,a
22°C	0.24 ± 0.03B,a	0.52 ± 0.24A,a	0.5 ± 0.07A,a	0.5 ± 0.21A,a	0.54 ± 0.03A,a	0.23 ± 0.02C,a	0.54 ± 0.04A,a	0.45 ± 0.15B,a	0.45 ± 0.18B,a	0.66 ± 0.1A,a
Set 9						F Value				
Temperature/Time in h	0	24	48	72	96	Time	Temperature	Time*Temperature		
30°C	0.23 ± 0.02B,a	0.5 ± 0.17A,a	0.53 ± 0.1A,a	0.48 ± 0.21A,a	0.59 ± 0.26A,a	31.12***	1.98*	0.44		
22°C	0.23 ± 0.03B,a	0.33 ± 0.09A,b	0.35 ± 0.06A,b	0.35 ± 0.07A,b	0.37 ± 0.11A,b					

Table 4.7**:** Texture profile analysis of the coconut water kefir fermented sourdough for dough gumminess. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Set 1						**Set 2**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	3.54 ± 0.1C,a	5.76 ± 0.11B,a	7.14 ± 0.36A,a	7.85 ± 0.23A,a	7.78 ± 0.25A,a	3.34 ± 0.09C,a	5.74 ± 0.06B,a	6.88 ± 0.46A,a	7.33 ± 0.34A,a	7.85 ± 0.1A,a
22°C	3.54 ± 0.1B,a	4.37 ± 0.02A,a	4.96 ± 0.05A,b	5.42 ± 0.05A,b	5.72 ± 0.05A,b	3.34 ± 0.09B,a	4.39 ± 0.07A,a	5 ± 0.07A,a	5.37 ± 0.03A,b	5.68 ± 0.06A,b
Set 3						**Set 4**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	3.56 ± 0.06C,a	5.72 ± 0.08B,a	6.63 ± 0.41A,B,a	7.29 ± 0.36A,a	7.48 ± 0.35A,a	3.56 ± 0.1C,a	5.6 ± 0.1B,a	6.96 ± 0.28A,B,a	7.27 ± 0.16A,a	7.37 ± 0.49A,a
22°C	3.56 ± 0.06B,a	4.34 ± 0.05A,a	4.97 ± 0.03A,b	5.38 ± 0.04A,b	5.7 ± 0.03A,b	3.56 ± 0.1B,a	4.37 ± 0.05A,a	4.98 ± 0.05A,b	5.38 ± 0.06A,b	5.78 ± 0.01A,b
Set 5						**Set 6**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	3.35 ± 0.07C,a	5.83 ± 0.25B,a	7.11 ± 0.27A,a	7.33 ± 0.55A,a	7.61 ± 0.07A,a	3.5 ± 0.01C,a	5.71 ± 0.19B,a	6.62 ± 0.2A,B,a	7.81 ± 0.36A,a	7.29 ± 0.27A,a
22°C	3.35 ± 0.07B,a	4.4 ± 0.1A,a	5 ± 0.04A,b	5.37 ± 0.07A,b	5.73 ± 0.04A,b	3.5 ± 0.01B,a	4.43 ± 0.07A,a	4.97 ± 0.04A,B,b	5.45 ± 0.05A,b	5.73 ± 0.11A,b
Set 7						**Set 8**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	3.4 ± 0.15C,a	5.79 ± 0.18B,a	6.51 ± 0.1A,B,a	7.57 ± 0.41A,a	7.53 ± 0.45A,a	3.41 ± 0.13C,a	5.64 ± 0.12B,a	6.76 ± 0.25A,B,a	7.25 ± 0.23A,a	7.71 ± 0.34A,a
22°C	3.4 ± 0.15B,a	4.46 ± 0.02A,a	5.03 ± 0.05A,b	5.36 ± 0.07A,b	5.75 ± 0.02A,b	3.41 ± 0.13B,a	4.45 ± 0.03A,a	5 ± 0.09A,B,b	5.41 ± 0.07A,b	5.76 ± 0.05A,b
Set 9						**F Value**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**Time**	**Temperature**	**Time*Temperature**		
30°C	3.32 ± 0.08C,a	5.82 ± 0.1B,a	6.78 ± 0.45A,B,a	7.58 ± 0.12A,a	7.58 ± 0.45A,a	164.72***	339.79***	22.12***		
22°C	3.32 ± 0.08B,a	4.46 ± 0.06A,a	5.02 ± 0.06A,b	5.41 ± 0.03A,b	5.69 ± 0.04A,b					

Table 4.8**:** Texture profile analysis of the coconut water kefir fermented sourdough for springiness. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Set 1						**Set 2**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	28.24 ± 0.04B,a	29.81 ± 4.98B,a	41.08 ± 0.48A,a	42.73 ± 0.47A,a	42.61 ± 0.56A,a	28.27 ± 0.04B,a	29.87 ± 5.46B,a	40.98 ± 0.31A,a	42.81 ± 0.27A,a	42.59 ± 0.51A,a
22°C	28.24 ± 0.04B,a	29.08 ± 0.05B,a	33.91 ± 0.16A,b	34.41 ± 0.39A,b	35.3 ± 0.26A,b	28.27 ± 0.04C,a	31.57 ± 0.25B,a	33.59 ± 0.29A,B,a	34.73 ± 0.07A,b	35.57 ± 0.28A,b
Set 3						**Set 4**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	28.26 ± 0.02C,a	33.22 ± 7.14B,a	41.17 ± 0.26A,a	42.45 ± 0.3A,a	42.79 ± 0.14A,a	28.26 ± 0.02B,a	42.9 ± 2.23A,a	42.82 ± 0.45A,a	42.83 ± 0.2A,a	42.95 ± 0.08A,a
22°C	28.26 ± 0.02C,a	31.8 ± 0.34B,b	33.63 ± 0.45A,B,b	34.65 ± 0.36A,b	35.65 ± 0.51A,b	28.26 ± 0.02C,a	31.73 ± 0.39B,a	33.83 ± 0.57A,B,b	34.43 ± 0.26A,b	35.53 ± 0.26A,b
Set 5						**Set 6**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	28.24 ± 0.03B,a	30.99 ± 4.25B,a	40.74 ± 0.31A,a	42.37 ± 0.58A,a	42.81 ± 0.3A,a	28.25 ± 0.05C,a	35.84 ± 0.18B,a	40.62 ± 0.17A,a	42.64 ± 0.31A,a	42.6 ± 0.48A,a
22°C	28.24 ± 0.03C,a	31.69 ± 0.4B,b	33.67 ± 0.47A,B,b	34.66 ± 0.47A,b	35.64 ± 0.4A,b	28.25 ± 0.05C,a	31.88 ± 0.1B,a	33.26 ± 0.23A,B,b	34.82 ± 0.26A,b	35.43 ± 0.24A,b
Set 7						**Set 8**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	28.26 ± 0.02C,a	35.75 ± 0.19B,a	40.99 ± 0.25A,a	42.42 ± 0.1A,a	42.58 ± 0.25A,a	28.23 ± 0.03C,a	35.84 ± 0.2B,a	41.22 ± 0.09A,a	42.55 ± 0.5A,a	42.88 ± 0.13A,a
22°C	28.26 ± 0.02C,a	31.95 ± 0.1B,b	33.39 ± 0.23A,B,b	34.71 ± 0.42A,b	35.8 ± 0.33A,b	28.23 ± 0.03C,a	31.58 ± 0.34B,a	33.57 ± 0.28A,B,b	34.7 ± 0.26A,b	35.5 ± 0.26A,b
Set 9						**F Value**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**Time**	**Temperature**	**Time*Temperature**		
30°C	28.26 ± 0.02C,a	35.75 ± 0.02B,a	40.85 ± 0.52A,a	42.28 ± 0.35A,a	42.83 ± 0.23A,a	2175.32***	3218.46***	225.45***		
22°C	28.26 ± 0.02C,a	31.55 ± 0.34B,b	33.77 ± 0.14A,B,b	34.47 ± 0.26A,b	35.58 ± 0.45A,b					

Table 4.9**:** Texture profile analysis of the coconut water kefir fermented sourdough for chewiness. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Set 1						**Set 2**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	1.47 ± 0.14D,a	3.69 ± 0.1C,a	4.61 ± 0.1B,C,a	5.93 ± 0.25A,B,a	6.04 ± 0.22A,b	1.38 ± 0.15D,a	3.63 ± 0.15C,a	4.84 ± 0.22B,a	6.07 ± 0.55A,a	6.03 ± 0.14A,b
22°C	1.47 ± 0.14D,a	3.03 ± 0.21C,a	5.19 ± 0.37B,C,a	6.76 ± 0.27A,B,a	7.25 ± 0.34A,a	1.38 ± 0.15D,a	2.9 ± 0.34C,a	5.54 ± 0.34B,a	6.64 ± 0.39A,B,a	7.7 ± 0.19A,a
Set 3						**Set 4**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	1.34 ± 0.16D,a	3.68 ± 0.15C,a	5.05 ± 0.44B,C,a	5.84 ± 0.42A,B,a	6.23 ± 0.24A,b	1.42 ± 0.14D,a	3.79 ± 0.12C,a	4.83 ± 0.25B,a	5.96 ± 0.5A,B,a	6.31 ± 0.22A,b
22°C	1.34 ± 0.16C,a	2.83 ± 0.32B,a	5.27 ± 0.46A,a	7.12 ± 0.3A,a	7.22 ± 0.43A,a	1.42 ± 0.14D,a	2.9 ± 0.42C,a	5.22 ± 0.31B,a	6.89 ± 0.4A,B,a	7.36 ± 0.35A,a
Set 5						**Set 6**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	1.46 ± 0.21C,a	3.74 ± 0.18B,a	5.14 ± 0.32A,a	5.84 ± 0.46A,a	6.17 ± 0.2A,b	1.48 ± 0.04D,a	3.64 ± 0.14C,a	4.57 ± 0.31B,a	6.25 ± 0.19A,a	6.29 ± 0.21A,b
22°C	1.46 ± 0.21D,a	3.05 ± 0.42C,a	5.7 ± 0.58B,a	6.87 ± 0.12A,B,a	7.29 ± 0.47A,a	1.48 ± 0.04D,a	2.72 ± 0.33C,a	5.73 ± 0.32B,a	6.8 ± 0.4A,B,a	7.5 ± 0.39A,a
Set 7						**Set 8**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	1.38 ± 0.2C,a	3.71 ± 0.23B,a	4.99 ± 0.38A,a	5.95 ± 0.39A,a	5.72 ± 0.18A,b	1.57 ± 0.05D,a	3.78 ± 0.16C,a	4.81 ± 0.52B,a	5.95 ± 0.38A,a	5.9 ± 0.13A,b
22°C	1.38 ± 0.2D,a	2.97 ± 0.5C,a	5.27 ± 0.26B,a	7.1 ± 0.34A,a	7.46 ± 0.21A,a	1.57 ± 0.05D,a	2.75 ± 0.33C,a	5.81 ± 0.13B,a	6.58 ± 0.45A,B,a	7.24 ± 0.12A,a
Set 9						**F Value**				
Temperature/Time in h	**0**	**24**	**48**	**72**	**96**	**Time**	**Temperature**	**Time*Temperature**		
30°C	1.34 ± 0.1D,a	3.69 ± 0.04C,a	5.05 ± 0.46B,a	6.36 ± 0.05A,a	6.16 ± 0.28A,b	3120.36***	113.89***	103.01***		
22°C	1.34 ± 0.1D,a	2.91 ± 0.41C,a	5.55 ± 0.3B,a	6.92 ± 0.49A,B,a	7.03 ± 0.27A,a					

4.3.5 Organic acids in sourdough

The organic acids in the dough were determined using the LC-MS method according to Lu et al., (2013). The dough samples were taken in triplicate for times, T=0, 24, 48, 72 and 96 h for all the sets incubated at 30°C. The incubation temperature of 30°C was chosen because the microbial cell counts for LAB, yeast and AAB were significantly high, compared to those at 4°C and 22°C (Figure 4.1).

Carboxylic acid analysis was carried out for sourdough fermented with CWK to determine changes in the concentration of acids such as lactic acid, acetic acid, pyruvic acid and succinic acid. Principal component analysis (PCA) was carried out to assess the variation in these acids for all nine sets with different concentrations of glucose and sucrose during fermentation for up to 96 h at 30°C. Samples were taken at time, T= 0, 24, 48, 72 and 96 h.

The PCA-2D score plot shown in Figure 4.9, described 91.8% and 4.3% of the total variation in Principal component 1 (PC1) and Principal component 2 (PC2) respectively. The scores for all the sets of sourdough fermented with coconut water kefir at different times (T=0, 24, 48, 72 and 96 h) are clearly shown. Five groups were clearly separated according to fermentation time (T=0, 24, 48, 72 and 96 h) indicating distinct changes in acids during fermentation. Samples at time, T=0 had negative scores along PC1. With increasing fermentation time (T= 24, 48, 72 and 96 h), sample scores became increasingly positive. Significantly high acid production was observed for all the sets incubated for 48, 72 and 96 h compared to all the sets at 0 and 24h ($p<0.05$).

The PCA biplot in Figure 4.10 shows both the scores and loadings plot for carboxylic acids in all the sourdough fermented with CWK sets at 0, 24, 48, 72 and 96 h. All the sets at T=0 h (control) and T=24 h had significantly high negative scores. At 48 h, all the sets had positive scores, which correspond to high positive loadings for lactic acid and pyruvic acid. This is supported by ANOVA results shown in Appendix 11 which show significantly high concentrations of lactic and pyruvic acid in the samples at 48 h. With increasing fermentation all the sets at time, T= 72 and 96 h had significantly high positive scores, which corresponded to the high positive loadings of acetic acid and succinic acid. These findings were supported by ANOVA results shown in Appendix 11 that showed significantly higher concentrations of lactic acid, acetic acid and pyruvic acid in fermented samples with increasing fermentation time.

Organic acids such as the short-chain fatty acids (SCFA), which were also found in the present study, contribute to flavour and aroma in sourdough (Gänzle, Vermeulen, & Vogel, 2007). High concentrations of acids are correlated to aroma formation in the sourdough during fermentation, especially lactic and acetic acid. This supports the increase in acids observed in this study.

The increase in succinic acid concentration and the low pyruvic acid concentration after 96 h of fermentation may be explained by the conversion of pyruvic acid to succinic acid by the LAB.

The results of the present study shown in Figure 4.10 with increased succinic acid production and low pyruvic acid concentration after 96 h of fermentation agree with those observed in a number of studies carried out on cheese and milk (Dudley & Steele, 2005).

Figure 4.9: PCA-2D score plots of PC1 and PC2 for the carboxylic acids found in the different sets of coconut water kefir fermented sourdough at 30°C with time (T=0, 24, 48, 72 and 96 h). The samples are colour coded as below:

Legends- : Represents time, T= 0 h; : Represents time, T= 24 h ; : Represents time, T=48 h; : Represents time, T=72 h; : Represents time, T= 96 h

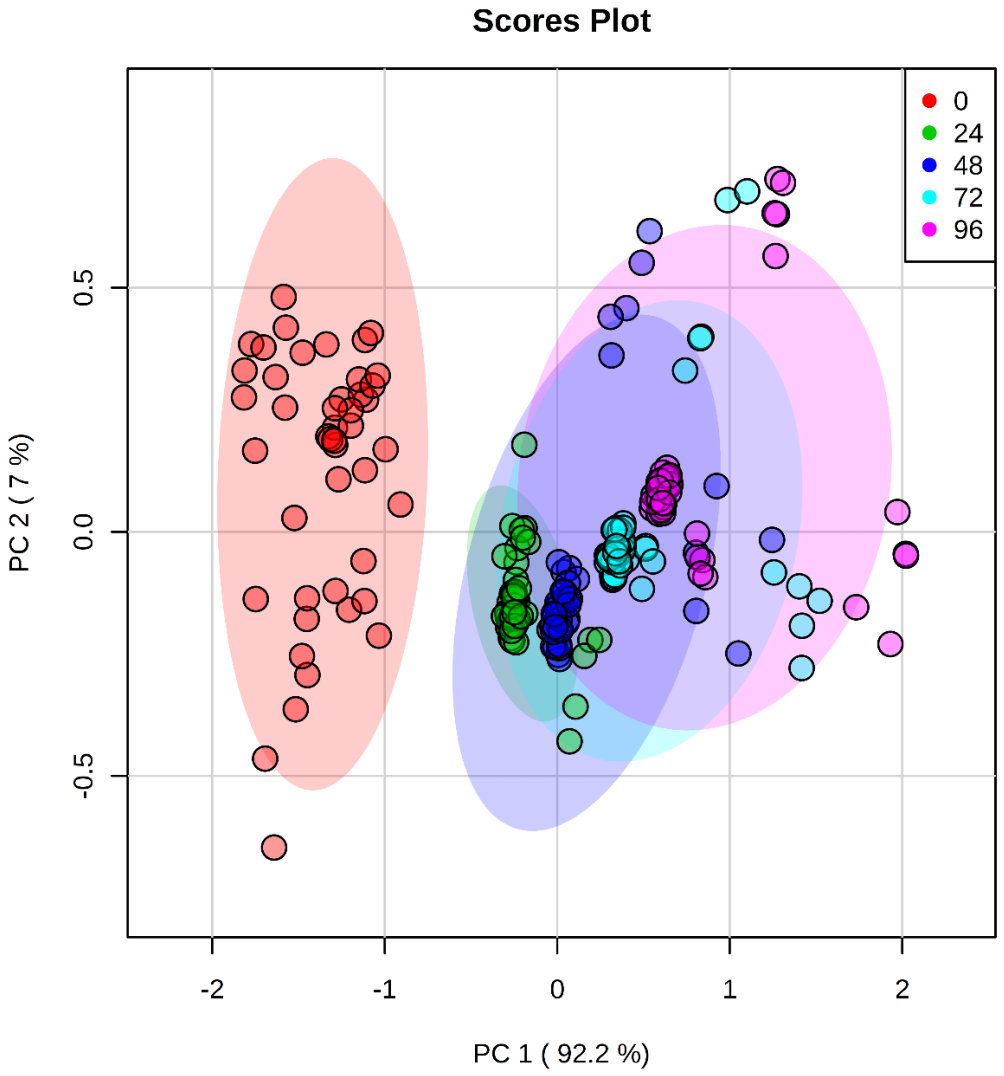
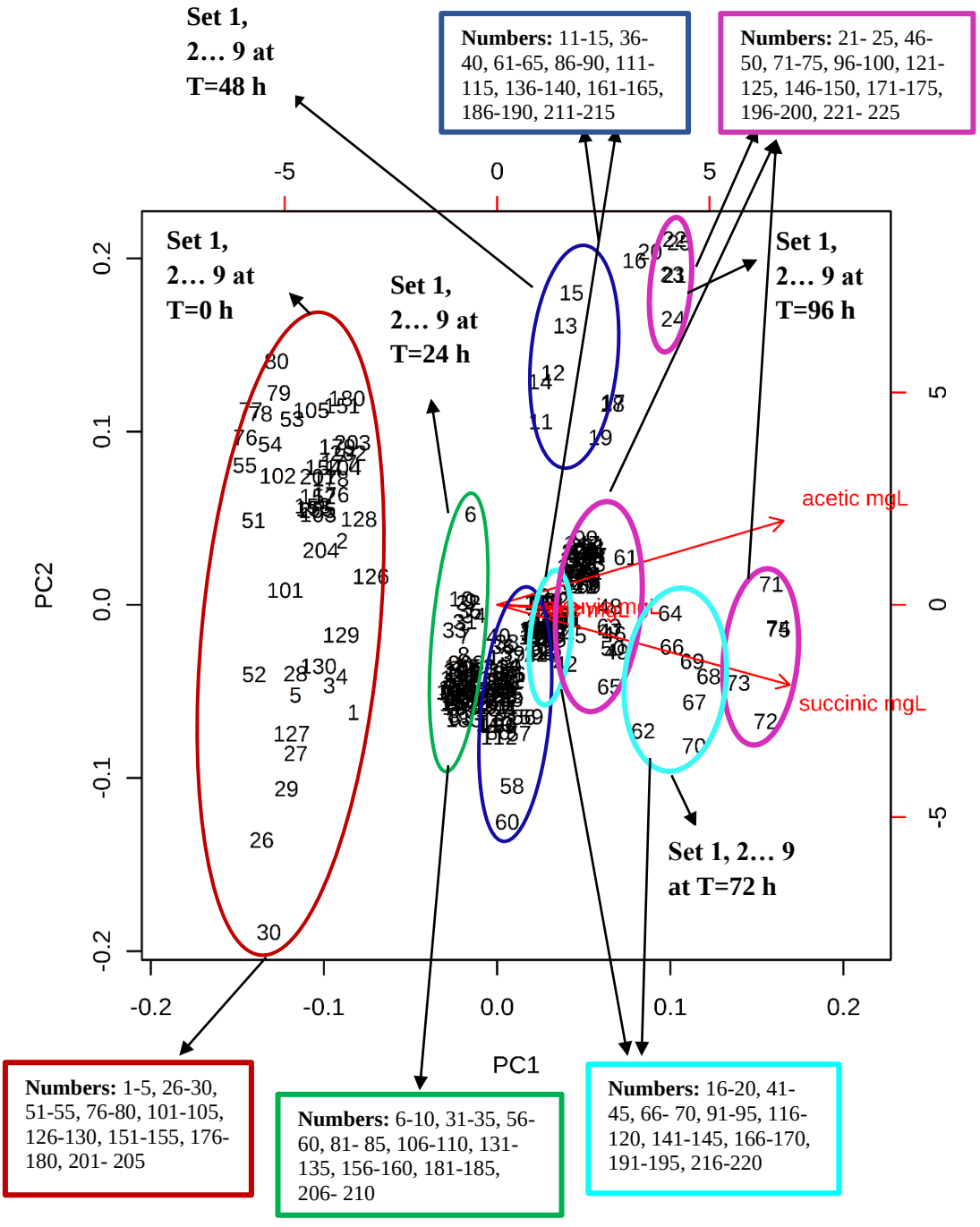


Figure 4.10: Principal components analysis (PCA) biplot for carboxylic acids in all the coconut water kefir fermented sourdough sets at different times (T=0, 24, 48, 72 and 96 h) at 30°C. The correlation loadings represent the individual carboxylic acids. The coconut water kefir samples are numbered as below:

Numbers 1-25: belong to Set 1, numbers 26-50: belong to Set 2, numbers 51-75: belong to Set 3, numbers 76-100: belong to Set 4, numbers 101-125: belong to Set 5, numbers 126-150: belong to Set 6, numbers 151-175: belong to Set 7, numbers 176-200: belong to Set 8, numbers 221-225: belong to Set 9.



4.3.6 Glutamic acid analysis for the dough by LC-MS

The level of glutamic acid was quantified in the dough sets using LCMS analysis (Figure 4.11 and Table 4.10). Significantly high concentrations of glutamic acid, 187 ± 1.58 mg/L were present in set 3 over the incubation time of 96 h, followed by set 1 and set 4 with 165.8 ± 2.86 mg/L and 161.96 ± 0.44 mg/L respectively after 96h of incubation ($p<0.05$).

The presence of glutamic acid observed in this study maybe attributed to the activity of the LAB in sourdough. In general, most LAB in fermented foods are capable of producing high concentration of glutamic acid (Zareian et al. (2012). Some LAB isolated from infants or grown on different media have been reported to produce glutamic acid (Tarek & Mostafa, 2010; Zalán, Hudáček, Štětina, Chumchalová, & Halász, 2010). Glutamic acid producing LAB have also been isolated from some fermented foods in Malaysia, as reported by Zareian et al. (2012). Many studies have reported the existence of the *gdh* gene in LAB which is responsible for the production of glutamic acid (Tanous, Chambellon, Sepulchre, & Yvon, 2005), which is consistent with this study reporting high amount of glutamic acid production in CWK fermented sourdough after 96 h of fermentation.

Figure 4.11: Bar graph for glutamic acid analysis for sourdough sets fermented with CWK. A,B,C,D,E,= Different alphabets for fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment, differ significantly using Fisher's least significant difference ($p < 0.05$). a,b,c: Different alphabets for different treatments for the same time interval, differ significantly using Fisher's least significant difference ($p < 0.05$). The error bars represent the standard deviation for the five replicate readings for each experimental set.

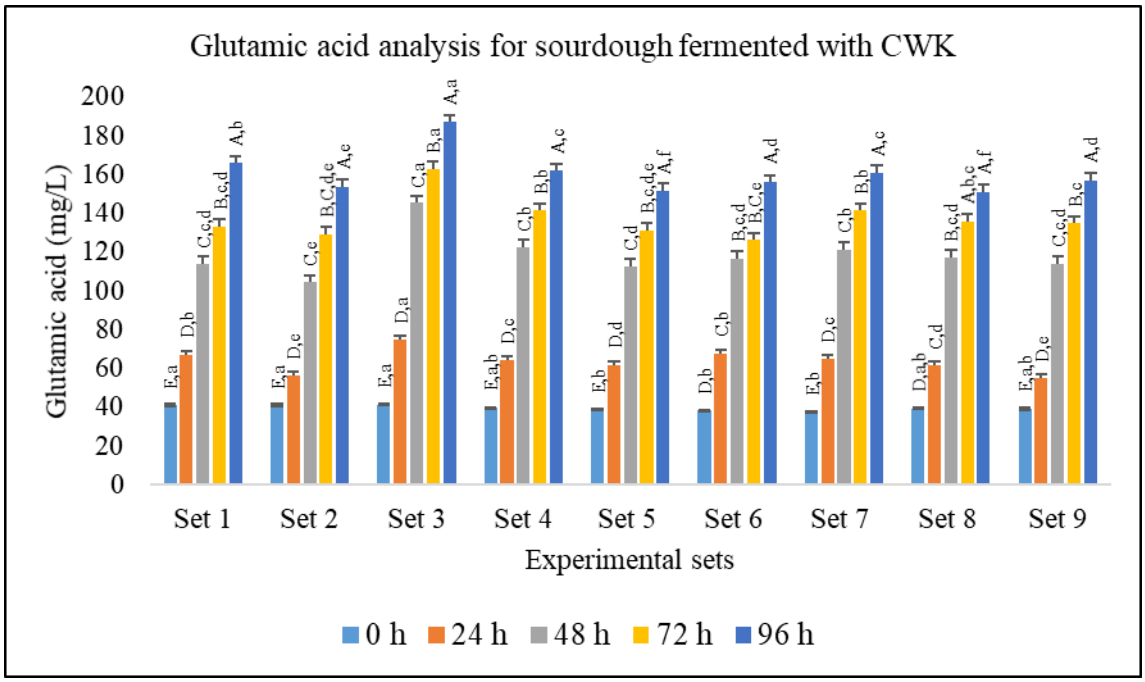


Table 4.10: Glutamic acid (mg/L) quantification for the coconut water kefir fermented sourdough using LCMS. A,B,C,D,E,= Different alphabets within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a,b,c: Different alphabets within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$).

	Glutamic acid (mg/L)				
Sourdough sets	**0 h**	**24 h**	**48 h**	**72 h**	**96 h**
Set 1	41 ± 2.5A,a	67 ± 1.5A,b	113.8 ± 6.2A,c,d	133.2 ± 1.6A,c,d	165.8 ± 2.8A,b
Set 2	41 ± 1.5B,C,a	56.2 ± 1.7C,e	104.4 ± 3.3B,e	129 ± 6.9B,C,d,e	153.6 ± 2.8A,e
Set 3	41.2 ± 1.9E,a	74.8 ± 2.5A,a	145.2 ± 3.3C,a	163.1 ± 11.1D,a	187.2 ± 1.5B,a
Set 4	39.6 ± 1.1C,a,b	64.1 ± 0.8C,c	122.2 ± 0.7D,b	141.3 ± 0.6A,b	161.9 ± 0.4B,c
Set 5	38.6 ± 2.8A,b	61.9 ± 1.1B,d	112.5 ± 0.5D,d	131.1 ± 1.2C,c,d,e	151.5 ± 0.8B,f
Set 6	38.2 ± 1.3C,b	67.5 ± 1.4C,b	116.6 ± 1.1B,c,d	126.1 ± 1.1B,C,e	155.9 ± 1.03A,d
Set 7	37.4 ± 0.8A,b	64.7 ± 0.7C,c	121.3 ± 1.2D,b	141.4 ± 1.1A,b	160.8 ± 0.39B,c
Set 8	39.4 ± 1.1E,a,b	61.7 ± 1.2B,d	117.7 ± 0.6D,c,d	135.9 ± 0.9A,b,c	150.8 ± 0.88C,f
Set 9	39 ± 1.5C,a,b	54.8 ± 0.9B,e	113.9 ± 0.7A,c,d	134.8 ± 0.9C,c	156.8 ± 0.9B,d

4.3.7 Determination of Fermentation Quotient (FQ)

FQ is a ratio that has been used to determine the ratio of acids produced during fermentation (Lactic acid: acetic acid). The concentration of lactic acid are higher than those of acetic acid, which depends on the biosynthesis of metabolites. Production of metabolites varies with the process parameters, LAB strain and their interaction with yeasts. The end products such as carbondioxide, lactate, acetate and ethanol are obtained when sourdough is fermented using mixed cultures (Ganzle et al., 1998). The homofermentative LAB strains convert more than 85% of glucose into lactic acid, while the heterofermentative LAB strains biosynthesize mainly lactic acid (50%) and important quantities of ethanol and CO_2 (Banu, Vasilean, & Aprodu, 2011). This, in turn, influences the total titratable acidity of the dough, which is an important factor for

determining the overall flavour profile of the final bread product upon baking (Vogelmann & Hertel, 2011).

FQ was determined for all the sourdough formulations incubated at 30°C for 96 h. Sourdough samples Set 3 and Set 1 had significantly highest FQ of 8.9 ± 0.2 and 8.8 ± 0.2, respectively ($p<0.05$) when compared with rest of the sourdough sets. The FQ values obtained in this study are between the range of 8-8.9, which has previously been reported by Barber, Ortolá, Barber, & Fernandez, 1992; Barber, Báguena, de Barber, & Martínez-Anaya, 1991; Salovaara & Spicher, 1987) as wide range of FQ for wheat sourdoughs. Spicher et al., (1981) and Spicher and Nierle (1984) reported that FQ depends largely on the type and concentration of LAB added to the sourdough.

Higher FQ values have been previously reported for sourdough in the range of 3.0 – 9.3 by Lattanzi et al. (2013). A study by Rossana Coda, Rizzello, & Gobbetti (2010) on sourdough, reported a high FQ value of 8.76. These results are consistent with the results of FQ obtained in this study for all the sourdough sets.

Table 4.11: FQ for sourdough formulations fermented at 30°C for 96 h. Letters a–g: Different superscript letters in columns following means signify significant differences ($p < 0.05$). P-value greater than $\alpha = 0.05$ indicates no significant difference.

Sourdoughs	**Lactic acid (mg/L)**	**Acetic acid (mg/L)**	**FQ**
Set 1	818.9 ± 8.9c	62.5 ± 4.4d	8.8 ± 0.2a
Set 2	820.9 ± 3.4b	68.1 ± 0.7a	8 ± 0.1c
Set 3	828.8 ± 4.8a	62.6 ± 1.9d	8.9 ± 0.2a
Set 4	812.3 ± 1.8d	66.3 ± 2.2b,c	8.2 ± 0.3b,c
Set 5	802.3 ± 0.8f,g	66.1 ± 1.9b,c	8.1 ± 0.2b,c
Set 6	802.7 ± 1.8f	65.7 ± 1.8c	8.1 ± 0.2b,c
Set 7	809.3 ± 1.4e	65.6 ± 0.6c	8.2 ± 0.1c
Set 8	801.4 ± 1.2g	66.6 ± 1.8b,c	8 ± 0.2b,c
Set 9	811.3 ± 1.8d	66.5 ± 1.6b,c	8.1 ± 0.2b,c
p-value	< 0.0001	< 0.0001	< 0.0001

4.4. Summary

In this chapter, a sourdough was developed using 48 h fermented coconut water kefir. Coconut water kefir used for developing the sourdough was supplemented with varying concentrations of glucose and sucrose. The 48 h fermented CWK set 3 used for preparing the sourdough had 4.84 ± 0.02 log CFU/ml of LAB, 3.86 ± 0.02 log CFU/ml of yeasts and 2.19 ± 0.53 log CFU/ml of acetic acid bacteria.

Overall, both factors – time and temperature - affected the cell counts. At longer incubation times at the temperature of 30°C, higher cell counts for LAB, AAB and yeasts were obtained compared to those from shorter incubation times at 22°C. The higher cell counts were accompanied by, a sharp decline in pH, increase in lactic acid production (D-/L- lactic acid) and increase in TTA, which consequently affected the overall viscosity of the CWK fermented sourdough. Due to this, there was an increase in dough volume with time, which was higher when incubated at 30°C temperature. The texture of the CWK fermented sourdough had higher values for hardness, resilience, chewiness, gumminess and springiness after incubation for 48, 72 and 96 h at 30°C, when compared to those at 22°C which had lower values. There were high quantities of organic acids such as lactic acid, pyruvic acid, succinic acid and malic acid produced in the CWK sourdough fermented for 48, 72 and 96 h at 30°C when compared to those fermented for 24h. The amino acid glutamic acid (a known flavour potentiator) was also produced in notably high quantities in the CWK fermented sourdough at 30°C after 96 h of incubation. The fermentation quotient of the sourdough sets fermented at 30°C for 96 h was obtained between the range of 8 – 8.9. Sourdough set 3 fermented at 30°C for 96 h had the significantly highest FQ of 8.9 ± 0.2.

In this chapter, a sourdough was developed using a 48h fermented coconut water kefir culture and the different sourdough types were characterised microbially and physico-chemically. It can also be concluded that since significantly high cell counts were obtained for sourdough sets fermented at 30°C, therefore, it was chosen as the incubation temperature for all the sourdough formulations.

Chapter 5 Identification of the microbiota in coconut water, kefir, CWK and CWK fermented sourdough using culture-dependent techniques and Illumina-MiSeq sequencing

5.2. Introduction

The objective of this chapter was to identify and characterise the microbiota such as LAB, yeast and AAB isolates from coconut water (CW), kefir grains, coconut water fermented with kefir (CWK) and CWK fermented sourdough (CWKS) (classified as laboratory fermented type 2 sourdough). Accurate characterisation and identification of microorganisms responsible for fermentation are important. The phenotypic identification of microorganisms contributes to the understanding of their physiological properties while the phylogenetic identification would reflect the natural relationships between the components of a microbial population. The microbial population during fermentation and leavening may change due to the changes in their environment such as a decrease in pH, an increase in carbon dioxide content and reduction in oxygen content. The dominant microorganisms during the early stages of kefir and sourdough fermentation could be replaced by completely different microorganisms during the later stages of fermentation. These microorganisms could contribute to the unique flavour, texture, aroma, shelf-life and nutritional quality of CWK- fermented sourdough. Hence, a detailed study of sourdough microflora would lead to the design and consistent production of starter cultures for industrial sourdough production.

Various microbial and molecular techniques and approaches have been used to identify sourdough and kefir microorganisms. Molecular techniques such as 16S ribosomal DNA (rDNA), PCR-denaturing gradient gel electrophoresis (DGGE), DNA sequencing, pulse field gel electrophoresis and pyrosequencing methods have been used to characterise the microbial community in the sourdough (Catzeddu, 2019). A recent study on traditional sourdough carried out by Xing et al., 2020 have identified isolates such as *L. crustorum, L. plantarum, L. mindensis, L. brevis, L. paralimentarius* and *Pediococcus pentosac* using 16S rRNA gene sequencing method. A similar study carried out by Çakır, Arıcı, Durak, & Karasu, 2020 on sourdough have identified and reported the dominant microorganisms such as *L. crustorum, Pediococcus, L. brevis, L. paraplantarum, L. plantarum, L. fermentum,* and *L. curvatus* using PCR 16S rDNA method.

DNA-based Illumina Miseq high throughput sequencing method, Sanger sequencing method, and culture dependent method such as Analytical profile index method (API) were used to carry out microbial characterization of isolates.

The LAB, AAB and yeast were isolated from kefir, CW, CWK and CWKS. At the start of the experiment, there were a total of 30 isolates which were classified based on their appearance and colonial morphology. Other phenotypic characterisation and screening based on Bergey's Manual of Determinative Bacteriology and biochemical characterisation using Analytical Profile Index identification method were then performed.

After carrying out the initial morphological screening, five putative isolates of LAB, three putative isolates for AAB and five putative isolates of yeast were confirmed using the API kits. The other isolates were eliminated based on morphological and biochemical characteristics such as Gram staining, motility test, colony and cell appearance, catalase test and production of CO_2 from glucose in MRS broth.

The isolates for LAB and AAB were confirmed by Sanger sequencing method. Sanger sequencing could not be applied to the yeast isolates due to operational limitations existing in New Zealand. All the sequences were confirmed by using the National Center for Biotechnology Information - The Basic Local Alignment Search Tool (NCBI-BLAST).

Illumina MiSeq sequencing was carried out to understand the microbial community dynamics between the different genera of microorganisms within the CWK-fermented sourdough during fermentations. Samples at each time, T=0, 24, 48, 72 and 96 h were taken from CWK and CWK – fermented sourdough for identifying the LAB, AAB and yeast genus dominant at a given time point. Changes in the microbial community during fermentation and the relative abundance of each genus were analysed.

5.2. Materials and methods

5.2.1 Phenotypic characterisation and identification of isolates

The samples used for identification were fresh young coconut water (Countdown, Auckland), kefir grains bought from Body Ecology™, New Zealand and CWK fermented sourdough.

One ml of uninoculated coconut water was spread on De Man, Rogosa and Sharpe agar medium (MRS), Malt Extract agar (ME) and acetic acid bacteria agar (AAB), (DIFCO, Fort Richard Laboratories, Auckland).

From the kefir grains, isolates were obtained from serial dilution plates in MRS agar, ME agar and AAB agar. Isolates from CWK fermentation were obtained from 300 ml of coconut water incubated with kefir grains (1.5 g/L) at 30°C up to 96 h. Samples were taken at 24 h time interval.

A CWK ferment (48h, 300ml) incubated in a food-grade LabServ incubator (Thermo Fisher Scientific, New Zealand), was used for the preparation of sourdough. Sourdough (600g) was prepared using CWK (300 ml), table salt (3 g) and oil (1.5 ml). One g of sourdough sample was taken every 24 h of fermentation. This was suspended in 9 ml of deionised water using a stomacher (or a mechanical mixer). Then 1 ml of this liquid sample was spread on MRS, AAB and ME agar (DIFCO, Fort Richard Laboratories, Auckland).

Phenotypic characteristics of isolates from kefir, CWK and CWK fermented sourdough were evaluated by determining the following: cell morphology, colonial morphology, cultural, biochemical and physiological characteristics.

Single colonies that differed in colonial characteristics were picked off from each plate and were initially Gram stained. Gram-positive microorganisms were further subdivided into spore formers and non-spore formers, using spore staining (Schaeffer-Fulton method with malachite green stain). Subsequent identification was performed using motility, and biochemical tests according to Bergey's Manual of Determinative Bacteriology (Garrity, Bell, & Lilburn, 2004) and API kits respectively.

The putative LAB isolates, which were Gram-positive, non-spore forming and catalase-negative, were further taken to cultural, biochemical and physiological characterisation. The characteristics included are shown in Table 5.1, Table 5.2 and Table 5.3.

The sugar fermentation characteristics were determined using the API CHL 50 biochemical test kit (Biomereuex, Mediray Laboratories, Auckland). After incubation all colonies from a sector of the highest dilution were further purified by successive streaking on the appropriate agar. For long term maintenance, the bacterial and yeasts isolates were stored at -80°C containing 15% glycerol. The LAB, AAB and yeasts were stored in MRS, AAB and ME broths, respectively. A total of 15

isolates including LAB, AAB and yeasts were identified, which were further subjected to identification using Analytical Profile Index (API) kits.

Putative acetic acid-producing bacteria were identified based on their growth on Acetic Acid Bacteria agar and in AAB broth. The Acetic Acid Bacteria agar was purchased from DIFCO, New Zealand. All the plates were incubated at 30°C for more than 48 h.

The putative AAB isolates were phenotypically identified using Bergey's Manual of Determinative Bacteriology (Garrity, Bell, & Lilburn, 2004) followed by biochemical identification, which was carried out using API 20 E kit (Mediray, New Zealand). The API package insert was supplied with test strips, incubation boxes and result sheets. Additionally, Carr's medium was used to confirm the presence of Acetobacter. Carr's medium was prepared using 30 g yeast extract, 20 g ethanol, 0.02 g bromocresol purple, and 20 g agar per litre, and the pH was adjusted to 5.5- 6.0.

Isolation of yeast isolates was performed using kefir grains, CWK and CWKS. Serial dilution plates on ME agar were prepared from kefir grains and CWK as described earlier. For CWKS, 1 g was suspended in 5 ml of deionised water, and placed in a stomacher bag for homogenization (Stomacher 400 Circulator, Thermofisher, New Zealand) for 3 cycles at 60 seconds each. This was followed by centrifugation for 5 min at 5000g. One ml of the supernatant was used for 10-fold serial dilutions which were plated on Malt Extract agar. The ME cultures were incubated at 29°C for 48-72 h. Colonies that were non-shiny and bigger than pin-point colonies were selected and were assumed to be yeast colonies (i.e. putative). These were purified using five phase streak plating on Malt Extract agar. For long term maintenance, pure cultures of isolates were grown in Malt Extract broth, at 29°C for 48-72 h, then mixed with 15% glycerol before storage at -80°C.

The API kit ID 20C AUX (Mediray, New Zealand) was used for the identification of putative yeast isolates. This system consisted of a single-use disposable plastic strip with 20 cupules. The identification of the yeasts was done as per the manufacturer's instructions. The positive or negative results (after incubation) were recorded (+) or (-) respectively, and the isolates were identified using API-web (AB bioMérieux).

5.2.2 Identification of lactic acid bacteria species and acetic acid bacteria species using Sanger Sequencing method

All the isolates that had been identified and confirmed using API were then confirmed using the Sanger sequencing method.

All the LAB and Acetic acid bacteria isolates were grown in triplicate in MRS broth at 30°C for 48 h. The broths were centrifuged at 5000 g for 10min (Heraeus Labofuge 200; Thermo Scientific). The pellet was collected followed by DNA isolation. For DNA isolation, the cell

pellets were first re-suspended in double distilled water and then microwaved for 30 secs at 150°C (Living and Co., New Zealand). This was followed by centrifugation at 1000 g for 5 min (Heraeus Labofuge 200; Thermo Scientific). The supernatant (about 5µl) was collected for carrying out polymerase chain reaction (PCR) for DNA amplification (Dashti, Jadaon, Abdulsamad, & Dashti, 2009).

For carrying out the PCR reaction, the primer sets: PCR1 forward (5' TCGTCGGCAGCGTCAGATGTGTATAAGAGA CAGCCTACGGGNGGCWGCAG 3') and PCR1 reverse (5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGA CTACHVGGGTATCTAATCC 3') with KAPA HiFi Hotstart Readymix (Kapa Biosystems, Wilmington, MA, USA) were used. The following thermocycling parameters were set: (1) 95°C for 3 min, (2) 25 cycles of 95°C for 30 secs, 55°C for 30 secs, 72°C for 30 secs, 72°C for 5 min, and (3) holding the samples at 4°C. Qubit double-stranded DNA high-sensitivity reagent kit (Life Technologies, Foster City, CA, USA) with a Qubit fluorometer (Thermo Fisher Scientific, Waltham, USA) was used to quantify the PCR output.

The quality and the size of the amplicon were assessed using a high sensitivity DNA kit on a 2100 bioanalyser instrument (Agilent Technologies, Santa Clara, CA, USA) with a DNA 100 chip. Once the size of about 500 base pairs was confirmed, the amplicon was used as a template for the index PCR.

The amplicons were then indexed using Nextera XT index kit with an additional eight PCR amplification cycles using the same PCR conditions as above. The indexed amplicons were purified and size-selected using AMPure XP beads (Beckman-Coulter, Brea, CA, USA) before sequencing by the Sanger sequencing method, with the 600 cycle V3 chemistry (300 base paired-end reads). A 5% PhiXspike-in was used, as per the manufacturer's recommendation.

Sequencing reactions to incorporate the fluorescent dyes were performed using the ABI PRISM® BIG DYE Terminator Sequencing Kits version 3.1. The following cycle sequencing was carried out in a PCR (Applied Biosystems GeneAmp® PCR System 9700). Agencourt® CleanSEQ® magnetic beads were used to remove the unincorporated fluorescent dyes. Sequencing products were then separated by capillary electrophoresis on the ABI PRISM® 3130XL Genetic Analyzer using POP7 polymer and 50cm capillaries.

In addition, Sanger sequencing was carried out at Auckland Genomics, DNA Sequencing Facility at the School of Biological Sciences, The University of Auckland. The raw data (DNA sequences) were received and analysed using Geneious prime Bioinformatics Software Pro 5.6 (Kearse et al., 2012) (Geneious, New Zealand). It is a tool that allows one to super-impose the forward and the reverse sequences for an isolate to obtain a complete sequence that can then be identified using

the National Center for Biotechnology Information- Basic local alignment search tool (NCBI) BLAST.

Sequence similarity searching was done using BLAST, available in the NCBI web site (Suntio and MÄKinen 2012). Multiple sequence alignments provided information about characters (nucleotide bases or amino acids) and regions (also called motifs or domains) that show similarities and differences. These also provided measures or scores of similarity which can help identify the percentage match of the sequence in question with those available in the database.

5.2.3 DNA isolation, PCR and MiSeq high-throughput sequencing methods (Illumina Sequencing)

Coconut water, kefir, coconut water kefir and sourdough prepared with coconut water kefir were used for this study. In order to understand the microbial ecology of coconut water kefir and sourdough prepared with coconut water kefir at a given time point, all the samples were prepared separately. Coconut water and kefir samples were used "as is" (DNA was extracted from 1g of the kefir powder dissolved in 5 ml of phosphate buffer saline). For coconut water kefir, 1.5 g/L of kefir grains were added to 300 ml coconut water and a time zero (T=0 hour) sample was taken and stored at -80°C. Coconut water kefir was then allowed to ferment at 30°C for next 24 h. Therefore, samples were collected at 0 h, 24 h, 48 h, 72 h and 96 h and were stored for a brief period of time at -80°C before DNA isolation. Coconut water kefir sample fermented for 48 h was used as an inoculum to prepare coconut water kefir sourdough, which was prepared by adding 300 ml of coconut water kefir mixture, 600g of flour, 3 gm of salt and 1.5 ml of oil.

Sourdough was proofed at 30°C at 60% humidity. Samples from coconut water kefir sourdough were taken at 0 h, 24 h, 48 h, 72 h and 96 h and were stored at -80°C for a brief period of time before DNA isolation. All the samples were taken in triplicates.

Samples from dough were taken as a pizza wedge slice to ensure that all the microflora- from the deep middle to the circumference of the dough were being sampled. In order to isolate the DNA from dough samples, a 1:1 ratio of dough: autoclaved Phosphate Buffer Saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) was mixed in a homogenizer (L5M-A Laboratory Mixer, Silverson®) and was allowed to digest for 1 h. This was done to make the dough mushy and soft in order to liberate the microbial DNA from the wheat matrix of the dough.

Thus, DNA was isolated each from coconut water, kefir, coconut water kefir (5-Samples* 3-triplicates) and Coconut water kefir sourdough (5-Samples* 3-triplicates) samples, using PowerFood DNA Isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA) following the

manufacturer's protocol. All extractions were carried out in a laminar flow hood under aseptic conditions. To improve cell lysis efficiency, tubes containing the samples and buffer solution were incubated in a water bath at 65°C for 30min before vortexing. An extraction blank was prepared, which served the purpose of blank used in this study. The extracted DNA was quantitated using NanoVue (GE Healthcare) followed by PCR.

Bacteria and yeasts were targeted for identification since these are the most abundant microorganisms in the sourdough (Ercolini et al., 2013). For carrying out the PCR reaction, primer set PCR was conducted with primer sets targeting the V3–V4 regions of the bacterial and archaeal 16S rRNA gene: PCR1 forward (5' TCGTCGGCAGCGTCAGATGTGTATAAGAGA CAGCCTACGGGNGGCWGCAG 3') and PCR1 reverse (5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGA CTACHVGGGTATCTAATCC 3') with KAPA HiFi Hotstart Readymix (Kapa Biosystems, Wilmington, MA, USA) were used and the following thermocycling parameters: (1) 95°C for 3 min, (2) 25 cycles of 95°C for 30 secs, 55°C for 30 secs, 72°C for 30secs, 72°C for 5 min, and (3) holding the samples at 4°C were set.

PCR quantification was carried out using Qubit double-stranded DNA high-sensitivity reagent kit (Life technologies, Foster City, CA, USA) with a Qubit fluorometer (Thermo Fisher Scientific, Waltham, USA). The quality and the size of the amplicon were assessed using a high sensitivity DNA kit on a 2100 bio-analyser instrument (Agilent Technologies, Santa Clara, CA, USA) with a DNA 100 chip. Once the size of about 500 base pairs was confirmed, the amplicon was used as a template for the index PCR.

The amplicons were then indexed using Nextera XT index kit (Illumina) with an additional eight PCR amplification cycles with the same PCR conditions as above. The indexed amplicons were purified and size-selected using AMPure XP beads (Beckman-Coulter, Brea, CA, USA) before sequencing on an Illumina Miseq (Illumina) with the 600 cycle V3 chemistry (300 base paired-end reads). A 5% PhiXspike-in was used, as per the manufacturer's recommendations.

Since the objective was to estimate the microbial diversity of the samples at a given time point during fermentation (results as shown in section 5.3.3), with respect to decreasing pH and increasing acidity, samples were sequenced to find out the operational taxonomic units (OTUs), indicative of the species of the microorganisms. Amplicon library preparation was performed according to recommended protocols (Illumina Demonstrated Protocol: 16S Metagenomic Sequencing Library Preparation -Amplicon, Clean-Up, & Index). Up to 96 uniquely indexed libraries were pooled per sequencing run, which was performed on an Illumina MiSeq using 300-cycle V2 chemistry (150 bp paired-end reads) following the manufacturer's recommendations. No-template and extraction reagent blanks (EXB) controls were also sequenced to establish

background bacterial populations. The method of analysis for Illumina output followed that published by Archer et al. (2019).

5.2.4 Bioinformatics analysis of MiSeq Illumina data and NCBI BLAST for acquired Sanger sequences

The microbial composition of each sample was examined by processing the raw sequence reads using USEARCH v 8.0.1623 (Edgar, 2013). The quality of these raw sequencing reads was analysed using USEARCH tool to remove abnormal sequences. First, the forward and reverse paired-end reads were combined, and the merged reads with lengths outside 200–500bp range or exceeding six homopolymers were removed by Mothurv 1.36.1 (Schloss et al., 2009). Next, the sequences were subjected to *Q* score filtering to remove reads with maximum expected error > 1. Singleton reads were then removed. Representative sequences of the operational taxonomic units (OTUs) were taxonomically assigned using the RDP classifier implemented in QIIME v1.9.1 (Caporaso et al., 2010). Greengenes release13_8 was used as the reference taxonomic database (McDonald et al., 2012).

5.2.5 Statistical analysis

The National Center for Biotechnology Information (NCBI) – BLAST (basic local alignment search tool) was used for identification of the sequences received from Sanger sequencing. The microbial composition for each sample was examined by processing the raw sequence reads using USEARCH v 8.0.1623 (Edgar, 2013). The quality of these raw sequencing reads was analysed using USEARCH tool to remove anomalous sequences.

5.3. Results and Discussion

5.3.1 Preliminary identification of LAB, AAB and yeast

Microbial growth was observed in the MRS agar and broth, AAB agar and broth and Malt extract agar and broth media for kefir grains, coconut water fermented with kefir and sourdough prepared with CWK (at different time intervals of 0, 24, 48, 72 and 96 h). The phenotypic characterisation of all the microorganisms was carried out to identify and group the isolates as LAB, AAB or yeast.

During the morphological screening, a total of 22 isolates of LAB (10 isolates), AAB (5 isolates) and yeasts (7 isolates) were identified from kefir (K), CWK and CWK fermented sourdough (CWKS), as discussed below. Upon completion of the phenotypic screening, only 13 isolates of all LAB, yeasts and AAB were confirmed.

All the preliminary 10 isolates were considered as putative LAB since the isolates were Gram positive, non-motile, catalase negative and non-spore forming. They occurred in short rods, singly, in pairs or short chains. All isolates tolerated the bile salt concentration 0.1-2% and grew at 15°C but not at 45°C. No growth was observed for any strains at 10% NaCl concentration, whereas at 6.5% NaCl, growth was positive for all except one isolate.

All the 5 preliminary isolates were identified as putative AAB since they were white-cream in colour and had opaque colonies with smooth surface. They were Gram negative and catalase positive. The cells were bacilliform, and further physiological and biochemical tests showed that they belonged to the genus Acetobacter.

Seven putative isolates of yeasts had white and cream coloured colonies and had an irregular surface appearance. The morphology of these cells was oval and had budding. The cell morphologies of these isolates are presented in Table 5.3.

The presumptive isolates were further characterised using growth at different temperatures (10°C, 30°C and 45°C), on media with different pH and salt conditions, and in different sugar media (using API kits, according to the manufacturer's instructions).

The results of the carbohydrate tests for LAB, AAB and yeast are shown in Tables 5.1, 5.2 and 5.3.

Based on the API results, fermentation occurred when there was a change in the colour of the medium from purple to yellow along with carbondioxide production. As mentioned in Table 5.1 for LAB, *L. fermentum* was able to ferment D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, maltose, lactose, melibiose, sucrose, starch and gluconate. *L. plantarum* was able to ferment all carbohydrates except D-xylose, amygdalin, melezitose, starch, glycogen and D-

lyxose. *L. fusant* fermented sucrose, trehalose, maltose, salicin, N-acetyl-glucosamine, Mannitol, D-mannose, D-fructose, D-glucose, galactose and ribose. *L. reuteri* fermented raffinose, D-ribose, sucrose, melibiose, lactose, maltose, esculin, arbutin, amygdalin, mannitol, D-glucose and D-galactose. *L. kunkeei* fermented trehalose, sucrose, melibiose, mannitol and D-fructose.

API results for AAB (Table 5.2) shows that *A. aceti* fermented glucose, rhamnose, melibiose, amygdalin and arabinose. *A. lovaniensis* fermented melibiose, amygdalin, arabinose, nitrogen dioxide and nitrite. *A. pasteurianus* fermented arginine dihydrolase, urease and acetoin.

Similarly, the API results for yeasts (Table 5.3) show that *Candida kefyr* fermented D-Glucose, glycerol, D-xylose, xylitol, D-galactose, D-sorbitol, D-lactose, D-saccharose and D-raffinose. *Rhodotorula mucilaginosa* fermented D-glucose, adonitol, xylitol, D-galactose, D-maltose, D-saccharose and D-raffinose. *Saccharomyces cerevisiae* fermented D-glucose, D-galactose, D-maltose, D-saccharose and D-raffinose. *Candida guilliermondii* fermented everything except inositol and D-lactose. *Candida colliculosa* fermented D-glucose, glycerol, D-sorbitol, D-saccharose, D-trehalose and D-raffinose.

Overall, out of the 22 isolates, only five species of LAB, three species of AAB were identified and 5 species of yeast were identified. Possibly, some of the 22 isolates were identical to each other. The species identified were *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus fusant, Lactobacillus reuteri, Lactobacillus kunkeei, Acetobacter aceti, Acetobacter lovaniensis, Acetobacter pasteurianus, Candida kefyr, Rhodotorula mucilaginosa, Saccharomyces cerevisiae, Candida guilliermondii* and *Candida colliculosa*.

All of the identified LAB utilized galactose, glucose and sucrose, and, thus, were the main organisms responsible for the fermentation. Such similar characterisation for kefir has been carried out by Diosma, Romanin, Rey-Burusco, Londero, & Garrote (2014), Witthuhn, Schoeman, & Britz (2005) and Zanirati et al., (2015). The microbial composition may vary according to kefir origin, the substrate used in the fermentation process and the culture maintenance methods. Tibetan kefir, which is used in China, is composed of *Lactobacillus, Lactococcus*, and yeast. Sun et al. (2015) have reported the presence of over 200 species of LAB in a traditionally fermented sourdough and have highlighted the commercially important LAB species. Various LAB species have also been identified in milk kefir and have been reported by Zanirati, Abatemarco, et al. (2015).

The presence of AAB in kefir has been reported by Garrote, Abraham, & De Antoni (2002). Additionally, AAB have been identified in Tibetan kefir, depending on the region in China from where it was obtained (Gao, Gu, Abdella, Ruan, & He, 2012). Furthermore, Tibetan kefir composition differs from that of Russian kefir, Irish kefir, Taiwan kefir, and Turkey fermented beverage with kefir; however, it is known that this microbial diversity is responsible for the

physicochemical features and biological activities of each kefir (Altay, Karbancıoglu-Güler, Daskaya-Dikmen, & Heperkan, 2013; Zhou, Liu, Jiang, & Dong, 2009; Kabak & Dobson, 2011)

Yeasts were identified according to the criteria of Kurtzman, Fell, & Boekhout (2011). Five species belonging to 3 genera were identified (Table 5.1). Despite many years of study, the microbiological composition of kefir grains is still being explored. The food standards of the Food and Agriculture Organization of the United Nations stated that kefir possesses lactose-fermenting yeasts (*Candida kefyr*) and non lactose-fermenting yeasts (*Rhodotorula mucilaginosa, Saccharomyces cerevisiae, Candida guilliermondii* and *Candida colliculosa*), but the yeast composition has not been fully defined although other authors have found additional species (Mainville et al. 2006). Among the yeasts registered in kefir grains from different origins the genera *Zygosaccharomyces, Candida, Kluyveromyces, Saccharomyces, Torulaspora, Issatchenkia, Pichia,* and *Debaryomyces* may be mentioned (Ahmed et al. 2013); with *Candida, Saccharomyces,* and *Rhodotorula* being isolated in the present work from the CWK and sourdough fermented with CWK. The composition of kefir microflora strongly depends on the origin of the grains, on the local conditions of culture, and on the storage and elaboration processes (Garrote et al. 2001, 2010).

Table 5.1: Results of cell and colony morphology, physiological and biochemical tests performed on LAB isolates. Isolates identified from multiple sources such as kefir (K), CWK and/or CWK fermented sourdough (CWKS) have been labelled accordingly.

Test	***Isolate 1*** **(Source-K, CWK, CWKS)**	***Isolate 2*** **(Source-K, CWK, CWKS)**	**Isolate 3 (Source-K, CWK, CWKS)**	***Isolate 4*** **(Source-K, CWK, CWKS)**	***Isolate 5*** **(Source-K, CWK, CWKS)**	***Isolate 6*** **(Source-K, CWK, CWKS)**	***Isolate 7*** **(Source-K, CWK, CWKS)**	***Isolate 8*** **(Source-K, CWK, CWKS)**	***Isolate 9*** **(Source-K, CWK, CWKS)**	***Isolate 10*** **(Source-K, CWK, CWKS)**
Colony colour	Transparent	Transparent	Transparent	Transparent	Transparent	Transparent	Transparent	Transparent	Transparent	Transparent
Surface appearance	Smooth/moist	Smooth/moist	Smooth/moist	Smooth/moist	Smooth/moist	Smooth/moist	Smooth/moist	Smooth/moist	Smooth/moist	Smooth/moist
Cell morphology	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli
Gram reaction	+	+	+	+	+	+	+	+	+	+
Catalase test	−	−	−	−	−	−	−	−	−	−
Spore formation	−	−	−	−	−	−	−	−	−	−
Nitrate reduction test	−	−	−	−	−	−	−	−	−	−
Indole test	−	−	−	−	−	−	−	−	−	−
6.5% NaCl growth test	−	−	−	+	+	+	+	+	+	+
10% NaCl growth test	−	−	−	−	−	−	−	−	−	−
4°C growth test	−	−	−	−	−	−	−	−	−	−
15°C growth test	+	+	+	+	+	+	+	+	+	+
45°C growth test	−	−	−	−	−	−	−	−	−	−
Growth at pH 3	−	−	−	−	−	−	−	−	−	−
Growth at pH 4	−	−	−	−	−	−	−	−	−	−
Growth at pH 5	−	−	−	−	−	−	−	−	−	−
0.1% Bile salt	+	+	+	+	+	+	+	+	+	+
0.9% Bile salts	+	+	+	+	+	+	+	+	+	+
1.5% bile salts	+	+	+	+	+	+	+	+	+	+
2% bile salts	+	+	+	+	+	+	+	+	+	+

Gas production during glucose fermentation in durhams tube	+	+	+	+	+	+	+	+	+	+
Glycerol	−	−	−	+	+	+	−	−	−	−
D-Ribose	−	−	−	+	+	+	+	+	+	−
D-xylose	+	+	+	−	−	−	−	−	−	−
D-Galactose	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	−	−	+
D-Mannose	+	+	+	+	+	+	+	−	−	−
Mannitol	−	−	−	+	+	+	+	+	+	+
Sorbitol	−	−	−	+	+	+	−	−	−	−
N-acetyl-glucosamine	−	−	−	+	+	+	+	−	−	−
Amygdalin	−	−	−	−	−	−	−	+	+	−
Arbutin	−	−	−	+	+	+	−	+	+	−
Esculin	−	−	−	+	+	+	−	+	+	−
Salicin	−	−	−	+	+	+	+	−	−	−
Cellobiose	−	−	−	+	+	+	−	−	−	−
Maltose	+	+	+	+	+	+	+	+	+	−
Lactose	+	+	+	+	+	+	−	+	+	−
Melibiose	+	+	+	+	+	+	−	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+
Trehalose	−	−	−	+	+	+	+	−	−	+
Melezitose	−	−	−	−	−	−	−	−	−	−
D-Raffinose	−	−	−	+	+	+	−	+	+	−
Starch	+	+	+	−	−	−	−	−	−	−
Glycogen	+	+	+	−	−	−	−	−	−	−
D-Gentiobiose	−	−	−	+	+	+	−	−	−	−
D-Turanose	−	−	−	+	+	+	−	−	−	−

D-Lyxose	–	–	–	–	–	–	–	–	–	–
D-Tagatose	–	–	–	+	+	+	–	–	–	–
D-Arabitol	–	–	–	+	+	+	–	–	–	–
Gluconate	+	+	+	+	+	+	–	–	–	+
Fermentation	Obligately Heteroferme ntative	Obligately Heteroferme ntative	Obligately Heteroferme ntative	Facultatively Heteroferme ntative	Facultatively Heteroferme ntative	Facultatively Heteroferme ntative	Obligately Heteroferme ntative	Obligately Heteroferme ntative	Obligately Heteroferme ntative	Obligately Heteroferme ntative
Isolate identified as	***Lactobacillus fermentum***	***Lactobacillus fermentum***	***Lactobacillus fermentum***	***Lactobacillus plantarum***	***Lactobacillus plantarum***	***Lactobacillus plantarum***	***Lactobacillus fusant***	***Lactobacillus reuteri***	***Lactobacillus reuteri***	***Lactobacillus kunkeei***

Table 5.2: Results of cell and colony morphologies, physiological and biochemical tests performed on AAB isolates. Isolates identified from multiple sources such as kefir (K), CWK and/or CWK fermented sourdough (CWKS) have been labelled accordingly.

Tests	***Isolate 1*** **(Source - K, CWK, CWKS)**	***Isolate 2*** **(Source- K, CWK, CWKS)**	***Isolate 3*** **(Source -K, CWK, CWKS)**	***Isolate 4*** **(Source- K, CWK, CWKS)**	***Isolate 5*** **(Source-K, CWK, CWKS)**
Colony colour	White and opaque	White and opaque	White and opaque	White and opaque	Cream colour and opaque
Surface appearance	Smooth	Smooth	Smooth	Smooth	Smooth
Cell morphology	Bacilli or oblong	Bacilli or oblong	Bacilli or oblong	Bacilli or oblong	Oblong
Gram reaction test	−	−	−	−	−
Catalase test	+	+	+	+	+
Oxidation of ethanol	+	+	−	−	−
Oxidation of acetic acid	−	−	−	−	−
Methyl red test	−	−	−	−	−
Voges-Proskauer test	−	−	−	−	−
30% glucose growth test	−	−	−	−	−
arginine dihydrolase	−	−	−	−	+
Lysine	−	−	−	−	−
ornithine decarboxylase	−	−	−	−	−
citrate utilization	−	−	−	−	−
Dihydrogen sulphide	−	−	−	−	−
Carr's medium	+	+	+	+	+
Urease test	−	−	−	−	+
tryptophane deaminase	−	−	−	−	−
Indole test	−	−	−	−	−
Acetoin	−	−	−	−	+
Fructose	+	+	+	+	+
Ribose	+	+	+	+	+
Xylose	+	+	+	+	+
Galactose	+	+	+	+	+
Mannitol	−	−	−	−	−
Raffinose	−	−	−	−	−
Inositol	−	−	−	−	−
Sorbitol	−	−	−	−	−
Rhamnose	+	+	−	−	−
Sucrose	−	−	−	−	−
Melibiose	+	+	+	+	−
Amygdalin	+	+	+	+	−
Arabinose	+	+	+	+	−
Nitrogen dioxide	−	−	+	+	−
Nitrite	−	−	+	+	−
Isolate identified as	***Acetobacter aceti***	***Acetobacter aceti***	***Acetobacter lovaniensis***	***Acetobacter lovaniensis***	***Acetobacter pasteurianus***

Table 5.3: Results of cell and colony morphology, physiological and biochemical tests performed on yeast isolates. Isolates identified from multiple sources such as kefir (K), CWK and/or CWK fermented sourdough (CWKS) have been labelled accordingly.

Test	***Isolate 1*** **(Source- K, CWK, CWKS)**	***Isolate 2*** **(Source-K, CWK, CWKS)**	***Isolate 3*** **(Source-K, CWK, CWKS)**	***Isolate 4*** **(Source-K, CWK, CWKS)**	***Isolate 5*** **(Source-K, CWK, CWKS)**	***Isolate 6*** **(Source-K, CWK, CWKS)**	***Isolate 7*** **(Source-K, CWK, CWKS)**
Colony colour	Cream	Pink – red	Milky white	Milky white	Milky white	White	White
Surface appearance	Glossy and smooth	Smooth and glossy	Smooth	Smooth	Smooth	Smooth	Smooth
Cell morphology	Oval	Oval	Oval	Oval	Oval	Oval	Oval
Vegetative propogation	Budding	Budding	Budding	Budding	Budding	Budding	Budding
Mycelium growth test	pseudohypha	pseudohypha	pseudohypha	pseudohypha	pseudohypha	pseudohypha	pseudohypha
Urease test	−	−	−	−	−	−	−
Nitrate reduction test	−	−	−	−	−	−	−
D-Glucose as a carbon source	+	+	+	+	+	+	+
Glycerol	+	−	−	−	−	+	+
Calcium 2-Keto-Gluconate	−	−	−	−	−	+	−
L-Arabinose	−	−	−	−	−	+	−
D-Xylose as a carbon source	+	−	−	−	−	+	−
Adonitol	−	+	−	−	−	+	−
Xylitol	+	+	−	−	−	+	−
D-Galactose as a carbon source	+	+	+	+	+	+	−
Inositol	−	−	−	−	−	−	−
D-Sorbitol as a carbon source	+	−	−	−	−	+	+
Methyl-α-D-Glucopyranoside	−	−	−	−	−	+	−
N-Acetyl-Glucosamine	−	−	−	−	−	+	−
D-Cellobiose as a carbon source	+	−	−	−	−	+	−
D-Lactose as a carbon source	+	−	−	−	−	−	−
D-Maltose as a carbon source	−	+	+	+	+	+	−

D-Saccharose as a carbon source	+	+	+	+	+	+	+
D-Trehalose as a carbon source	–	–	–	–	–	+	+
D-Melezitose as a carbon source	–	–	–	–	–	+	–
D-Raffinose as a carbon source	+	+	+	+	+	+	+
Isolate identified as	***Candida kefyr***	***Rhodotorula mucilaginosa***	***Saccharomyces cerevisiae***	***Saccharomyces cerevisiae***	***Saccharomyces cerevisiae***	***Candida guilliermondii***	***Candida colliculosa***

5.3.2 Molecular identification of LAB and AAB isolates using Sangers sequencing

The LAB and AAB isolates obtained above were sequenced using the Sanger sequencing method. DNA was isolated from each of the identified isolates (pure culture colonies of each isolate) (Section 5.3.1) and was purified before Sanger sequencing.

The short sequence 16s rDNA which was obtained, helped confirm the identity of LAB and AAB identified using API kits. The species were confirmed as shown in Table 5.4. The partial sequences for both LAB and AAB are as shown in Appendix 13, and which were the final output of a completed alignment of forward and reverse sequences received. The sequences were aligned using Geneious® 11.1.4 after manually removing the noise on both the ends of the forward and reverse sequences. These partial sequences were then confirmed using NCBI BLAST and the identification of species for the organisms was selected for the one with the highest maximum score and the lowest E – value (for high significance).

The yeast sequences could not be confirmed by using Sanger sequencing method since no eukaryotic (yeast specific) primers were available in the laboratory for sequencing the isolates. Therefore, the yeast isolates were identified using phenotypic characteristics only.

Most of the LAB and AAB which have been identified in this study such as *L. fermentum, L. plantarum, L. fusant, L. reuteri, L. kunkeei, A. aceti, A. lovaniensis* and *A. pasteurianus* have been identified and reported for kefir in previous studies. Studies carried out by Sakamoto, Tanaka, Sonomoto, & Nakayama, (2011) and Miyashita et al. (2012) identified *L. plantarum, L. rueteri* and *L. fermentum* from sourdough by using 16s rRNA gene sequencing and pyrosequencing-based methods. *Acetobacter aceti* and *Acetobacter pasteurianus* have been previously used in the preparation of sourdough in a study carried out by De Pauw (2018). Acetobacter species have also occasionally been identified and detected in sourdough (Minervini et al., 2012; Ripari et al., 2016). The identification of *Acetobacter aceti*, *Acetobacter lovaniensis* and *Acetobacter pasteurianus* has been carried in kefir and kefir beverages by Rattray and O'Connell (2011), Rosa et al. (2017), Garofalo et al. (2015), Prado et al. (2015), Bourrie et al. (2016) and Walsh et al. (2016). In a separate study carried out on Brazilian kefir grains, Magalhães, Pereira, Campos, Dragone, & Schwan (2011) have reported that the acetic acid bacteria, *Acetobacter lovaniensis*, has been isolated from kefir along with some LAB and yeast species such as *Lactobacillus paracasei, Lactobacillus parabuchneri, Lactobacillus casei, Lactobacillus kefiri, Lactococcus lactis* and *S. cerevisiae* using 16s rRNA gene sequencing methods.

A common sourdough LAB, *L. sanfranciscensis* was not identified in this study, which could be due to the sourdough formulated in this study being a laboratory type 2 fermented sourdough prepared using kefir powder. The coconut water was used as a growth medium for fermenting the kefir grains. Perhaps, due to lack of nutrients, the *L. sanfranciscensis* strain might have been lost.

The absence of this species might also be due to the presence of competitive bacterial strains. The complexity and stability of the sourdough microbiota depend on a number of determinants, which include environmental microbiota (e.g. microbiota of flour and other ingredients and house microbiota) and their potential metabolic activities (e.g. cofactor regeneration capability and energy synthesis from various sources), and technology parameters (e.g. chemical and enzyme composition of the flour, leavening temperature, pH and redox potential, dough yield, and number and length of sourdough refreshments) (De Vuyst, Vrancken, Ravyts, Rimaux, & Weckx, 2009; Gänzle, Vermeulen, & Vogel, 2007; Gänzle & Vogel, 2003; Gobbetti, De Angelis, Corsetti, & Di Cagno, 2005; Hammes & Gänzle, 1998). Bessmeltseva et al., (2014), Ercolini et al. (2013), Minervini et al. (2015), Rizzello et al. (2015) and Van der Meulen et al. (2007) have reported that *L. sanfranciscensis* is generally absent in laboratory-made sourdoughs started with flour only.

Table 5.4: Sanger sequencing of each isolate of LAB and AAB species.

The E – value*- determines the number of hits one can "expect" to see by chance when searching a database of a particular size, which in this case is 0. Therefore, this implies that there were no random hits in the identification of LAB and AAB species on NCBI BLAST. Accession number*- is the unique identification number on the NCBI BLAST database. Maximum score*- is the total score which has been identified as that particular microorganism, which is the highest alignment score of a set of aligned segments from the same subject (database) sequence. The percentage of identity was determined by performing multiple sequence alignments in BLAST.

Identified organisms (% identity match)	**Accession number***	**E-value***	**Maximum score***
Lactobacillus fermentum strain CAU6479 16S ribosomal RNA gene, partial sequence (100)	MF582851.1	0.0	2619
Lactobacillus plantarum strain RS66X 16S ribosomal RNA gene, partial sequence (100)	MN450268.1	0.0	2536
Lactobacillus Fusant XU1 16S ribosomal RNA gene, partial sequence (99)	KT335719.1	0.0	2687
Lactobacillus reuteri DSM 20016, 16S ribosomal RNA gene, partial sequence (100)	NR_119069.1	0.0	2883
Lactobacillus kunkeei strain H14_2_1BCO2 16S ribosomal RNA gene, partial sequence (100)	KF599421.1	0.0	2582
Acetobacter aceti strain W1 16S ribosomal RNA gene, partial sequence (100)	KC662508.1	0.0	2676
Acetobacter lovaniensis strain NBRC 13753 16S ribosomal RNA, partial sequence (100)	NR_040832.1	0.0	2665
Acetobacter pasteurianus strain bh12 16S ribosomal RNA gene, partial sequence (100)	FJ227313.1	0.0	2326

5.3.3 Characterisation of the relative abundance of microorganisms using Miseq high-throughput sequencing method

To understand the diversity of the sourdough fermented with CWK and CWK during fermentation, 16s rDNA analysis was carried out. Samples from CWK fermented sourdough and CWK were taken at 0, 48 and 96 h time intervals to understand the ecological succession of microorganisms. Samples of kefir and coconut water were also investigated using the high throughput Illumina sequencing method in order to understand the microbial community present in them.

Results of the sequencing in Figure 5.1, show that the LAB were dominant throughout the duration of fermentation of CWK and CWK-fermented sourdough, as represented by the Family: Lactobacillaceae, Genus: Lactobacillus with a dark orange – light brown and a sky blue shade.

The relative abundance of the microorganisms using Illumina sequencing output was studied and it was found that overall, from all the samples, 8 LAB species (*Lactococcus lactis, Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus delbrueckii, Lactobacillus paracasei, Lactobacillus brantae, Lactobacillus reuteri* and *Lactobacillus kunkeei*) were highly abundant amongst all the samples, but was highest for CWK fermented sourdough (Figure 5.2).

It is interesting to note that five out of the 8 species identified using the Illumina sequencing method, which are *Lactococcus lactis, Lactobacillus rhamnosus, Lactobacillus delbrueckii, Lactobacillus paracasei* and *Lactobacillus brantae,* were not identified using culture – dependent phenotypic identification method. This might be due to the bacteria not being properly isolated and purified. Moreover, species with a large population size in the sample might give greater amounts of template DNA, and therefore they had a higher probability of detection by Illumina sequencing method compared to the microbial identification methods (Prakitchaiwattana et al., 2004).

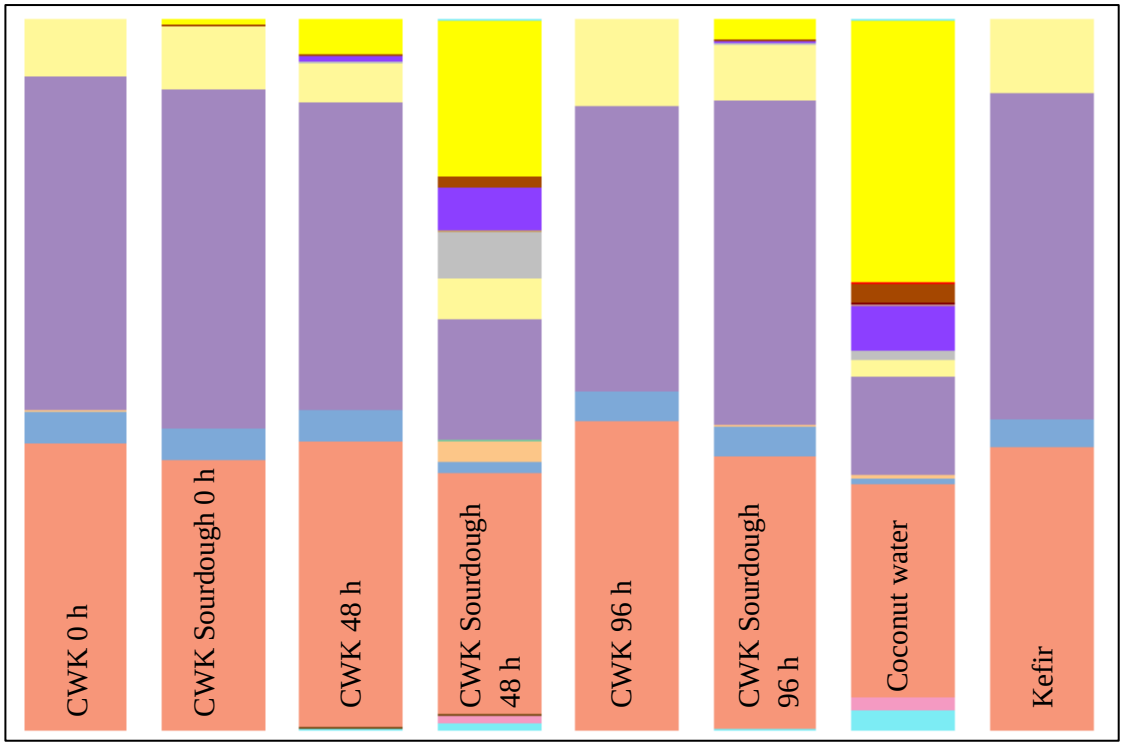
LAB species were most abundant in fermented sourdough and fermented CWK but less so in the kefir powder (Figure 5.2). However, they were detected in all the samples. The only exception is for *Lactococcus lactis* and *Lactobacillus plantarum*, which were equally abundant in CWK - fermented sourdough and kefir, then followed by CWK. It is well known that *Lactobacillus plantarum* is an endophytic LAB. It was also observed that *Lactobacillus reuteri, Lactobacillus plantarum, Lactococcus lactis* and *Lactobacillus brantae* were more highly abundant LAB compared to the rest of the LAB species.

Lactobacillus plantarum and *Lactococcus lactis* are commonly found in kefir grains and sourdough (Farnworth & Mainville, 2008; Witthuhn, Schoeman, & Britz, 2005). A study carried out by Chen, Wang, & Chen (2008) has reported the presence of various LAB species in kefir including *Lactobacillus rhamnosus*, *Lactobacillus delbruecki* and *Lactobacillus paracasei*, using a polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) method. Detection of *Lactobacillus reuteri* in kefir has been reported by Bourrie, Willing, & Cotter (2016) and Rosa et al. (2017). These species have also been identified in sourdough during fermentation, as reported by Huys et al. (2013) and Minervini et al. (2014). A sequencing study carried out on wholemeal flour-based sourdough carried out by De Angelis, Minervini, Siragusa, Rizzello, & Gobbetti, (2019) has identified several LAB species including *Lactobacillus brantae*.

As discussed earlier, the most common types of LAB that have been previously identified for laboratory type 2 sourdough were *Lactobacillus sanfranciscensis, Lactobacillus brevis,*

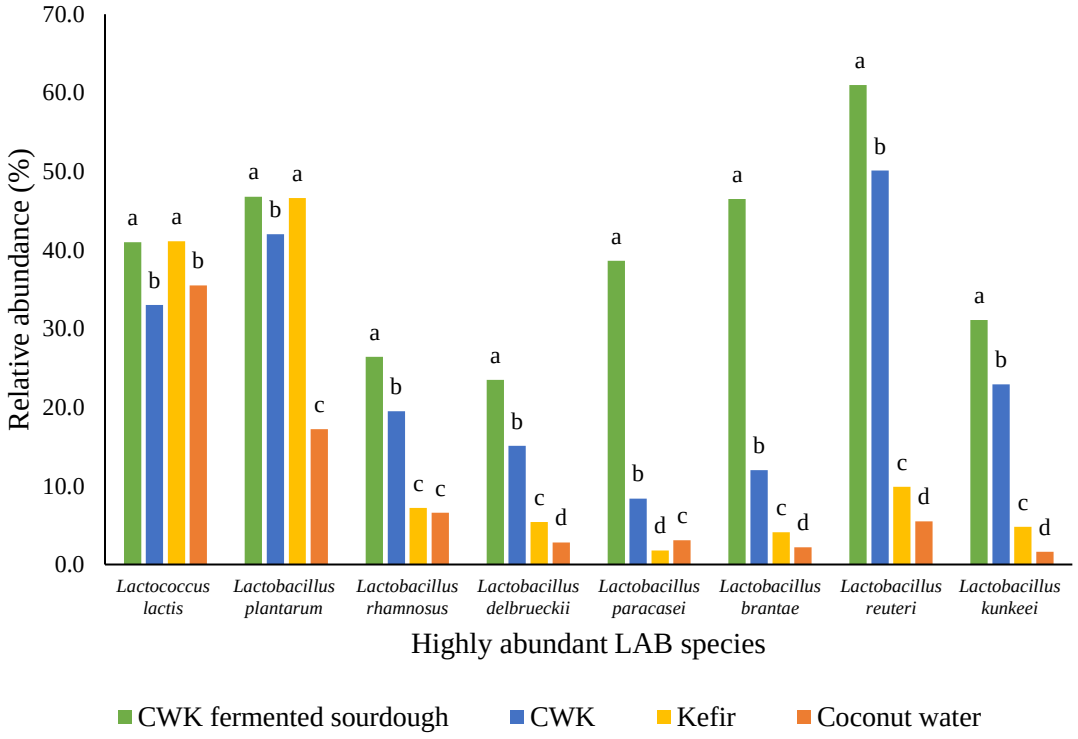
Lactobacillus fermentum, Lactobacillus reuteri, Lactobacillus pontis, Lactobacillus panis and *Weissella spp.* (Corsetti and Settanni, 2007; De Vuyst and Neysens, 2005; De Vuyst and Vancanneyt, 2007; Ehrmann and Vogel, 2005; Ganzle and Gobbetti, 2013; Huys et al., 2013). Two out of the above mentioned LAB, which were *Lactobacillus fermentum* and *Lactobacillus reuteri* have been identified in the present study, and which were both obligately heterofermentative microorganisms. *L. plantarum*, which has also been identified in this study is a facultative heterofermentative LAB which is usually isolated and identified from type three backslopped sourdough (Papadimitriou et al., 2019). This could also be attributed to the acids produced upon fermentation of CWK and CWKS as mentioned in section 3.3.5 and section 4.3.5 in Chapter 3 and Chapter 4 respectively.

Figure 5.1: Stacked bar graph representing the bacterial compositions of CWK fermented sourdough (at times, T=0, 48 and 96h), fermented CWK (at times, T = 0, 48 and 96h), coconut water and kefir determined by partial 16S rRNA gene sequencing.



Legend	Taxonomy
	Family: Corynebacteriaceae, Genus: Corynebacterium
	Family: Microbacteriaceae, Genus: Salinibacterium
	Family: Nocardiaceae, Genus: Rhodococcus
	Family: Propionibacteriaceae, Genus: Propionibacterium
	Family: Sphingobacteriaceae, Genus: Pedobacter
	Family: Bacillaceae, Genus: Anoxybacillus
	Family: Bacillaceae, Genus: Bacillus
	Family: Planococcaceae, Genus: Lysinibacillus
	Family: Enterococcaceae;Other,
	Family: Lactobacillaceae
	Family: Lactobacillaceae, Genus: Lactobacillus
	Family: Leuconostocaceae, Genus: Leuconostoc
	Family: Leuconostocaceae, Genus: Weissella
	Family: Streptococcaceae, Genus: Lactococcus
	Family: Streptococcaceae, Genus: Streptococcus
	Family: Clostridiaceae, Genus: Clostridium
	Family: [Tissierellaceae], Genus: Anaerococcus
	Family: [Tissierellaceae], Genus: Finegoldia
	Family: Rhizobiaceae, Genus: Agrobacterium
	Family: Rhizobiaceae, Genus: Rhizobium
	Family: Sphingomonadaceae, Genus: Sphingomonas
	Family: Burkholderiaceae, Genus: Burkholderia
	Family: Comamonadaceae, Genus: Delftia
	Family: Comamonadaceae, Genus: Variovorax
	Family: Oxalobacteraceae, Genus: Cupriavidus
	Family: Oxalobacteraceae, Genus: Herbaspirillum
	Family: Ectothiorhodospiraceae
	Family: Enterobacteriaceae, Genus: Escherichia
	Family: Moraxellaceae, Genus: Acinetobacter
	Family: Pseudomonadaceae, Genus: Pseudomonas
	Family: Xanthomonadaceae, Genus: Dyella
	Family: Xanthomonadacea, Genu: Luteibacter

Figure 5.2: Represents the relative abundance of 8 LAB species for CWK fermented sourdough, CWK, CW and kefir. These 8 LAB species are the highly dominating species across all the samples. Alphabets a-d represent significant changes for each isolate for CKW fermented sourdough, CWK, kefir and coconut water.



5.4. Summary

To conclude this section, the results obtained for identification and characterisation using API combined with Sanger sequencing confirm the species level identification for LAB and AAB in CWK and sourdough fermented with coconut water kefir. The species identified were *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus fusant, Lactobacillus reuteri, Lactobacillus kunkeei, Acetobacter aceti, Acetobacter lovaniensis, Acetobacter pasteurianus, Candida kefyr, Rhodotorula mucilaginosa, Saccharomyces cerevisiae, Candida guilliermondii* and *Candida colliculosa*.

The diversity of microorganisms in the sourdough fermented with CWK and CWK (fermented upto 96 h) was studied with time and these results provide important insights into the relative abundance of the microorganisms present at a given time point. The organisms present at 0, 24, 48 h in CWK and after 96 h in CWK sourdough fermentation could be tolerant to low pH and highly acidic conditions. Additionally, the coconut water and kefir microflora were also sequenced, suggesting that it harboured diverse genera of microorganisms but the majority of which were LAB.

The results obtained in this study show that a combination of culture-dependent and culture-independent technique (Sanger sequencing and high-throughput Illumina sequencing method) could successfully identify the microorganisms present in kefir, CWK and CWK fermented sourdough. Several LAB strains identified using Illumina sequencing method were not identified using Sanger sequencing method, additional LAB species were revealed upon using Illumina MiSeq high-throughput sequencing technology. However, these species detected by the latter technique might not be culturable.

The lactococci were detected and identified using SEM and Illumina technique, respectively. However, the culture-dependent and phenotypic methods failed to identify the lactococci. This demonstrates the high-power ability of electron microscopy and the DNA sequencing methods. The culture-dependent methods of isolation and identification were probably either not able to distinguish the lactococci colonies from the lactobacilli or not able to enrich the growth of lactococci such that they manifest as distinct colonies of their own. It is probable that in the cultivation media used, the growth of lactobacilli dominated that of the lactococci, thus, preventing the latter to produce sufficient colonies. Another probable reason for the absence of detectable lactococci during cultivation is the actual absence in the commercial kefir powder used at the time of isolating the different microorganisms due to conditions of transport and storage

Chapter 6 Functional properties of microorganisms isolated from the formulated sourdough, CWK and kefir

6.3. Introduction

Sourdough fermentation can impart health-benefiting properties to bread beyond being a source of nutrients. Such functional properties of sourdough may include increased bioavailability of phytonutrients, decreased glycaemic response and an increase in the dietary fibre (Gobbetti et al., 2014, Poutanen et al., 2009). Within the cereal matrix, microbial activities of LAB and yeasts can result in the production of nutritionally active compounds such as peptides, amino acids (and amino acid derivatives such as γ-amino butyric acid) and exopolysaccharides (Anson et al., 2012, Katina et al., 2005, Liukkonen et al., 2003, Wang et al., 2014).

Sourdough fermentation leads to phytase-dependent dephosphorylation of phytic acid (Lioger et al., 2007, Moslehi-Jenabian et al., 2010, Reale et al., 2004). Phytic acid [myo-inositol hexakis (dihydrogenphosphate)] constitutes 1–4% by weight of cereal grains, being a source of myo-inositol and the major storage form of phosphorus. This molecule is highly charged with six phosphate groups extending from the central myo-inositol ring. However, phytic acid chelates several divalent nutritional minerals, hence, it is considered an antinutritional factor. Phytic acid also complexes the basic amino acid group of proteins, thus decreasing the dietary bioavailability of these nutrients (Dvorakova, 1998; Wodzinski and Ullah, 1995). Therefore, its degradation improves the availability of nutrients and minerals such as phosphorous, magnesium, iron, calcium and zinc. Some of the phytase activity are endogenous to cereals but activated by LAB acidification, whereas other phytase activities are directly produced by both yeasts and LAB (Gobbetti et al., 2014). In addition, the LAB in the sourdough produce bioactive compounds, volatile fatty acids, lactic acid and amino acids during the fermentation process (Vinderola, Ouwehand, Salminen, & von Wright, 2019).

Glutamic acid is an amino acid that plays a very important role in taste perception, intermediary metabolism and excitatory neurotransmission. It plays a pivotal role in gastrointestinal tract during gastric phase digestion and enhances gastric exocrine secretion. It is also a precursor for γ-amino butyric acid (GABA), glutathione, arginine and proline. GABA possesses several well-known physiological functions (i.e., anti-hypertension and anti-diabetic) and glutathione plays a key role in the protecting the mucosa from peroxide damage and dietary toxins. A study by Zareian, Ebrahimpour, Mohamed, & Saari (2013) reported that *L. plantarum* has the potential to synthesize both glutamic acid and GABA in fermented foods. LAB also play a role in increasing

the shelf-life and safety of foods, improve food texture, and contribute to the nutritional value of food products. The LAB can impart pleasant sensory properties to sourdough bread. Li and Cao et al., 2010 have reported that production of L-glutamic acid and GABA (γ-aminobutyric acid) were of utmost importance to produce a functional food product. Komatsuzaki, Shima, Kawamoto, Momose, & Kimura (2005) have reported that during fermentation, the production of glutamic acid was positively correlated with increase in GABA concentration. Thus, using LAB capable of producing glutamic acid might facilitate production of functional foods rich in bioactive molecules such as GABA (Zareian et al., 2012).

The main metabolic activities of sourdough LAB that influence the nutritional importance of sourdough are their proteolytic activity (Gobbetti et al., 1995), formation of volatile compounds, antibacterial and anti-mould compounds (Corsetti & Settanni, 2007) as well as their exopolysaccharide (EPS) producing characteristics (Galle & Arendt, 2014). Exopolysaccharides (EPS) are microbial polysaccharides produced outside the cell wall (Madiedo & Gavilán, 2005; Korakli et al., 2001). They consist of dietary oligosaccharides which are non-digestible and aid in modulating the activity and composition of the intestinal microflora. EPS may also contain a variety of proteins, glycoproteins, glycolipids, and in some cases, surprising amounts of extracellular DNA (e-DNA) (Flemming, Neu, & Wozniak, 2007).

Sourdough LAB synthesize a variety of EPS through the activity of glycosyltransferases. A previous study carried out on EPS suggests that some of the polymers from the fermentation of cereal foods by LAB may be available for food applications and processing (Bounaix et al., 2009; Tieking and Gaenzle, 2005). Synthesis of EPS (glucans and fructans) with prebiotic potential has been reported for sourdough fermentation of sorghum and wheat flours by LAB such as *L. frumenti, L. pontis, L. acidophilus* and *L. reuteri* (Schwab, Mastrangelo, Corsetti, & Gänzle, 2008; Tieking, Kaditzky, Gänzle, & Vogel, 2003; Tieking, Korakli, Ehrmann, Gänzle, & Vogel, 2003). Kralj et al. (2002) reported that by adding 12% sucrose to the dough during fermentation, *L. reuteri* strain 121 can form two types of EPS, a 4,6-disubstituted ά-glucan (reuteran) and a levan. Glucansucrase and fructosyltransferase are the enzymes responsible for the synthesis of above EPS (Van Hijum, van Geel-Schutten, Rahaoui, Van Der Maarel, & Dijkhuizen, 2002).

EPS acts as a hydrocolloid to improve the overall textural properties of the sourdough bread produced. Cereal-based LAB species have been associated with the formation of homopolymeric EPS (Tieking & Gänzle, 2005) but recent studies also showed the production of heteropolymeric EPS by sourdough isolates which were shown to alter the physicochemical properties of sourdough (Galle et al., 2011). Identification of the EPS- producing LAB in sourdough LAB could help improve the quality of sourdough.

Therefore, the objective of this study was to establish the functional properties of the LAB, yeast and acetic acid bacteria isolated from the coconut water kefir-fermented sourdough, CWK and kefir. The LAB, yeast and AAB isolates that were used in this study had been isolated in the previous chapter (Chapter 5).

This chapter specifically aims to determine the following properties of the isolates, such as glutamic acid production, phytase enzyme activity and exopolysaccharide production.

6.2. Materials and methods

6.2.1 LAB, AAB and yeast species

All the LAB, AAB and yeast strains used in this study (Table 6.1) were isolated and identified from kefir, CWK and CWK fermented sourdough. Since they were stored at -80°C with 20% glycerol, they were first revived by growing in MRS broth (Difco, New Zealand) and acetic acid bacteria broth (DIFCO, New Zealand) at incubated for 48 h at 30°C (LabServe incubator, New Zealand).

Table 6.1: Isolates used in the study (as identified in chapter 3)

Type	Isolates
LAB	*Lactobacillus fermentum* *Lactobacillus plantarum* *Lactobacillus fusant* *Lactobacillus reuteri* *Lactobacillus kunkeei*
AAB	*Acetobacter aceti* *Acetobacter lovaniensis* *Acetobacter pasteurianus*
Yeast	*Candida kefyr* *Rhodotorula mucilaginosa* *Saccharomyces cerevisiae* *Candida guilliermondii* *Candida colliculosa*

6.2.2 Cultures and culture media

All the identified species of LAB, acetic acid bacteria and yeasts (Section 3.1) were included in this study. Stock cultures of LAB, AAB stored as MRS broth and AAB broth cultures containing 20% glycerol were revived in their appropriate media.

All the identified species of yeast, which were stored as stock cultures in 20% glycerol, were revived on Malt Extract (ME) agar and then grown in minimal medium containing phosphate, 6.7 g/L of yeast nitrogen base without amino acids (Difco, New Zealand) and 20 g/L of glucose (Thermo Fisher, New Zealand). The phytic acid containing medium was prepared from phosphate-free minimal medium, supplemented with 2 g/L of phytic acid dipotassium salt of the highest available purity (Sigma-Aldrich Inc., New Zealand). The quantity of free phosphate in the phytic acid was established to be <5% (w/w) in all formulations used, in accordance with the manufacturer's instructions. All media were prepared as 10-fold concentrated stock solutions and

filter sterilized (Filtropure, 0.45 μm, Sarstedt, Germany) instead of autoclaving which releases orthophosphate from phytate (Bae, Yanke, Cheng, & Selinger, 1999; Fredrikson, Andlid, Haikara, & Sandberg, 2002). To obtain the corresponding solid medium, Bacteriological Agar without phosphorus (15 g/L) (Oxoid Ltd, New Zealand) was suspended in water (90% of the final volume) and autoclaved at 121 °C for 15 min, which was used for yeast isolates. After cooling to 45°C, 10-fold concentrated phosphate containing minimal medium, phosphate-free minimal medium or phytic acid containing medium was added. All media were formulated with deionised water.

All the yeast isolates were grown on 10 ml of phosphate containing minimal medium and were incubated at 25°C for 48 h in LabServ incubator (Thermo Fisher, New Zealand). Yeast cells were harvested by centrifugation (DuPont Sorvall instruments, RC5C) at 4000 g for 5 min. The pellet obtained was washed three times with 10 ml sterile sodium chloride solution (9 g/L). The cell suspensions were standardised at an optical density $(O.D.)_{540nm}$ reading of 2.0 using a spectrophotometer (Pharmacia Biotech Ultrospec 2000) (Schnürer, Olstorpe, & Passoth, 2009).

6.2.3 Determination of phytase production by LAB, AAB and yeast isolates

6.2.3.1 Phytase assay- agar assay for LAB and AAB

The pure cultures of LAB and AAB strains were grown respectively on MRS broth and acetic acid bacteria broth (Difco, New Zealand) then incubated for 48 h at 35°C. The phytase studies were carried out on modified MRS broth (MRS-MOPS), in which the inorganic phosphate (potassium dihydrogen phosphate) was replaced by 0.65 g/l of sodium phytate and 0.1 M 3-[N-Morpholino] propane sulfonic acid (MOPS, Thermo Fisher, New Zealand). In the MRS broth, the contents of beef extract, glucose and yeast extract were reduced to 4g/L, 10g/L and 2g/L respectively to reduce the final phosphate concentration and promote enzyme synthesis (Palacios, Haros, Rosell, & Sanz, 2008). Additionally, MRS-MOPS broth with added arginine and glycine (10 g/L), and supplemented with lactose (10 g/L) instead of glucose (MRS-MOPS + Amino acid) was inoculated with each active culture (Palacios, Haros, Rosell, & Sanz, 2005). MRS-MOPS and MRS-MOPS + Amino acid agar plates were prepared using the same broth recipe but with the addition of agar at 1%. .

No calcium carbonate was present in MRS-MOPS and MRS-MOPS + Amico acid agar media, but they were all supplemented with 1% of hexacalcium phytate (Sigma-Aldrich, New Zealand).

The MRS-MOPS and MRS-MOPS + Amino acid agar media were thereafter inoculated with 5% v/v overnight cultures of LAB and AAB strains (with absorbance of 2 O.D recorded at 600 nm wavelength). The inoculum was spread across a uniform area, in a circular manner, over a diameter of 5cm). The plates were incubated for 48 h at 30°C. After incubation, the colonies were

washed from the agar surface using double distilled water and Petri plates were flooded with 2% (w/v) aqueous cobalt chloride solution (Bae, Yanke, Cheng, & Selinger, 1999). This was allowed to stand for 5 min at room temperature, after which the cobalt chloride solution was replaced with a newly formulated solution containing equal volumes of a 6.25% (w/v) aqueous ammonium molybdate solution and 0.42% (w/v) ammonium metavanadate solution. After 5 min incubation, the ammonium molybdate/ammonium vanadate solution was separated and the plates were studied for zone of phytate hydrolysis.

6.2.3.2 Phytase assay- Enzyme activity assay for LAB and acetic acid bacteria

All the strains of LAB and AAB that exhibited some ability to hydrolyse phytase in the solid medium were chosen in performing the phytase enzyme activity using liquid medium. Three different liquid media, namely, Chalmers broth, Chalmers broth supplemented with 1% of sodium phytate, and Chalmers broth supplemented with 2% of Calcium chloride were used for this assay (Sigma-Aldrich, New Zealand). Each isolate of LAB and AAB was inoculated into triplicate bottles of each broth.

After 24 h of incubation at 30°C, all the broths were centrifuged at 10,000 g for 10 min at room temperature and 1ml of supernatant was collected. This was mixed with 600 µL of a reaction substrate (3 mM sodium phytate in 0.2 M sodium acetate buffer, pH 4.0) and was incubated at two different temperatures of 30°C and 44°C for 45 min and 75 min, respectively. The reaction was stopped by adding 750 µL of a 5% weight/volume trichloroacetic acid (Sigma T6399) solution. The released inorganic phosphate was evaluated by measuring the absorbance at 700 nm after addition of 750 uL of fresh colour reagent. The colour regant consisted of four volumes of 1.5% w/v ammonium molybdate (Sigma A7302) in a 5.5% v/v sulphuric acid solution and one volume of a 2.7% w/v ferrous sulphate (Sigma F7002) solution.

Phytase activity was measured in terms of inorganic orthophosphate released from phytate (µmol for min). One unit of phytase activity (U/mL) was defined as the amount of enzyme required to liberate 1 µmol of inorganic phosphate per minute under assay conditions (Cheryan & Rackis, 1980).

All the results were compared to a standard curve prepared with inorganic phosphate- di-potassium hydrogen phosphate (K2HPO4).

6.2.4 Determination of phytase activity for yeast isolates

6.2.4.1 Extraction of phytase enzyme

All the yeast isolates were grown in 10 ml of phosphate-containing minimal medium, incubated at 25°C for 48 h in LabServ incubator (Thermo Fisher, New Zealand). All the yeast cells were harvested by centrifugation (DuPont Sorvall instruments, RC5C) at 4000 g for 5 min. The pellet

obtained was washed three times with 10 ml sterile sodium chloride solution (9 g/L). The absorbance at 600 nm was determined using spectrophotometer (Pharmacia Biotech Ultrospec 2000 Spectrophotometer) (Schnürer, Olstorpe, & Passoth, 2009). Extracellular phytase activity (activity of the enzymes which are not bound to the yeast cells, but are present in the medium) was measured by collecting the supernatant after centrifugation of the yeast cells grown in the media and filter sterilising (0.2 μm pore size, Filterpure, New Zealand) to remove any cell debris present in the medium.

Intracellular phytase enzyme (present inside the yeast cell wall and cytoplasm) was extracted by a slight modification to a method described by Ciriacy & Breitenbach (1979) (Ciriacy & Breitenbach, 1979). The yeast cell pellet was resuspended in 20 mL ice-cold crude extract buffer) [50 mM imidazole, 0.5 mM dithiothreitol (Sigma-Aldrich Inc., New Zealand), pH 7] and washed twice in 20 ml of ice-cold crude extract buffer. The suspensions were transferred to Eppendorf tubes and centrifuged in an Eppendorf centrifuge 5810 R (Eppendorf AG, Hamburg, Germany) at 10,000 g, 4°C for 20 min to eliminate all cell debris. The supernatants were filtered (pore size 0.2 μm) and concentrated to 250 μL in an Amicon Ultra centrifugal device and placed on ice. For all tested yeasts strains, triplicates of crude extract were prepared.

6.2.4.2 Enzyme assay

A 10 μL of the sample was mixed in an Eppendorf tube with 40 μL 0.1 M sodium acetate buffer (0.2 M acetic acid and 0.2 M sodium acetate, pH 5.0), with added phytic acid dipotassium salt (with final phytic acid concentration of 2 g/L). Negative controls were prepared from samples mixed with sodium acetate buffer without phytic acid.

Samples were incubated at 30°C and the reactions were halted immediately (at Time, T=0 min), and after 5, 10, 15, 30 and 60 min by adding 50μL 10% trichloracetic acid. The phosphate liberated was determined by a method described by Heinonen & Lahti (1981) (Heinonen & Lahti, 1981) by adding 800 μL acid molybdate reagent (1 volume of 10 mM ammonium heptamolybdate-tetrahydrate, 1 volume of 2.5 M sulphuric acid and 2 volumes of acetone).

Absorbance reading at 335nm was measured by using sodium acetate buffer (without phytic acid) and trichloroacetic acid as blank. A standard curve of phosphate (range of 0 μmol/ml to 3μmol/ml) was prepared with sodium acetate buffer and measured in the same conditions as the enzyme samples (Sano, Fukuhara, & Nakamura, 1999; Vohra & Satyanarayana, 2001).

6.2.4.3 Calculation of enzyme activity for yeast cells

One unit of phytase activity is defined as the amount of protein that releases 1 μmol inorganic phosphate within 1 min. Activities were calculated as both specific activity (Unit/mg of protein)

and as volumetric activity (Unit/mL of yeast culture). All assays were performed in triplicate and the mean values presented.

The activities were calculated according to Bergmeyer (1974) (Bergmeyer, 1974):

Specific activity (Unit/mg protein) $=V\times\Delta cPO4\times v^{-1}\times cprot^{-1}\times\Delta t^{-1}$

Volumetric activity (Unit/ mL of culture liquid) $=V\times\Delta cPO4\times v^{-1}\times\Delta t^{-1}\times (100\ mL)^{-1}$

Where – "V" is total sample volume measured in mL; "ΔcPO4" is phosphate concentration that has changed with time interval measured in µmol; "v" is volume of the crude extract measured in ml; "cprot" is protein concentration measures in mg/mL and "Δt" is the time interval measured in min

6.2.5 Determination of glutamic acid concentration produced by the individual strains of LAB, yeast and acetic acid bacteria

All the identified strains of LAB, AAB and yeasts were grown in MRS broth, acetic acid bacteria broth and Malt extract broth, respectively. The LAB and AAB were incubated at 30°C and yeast strains were incubated at 25°C in LabServ incubator, New Zealand for 48 h. All the cultures were centrifuged at 5000g for 10 min to collect the cell-free supernatant. This was sampled in triplicate to carry out the LC-MS chromatographic analysis for determining the glutamic acid concentration produced by individual strain/isolate.

A 1.4 mL volume of 50% methanol containing 10 mg L-1of 2,3,3,3-d4-alanine [d4A] was added to the samples and mixed in a microcentrifuge tube. The tubes were vortexed to obtain a homogenous mixture. This was followed by centrifugation at 10,000 g for 5 min at 4°C (Z216MK, HERMLE Labortechnik GmbH, Germany). A 10 µl volume of extract was used to perform pre-column derivatisation with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate following a method adapted from Salazar et al (Salazar, Armenta et al. 2012). The LC-MS was an Agilent 1260 Series liquid chromatograph comprising a G1311B quaternary pump, G1329B thermostatted autosampler and a G1330B thermostatted column compartment (Agilent Technologies, Santa Clara, USA). Mobile phase A was 0.6% formic acid in ultrapure water and mobile phase B was 0.1% formic acid in acetonitrile. The column used was a Phenomenex Kinetex EVO C18 column, measuring 2.1x150 mm with 1.7 µm diameter packing material, maintained at 25 °C. The chromatographic gradient was held for 1 minute at 1.5% B and then ramped to 13% B at eight minutes, 17% B at 15 minutes and 80% B at 16 minutes before returning to 1.5% B at 17.5 minutes. The flow rate was 300 µL minute-1, the total run time was 28 minutes and the injection volume was 5 µL. For detection an Agilent 6420 triple quadrupole mass spectrometer fitted with an Agilent Multimode Ionisation source was operated in positive electrospray mode. Optimum Multiple Reaction Monitoring [MRM] transitions were established

using Agilent MassHunter Optimiser B06.00 software. Derivatised amino acid peak areas were normalised to the recovery of d4A and quantified by reference to a dilution series of external standards prepared from a commercial glutamic acid standard (Sigma product A9906, Sigma-Aldrich Pty. Ltd., Sydney, Australia). Data were collected and processed using Agilent MassHunter software.

6.2.6 Determination of exopolysaccharide production in LAB and acetic acid bacteria strains

6.2.6.1 Screening for exopolysaccharide (EPS) producing microorganisms

Verification of EPS production by the strains was done by observing growth on Modified Chalmers agar (Pepe, Villani, & Coppola, 2001). Modified Chalmers agar was prepared by addition of sucrose (5% weight/volume). No calcium carbonate was included in the agar, when made. All the carbohydrates were filter sterilised before they were added to the medium by using 0.45μm pore size filters (Swinney syringe filters, New Zealand).

Production of EPS was detected by the presence of slimy colonies on the agar plates after incubation at 30°C for 72 h (LabServe incubator, New Zealand). The colonies were examined by touching them with a sterile toothpick to observe the ropy EPS phenotype that produces long visible strings (Ruas-Madiedo, Abraham, Mozzi, & De Los Reyes-Gavilán, 2008; Vescovo, Scolari, & Bottazzi, 1989). The positive strains detected by the primary assay were also tested on modified Chalmers agar without the addition of calcium carbonate and supplemented with 5% sucrose. The strains were incubated at 30°C for 3 days, and the ropy colonies were evaluated again, as described above. All the tests were done in triplicate for each strain of LAB and AAB.

6.2.6.2 Quantification of EPS production

All the strains of LAB, AAB and yeasts that tested positive for EPS production in a solid substrate, were selected for quantitative analysis for EPS production. All the strains were grown in their respective broths (MRS broth for growing LAB and acetic acid bacteria broth for growing AAB, manufactured by Difco, NewZealand) for 48 h at 30°C in LabServe incubator (New Zealand).

All the inocula were standardised by using a spectrophotomer, on which the absorbance reading was taken and was adjusted to 2 O.D. by dilution or concentration of all the samples (in triplicate). Pharmacia Biotech Ultrospec 2000 Spectrophotometer UV/Visible light Reader was used at the wavelength of 600nm. One ml of the culture acquired from each of the isolate had approximately $5x10^7$ LAB and AAB isolates. This was determined using a spread plate technique. Therefore, 1 ml was taken from each strain in triplicate, and streaked on to a Chalmers agar with 5% sucrose with and without the addition of 0.5% w/v of yeast extract. This was done to ascertain the effect

of yeast extract on EPS production. The plates were incubated at 30°C for 48 h. After incubation, all the colonies that produced EPS were washed repeatedly at 5000g for 5 min at room temperature (DuPont Sorvall instruments RC5C, Fiberlite F21–8x50y rotor, Thermofisher USA) with 30ml of deionised water until the visible ropiness disappeared. The ropy colonies were collected in sterile 100 ml falcon tubes.

The falcon tubes were centrifuged at 5000g for 10 min at room temperature to collect the cell pellet (DuPont Sorvall instruments RC5C, Fiberlite F21–8x50y rotor, Thermofisher USA). This was followed by addition of two volumes of 98% (v/v) ethanol, in order to precipitate the EPS. This was left to stand overnight at 4°C followed by centrifugation at 5000g for 10 min at 4°C temperature. The pellet was retained and was re-suspended in 1 ml of double distilled water. The suspension was freeze-dried on a benchtop freeze drier (Virtis Advantage Pro from SP Scientific, United States) for 24 h, and was weighed to quantify the EPS using an analytical balance (Thermo Fisher, New Zealand).

The amount of EPS obtained was expressed as polymer dry mass in mg/kg of the wet medium (Minervini et al., 2010; Vuyst & de Ven, 1998).

6.2.7 Statistical analysis

At least five individual replicates of each of the experimental sets were prepared for statistical analysis. All the results are expressed as means of values ± standard deviation of three separate determinations. One-way and two- way analysis of variance (ANOVA) were applied to determine level of significance. When the ANOVA was significant, the means were separated by pairwise comparison using Tukey's (HSD) test or Fisher (LSD) test at 5% significance level. All statistical analyses were performed using the Statistical Analysis System – XLSTAT-MX version 2012.4.02 (Addinsoft, New York, NY, USA).

6.3. Results and Discussion

6.3.1 Determination of glutamic acid concentration for LAB, AAB and yeast isolates using LCMS analysis method

In order to investigate the concentration of glutamic acid produced by each isolate of LAB, ABB and yeast, LCMS analysis was carried out. From Table 6.2 and Figure 6.1, it can be observed that significantly high concentration of glutamic acid was produced by *Lactobacillus plantarum* (264.89 ± 8.57 µMoles/L) followed by *Lactobacillus fermentum* (260.38 ± 12.09 µMoles/L), and *Lactobacillus reuteri* (251.16 ± 10.61 µMoles/L) $p<0.05$. Gobbetti et al. (1994) have reported that by using *Lactobacillus plantarum* in sourdough fermentation caused a considerable increase in total concentration of free amino acids. Zareian et al. (2012) reported *Lactobacillus plantarum* as a glutamic acid producer. A similar study by Pozo-Bayón et al. (2005) has detected the presence of glutamic acid by *Lactobacillus plantarum* strains J-39 (19.17 mg/L) and J-51(16.82 mg/L) in wine after malolactic fermentation by LAB. A study carried out by Coda, Rizzello, & Gobbetti (2010) has reported considerably high glutamic acid concentrations of 107 ± 20 mg/L in sourdoughs made with common wheat flour and fermented with *Lactobacillus plantarum* strain C48.

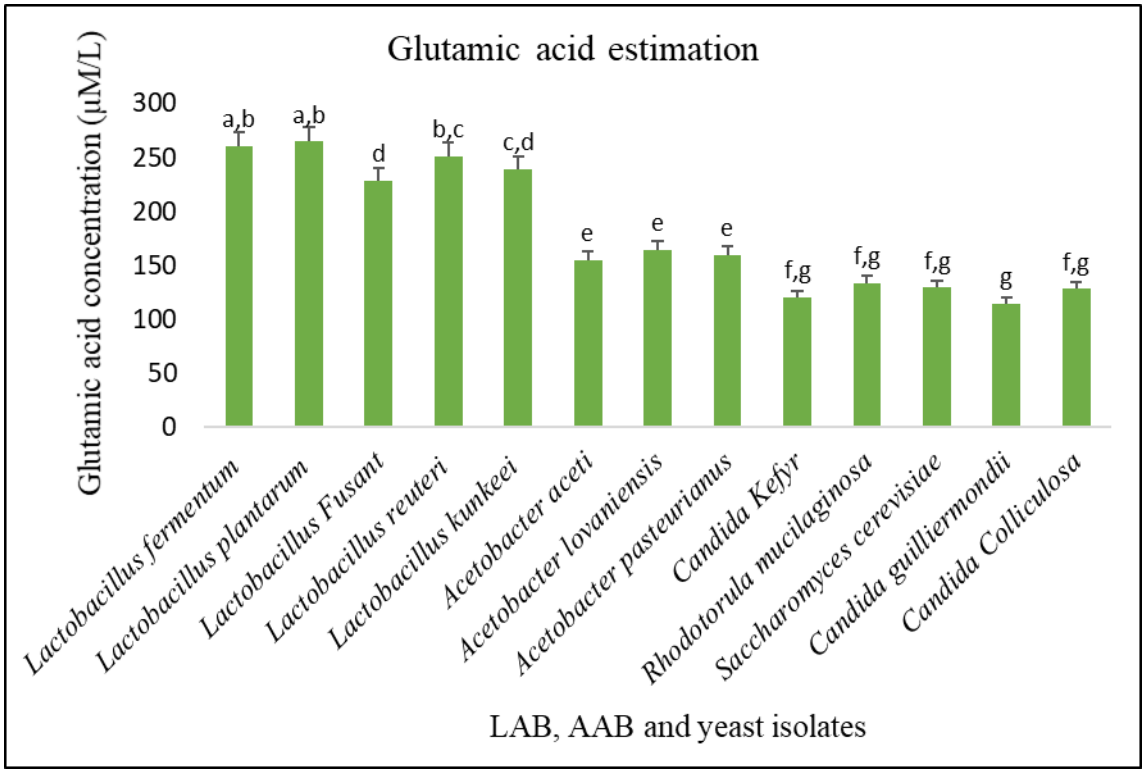
AAB strains (*Acetobacter aceti, Acetobacter lovaniensis* and *Acetobacter pasteurianus*) produced average quantities between 155.28 ± 4.84 µMoles/L - 164.37 ± 4.11 µMoles/L of glutamic acid (as shown in Table 6.2). Significantly low concentrations of glutamic acid is produced by all the individual yeast species with *Candida guilliermondii* producing significantly the lowest (114.29 ± 1.95, $p<0.05$).

Gobbetti et al. (1994) reported production of enhanced amounts of glutamic acid by *S. cerevisiae* strain 141 during sourdough fermentation.

Table 6.2: Glutamic acid concentration determined using LCMS for each isolate in µmoles/L. (a,b,c,d determine significant difference within a column)

Isolates	**Glutamic acid concentration (µmoles/L)**
Lactobacillus fermentum	260.38 ± 12.1a,b
Lactobacillus plantarum	264.89 ± 8.5a,b
Lactobacillus fusant	228.96 ± 12.7d
Lactobacillus reuteri	251.16 ± 10.6b,c
Lactobacillus kunkeei	239.08 ± 10.8c,d
Acetobacter aceti	155.28 ± 4.8e
Acetobacter lovaniensis	164.37 ± 4.1e
Acetobacter pasteurianus	159.52 ± 0.7e
Candida kefyr	120.83 ± 0.9f,g
Rhodotorula mucilaginosa	133.35 ± 0.8f,g
Saccharomyces cerevisiae	129.37 ± 3.8f,g
Candida guilliermondii	114.29 ± 1.9g
Candida colliculosa	128.18 ± 7.1f,g

Figure 6.1: Glutamic acid production in each isolate. Letters a-g determine the significant difference between each isolate producing glutamic acid. The error bars represent the standard deviation for the five replicate readings for each experimental set.



6.3.2 Phytate zone of hydrolysis

All LAB, AAB and yeast strains isolated from kefir, CWK and CWK-fermented sourdough were screened for their phytate degrading ability using two different types of media: modified MRS agar (MRS-MOPS) in which inorganic phosphate was replaced by sodium phytate (0.65 g/L, Sigma) and 0.2% $CaCl_2$, and MRS-MOPS + AA supplemented with 10g/L of arginine and glycine.

All the isolates were incubated for 48h at 30°C. As shown in Table 6.3, all were positive for phytate hydrolysis by exhibiting a zone of clearance (translucent) around the inoculated area on the agar. A counterstaining method was used by flooding the agar with aqueous cobalt chloride, which helps determine the false positives for phytate negative microorganisms. The exact mechanism for the counterstaining procedure is not yet known. However, it is estimated that phytate can form complexes with cobalt and metals and has a pH dependence of these complexes, with relative binding of different cations with phytate (Bae et al., 1999; Vohra et al., 1965).

After counterstaining, all the phytase positive LAB, AAB and yeast displayed a halo ranging between 2.3 mm to 14.24 mm in diameter. *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus fusant, Lactobacillus reuteri* and *Lactobacillus kunkeei* had significantly larger zone of hydrolysis that ranged between 14.24 ± 0.12mm and 13.73 ± 0.08mm ($p<0.05$). LAB, AAB and yeast were tested for their ability to degrade sodium phytate in the presence of calcium chloride. The supplemented calcium aids in the phytase enzyme activity and does not itself take part in the reaction (De Angelis et al., 2003). The phytate degradation ability of all these isolates can be attributed to the presence of phytase enzyme which degrades the available phytate (sodium phytate) in the agar in the presence of calcium chloride since no white precipitate was observed around the zone of enzyme-specific phytate hydrolysis (Bae, Yanke, Cheng, & Selinger, 1999).

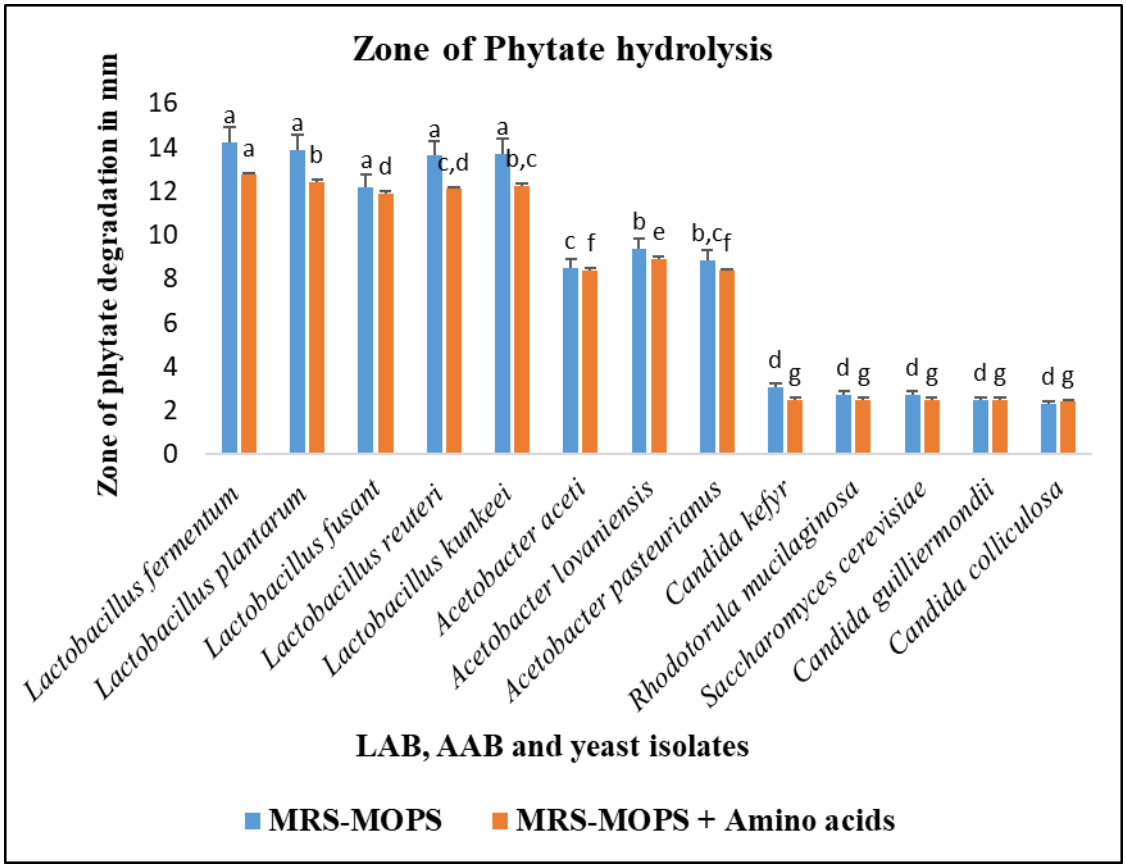
Acetobacter species had significantly smaller hydrolysis zone compared to those of LAB, with values between 8.5 ± 0.2 mm and 9.37 ± 0.15mm ($p<0.05$). No previous study has reported phytate hydrolysis by AAB according to previous literature. The ability to utilise phytate can thus be attributed to the production of phytase enzyme by AAB.

Significantly smaller zone of hydrolysis was obtained for all yeast species that ranged between 2.3 ± 0.17mm and 3.07 ± 0.15mm ($p<0.05$). Positive yeast strains for phytate hydrolysis have been previously reported by Howson & Davis (1983) for *S. cerevisiae*, a GRAS (Generally Recognized as Safe) organism used in food for human consumption. Degradation of phytate by phytase produced by *S. cerevisiae* is valuable in the industrial production of food from plant seed meals (Han et al. 1999). Tsang (2011) showed that 2 out of the 3 isolates of *C. kefyr*, and 3 out of 4 isolates of *C. guilliermondii* tested positive for phytate hydrolysis. Additionally, Caputo, Visconti, & De Angelis (2015) reported that *C. colliculosa* possessed phytate hydrolysis ability. Phytase activity has also been reported to be positive for *Rhodotorula mucilaginosa* (Yu, Wang, & Liu, 2015).

Table 6.3: Phytate zone of hydrolysis by LAB, AAB and yeast on MRS-MOPS and MRS-MOPS supplemented with Amino acids grown for 48h at 30°C. Different letters on each column mean statistical differences between strains ($P < 0.05$). The data are expressed as the mean and standard deviation for the two independent experiments.

	Zone of hydrolysis (Diameter of the zone of hydrolysis in mm)	
LAB, AAB and yeast isolates	**Average MRS-MOPS**	**Average MRS-MOPS + Amino acids**
Lactobacillus fermentum	14.2 ± 0.1a	12.7 ± 0.1a
Lactobacillus plantarum	13.8 ± 0.1a	12.4 ± 0.1b
Lactobacillus fusant	12.1 ± 0.5a	11.9 ± 0.1d
Lactobacillus reuteri	13.6 ± 0.1a	12.1 ± 0.1c,d
Lactobacillus kunkeei	13.7 ± 0.1a	12.2 ± 0.1b,c
Acetobacter aceti	8.5 ± 0.2c	8.4 ± 0.1f
Acetobacter lovaniensis	9.3 ± 0.1b	8.9 ± 0.2e
Acetobacter pasteurianus	8.8 ± 0.2b,c	8.3 ± 0.1f
Candida kefyr	3.0 ± 0.1d	2.5 ± 0.2g
Rhodotorula mucilaginosa	2.7 ± 0.1d	2.4 ± 0.3g
Saccharomyces cerevisiae	2.73± 0.2d	2.5 ± 0.1g
Candida guilliermondii	2.4 ± 0.2d	2.5 ± 0.1g
Candida colliculosa	2.3 ± 0.1d	2.4 ± 0.2g

Figure 6.2: Bar graph for phytate zone of hydrolysis of LAB, AAB and yeast on MRS-MOPS and MRS-MOPS supplemented with Amino acids grown for 48h at 30°C. Different letters on each column show statistical differences between strains ($P < 0.05$). The data are expressed as mean and standard deviation for the two independent experiments. The error bars represent the standard deviation for the five replicate readings for each experimental set.



6.3.3 Phytase enzyme activity assay

Each of the LAB, AAB and yeast isolates that had tested positive for phytate-hydrolysis (Figure 6.2 and Table 6.3), were subjected to phytate assay in liquid medium. In this assay, sodium phytate in the presence of calcium was used to modulate the enzymatic activities of phytate degrading enzymes in microorganisms (Gibson & Ullah, 1988; Hubel & Beck, 1996; Kim, Kim, Bae, Yu, & Oh, 1998; Shimizu, 1992). One percent of sodium phytate added to each medium of growth tested, did not act as an inhibitor of phytate-degrading enzymes. In fact, the phytase activity of all the pytate-degrading strains was detected in all of the 3 media used, as reported in Table 6.4 and Figure 6.3. Among LAB, AAB and yeast, the highest phytase values were found for *L. fermentum* (4052.53 ± 171.14 U/ml; $p \leq 0.05$) and *L. plantarum* (3151.83 ± 383.07 U/min; $p \leq 0.05$) grown on Chalmers broth, Chalmer broth supplemented with either 1% sodium phytate or 2% calcium chloride (Table 6.4).

It has been reported previously that all the tested strains of LAB exhibited the production of phytase between 2.6-146 U/ml in terms of inorganic orthophosphate released (Sreeramulu, Srinivasa, Nand, & Joseph, 1996; Zamudio, Gonzalez, & Medina, 2001). Positive phytase production between 0.53 – 0.74 U/ml has been reported for *L. plantarum* T211, *L. plantarum* H10, *L. plantarum* H5 and *L. plantarum* L3 has been previously reported by Anastasio et al. (2010) for its growth on all the three types of Chalmers broths (Chalmers broth, Chalmers broth supplemented with either 1% sodium phytate or 2% calcium chloride). Phytase production of 110.6 U/ml by *L.fermentum* DC400 incubated for 24 h at 37°C has been reported by De Angelis et al., (2003), along with its capability to hydrolyse the available phytate in the medium. The phytase activity in this study is higher when compared to literature, which could be due to longer incubation time of 48 h in contrast to 24 h used by De Angelis et al., (2003). Yildirim & Arici (2019) have reported phytase activity for *Lactobacillus plantarum* ELB78 of 797.88 U/mL. Sumengen, Dincer, and Kaya (2013) reported extracellular phytase activity of 984.50 U/mL from *Lactobacillus plantarum* isolated from a fermented product known as "shalgam". This is significantly lower than the value obtained in this study for *L. plantarum*, which could be due to the longer fermentation time of the sourdough sets.

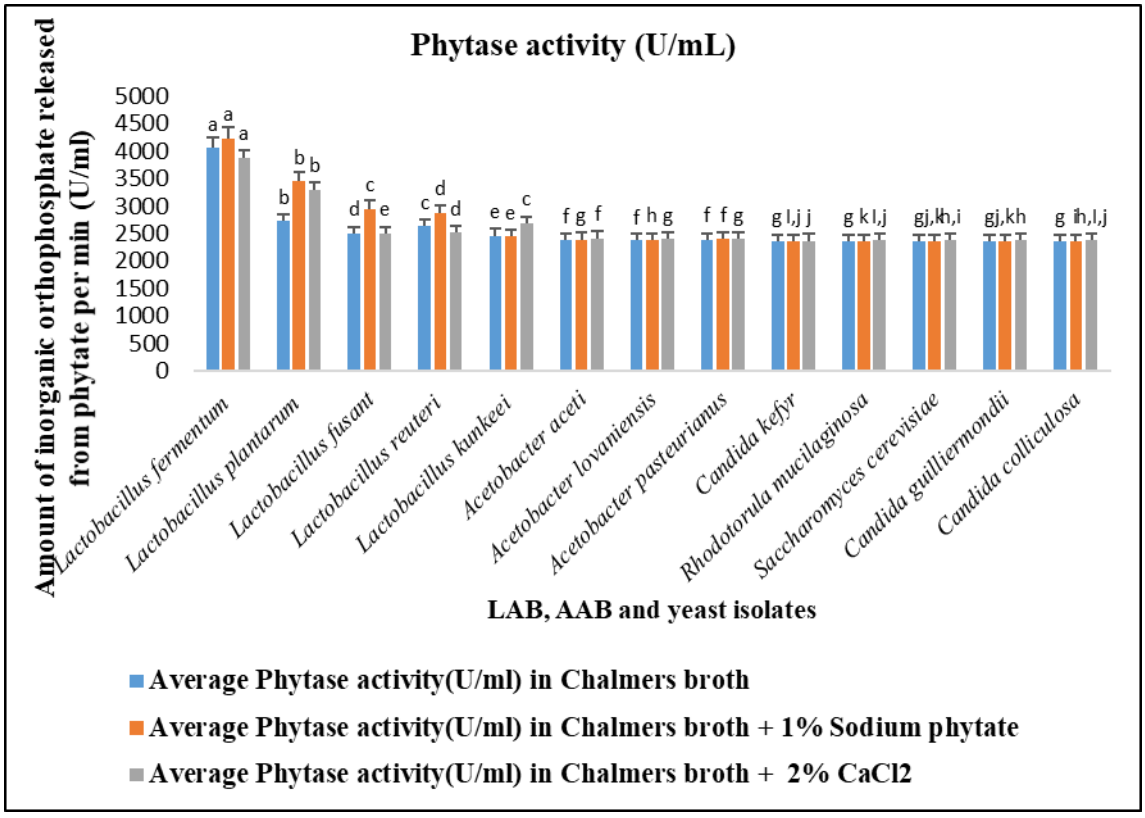
When compared with LAB, AAB strains showed significantly lower phytase activity ranging between 2389.02 ± 14.84 U/ml for *A. aceti* to 2383.86 ± 10.74 U/ml for *A. lovaniensis* ($p \leq 0.05$), followed by yeast strains with phytase activity ranging between 2360.21 ± 6.36 U/ml for *R. mucilaginosa* to 2364.08 ± 5.82 U/ml for *C. colliculosa* ($p \leq 0.05$).

Phytase activity has been reported for *S. cerevisiae* by Howson and Davis (1983), Han et al. (1999) and Turk et al. (2000), and for various other Candida species including *C. kefyr*, *C. guilliermondii* and *C. colliculosa* (Caputo, Visconti, & De Angelis, 2015; Tsang, 2011). Maximum phytase activity reached 205.4 U/ml for *Rhodotorula mucilaginosa* as reported by Yu, Wang, & Liu (2015).

Table 6.4: Phytase enzyme activity of LAB, AAB and yeast on Chalmers broth, Chalmers broth supplemented either with 1% sodium phytate or 2 % calcium chloride. Alphabets a-j: represent significant difference among each isolate within the column.

Isolates	**Average Phytase activity(U/ml) in Chalmers broth**	**Average Phytase activity(U/ml) in Chalmers broth + 1% Sodium phytate**	**Average Phytase activity(U/ml) in Chalmers broth + 2% $CaCl_2$**	**Overall Averge for phytase activity (U/ml)**
Lactobacillus fermentum	4052.2a	4223.8a	3881.5a	4052.5 ± 171.1a
Lactobacillus plantarum	2717.9b	3443.3b	3294.1b	3151.8 ± 383.0b
Lactobacillus fusant	2497.3d	2948.0c	2495.2e	2646.8 ± 260.8c
Lactobacillus reuteri	2628.5c	2875.3d	2510.7d	2671.5 ± 186.0c
Lactobacillus kunkeei	2456.5e	2437.1e	2678.4c	2524.0 ± 134.0c,d
Acetobacter aceti	2373.9f	2389.4g	2403.6f	2389.0 ± 14.8d
Acetobacter lovaniensis	2375.2f	2380.4h	2395.9g	2383.8 ± 10.7d
Acetobacter pasteurianus	2376.5f	2394.6f	2392.0g	2387.7 ± 9.7d
Candida kefyr	2356.8g	2361.0i,j	2366.2j	2361.3 ± 4.7d
Rhodotorula mucilaginosa	2355.9g	2357.2k	2367.5i,j	2360.2 ± 6.3d
Saccharomyces cerevisiae	2354.6g	2358.4j,k	2371.3h,i	2361.5 ± 8.7d
Candida guilliermondii	2356.8g	2359.7j,k	2372.6h	2363.9 ± 8.4d
Candida colliculosa	2358.4g	2363.6i	2370.1h,i,j	2364.8 ± 5.8d

Figure 6.3: Phytase activity for LAB, AAB and yeast isolates grown in Chalmers broth supplemented with 1% sodium phytate and 2 % calcium chloride. The error bars represent the standard deviation for the five replicate readings for each experimental set. Alphabets a-l: represent significant difference among each isolate within the column.



6.3.4 Screening for EPS production

A method reported by Ruas-Madiedo, Abraham, Mozzi, & De Los Reyes-Gavilán (2008) and Vescovo, Scolari, & Bottazzi (1989) for detecting the EPS production was used. EPS was detected through the examination of slimy colonies on the plate until 72 h of incubation at 30°C on the above mentioned media. The positive colonies were further confirmed phenotypically by the formation of strings upon the touching the colonies with a toothpick aseptically.

LAB, AAB and yeast strains were considered positive for EPS production if they formed slimy colonies on Chalmers agar (CA) in the presence of 5% sucrose and without $CaCO_3$ (CA+S), CA supplemented with 5% sucrose and no yeast extract (YE) and CA supplemented with 5% sucrose and 0.5% YE (Table 6.5).

All the strains tested positive for EPS production on CA+S were then grown on CA+S-YE and CA+S+YE for quantification of EPS produced by all the individual isolates.

In the presence of only $CaCO_3$, without any sucrose supplementation, there was no EPS production. *L. fermentum*, *L. plantarum* and *L. reuteri* produced EPS after 24 h of incubation at

30°C with 5% CO_2 on CA+S, CA+S-YE and CA+S+YE. However, *L. kunkeei* produced EPS after 48 h with 5% CO_2 on incubation at 30°C. *L. fusant* did not produce EPS on all the three agars.

Chalmers agar supplemented with 5% sucrose and without $CaCO_3$ is the most suitable medium for the growth of EPS- producing LAB, which can be visualised in the form of slimy colonies. EPS production depends on the medium composition (i.e., nitrogen and carbon source and growth factors), temperature, pH, incubation time and the available oxygen, to which the microorganisms are exposed. All LAB, AAB and yeast showed growth on CA+S-$CaCO_3$, CA+S-YE and CA+S+YE agar. Homopolysaccharide- producing LAB strains such as *Lactobacillus reuteri* often originate from cereal products and heteropolysaccharide producing LAB strains are present in high proportions in dairy products which are fermented (Kralj et al., 2004; Van der Meulen et al., 2007; Wszolek, Kupiec-Teahan, Guldager, & Tamime, 2006). Synthesis of homopolysaccharide is correlated to the presence of sucrose in the media, which is the sole substrate for utilisation by glycansucrase, while on the other hand, heteropolysaccharide production is independent of the sucrose source (Gänzle & Schwab, 2009).

Acetobacter aceti and *Acetobacter pasteurianus* produced EPS after 48 h of incubation at 30°C with 5% CO_2 on CA+S-$CaCO_3$, CA+S-YE and CA+S+YE agar (Table 6.5). No EPS was produced by *A. lovaniensis*. AAB are Gram-negative obligate aerobes and belong to the subdivision of α-proteobacteria (well-known vinegar producers). Almost all AAB species have the ability to grow in static culture by 'floating' as they produce a pellicle on the surface of the culture medium. This pellicle is an aggregate of all the cells suspended in the liquid-air interface and are strongly bound to one another by polysaccharide or other extracellular matrices on the AAB cell surface. The pellicle polysaccharides occur as homopolysaccharide of cellulose or as heteropolysaccharides such as capsular polysaccharide of *Acetobacter aceti* strain IFO3284 (Moonmangmee, Kawabata et al., 2002). *Acetobacter pasteurianus* strain produces two different types of colony on agar surface; rough surfaced colony and smooth surfaced colony. The rough surfaced colony producer strain can produce pellicle which allows it to float on the medium surface in static culture (Matsushita, Ebisuya, Ameyama, & Adachi, 1992).

In terms of acetic acid bacteria, contradictory results have been reported concerning the influence of carbon source on EPS yields. Fructose, sucrose, and glucose (Embuscado, Marks, & Bemiller, 1994; Kojima, Seto, Tonouchi, Tsuchida, & Yoshinaga, 1997; Masaoka, Ohe, & Sakota, 1993; Tonouchi, Tsuchida, Yoshinaga, Beppu, & Horinouchi, 1996; Weinhouse & Benziman, 1974; Yang, Park, Hwang, Pyun, & Kim, 1998) have been reported to provide the highest bacterial cellulose yield. There is, however, no information about the influence of the carbon source on the

chemical composition of heteropolysaccharides, such as acetan or gluconacetan, produced by Acetobacter strains (Degeest & De Vuyst, 2000).

Candida guilliermondii and *Rhodotorula mucilaginosa* were positive for EPS production after 48 h of incubation at 28°C for all CA+S, CA+S-YE and CA+S+YE whereas *C. kefyr, S. cerevisae* and *C. colliculosa* were negative for phenotypic EPS production (Table 6.5).

The majority of the Candida species have the ability to form surface-attached microbial communities by producing extracellular polymeric substances (Costerton et al, 1995). Gientka et al. (2016) have reported the production of EPS by *Candida guilliermondii* isolated from kefir. A study carried out by Garza et al. (2016) has reported that EPS is produced by *Rhodotorula mucilaginosa*. In most cases, EPS are produced when microorganisms are under conditions of stress in order to create a shell-like structure that prevents toxic reagents from reaching the cell (Nogueira, Melao, Lombardi, & Vieira, 2005; Pérez et al., 2008; Zhou, Shen, Meng, Liu, & Zhang, 2010).

Table 6.5: The table reports only the species that showed EPS production in CA+S, CA+S-YE and/or in CA+S+YE.

CA+S: modified Chalmers agar without $CaCO_3$ and with sucrose (5%, wt/vol) as the carbon source.

CA+S-YE: modified Chalmers agar without $CaCO_3$ and with sucrose (5%, wt/vol) as the carbon source without additional YE

CA+S+YE: modified Chalmers agar without $CaCO_3$ and with sucrose (5%, wt/vol) as the carbon source with 05.% wt/vol YE.

LAB and AAB: incubated at 30°C with 5%CO_2 and yeast incubated at 28°C

"++": EPS (slime) production after 24 h;

"+": EPS (slime) production after 48 h ;

"-": no EPS (slime) production.

Isolates	Qualitative EPS			
	Chalmers agar (CA) + $CaCO_3$	**Chalmers agar (CA) + 5% Sucrose without $CaCO_3$**	**Chalmers agar + 5% sucrose and no yeast extract**	**Chalmers agar + 5% sucrose + 0.5% wt/vol yeast extract**
Lactobacillus fermentum	-	++	++	++
Lactobacillus plantarum	-	++	++	++
Lactobacillus fusant	-	-	-	-
Lactobacillus reuteri	-	++	++	++
Lactobacillus kunkeei	-	+	+	+
Acetobacter aceti	-	+	+	+
Acetobacter lovaniensis	-	-	-	-
Acetobacter pasteurianu	-	+	+	+
Candida kefyr	-	-	-	-
Rhodotorula mucilagino	-	+	+	+
Saccharomyces cerevisic	-	-	-	-
Candida guilliermondii	-	+	+	+
Candida colliculosa	-	-	-	-

6.3.5 EPS quantification

All the isolates that tested positive for EPS production on CA+S-YE and CA+S+YE agar (Figure 6.4 and Table 6.5), were further quantified for EPS production as polymer dry mass in mg/kg. All the LAB, AAB and yeast strains show higher EPS production on Chalmers agar supplemented with 5% sucrose and 0.5% YE compared to CA+5% sucrose without YE (Table 6.6).

Three out of the four LAB strains showed the significantly highest EPS production on CA+S+YE, between 85.21 ± 2.35 mg/kg - 94.01 ± 1.65 mg/kg polymer dry mass (PDM) ($p \leq 0.05$), when compared to EPS production by AAB and yeast.

EPS quantification for LAB is often tested using broth medium (modified broth with any simple sugar) and/or complex media. Therefore, a simple and advantageous method was applied in this study. The other reason for using a solid medium was to reduce the chances of contamination by polysaccharides, such as some proteins and/or other high-molecular-mass molecules present in the liquid culture medium. Moreover, solid surfaces of agar culture media stimulate attached bacteria to synthesize EPS with respect to planktonic cells in broth media (Minervini et al., 2010).

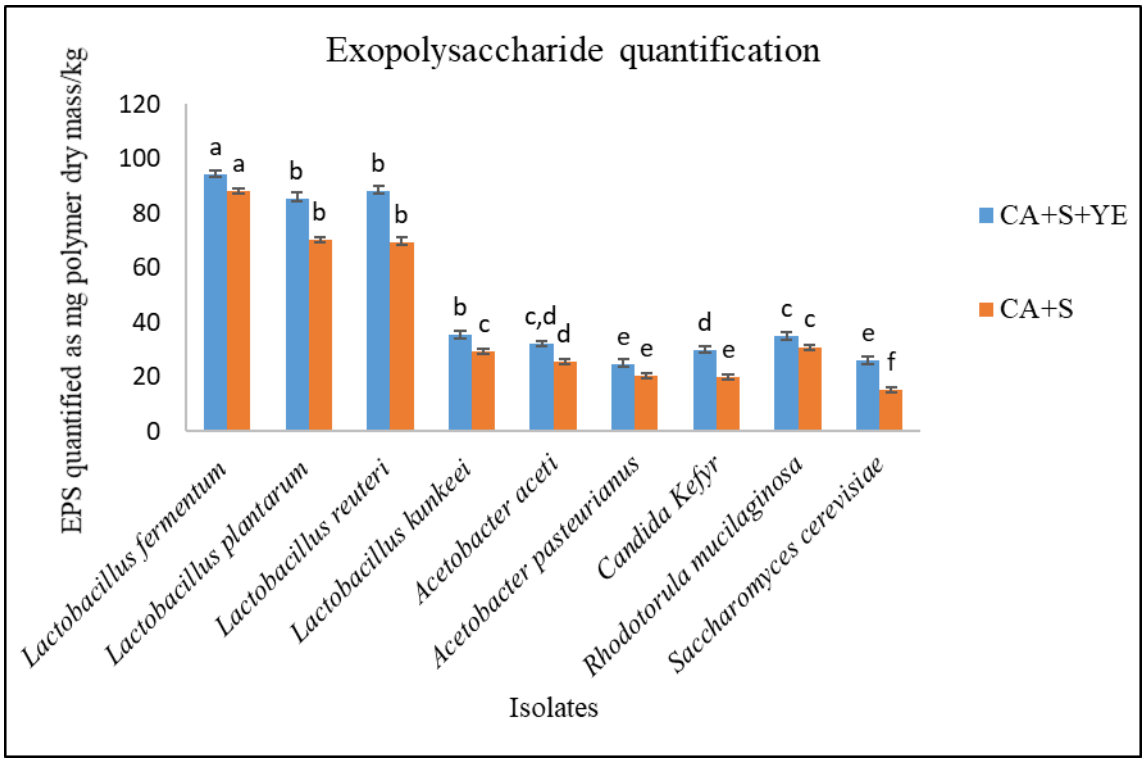
Acetobacter aceti and *Acetobacter pasteurianus* produced higher EPS on CA+S+YE of 24.55 ± 1.99 and 32.27 ± 0.98 mg/kg PDM respectively ($p \leq 0.05$). The genus Acetobacter is known for its capacity to produce cellulose (a type of EPS), which is used for some of the fermented food products (Blanc, 1996; Chung & Shyu, 1999; De Iannino, Couso, & Dankert, 1988; Kent, Stephens, & Westland, 1991). A study carried out by Matsushita, Ebisuya, Ameyama, & Adachi (1992) provides evidence that *A. aceti* (R-strain or rough surfaced colony) produces EPS, however, no quantitative data were presented.

Candida guilliermondii and *Rhodotorula mucilaginosa* produced 29.66 ± 1.44 and 34.73 ± 1.58 mg/kg PDM respectively ($p \leq 0.05$). Vazquez-Rodriguez et al. (2018) have reported that *Rhodotorula mucilaginosa* strain UANL-001L produced 19 mg/ml of EPS after 96 h of incubation at 28°C. A study carried out by Gientka et al. (2016) shows that *Candida guilliermondii* was able to synthesize 2.9 g/L of EPS after 72 h of incubation and with maltose as the carbon source.

Table 6.6: Quantification of EPS produced by LAB, AAB and yeast isolates.Alphabets a-f: represent significant EPS production by LAB, AAB and yeast within the column.

Isolate	**Chalmers agar + 5% sucrose and no yeast extract (Polymer dry mass in mg/kg)**	**Chalmers agar + 5% sucrose + 0.5% wt/vol yeast extract (Polymer dry mass in mg/kg)**
Lactobacillus fermentum	87.89 ± 1.1a	94.01 ± 1.6a
Lactobacillus plantarum	70.36 ± 0.9b	85.21 ± 2.3b
Lactobacillus reuteri	69.31 ± 1.8b	87.92 ± 2.0b
Lactobacillus kunkeei	29.33 ± 0.8c	35.16 ± 1.8c
Acetobacter aceti	25.7 ± 0.6d	32.27 ± 0.9c,d
Acetobacter pasteurianus	20.5 ± 0.6e	24.55 ± 1.9e
Candida guilliermondii	19.97 ± 0.6e	29.66 ± 1.4d
Rhodotorula mucilaginosa	30.92 ± 0.6c	34.73 ± 1.5c

Figure 6.4: EPS quantification (polymer dry mass/kg) for all the LAB, AAB and yeast strains tested positive for EPS production (Table 6.5) The error bars represent the standard deviation for the five replicate readings for each experimental set. Alphabets a-f: represent significant difference among each isolate within the column.



6.4. Summary

The biological and enzymatic phytase assays have shown that *L. fermentum* and *L. plantarum* exhibited the highest phytase production and phytate-hydrolysing properties.

Similarly, these two isolates exhibited the highest EPS-producing-capability.

Except for *Lactobacillus fusant, Lactobacillus reuteri* and *Lactobacillus kunkeei*, all the LAB produced high glutamic acid concentration. Production of glutamic acid was observed for AAB and yeast isolates, but it was significantly lower when compared with those of LAB isolates. Interestingly, among all the yeast isolates, *Candida guilliermondii* had significantly the lowest glutamic acid producing capability.

This chapter has demonstrated that the microorganisms associated with the sourdough fermentation in this study possess functional properties that could improve the sourdough**.**

Chapter 7 Functional properties of sourdough bread prepared with coconut water kefir

7.4. Introduction

Sourdough has been used in bread production for dough leavening (Roussel & Chiron, 2002), but it also extends shelf life, improves nutritional properties, increases bioactive compound contents and improved bread flavour (Banu et al., 2011, De Vuyst et al., 2013; Gänzle & Gobbetti, 2013). Sourdough is a complex ecosystem in which lactic acid bacteria (LAB) and yeast interact together and with the ingredients depending on the process parameters (Zotta, Piraino, Ricciardi, McSweeney, & Parente, 2006). The endogenous factors in flours and technological parameters employed for sourdough processing have a key role in influencing the microbial communities (Hammes and Gänzle, 1998). Sourdough breads are considered nutraceutical due to their health-promoting attributes. These may include reduction of risk with colorectal cancer, cardiovascular disorders, diabetes and obesity. They could alleviate gluten intolerance and celiac disease. Most importantly, it is also very useful for those people who are gluten intolerant (celiac disease). The sourdoughs used in bread production usually contain wide range of probiotics which bestows positive effects on health (Slavin, 2004). Various functional and nutritional features of sourdough fermentation such as its application for salt reduction, resolving irritable bowel syndrome (IBS), synthesis or release of bioactive compounds, metabolism of phenolic compounds, ability to lower the glycaemic index, increase mineral bioavailability and decrease the gluten content have been proven (Antognoni et al., 2017; Cosola et al., 2018; Cozma-Petruț, Loghin, Miere, & Dumitraşcu, 2017; Gobbetti et al., 2019; Zannini & Arendt, 2018; Zhao, Kinner, Wismer, & Gänzle, 2015).

Phytase, glutamic acid and glycaemic index are important functional properties that can help validate the nutritional quality of the sourdough bread. Phytase enzyme sequentially dephosphorylates phytic acid to products of lower chelating capacity and higher solubility, and in turn, increasing the mineral absorption (Haros et al., 2009). Endogenous phytase enzyme activity in cereals can be enhanced by low pH during sourdough fermentation but it is not adequate to effectively degrade phytate (Gobbetti et al., 2014; Greiner and Konietzny, 2006, Sanz-Penella et al., 2012). Anastasio et al. (2010) and Nuobariene et al. (2015) have reported that *L. plantarum* and *L. fermentum* are capable of high extracellular phytase activity. Verni et al., (2017) have also reported that fermented sourdough is capable of decreasing the phytate content of bread, increasing the nutritional and functional properties of bread, possessing intense phytase and antioxidant activities which are responsible for the nutritional, textural, and sensory advantages of the final product and increasing total free amino acids.

Glutamic acid is a precursor to other important amino acids such as proline and arginine as well as to some bioactive precursors like glutathione and γ-aminobutyric acid (GABA). GABA possesses several well known physiological functions, that is, it is anti-hypertension (Inoue et al., 2003) and anti-diabetic (Hagiwara, Seki, & Ariga, 2004). GABA acts as the major inhibitory neurotransmitter in the mammalian central nervous system and is known to improve the plasma concentration, growth hormones and the protein synthesis in the brain (Cho, Chang, & Chang, 2007). In addition, GABA has hypotensive, tranquillizing, diuretic and antidiabetic effects. Furthermore, Hagiwara et al. (2004) reported that GABA acted as a strong secretagogue of insulin from the pancreas, therefore, effectively preventing diabetes. GABA intake can regulate the sensations of pain and anxiety, and lipid levels in serum (Kono & Himeno, 2000; Miura et al., 2006). Furthermore, consumption of GABA-enriched foods can inhibit cancer cell proliferation and improve memory and the learning abilities (Miura et al., 2006; Park & Oh, 2007). Therefore, GABA has been classified as a bioactive component in foods and pharmaceuticals (Adeghate & Ponery, 2002; Capitani et al., 2003; Chou & Weimer, 1999).

Sourdough fermentation of wheat flour dough significantly lowers the GI of bread by reducing the rate of starch digestion, mostly through the formation of organic acids that delay the absorption of starch (Björck & Elmståhl, 2003; Poutanen, Flander, & Katina, 2009). According to the Harvard Medical School recommendation, food with a GI $\leq$ 55 are ranked as low, and GI between 56 and 69 are ranked as moderate and $\geq$70 are ranked as high GI foods. Sourdough wheat bread is, therefore, recognized as a low GI food (D'Alessandro and De Pergola, 2014). Coda et al. (2015) have reported that phytase activation and decrease in starch digestibility could lower the glycaemic response of humans to sourdough bread. Sensory analysis was carried out on baked breads to determine the top three most liked breads. On those breads, all the physico-chemical tests were carried out including carboxylic acid analysis and total amino acid analysis. The glycaemic index of bread produced in this study was also analysed since this is considered an important functional attribute. Amino acid production in the sourdough bread is quantified to understand the formation of amino acids during sourdough fermentation. Some amino acids are precursors of aromatic compounds which are a major factor affecting taste. Thiele et al (2002) and Gassenmeier & Schieberle (1995) reported that the amino acids leucine and proline are flavour precursors produced by yeast during sourdough proofing.

This study was aimed at developing a sourdough bread produced from a dough fermented with kefir coconut water and supplemented with phytase and glutamic acid producing microorganisms. Two LAB strains (Lactobacillus fermentum and Lactobacillus plantarum) that produced the highest concentration of phytase and glutamic acid in Chapter 4 were chosen for product development for creating a functional sourdough bread.

The phenomenon that foods turn progressively brown during baking or storage is the result of the well-known Maillard reaction. This reaction, also called non-enzymatic browning or glycation, is of outstanding importance for the formation of colour, aroma and flavour precursors in foods such as sourdough breads (Prost et al., 2012). Fermentation is the major route for volatile formation in sourdough and bread crumb. It produces mainly acids, alcohols, aldehydes, esters and ketones (Pico et al., 2015).

However, desired flavour attributes (intensity of overall flavour, roasted flavour and intensity of aftertaste) correlated in general strongly with undesired flavour attributes such as pungent flavour and reduced fresh flavour. This is due to the fact that acidification (especially formation of acetic acid) is the main factor enhancing pungent flavour (Spicher & Stephan, 1993) and diminishing fresh flavour (Heiniö, Liukkonen, Katina, Myllymäki, & Poutanen, 2003). On the other hand, acidification has been shown to be also a key factor in the induction of proteolysis, the main factor enhancing e.g. roasted flavour, during sourdough fermentation (Thiele et al., 2002).

The Fermentation Quotient (FQ) is defined as the molar ratio of lactic acid to acetic acid that is used to determine the potential effects in the aroma and texture profiles of the sourdough (Spicher 1983). The total titratable acidity and FQ are considered to affect the sensory characteristics and shelf-life of breads (Röcken et al., 1992). Lactic acid contributes to forming a more elastic dough, while acetic acid is responsible for a shorter and harder dough and, therefore, a greater concentration of the first is desirable (Settanni et al., 2013). Added sodium chloride and glutamic acid produced during fermentation, could act as taste-enhancers and thus alter sour taste perception (Miller and Hoseney, 2008, Stromeck et al., 2011). Many volatile compounds have been identified in sourdough bread (Pétel et al., 2017, Pico et al., 2015, Quílez et al., 2006).

7.2. Materials and methods

7.2.1 Microorganisms used to formulate the sourdough

Pure cultures of two isolates with functional attributes (Chapter 6) were supplemented at known cell concentrations into the sourdough. The isolates were: isolate 1 (*L. fermentum*) and isolate 2 (*L. plantarum*). Each isolate, stored with glycerol (20%) at -80°C, was revived in MRS broth (100 ml) and incubated for 48 h at 30°C in a 5% CO_2 incubator. The broth cultures were spun down in a GYROZEN centrifuge, model 1580R (Bio-strategy, New Zealand) at 4000 x g for 15 min to obtain a cell pellet. The cell pellet for each isolate was washed with sterile de-ionised water and re-suspended in fresh sterile de-ionised water. The cells were washed three times to remove all traces of the MRS broth. The cell suspensions were prepared fresh on the same day the dough was produced. The concentration of each isolate was measured using a spectrophometer (Ultrospec 2100 pro UV/visible spectrophotometer). The absorbance reading was converted to a unit of log CFU/ml by reference to a standard curve drawn between absorbance values (600 nm) and known colony units (log CFU/ml). Once the required concentration of each isolate was obtained, the cells were again spun down for 15 min at 4000 g to obtain a cell pellet. The cell pallet was directly added to the flour and CWK mixture to prepare a dough, as shown in Table 7.2.

The baker's yeast (Edmond's instant dry yeast, New Zealand) used in the dough was purchased from Countdown, Auckland. The proportions of the isolates and baker's yeast added to the dough are shown in Table 7.2.

7.2.2 Production and formulation of sourdough breads using factorial analysis

A D-Optimal Design (Table 7.1) was used to generate the formulations of sourdough breads using high and low concentrations of *L. plantarum* and *L. fermentum* as variables, along with the addition of baker's yeast as an independent variable (Shiby, Radhakrishna, & Bawa, 2013). The D-Optimal Design was generated using The Unscrambler 10.1 (Camo Analytics AS, Norway).

Table 7.1: Experimental ranges and levels of independent variables used in the D-optimal mixture design for the formulation of top 8 sourdough breads.

Variables	**Range of levels**	
	Low concentration	**High concentration**
Isolate 1 (*L. fermentum*)	4.9 Log CFU/ml	8.3 Log CFU/ml
Isolate 2 (*L. plantarum*)	5.0 Log CFU/ml	9.6 Log CFU/ml
Baker's yeast	0 g	1.16 g

Time of fermentation	18 h	24 h

The fermented mixture of coconut water kefir used in this recipe contained 12g/L of sucrose (Set 3 as shown in Chapter 3, Table 3.3) as it produced the highest cell counts for LAB and yeast, and faster sugar utilization that resulted in low concentration of residual sugar at the end of 96 h of fermentation. Coconut water kefir was allowed to ferment for up to 48 h at 30°C in a LabServ incubator (Thermo Fisher, New Zealand) before being used in bread preparation.

The flour used for this recipe was a high-grade flour (Home brand, Countdown, New Zealand) and Canola oil bought from Countdown, New Zealand was used. No water was used in preparing the sourdoughs. All the sourdough breads were made by mixing salt, dry yeast, 48 h fermented CWK and inoculum specified (Table 7.1) to form a dough. The dough was prepared at 27 ± 2°C, using Breville BBM100WHT Baker's Oven Breadmaker, New Zealand. This dough was then kept in the proofer to ferment at 30°C and 70% humidity for 18 – 24 h, depending on the time of fermentation mentioned in Table 7.2. The proofer used was a Unox linemiss proofer XL193-B, Hospitality Supply, New Zealand. Table 7.2 shows the formulations for eight bread types based on the D-optimal mixture design. The doughs were baked at 175°C for 25 min in a 10% humidity oven (Piron, New Zealand) that was pre-heated at 175°C for 30 min.

Table 7.2: Formulation of sourdough bread using varying concentrations of isolates, yeast, fermentation time.

Breads	**Flour (g)**	**Coconut water kefir with 12 g/L of added sucrose (mL)**	**Sourdough (LAB) (log CFU/mL)**		**Salt (g)**	**Dry yeast (*Saccharomyces cerevisiae*) (g)**	**Time of fermentation (hr)**
			Isolate 1 *Lactobacillus fermentum*	**Isolate 2** *Lactobacillus plantarum*			
Bread 1 A	500	300	8.3		8.18	1.16	18
Bread 2 B	500	300	8.3		8.18	0	24
Bread 3 C	500	300	4.9		8.18	1.16	18
Bread 4 D	500	300	4.9		8.18	0	24
Bread 5 E	500	300		9.6	8.18	1.16	18
Bread 6 F	500	300		9.6	8.18	0	24
Bread 7 G	500	300		5.0	8.18	1.16	18
Bread 8 H	500	300		5.0	8.18	0	24

7.2.3 Sensory analysis for breads

7.2.3.1 Ethics statement and location

The Auckland University of Technology Ethics Committee approved the sensory study (AUTEC ethic application 16/340) carried out in this research. The trained panellists gave written and informed consent prior to the commencement of the study. Sensory testing took place at the AUT University Sensory Suite, Auckland, New Zealand. Sensory sessions were approximately 60 min in duration and as compensation for their time, participants received cash incentives.

7.2.3.2 Sensory attributes

The sensory attributes evaluated in this study are listed in Table 7.3. Each food reference consisted of either a 20 mL of solution or a 25 g of solid reference food, which was served at room temperature (22°C) in a 30 mL black plastic cup or a paper plate. The samples were coded with three-digit random numbers.

Table 7.3: List and description of sensory attributes used in sensory testing using CATA (Callejo, 2011; Song, Perez-Cueto, & Bredie, 2018).

Attributes	Food reference	Definition	References
Buttery	Mainland unsalted butter (Countdown, NZ)	The aromatics commonly associated with natural, fresh, unsalted butter	Heenan, Dufour, Hamid, Harvey, & Delahunty, 2008
Nutty	Coconut meat – dry (Countdown, NZ)	Taste associated with nuts, like coconut	Lotong, IV, & Chambers, 2000
Sour	0.1% citric acid solution	Sour taste, like that elicited by citrus fruits	Heenan, Dufour, Hamid, Harvey, & Delahunty, 2008
Coconut	Concentrated coconut water (UFC, NZ)	Coconut water taste	Heenan, Dufour, Hamid, Harvey, & Delahunty, 2008
Roasted	Roasted bread (White bread, Countdown, NZ)	Aroma associated with roasted bread	Lotong, IV, & Chambers, 2000
Sweet	3% sucrose solution	Sweet taste, like that elicited by sugar	Heenan, Dufour, Hamid, Harvey, & Delahunty, 2008
Salty	0.2% sodium chloride solution	Salty or umami taste – elicited by glutamic acid or NaCl solution	
Floury	Typical of wholemeal flour of wheat mixed with boiling water in proportion 1:2	Aromatics associated with standard baking flour (example: wheat flour)	
Yeasty	A teaspoon of yeast in 250 ml of water	Odour associated with yeast fermentation in bread	

Developed	Loaf of fresh bread from Deli (Countdown, NZ)	When the bread is leavened properly and develops small air pockets/small holes	
Overcooked	Overcooked bread	When the bread is cooked beyond the normal time and tastes a bit burnt	Ella, 2011
Light	Undercooked bread	Very light coloured crust	
Homogenous		Homogeneity of the pores in the crumb	
Heterogenous		Unevenly distributed pores in the crumb	
Golden		When the colour of the crust is light brown/golden upon baking	
Flat		Due to less aeration and leavening of the dough	
Dark		Degree of color darkness in the crumb ranging from white to dark brown	
Thin		Width of the crust is less	
Thick		Width of the crust is bigger	
Smooth		Smoothness/evenness of the crust	
Shiny	Croissant (Countdown, NZ)	Reflection of light on the piece	
Greasy		The appearance of the bread which is covered with oil/animal fat later	
Cracked		The split crust due to increased leavening	
Brown/ Beige		The colour of the bread after baking	
Blister		Formation of tiny bubbles on the bread crust after baking	
Porous		Air pockets in the leavened bread	
Sticky		Bread particles stick to the pallet and during mastication and take a longer time to be washed down by the saliva	
Small holes		Pores in the leavened bread	

Dry		Sensation of dryness due to lack of saliva; absence of water	Gao, Tay, Koh, & Zhou, 2018
Compact/ Dense crumb		Bread containing little cells filled with gas; high density	
Airy crumb/ Big hole		Bread containing cells filled with gas; low density	Gao, Tay, Koh, & Zhou, 2018
Toasted	Toasted white bread, (Countdown NZ)	The odour impression of bread and crumb after baking/heating	Lotong, IV, & Chambers, 2000
Fruity	Mixed fruit platter from (Countdown, NZ)	An aromatic impression of dark fruit that may include sweet, slightly brown, overripe. and somewhat sour	Lotong, IV, & Chambers, 2000
Supple		Degree to which particles reside on the palate during consumption	Heenan, Dufour, Hamid, Harvey, & Delahunty, 2008
Spongy	Sponge cake (Countdown, NZ)	Like a sponge, especially in being porous, compressible, or absorbent.	Gao, Tay, Koh, & Zhou, 2018
Soft	Sandwich bread (Countown, NZ)	Less force required to bite through sample between teeth	
Crunchy		Low pitch sound produced on crust fracture during mastication	
Moist		Amount of moisture perceived on the surface of the product, when in contact with the oral cavity	Heenan, Dufour, Hamid, Harvey, & Delahunty, 2008
Hard		Force required to bite completely through sample placed between the molars	
Fresh		Freshly or recently baked bread loaf	Ella, 2011
Dry		Bread baked due to loss of moisture	
Doughy		Under baked bread dough	
Chewiness		Longer time required to chew the bread to reduce	

		it to a consistency suitable for swallowing	
Crumbly		Bread particles fall apart easily	Gao, Tay, Koh, & Zhou, 2018

7.2.3.3 Panellists

Forty-nine trained panellists (a mix of males and females) between 21 and 39 years of age participated in this study (Mean age = 30, standard deviation = 9.81). They were recruited online through an advertisement posted on social networking services (i.e., Facebook and Instagram), or on university bulletin boards and were compensated for their participation. None of the panellists were smokers or suffered from hearing, eating disorders, or other health problems associated with food that may interfere with hearing or understanding the instructions or during gustation. All data were collected between the hours of 10:00 am and 2:00 pm, and participants were directed not to eat anything 2 h prior to the commencement of the sensory study.

7.2.3.4 Panellist training

Panelists were invited to attend a sensory training session for Check-All-That-Apply (CATA) for product sensory assessment. They were trained over three 5 h sessions, with a total training time of 15 h. Sensory attributes associated with CWK sourdough bread were generated, as shown in Table 7.3. Participants were asked to familiarize themselves with the definitions of the attributes. The panelists were then instructed to select multiple attributes that they perceived when consuming the bread samples.

7.2.3.5 CATA

The procedure used for CATA was described by Castura, Antúnez et al. (2016) and Baker, Castura, and Ross (2016). Assessors were instructed to review the attributes prior to the evaluation, to get familiar with the attribute distribution on the screen. The CATA list included a list of attributes. Assessors were asked to check the attributes that described the sensory characteristics of samples at each moment of the evaluation. Panellists were also asked to familiarise themselves with sensory attribute definitions (Table 7.3). Assessors were asked to check the attributes that described the sensory characteristics of samples during evaluation.

7.2.3.6 Tasting conditions

The William Latin square design (MacFie, Bratchell, Greenhoff, & Vallis, 1989) was used to present the samples, and ensured that the order effects were balanced out. Three-digit random numbers were used to code the product, which were cut in the dimensions of 1cm x 1cm x 1cm (length x breadth x height) and weight 5 ± 0.5 gm. The panellists were requested to rinse their palate with water and unsalted crackers during a 60 s break. The testing was carried out in a temperature-controlled room (18 °C), under white light in individual booths.

7.2.4 Nutritional composition and physicochemical analysis of the sourdough bread

Sourdough breads with desirable sensory characteristics based on CATA testing were subjected to further nutritional and chemical analysis.

7.2.4.1 Sample preparation

The sourdough samples B, D and F were used for this analysis. Sample F was composed of 2.7 CFU/ml of *L. plantarum*, without dry yeast and was fermented for 24 h. Sample B was composed of 2.2 CFU/ml of *L. fermentum*, without dry yeast and fermented for 24 h. Sample D was composed of 1.1 CFU/ml of *L. fermentum*, without dry yeast and was fermented for 24 h

7.2.4.2 Ash content

The ash content was determined by igniting the sample at 550 °C in an electric furnace, AOAC 923.03 (Official Method 923.03, 2005, AOAC, 2005). One g bread samples were dried in an oven (MOV-112-PE Laboratory Oven) at 135°C for 3 h. After 3 hours, the samples were weighed and then placed in a retort furnace (Perfect Fire III, HDTP-56-55) at 550°C for 18 h. At the end of ashing, samples were cooled in the desiccator before reweighing. The analysis was carried out in triplicate for each sample. Ash content was calculated using the formula below.

$$\text{Ash content} = \frac{\text{weight of samples before ashing} - \text{weight of samples after ashing}}{\text{weight of oven dried sample}}$$

7.2.4.3 Carbohydrates

The carbohydrate content was determined according to 1.2.8 FSANZ Food Standards Code, by subtracting the values for moisture content, total fat, ash, dietary fibre and protein content from 100%.

Carbohydrate content = 100 – (moisture content + total fat + ash + dietary fibre + protein content)

7.2.4.4 Dietary fibre

To determine the dietary fibre in the bread samples, AOAC method 985.29 (AOAC, 2005) was used. Briefly, duplicate portions of bread sample were gelatinized using Termamyl (heat-stable α-amylase) and enzymatically digested with protease and amyloglucosidase to remove protein and starch. The fat content was determined before determining the total dietary fibre for the bread samples. Four volumes of ethyl alcohol were added to precipitate the soluble dietary fibre. The total residue was filtered and washed with 78% ethyl alcohol, 95% ethyl alcohol and acetone. After drying, the residue was weighed. One duplicate is analysed for protein and the other is incinerated at 525°C to determine the quantity of ash. Total dietary fibre was calculated using the equation below.

Total dietary fibre = weight of the residue – weight of (protein + ash)

7.2.4.5 Fat Content

To determine the fat content in breads, AOAC acid hydrolysis method 922.06 (AOAC, 2016) was used. Two g of bread sample was placed in a 50 ml beaker to which 2 ml ethanol was added. This mix was stirred properly to avoid lumping of bread particles upon the addition of 8 M hydrochloric acid (HCl). Thereafter, 10 ml HCl was added and mixed well. This was allowed to incubate at 75°C for 3 h, with frequent stirring after every 30 min. This mixture was then transferred to a Mojonnnier fat-extraction apparatus. The beaker was rinsed into extraction tube with 25 ml ether, added in three proportions, into a glass stopper flask. This was followed by vigorous mixing for 1 min. Twenty five ml of redistilled petroleum ether (with a boiling point <60°C) was added and the mixture was vigorously mixed for 1 min. This mixture was allowed to stand until the upper liquid was clear. Collect all the ether-fat solution through a filter consisting of a cotton plug packed firmly in a funnel stem. The clear liquid was allowed to pass through the filter, into a beaker containing 125 ml of porcelain chips. Before weighing the beaker flask, it was dried in the oven at 100°C. The liquid in the tube was re-extracted twice with 15 ml ether, each time. The clear ether solution was drawn through the filter into the same flask. The tip of the funnel, end of the funnel and the spigot were washed with 2 ml of ether. Ethers were allowed to evaporate in steam bath and then dried in the oven at 100°C for 90 min. This dried fat was then weighed to determine the fat content.

7.2.4.6 Moisture

To determine the moisture content of the bread samples, AOAC method 925.09 (AOAC, 2016) was used. Firstly, 2 g of bread sample was weighted and then the sample bread sample was then dried at 100°C for 5 h, then cooled and placed in a desiccator. This was then weighed to determine the loss in moisture.

7.2.4.7 Determination of protein

7.2.4.7.1 Reagents used

The reagents used for this assay include the catalyst tablets – these tablets contained 3.5 g of K_2SO_4 and 0.175 g of HgO (Tecator Inc., USA). Boric acid solution (4%) – 4 g of H_3BO_3 was dissolved in water containing 0.7ml of 0.1% alcoholic solution of methyl red and 1 ml of 0.1% alcohol solution of bromocresol green diluted to 100 ml with water. Sodium hydroxide – sodium thiosulfate solution – 2000g of NaOH and 125 g $Na_2S_2O_3$ in H_2O and dilute to 5 L. Hydrochloric acid (HCl) standard solution (0.2M), hydrogen peroxide (30-35%) and concentrated sulphuric acid.

7.2.4.7.2 Assay procedure

Two g of homogenised bread sample was added to the 250 ml digestion tube. The tubes were placed in the fume hood and 3 boiling chips, 2 catalyst tablets, 15 ml of H_2SO_4 and 3 ml of 35% H_2O_2 was added. The reaction was allowed to subside and then the tubes were transferred to a block digestor preheated at 410°C. This was allowed to digest for 45 min at 410°C until the reaction was clear and then the tubes were removed and were allowed to cool for 20 min by adding 75 ml of water.

In an alkali tank of steam distillation unit, NaOH-$Na_2S_2O_3$ solution was placed. The digestion tube containing the diluted digest was attached. Receiving flask containing 25 ml of H_3BO_3 mixed with indicator, was placed on the platform, with a tube from condenser which extended below the surface of absorbing solution. This was allowed to steam-distill until 125 ml solution was collected and the absorbing solution turned green from liberated NH_3. The digestion tube and the receiving flask units were removed.

Absorbing solution was titrated with 0.2M HCl to a neutral grey end point and the volume acid required was recorded to 0.01ml. The reagent blank was titrated in the same way. The protein content (%) was calculated using the equation below.

Protein % = (VA-VB) * 1.4007 * M * (6.25/g test portion)

7.2.4.8 Total Titratable acidity

The total titratable acidity was determined by AOAC method 942.15 (AOAC, 2016). Briefly, 5 g of bread sample was homogenised and then was added to 50 ml of neutralised water. One ml of phenolphthalein was then added to this mixture and was titrated against 0.1 M NaOH solution until the end point was reached (solution became pale). Titratable acidity was calculated as g/100g of the acid in the sample.

7.2.4.9 Shelf life study - enumeration of Yeasts and Moulds

The method used to enumerate the yeasts and moulds for determining the shelf life of the bread products was from Compendium of Methods for the Microbial Examinations of Foods, Chapter 21.51, American Public Health Association, 5th Edition (2015).

Briefly, Dichloran Rose Bengal Chloramphenicol (DRBC) agar, which contained 100µg of chloramphenicol (added to the agar before autoclaving), Acetic Dichloran Yeast extract Sucrose agar (ADYS), Malt Extract agar (ME), Dichloran glycerol(18% added glycerol) agar (D18) and Tryptone Glucose Yeast Extract agar (TGY) were used for this assay.

All the agar media were autoclaved for 121°C for 15 mins and were poured in Petri plates then allowed to cool overnight at a temperature of 25°C, under dark environment.

One g of bread sample was completely homogenised and added 9ml peptone water. This was further diluted 9-folds and 0.1ml of these dilutions were spread plated on DRBC, D18, ADYS, ME and TGY agar media.

The plates were incubated for 5 days at 25°C and the colonies were counted and reported as CFU/g for each bread sample.

7.2.5 Texture analysis

Texture Profile Analysis (TPA) was performed to determine sourdough bread texture in terms of hardness, springiness, cohesiveness, chewiness, gumminess and resilience. All samples were prepared and baked on the day of the test. Each bread sample was cut using a knife to obtain a cube (2cm *2cm* 2cm). The cube was taken from the middle of each loaf to evaluate the crumb texture. Analysis was performed using a Stable Micro Systems TA.XT plus Texture Analyser equipped with a Film Support Rig (HDP/FSR) on a Heavy Duty Platform (HDP/90) with a 5 mm stainless steel probe (P/55) and a 5 kg load cell. The texture analyser was set to measure force in the compression mode with a pre-test speed of 2.0 mm/s, a test speed of 1.0 mm/sec, and a post-test speed of 10.0 mm/sec time 30s; load cell: 50 Kg; trigger force: auto-10g. Target mode was set to distance set at 5 mm. Data acquisition rate was set at 500 pps.

7.2.6 Determination of Glycaemic Index

For this study, recruitment of 30 participants was done in the Auckland region. Fifteen healthy males and 15 females between the ages of 25-35 years of age were selected for this study with no pre-existing diabetic conditions. The exclusion criteria included those with medical conditions (including type 2 diabetes), those on medications interfering with glucose metabolism, those who are allergic or intolerant to high grade wheat flour, kefir or coconut water.

Each participant gave a written consent to participate. The ethics approval letter was obtained from the Auckland University of Technology Ethics Committee (AUTEC) on 23 August 2018 (Ethics application 16/340) (Appendix 14).

All participants were instructed to fast for anytime between 8 h to 12 h before the start of experiment. The CareSens-NPOP blood glucose monitoring device (Pharmaco NZ Limited, New Zealand) was used. CareSens-N blood glucose test strips (Pharmaco NZ Limited, New Zealand) were used along with the lancets to puncture the skin surface of the finger, for accurate reading. Once the skin surface was punctured, the drop of blood was collected for each participant, measuring fasting blood glucose at 0 min (at the start of the experiment), which was used as baseline blood glucose concentration, expressed in millimoles per litre.

A slice of bread weighing 50g was given to the participants to consume within the first 15 min. Blood glucose reading was taken after every 15 min until the end of 120 min (or until required). Participants were provided with water during the experiment and remained seated throughout the time readings were taken.

Glycaemic index (GI) value of the bread was measured according to ISO 26642:2010-E. Anhydrous glucose powder (50gm) was used as reference for this experiment, such that, a saturated solution of the anhydrous glucose powder was mixed with water and given to the same participants for consumption. Saturated solution of the anhydrous glucose powder was mixed with water and given to the same participants to consume. A blood glucose response curve for the two-hour period was constructed using the blood samples obtained.

The blood samples were taken by pricking the thumb or middle finger using a sterilised disposable lancet provided in the CareSens-NPOP blood glucose monitoring device. The test strip attached to the blood glucose monitor was exposed to blood to provide a reading. The readings from blood samples taken over time were used to construct a blood glucose response curve for the two-hour period. The Incremental Area under the Curve (IAUC) for blood glucose was calculated for each bread sample using the trapezoidal method (Wolever & Jenkins, 1986), and reflected the total rise in blood glucose levels after eating the bread samples. The IAUC for each bread sample taken by each panellist was expressed as a percentage of the mean IAUC for the reference food (glucose) taken by the same subject, and the mean of the resulting values was the bread GI value.

The GI has been defined as formula below (Wolever & Jenkins, 1986):

$$GI(\%) = \frac{\text{Area under the curve for 50g carbohydrate from test food}}{\text{Area under the curve for 50g carbohydrate from the reference food (glucose)}} \times 100$$

7.2.7 Determination of fermentation quotient

The fermentation quotient was calculated as a molar ratio between lactic acid and acetic acid (Galli et al., 2019). The determination of lactic and acetic acid from the dough was carried out using the LC-MS method as mentioned in section 4.2.11, materials and method section for Chapter 4.

7.2.8 Determination of Carboxylic acids using Liquid Chromatography – Mass Spectrometry (LC-MS)

The bread samples in triplicate were first ground. Then 50±0.1 mg was weighed and added to ice-cold 50% MeOH, containing 10 mg/L 4-chlorobenzoic acid (CBA). CBA was used as an internal

standard. The mixture was centrifuged at 10,000 x g for 5 minutes at 4°C (Z216MK, HERMLE Labortechnik GmbH, Germany). The 2-hydrazinoquinoline (HQ) derivatization reaction was conducted by mixing 5 µL of the bread sample with 100 µL of acetonitrile solution containing 1 mM 2,2′-dipyridyl dipyridyl disulfide (DPDS), 1 mM triphenylphosphine (TPP) and 1 mM HQ. The reaction mixture was incubated at 60 °C for 60 min. The HQ derivatives in the reaction mixture were analyzed using LC-MS.

The carboxylic acids used as standards for this assay were lactic acid, succinic acid, acetic acid and pyruvic acid prepared with 50% methanol, and 20mM sodium hydroxide. The LC-MS was an Agilent 1260 Series liquid chromatograph comprising a G1311B quaternary pump, G1329B thermostatted autosampler and a G1330B thermostatted column compartment (Agilent Technologies, Santa Clara, USA). Mobile phase A was 0.1% formic acid in ultrapure water and mobile phase B was 0.1% acetic acid in acetonitrile. The column was a Phenomenex Kinetex EVO C18 column, measuring 2.1x150 mm with 1.7 µm diameter packing material, maintained at 25 °C. The chromatographic gradient was held for 0.1 min at 3% B and then ramped to 15% B at 6 min and 90% B at 9 min before returning to 3% B at 11 min. The flow rate was 200 µL min-1, the total run time was 16 minutes and the injection volume was 5 µL.

For detection, an Agilent 6420 triple quadrupole mass spectrometer fitted with an Agilent Multimode Ionisation source was operated in positive electrospray mode. Derivatised carboxylic acid peak areas were normalised and quantified by reference to a dilution series of external standards prepared. Data was collected and processed using the Agilent MassHunter software.

7.2.9 Determination of amino acids using Liquid Chromatography – Mass Spectrometry (LC-MS)

One g of bread sample, in triplicate, with a mass of 45 ± 0.1 mg were added to 1.4 mL volume of 50% methanol containing 10 mg L-1of 2,3,3,3-d4-alanine [d4A] in a microcentrifuge tube. The tubes were vortexed to obtain a homogenous mixture. This was followed by centrifugation at 10,000 g for 5min at 4°C (Z216MK, HERMLE Labortechnik GmbH, Germany). A 10 µl volume of extract was used to perform pre-column derivatisation with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate following a method adapted from Salazar et al (2012). The LC-MS was an Agilent 1260 Series liquid chromatograph comprising a G1311B quaternary pump, G1329B thermostatted autosampler and a G1330B thermostatted column compartment (Agilent Technologies, Santa Clara, USA). Mobile phase A was 0.6% formic acid in ultrapure water and mobile phase B was 0.1% formic acid in acetonitrile. The column used was a Phenomenex Kinetex EVO C18 column, measuring 2.1x150 mm with 1.7 µm diameter packing material,

maintained at 25 °C. The chromatographic gradient was held for 1 min at 1.5% B and then ramped to 13% B at eight min, 17% B at 15 min and 80% B at 16 min before returning to 1.5% B at 17.5 min. The flow rate was 300 µL/min, total run time was 28 min, and the injection volume was 5 µL. For detection, an Agilent 6420 triple quadrupole mass spectrometer fitted with an Agilent Multimode Ionisation source was operated in positive electrospray mode. Optimum Multiple Reaction Monitoring [MRM] transitions were established using Agilent MassHunter Optimiser B06.00 software. Derivatised amino acid peak areas were normalised to the recovery of d4A and quantified by reference to a dilution series of external standards prepared from a commercial amino acid mix (Sigma product A9906, Sigma-Aldrich Pty. Ltd., Sydney, Australia). Data were collected and processed using Agilent MassHunter software.

7.2.10 Statistical analysis

Significant differences between texture readings of the different samples were tested using One-way ANOVA on XLSTATS with Tukey's post hoc comparison tests, where $p < 0.05$ ($\alpha = 0.05$) indicates significant differences between means.

Principle coordinate analysis was carried out using XLSTAT version 2019.1.2, StatSoft Inc., USA (Anderson & Braak, 2003). The influence of variables (types of isolates: isolate 1 and isolate 2, and the concentration of isolate 1 and isolate 2) on coconut water kefir fermentation was studied by employing a full factorial design (Myers, Montgomery, & Anderson-Cook, 2016). Runs were performed at random. Results were analyzed using XLSTAT (version 2019.1.2, StatSoft Inc., USA) software.

Canonical Variate Analysis (CVA) of duration and frequency of sensations was conducted with dominance duration as the dependent variable to evaluate the differences between sourdough samples based on the sensory attributes as carried out by Jager et al. (2014). In addition, Hotelling-Lawley trace Multivariate Analysis of Variance (MANOVA) tested for significant differences between each product loadings at the 5% level. CVA was used in this study as it can maximize the distances between products, while minimizing residual variability (Monrozier & Danzart, 2001).

For CATA analysis, the Cochran's Q test (Manoukian, 1986) was carried out to identify significant differences among samples for each of the sensory terms. In this study, Cochran's Q test was used to evaluate the differences between treatments (samples) with binary responses (Torres et al., 2017).

Correspondence analysis (CA) was performed on the frequency table from each experimental treatment considering chi-square distances. CA was used to visualize the frequency table as a

generalization of Principal Component Analysis (PCA) for the attributes. The method projects the data into orthogonal components such as to maximize the sequential representation of the variation in the data (Fay, Boyd, & Salkind, 2010; Greenacre, 2017). Multidimensional Alignment (MDA) was applied to determine the cosine values between the bread products and sensory attributes originating in the CA of the CATA questions.

7.3. Results and Discussion

7.3.1 Development of coconut water kefir fermented sourdough bread supplemented with different concentrations of *Lactobacillus fermentum, Lactobacillus plantarum* and baker's yeast.

A CWK sourdough bread was formulated using *Lactobacillus fermentum* and *Lactobacillus plantarum*. The isolates from Chapter 6 with high glutamic acid and phytase producing ability were chosen to formulate this product. As *Lactobacillus fermentum* and *Lactobacillus plantarum* produced significantly high concentrations of glutamic acid and phytase enzyme (Chapter 6, Figure 6.1 and Figure 6.3) both a high and a low cell concentration of both these isolates were used to develop a CWK fermented sourdough bread. Other factors were kept constant such as the addition of dry yeast (at 1.16 g/L) and fermentation time (18 h and 24 h).

7.3.2 Sensory analysis of sourdough breads using Check-all-that-apply (CATA)

Flavour perception for CWK- fermented sourdough bread samples supplemented with different concentrations of *Lactobacillus fermentum, Lactobacillus plantarum* and baker's yeast was determined using CATA. Cochran's Q test was carried out to identify if significant differences among samples for each of the descriptors in the CATA questionnaire exist (Manoukian, 1986). Frequency of attributes across each sourdough samples was calculated. Results from Cochran's Q test showed significant differences for 36 out of 50 attributes among the 8 samples (A-H) ($p<0.05$) (Table 7.4).

The citation frequency of each attribute is summarized in Table 7.4. B, C, D, G and H breads were significantly more developed compared to breads A, E and F. 'Developed' is positively correlated with the breads as reported by Plessas et al. (2005) and Katina et al. (2005). All the breads were highly porous except bread sample E. In fact, breads A, F and H were significantly more porous compared to all the other bread samples. Porosity is related to the amount of carbon dioxide produced during the dough fermentation, which in turn is related to the presence of many non-volatile and volatile compounds being produced during dough fermentation. Therefore, porosity has been directly linked to improving elasticity, softness and springiness, which are the main parameters to determine the overall quality of the bread (Rathnayake, Navaratne, & Navaratne, 2018). Porous (big holes) and soft bread could be due to the gas trapped in the gluten during fermentation, thus affecting the viscoelastic properties of dough (Palomba et al. 2012; Pepe et al. 2013), by increasing gas retention during proofing and baking (Kaditzky and Vogel, 2008; Katina et al. 2009; Torrieri et al. 2014).

The frequency of the 'light' attribute, for rating the appearance of the bread, was significantly high for all breads, except bread C, E and F. Light brown colour is positively related to the

sourdough breads as reported by Ua-Arak, Jakob, & Vogel (2017). Bread crust experiences Maillard reactions and caramelisation, which are influenced by quantity and quality of the precursors in the dough, pH changes and the quantitative ratio of amino nitrogen to reducing sugar during baking (Martins, Jongen, & Van Boekel, 2000; Mohd Jusoh, Chin, Yusof, & Abdul Rahman, 2009). All the breads were significantly smoother in terms of appearance except bread B and E. Bread F had significantly higher shiny attribute compared to bread. D. Amina, Ismail, & Abdelkader, (2018) have reported that the overall appearance of the bread is more likeable when the crust appears smooth, shiny, regularly coloured (brown) and was free of blisters.

Bread samples A, B, C, D, F, G and H were sour compared to bread E. Sour flavour in the breads could be attributed to the addition of lactic acid bacteria like *L. plantarum* and *L. fermentum*. It is well known that during dough fermentation, many acids like lactic acid and acetic acid are produced, which contribute to the perceived sourness of bread (results as shown in Chapter 4, section 4.3.5). Samples G, D and E had significantly higher frequency for freshness compared to the rest of the breads. Generally, the status of bread freshness as perceived by consumers depends on flavour (taste and aroma), appearance and crispness of crust, firmness of crumb, and bread volume. Breads A, D and E had significantly higher frequency for spongy compared to bread sample C. The differences obtained between breads A, D, E and rest of the bread samples could be related to the change in dough properties during and at the end of fermentation. This could be influenced by different structural components such as gluten and by changes in the pH value of the dough system (Clarkston et al., 1996).

Bread samples B and F had significantly firmer texture compared to bread sample D. Both sourdough breads B and F did not have any additional baker's yeast added to them during the dough fermentation. A study carried out by Plessas, Pherson, Bekatorou, Nigam, & Koutinas, (2005) reported that breads made with added baker's yeast were softer and less firm, when compared to breads made with kefir only. Breads C, G and H had significantly higher frequency for roasted flavour, compared to bread sample A. According to Katina et al. (2004), the roasted flavour in the sourdough correlates with the type and quantity of amino acids produced during sourdough fermentation. Samples A, F, G and H had a significantly higher frequency for yeasty compared to breads B, C, D and E. Thiele, Gänzle, & Vogel, (2002) explained that sourdough breads which contained *S. cerevisiae* (commonly known as baker's yeast), could be perceived as 'yeasty'. Although yeasty odour is generally not associated with sourdough breads, the presence of *S. cerevisiae* in the fermented coconut water kefir or the added baker's yeast to the sourdough, could lead to the sourdough breads A, F, G and H being perceived as more yeasty over the other breads.

Breads E, F, G and H were perceived as being significantly overcooked compared to breads A, B, C and D. 'Overcooked' is the appearance of the bread samples, which is influenced by cooking temperature (Khan, 2002). Interestingly, all the breads fermented with *L. plantarum* breads E-H) (addition of low and high concentration of cells) were perceived as overcooked compared to all breads fermented with *L. fermentum* (breads A-D). Samples B, C, G and H had significantly hard texture compared to samples A, D, E and F. This could be due to the change in the specific volume of the dough. Generally, the breads are hard if there is less leavening activity and less acidity in the dough. The impact of sourdough on bread volume has been suggested to be principally due to enzymatic reactions taking place throughout the fermentation (Corsetti et al., 1988; Clarke et al., 2004; Katina et al., 2006). In addition, bread samples A, D and E had highly significant frequency for doughy, coarse, chewy and crumbly texture attributes compared to bread samples B, C, F, G and H.

Correspondence analysis

Correspondence analysis (CA) that was carried out to evaluate the CATA questionnaire produced a bi-dimensional map (Figure 7.1) that explained 67.89% variance in the first two dimensions as seen in Figure 7.1, with 43.98% and 23.91% variance explaining the first and second dimensions, respectively.

In addition, Multidimensional Alignment (MDA) was applied to determine the cosine values between the bread products and sensory attributes originating in the CA of the CATA questions. Table 7.5 highlights the attributes which are positively and negatively correlated with the bread samples using the first two dimensions of CA bi-dimensional map.

As shown in Figure 7.1, the first dimension was positively correlated to samples C, H and G and negatively correlated to samples E, D and A. On the other hand, the second dimension positively correlated to samples F and B. The sensory map generated by CA further showed the relationship between the sensory attributes and the bread products and exhibit two dimensions.

Bread samples C, G and H had high positive scores along Dimension 1 (Figure 7.1). These samples were highly positively correlated with the attributes namely, developed, shiny, greasy, dense crumb, beige, sweet, roast, supple, and hard (Table 7.5). Attributes such as crumbly, doughy, blister and overcooked are negatively correlated to the sourdough bread as reported by Katina, Heiniö, Autio, & Poutanen, 2006.

Bread samples A and D had high negative scores along Dimension 1 (Figure 7.1). These samples were highly positively correlated to the attributes, heterogenous, dark, airy crumb, buttery, spongy, dry, doughy, coarse, chewy and crumbly (Table 7.5). Flavour attributes such

as nutty and buttery are positively related to the sourdough bread (Katina et al., 2006). The heterogenous and light appearance of the bread are dependent on the baking temperature of bread. Bread sample E also had high negative loadings along Dimension 1 (Figure 7.1). This sample was highly positively correlated to the attributes, airy crumb, spongy, fresh, doughy, coarse, chewy and crumbly (Table 7.5). Attributes such as doughy, crumbly and coarse have been negatively correlated with sourdough bread samples, as reported by Katina, Heiniö, Autio, & Poutanen, (2006).

Bread samples B and F that were not well explained along Dimension 1 had high negative scores along Dimension 2 (Figure 7.1). These samples were highly positively correlated with the attributes, light, blister, porous, small holes, big hole, nutty and moist (Table 7.5). Starter culture, ash content of flour, fermentation temperature and dough yield have been reported to influence bread flavour. The known liked attributes for sourdough breads include attributes like springiness, porous, sour, fresh and roasted (Bartkiene et al., 2017; Di, Jinshui, Feng, & Changfu, 2018; Di Monaco, Torrieri, Pepe, Masi, & Cavella, 2015; Katina, Heiniö, Autio, & Poutanen, 2006; Lotong, Iv, & Chambers, 2000).

From the present study, bread samples C, G, and H were eliminated from further studies due to their correlation with negative attributes. Bread samples, B, D and F were chosen for further study due to their correlation with positive attributes. Bread sample E was not chosen for further study as the objective of this study was to create a sourdough using only LAB and fermented coconut water kefir. Since bread sample E had 1.16 g/L of added baker's yeast in the dough, which does not agree with the objective of this chapter, this bread sample was eliminated.

Table 7.4: CATA data by citation frequency and Cochran's Q test associated with each sample of CWK fermented sourdough bread (sample A-H) by consumers (n = 50) on check-all-that-apply questions. a–e: Different superscript letters in rows denote frequencies of attribute that are significantly different ($p < 0.05$) across the sourdough bread samples. P-value greater than α = 5% indicates no significant difference.

Products\ Dimensions	**p-values**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Developed	0.018	4a	5a,b	4a,b	5a,b	2a	3a	9a,b	12b
Overcooked	0.001	7a	11a,b	8a	9a,b	22c	18b,c	17b,c	18b,c
Light	0.001	12b,c	16c	9a,b, c	15c	4a,b	2a	9b,c	10b,c
Heterogenous	0.000	19c	16b,c	1a	17c	7a,b	7a,b	3b,c	5a

Dark	0.000	27e	17d	7a,b,c	20de	3a	6a,b	15b,c	12b,c,d
Smooth	0.002	10b,c	6a,b	9b,c	8b,c	1a	15b,c	16b,c	13b,c
Shiny	0.029	15b,c	7a,b	14a,b,c	6a	12a,b,c	19c	14b,c	10a,b,c
Greasy	0.000	3a	5a	19c	3a	8a,b	6a,b	14b,c	18c
Blister	0.000	23d	22d	0a	12c	8c	22d	7b,c	1a,b
Porous	0.000	16d	6a,b,c	3a	5a,b	1a	13c,d	12b,c	14c,d
Small holes	0.002	13c	10b,c	4a,b	11b,c	1a	15c	10b,c	10b,c
Dry	0.000	13b,c	19c	4a	17c	7a,b	15b,c	4b,c	6a
Dense crumb	0.034	8a,b	15b	17b	14b	8a,b	5a	12b,c	12a,b
Compact	0.002	0a	3a,b,c	8b,c,d	2a,b	9c,d	3a,b,c	11b,c	6b,c,d
Big hole	0.001	15b,c	22c	10a,b	13b,c	4a	13b,c	11b,c	6a,b
Beige	0.000	5a,b	0a	13c,d	0a	12b,c	3a	23b,c	24d
Airy crumb	0.000	13b	0a	0a	0a	10b	10b	0b,c	0a
Yeasty	0.002	10a,b,c	5a,b	4a	5a,b	3a	15c	13b,c	9a,b,c
Sweet	0.044	5a,b	5a,b	9b	6a,b	1a	9b	11b,c	11b
Sour	0.000	5a,b	12b,c	11a,b,c	6a,b,	3a	17d	15d	16d
Roast	0.000	2a	4a,b	17c	4a,b	6a,b	6a,b	15c	11b,c
Nutty	0.000	17b	21b	18b	19b	7a	14a,b	16b,c	7a
Buttery	0.000	15b,c	10b	0a	16b,c	13b,c	22c	2b,c	1a
Supple	0.000	4a,b	3a	8a,b,c,d	5a,b,c	2a	13c,d	11b,c	14d
Spongy	0.004	21b	11a,b	7a	20b	16b	12a,b	9a,b	11a,b
Soft	0.000	1a	14c	4a,b	2a	0a	15c	14b,c	10b,c
Moist	0.000	9b	20d	3a	6a,b	9b	13c	11b,c	4a
Hard	0.005	11a	14a,b	22b	10a	8a	9a	8b,c	16a,b
Fresh	0.050	6a	5a	8a,b	12b	11b	3a	15c	4a
Firm	0.004	9a,b,c	15c	9a,b,c	3a	4a,b	16c	13b,c	12b,c
Elastic	0.000	13c,d,e	0a	15de	9b,c,d	10b,c,d	5a,b,c	4b,c	22e
Doughy	0.000	12b	0a	0a	9b	7b	0a	0a	0a
Coarse	0.000	9b	0a	0a	10b	13b	0a	0a	0a
Chewiness	0.000	10b	0a	0a	12b	14b	0a	0a	0a
Crumbly	0.006	4b	0a	0a	3b	4b	0a	0a	0a
Developed	0.018	4a	5a,b	4a,b	5a,b	2a	3a	9a,b	12b
Overcooked	0.001	7a	11a,b	8a	9a,b	22c	18b,c	17b,c	18b,c
Light	0.001	12b,c	16c	9a,b,c	15c	4a,b	2a	9b,c	10b,c
Homogenous	0.618	11	16	10	11	10	11	14	15
Heterogenous	0.000	19c	16b,c	1a	17c	7a,b	7a,b	3b,c	5a
Golden	0.426	14	13	18	14	9	11	12	16
Flat	0.068	3	5	8	7	4	10	8	1

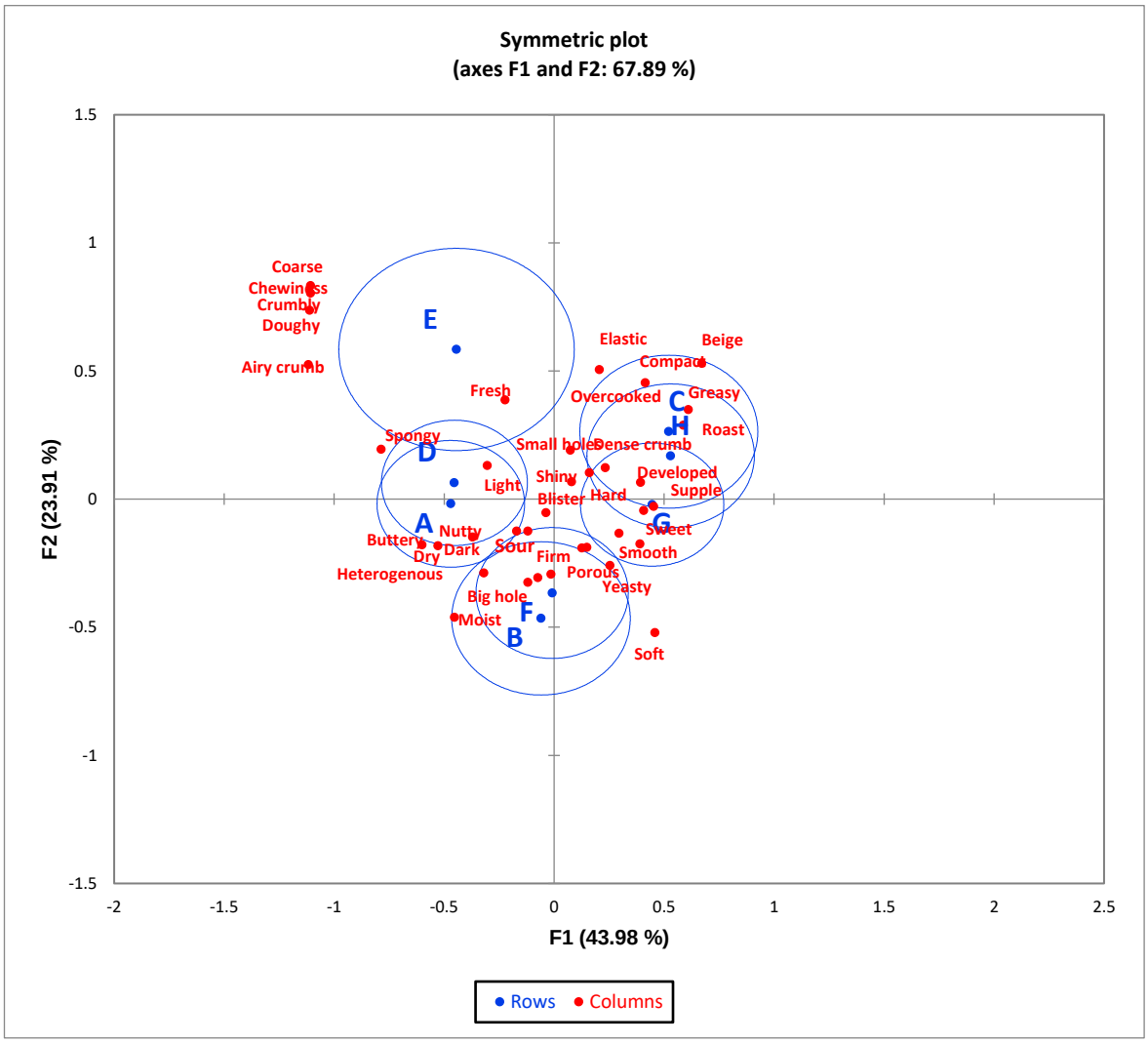
Products\ Dimensions	p-values	A	B	C	D	E	F	G	H
Dark	0.000	27e	17d	7a,b,c	20de	3a	6a,b	15b,c	12b,c,d
Thin	0.244	9	6	9	5	5	1	10	6
Thick	0.067	15	9	10	4	15	14	13	11
Smooth	0.002	10b,c	6a,b	9b,c	8b,c	1a	15b,c	16b,c	13b,c
Shiny	0.029	15b,c	7a,b	14a,b,c	6a	12a,b,c	19c	14b,c	10a,b,c
Greasy	0.000	3a	5a	19c	3a	8a,b	6a,b	14b,c	18c
Golden	0.103	15	18	15	16	12	14	7	8
Cracked	0.221	9	11	6	11	8	5	5	4
Brown	0.772	9	11	13	8	7	12	11	11
Blister	0.000	23d	22d	0a	12c	8c	22d	7b,c	1a,b
Porous	0.000	16d	6a,b,c	3a	5a,b	1a	13c,d	12b,c	14c,d
Sticky	0.130	14	9	5	7	14	12	13	11
Small holes	0.002	13c	10b,c	4a,b	11b,c	1a	15c	10b,c	10b,c
Grey	0.523	11	14	7	14	9	11	11	15
Dry	0.000	13b,c	19c	4a	17c	7a,b	15b,c	4b,c	6a
Dense crumb	0.034	8a,b	15b	17b	14b	8a,b	5a	12b,c	12a,b
Compact	0.002	0a	3a,b,c	8b,c,d	2a,b	9c,d	3a,b,c	11b,c	6b,c,d
Big hole	0.001	15b,c	22c	10a,b	13b,c	4a	13b,c	11b,c	6a,b
Beige	0.000	5a,b	0a	13c,d	0a	12b,c	3a	23b,c	24d
Airy crumb	0.000	13b	0a	0a	0a	10b	10b	0b,c	0a
Yeasty	0.002	10a,b,c	5a,b	4a	5a,b	3a	15c	13b,c	9a,b,c
Toasted	0.085	11	6	11	6	15	16	9	13
Sweet	0.044	5a,b	5a,b	9b	6a,b	1a	9b	11b,c	11b
Sour	0.000	5a,b	12b,c,d	11a,b,c,d	6a,b,c	3a	17d	15b,c	16d
Roast	0.000	2a	4a,b	17c	4a,b	6a,b	6a,b	15c	11b,c
Nutty	0.000	17b	21b	18b	19b	7a	14a,b	16b,c	7a
Fruity	0.057	14	7	6	9	9	6	3	5
Coconut	0.126	11	13	18	6	9	10	11	10
Buttery	0.000	15b,c	10b	0a	16b,c	13b,c	22c	2b,c	1a
Supple	0.000	4a,b	3a	8a,b,c,d	5a,b,c	2a	13c,d	11b,c	14d
Spongy	0.004	21b	11a,b	7a	20b	16b	12a,b	9a,b	11a,b
Soft	0.000	1a	14c	4a,b	2a	0a	15c	14b,c	10b,c
Crunch	0.110	5	7	6	13	10	13	12	14
Moist	0.000	9b	20d	3a	6a,b	9b	13c	11b,c	4a
Hard	0.005	11a	14a,b	22b	10a	8a	9a	8b,c	16a,b
Fresh	0.050	6a,b	5a,b	8a,b	12b	11b	3a	15b,c	4a,b
Firm	0.004	9a,b,c	15c	9a,b,c	3a	4a,b	16c	13b,c	12b,c

Elastic	0.000	13c,d,e	0a	15de	9b,c,d	10b,c,d	5a,b,c	4b,c	22e
Doughy	0.000	12b	0a	0a	9b	7b	0a	0a	0a
Coarse	0.000	9b	0a	0a	10b	13b	0a	0a	0a
Chewiness	0.000	10b	0a	0a	12b	14b	0a	0a	0a
Crumbly	0.006	4b	0a	0a	3b	4b	0a	0a	0a

Composition of bread samples A-H is shown below:

A- Composed of 8.3 log CFU/ml of *L. fermentum*, 1.16 g of dry yeast and was fermented for 18h
B- Composed of 8.3 log CFU/ml of *L. fermentum*, without dry yeast and fermented for 24h
C- Composed of 4.9 log CFU/ml of *L. fermentum*, 1.16 g of dry yeast and was fermented for 18h
D- Composed of 4.9 log CFU/ml of *L. fermentum*, without dry yeast and was fermented for 24h
E- Composed of 9.6 log CFU/ml of *L. plantarum*, 1.16 g of dry yeast and was fermented for 18h
F- Composed of 9.6 log CFU/ml of *L. plantarum*, without dry yeast and was fermented for 24h
G- Composed of 5.0 log CFU/ml of *L. plantarum*, 1.16 g of dry yeast and was fermented for 18h
H- Composed of 5.0 log CFU/ml of *L. plantarum*, without dry yeast and was fermented for 24h

Figure 7.1: Correspondence analysis plot (with CI 95% based on chi-square distance) of CWK fermented sourdough bread samples A-H, using check-all-that-apply results.



Composition of bread samples A-H is shown below:

A- Composed of 8.3 log CFU/ml of *L. fermentum*, 1.16 g of dry yeast and was fermented for 18h
B- Composed of 8.3 log CFU/ml of *L. fermentum*, without dry yeast and fermented for 24h
C- Composed of 4.9 log CFU/ml of *L. fermentum*, 1.16 g of dry yeast and was fermented for 18h
D- Composed of 4.9 log CFU/ml of *L. fermentum*, without dry yeast and was fermented for 24h
E- Composed of 9.6 log CFU/ml of *L. plantarum*, 1.16 g of dry yeast and was fermented for 18h
F- Composed of 9.6 log CFU/ml of *L. plantarum*, without dry yeast and was fermented for 24h
G- Composed of 5.0 log CFU/ml of *L. plantarum*, 1.16 g of dry yeast and was fermented for 18h
H- Composed of 5.0 log CFU/ml of *L. plantarum*, without dry yeast and was fermented for 24h

Table 7.5: Values of cosine between bread product and the attributes used to describe the samples, obtained by Correspondence Analysis (CA) for eight sourdough bread samples using CATA questions.

	A	B	C	D	E	F	G	H
Developed	-0.992	-0.288	0.955	-0.954	-0.466	-0.188	0.977	0.990
Overcooked	-0.398	-0.971	0.743	-0.229	0.523	-0.941	0.317	0.628
Light	0.602	0.887	-0.879	0.451	-0.309	0.835	-0.530	-0.793
Heterogenous	0.957	0.445	-0.991	0.890	0.310	0.351	-0.928	-1.000
Dark	0.828	0.690	-0.986	0.716	0.015	0.612	-0.776	-0.947
Smooth	-0.894	0.294	0.627	-0.959	-0.880	0.391	0.929	0.742
Shiny	-0.790	-0.736	0.973	-0.669	0.050	-0.662	0.734	0.925
Greasy	-0.887	-0.603	0.999	-0.791	-0.130	-0.517	0.843	0.978
Blister	0.727	0.797	-0.947	0.594	-0.145	0.731	-0.665	-0.884
Porous	-0.518	0.758	0.116	-0.661	-0.998	0.821	0.590	0.272
Small holes	0.087	0.997	-0.492	-0.091	-0.767	1.000	0.001	-0.349
Dry	0.767	0.760	-0.964	0.641	-0.086	0.689	-0.708	-0.910
Dense crumb	-0.861	-0.644	0.995	-0.758	-0.078	-0.562	0.814	0.966
Compact	-0.703	-0.818	0.935	-0.566	0.180	-0.754	0.638	0.867
Big hole	0.272	0.994	-0.647	0.097	-0.633	0.978	-0.187	-0.518
Beige	-0.809	-0.713	0.980	-0.693	0.017	-0.637	0.755	0.937
Airy crumb	0.961	-0.114	-0.759	0.995	0.778	-0.216	-0.981	-0.852
Yeasty	-0.590	0.698	0.202	-0.724	-1.000	0.768	0.658	0.354
Sweet	-0.989	-0.019	0.839	-1.000	-0.687	0.084	0.998	0.914
Sour	-0.896	0.289	0.631	-0.960	-0.877	0.386	0.931	0.745
Roast	-0.914	-0.551	1.000	-0.828	-0.192	-0.462	0.876	0.989
Nutty	0.715	0.808	-0.941	0.580	-0.163	0.743	-0.652	-0.875
Buttery	0.969	0.405	-0.984	0.910	0.353	0.308	-0.944	-1.000
Supple	-0.995	-0.065	0.863	-0.997	-0.653	0.038	1.000	0.932
Spongy	0.903	-0.275	-0.643	0.965	0.870	-0.372	-0.937	-0.756
Soft	-0.630	0.661	0.251	-0.758	-0.997	0.735	0.695	0.401
Moist	0.380	0.975	-0.730	0.210	-0.540	0.947	-0.298	-0.613
Hard	-0.904	-0.572	1.000	-0.813	-0.167	-0.485	0.863	0.985
Fresh	0.465	-0.796	-0.056	0.615	0.992	-0.854	-0.540	-0.213
Firm	-0.671	0.620	0.302	-0.791	-0.992	0.698	0.733	0.449
Elastic	-0.414	-0.966	0.754	-0.246	0.509	-0.935	0.333	0.641
Dry	0.942	0.488	-0.996	0.867	0.264	0.396	-0.909	-0.997
Doughy	0.888	-0.306	-0.617	0.955	0.886	-0.403	-0.925	-0.733
Coarse	0.775	-0.495	-0.443	0.875	0.962	-0.582	-0.827	-0.579
Chewiness	0.786	-0.480	-0.458	0.883	0.957	-0.568	-0.836	-0.593
Crumbly	0.811	-0.443	-0.495	0.902	0.944	-0.532	-0.859	-0.626

[a] Values in green indicates high positive correlation of the sensory attribute with the respective sample.
[b] Values in red indicates high negative correlation of sensory attribute with the respective sample.

Composition of bread samples A-H is shown below:

A- Composed of 8.3 log CFU/ml of *L. fermentum*, 1.16 g of dry yeast and was fermented for 18h
B- Composed of 8.3 log CFU/ml of *L. fermentum*, without dry yeast and fermented for 24h
C- Composed of 4.9 log CFU/ml of *L. fermentum*, 1.16 g of dry yeast and was fermented for 18h
D- Composed of 4.9 log CFU/ml of *L. fermentum*, without dry yeast and was fermented for 24h
E- Composed of 9.6 log CFU/ml of *L. plantarum*, 1.16 g of dry yeast and was fermented for 18h
F- Composed of 9.6 log CFU/ml of *L. plantarum*, without dry yeast and was fermented for 24h
G- Composed of 5.0 log CFU/ml of *L. plantarum*, 1.16 g of dry yeast and was fermented for 18h
H- Composed of 5.0 log CFU/ml of *L. plantarum*, without dry yeast and was fermented for 24h

7.3.3 Proximate and texture analysis

Proximate and texture analyses were carried out for the sourdough bread samples, B, D and F. The results are shown in Table 7.6.

7.3.3.1 Determination of protein content

The protein content of bread sample D (8.5 ± 0.5 g/100g) was significantly higher than that of bread sample F (8.2 ± 0.4g/100g) ($p<0.05$).

This could be explained by the fact that the proteolytic activity of bread sample F (composed of 9.6 log CFU/ml of *L. plantarum*) might have been higher than that of bread sample D (composed of 4.9 log CFU/ml of *L. fermentum*). The decrease in the concentration of total protein in sourdough was a consequence of degradation of wheat flour proteins owing to the action of proteolytic enzymes (proteases) and LAB. Generally, the proteases of wheat flours are grouped as proteinases and peptidases (Gänzle, Loponen, & Gobbetti, 2008). Proteinases catalyze protein degradation into smaller peptide fractions; peptidases hydrolyze specific peptide bonds or completely break down peptides to amino acids. A study carried out by Yin et al. (2015) states that sourdough fermented with *L. plantarum* M616 showed an overall decrease in the total protein content of the bread after 24 h time period. A study carried out by Rollán, De Angelis, Gobbetti, & De Valdez, (2005) reported that *Lactobacillus plantarum* CRL 759 and 778 used to prepare sourdough had a wide spectrum of peptidase activity during fermentation. They also reported that the degree of proteolysis was higher for dough started with *L. plantarum CRL 778*, producing significant levels of basic, aliphatic, and aromatic amino acids. These results agree with the result obtained in this study.

7.3.3.2 Total dietary fibre

The dietary fibre content of bread samples F and B (3.2 ± 0.2 g/100g and 3.1 ± 0.1g/100g respectively) were higher than that of bread sample D (2.9 ± 0.1 g/100g). There was no significant change in the dietary fibre content for these bread samples, which could be due to the same flour quantity (500 g) used to formulate all the breads.

7.3.3.3 Ash content

The ash content for bread samples F, B and D did not show any significant difference between the bread samples (1.9 ± 0.2g/100g, 1.8 ± 0.1g/100g and 1.9 ± 0.1g/100g respectively). The non-significant change in the ash content of the breads could be explained by the use of the same quantity of flour (500 g) to prepare the breads.

7.3.3.4 Carbohydrate content

The carbohydrate content for bread samples F, B and D did not show any significant difference between the bread samples (51.9 ± 0.3g/100g, 51.2 ± 0.2g/100g and 53.1 ± 0.4g/100g respectively). The non-significant change in the carbohydrate content could be due to the same quantity of sucrose (12 g/L) added to coconut water kefir during the 48 h fermentation period, which was added to breads D, F and B. The quantity of the added flour (500 g) was also kept constant for all the formulations, due to which the activity of cereal amylases on the flour would have been comparable amongst all the bread samples (Mathewson, 2000).

7.3.3.5 Moisture content

The moisture content of bread sample F (34.3± 0.1g/100g) was higher than that of bread sample B (33.6 ± 0.1g/100g). Bread sample D had the lowest moisture content (32.6 ± 0.1g/100g). The results for moisture analysis were not significant and this could be due to the addition of same quantity of fermented coconut water kefir (300ml) added to all the three breads.

7.3.3.6 Total fats

The fat content for bread samples F, B and D did not show any significant difference between the bread samples (1.4 ± 0.0g/100g, 1.2 ± 0.0g/100g and 1.2 ± 0.0g/100g respectively). The non-significant change in the fat content of the breads could be explained by similar formulations of the breads. No additional fats were added to the recipe.

7.3.3.7 Total titratable acidity (TTA)

The TTA of bread sample F (0.6 ± 0.1g/100g, $p<0.05$) was significantly higher than bread sample B and D (0.4 ± 0.0g/100 g and 0.4 ± 0.1g/100g respectively, $p<0.05$).

Bello et al., (2007) have reported that the sourdough bread fermented with *L. plantarum* had a higher acidification rate than the chemically acidified sourdoughs. Their results support the observations in the present study where bread sample F contained significantly higher concentrations of lactic acid, acetic acid, pyruvic acid and succinic acids (Table 7.10) compared to those in breads B and D.

7.3.3.8 Shelf life

The shelf life study was carried out over two weeks by counting the CFU/g of yeast and moulds. The yeast and mould count was <10 CFU/g for all three bread samples F, B and D.

The delay of yeast mould growth is likely associated with the presence of organic acids (Lynch et al., 2014). Sourdough-associated LAB produce many antimicrobial substances, such as organic acids, CO_2, ethanol, hydrogen peroxide, diacetyl, fatty acids, phenyllactic acid, reuterin and fungicins (Messens & De Vuyst, 2002; Schnürer & Magnusson, 2005). Among the organic acids, acetic and propionic acid produced by heterofermentative LAB are more effective than lactic acid (Schnürer and Magnusson 2005). Also, *L. plantarum* shows very broad antimicrobial activity, and the antifungal compounds 4-hydroxyphenlyllactic and especially phenyllactic acids have been identified as responsible for fungal inhibition (Bello, Walter, Hertel, & Hammes, 2001; Lavermicocca et al., 2000; Ryan, Dal Bello, & Arendt, 2008).

7.3.3.10 Texture analysis

The texture was characterised based on hardness, resilience, cohesiveness, gumminess, springiness and chewiness, as shown in Table 7.7.

Bread sample F had the significantly highest hardness value (1137.1±18.3, $p<0.05$) compared to bread samples D and B (966.3±20.2 and 937.3±26.4 respectively, $p<0.05$), with significantly low hardness values. Bread sample B significantly low value (76.7±6.3, $p<0.05$) for springiness when compared to bread samples D and F (87.2±2.4 and 87.9±1.9 respectively, $p<0.05$). The high hardness and springiness values for sample F could be attributed to high acidity in the bread sample, as explained above in section 7.3.3.7. The higher concentration of *L. plantarum* F could be responsible for the higher acidification and thus, the higher hardness and springiness value. These results are supported by a study carried out by Bello et al., (2007), which reports a higher acidification rate for sourdough fermented with *L. plantarum* when compared to the chemically acidified sourdoughs.

Bread samples D and B had significantly high values for adhesiveness (0.5 ±0.0 and 0.4 ±0.0 respectively, $p<0.05$) when compared with sample F (0.1±0.0, $p<0.050$). Sample F had a significantly high resilience value (44.8±3.5, $p<0.05$) when compared to sample D and B (34.9±1.4 and 37.8±0.6 respectively, $p<0.05$). No significant differences were observed for texture parameters namely, cohesiveness, gumminess and chewiness for bread samples D, F and B.

Table 7.6: Proximate analysis for coconut water fermented sourdough bread samples B, D and F. Means in the rows for each determined parameter followed by different letters (a,b,c) are significantly different ($p < 0.05$), as determined by the ANOVA. Composition of Sample B, D and F is as shown in the footnote.

Test Name	Bread sample F	Bread sample B	Bread sample D	p-value
Ash Content (g/100 g)	1.9 ± 0.2 a	1.8 ± 0.1a	1.9 ± 0.1a	0.422
Carbohydrates (g/100 g)	51.9 ± 0.3a	51.2 ± 0.2a	53.1 ± 0.4a	0.455
Dietary fibre (g/100 g)	3.2 ± 0.2a	3.1 ± 0.1a	2.9 ± 0.1b	0.064
Fat Content (g/100 g)	1.4 ± 0.0a	1.2 ± 0.0a	1.2 ± 0.0a	0.079
Moisture (g/100 g)	34.3± 0.1a	33.6 ± 0.1b	32.6 ± 0.1c	0.551
Protein Content (g/100 g)	8.2 ± 0.4b	8.3 ± 0.3a,b	8.5 ± 0.5a	0.047
Titratable acidity (g/100 g)	0.6 ± 0.1a	0.4 ± 0.0b	0.4 ± 0.1b	0.011
Enumeration of Yeasts and Moulds (CFU/g)	< 10	< 10	< 10	

Composition of sourdough bread samples:

F- Composed of 9.6 log CFU/ml of *L. plantarum*, without dry yeast and was fermented for 24h

B- Composed of 8.3 log CFU/ml of *L. fermentum*, without dry yeast and fermented for 24h

D- Composed of 4.9 log CFU/ml of *L. fermentum*, without dry yeast and was fermented for 24h

Table 7.7: Texture analysis for the sourdough bread samples F, B and D. Means in the columns for each determined parameter followed by different letters (a,b,c) are significantly different ($p < 0.05$), as determined by the ANOVA. Composition of Sample B, D and F is as shown in the footnote.

Bread samples	Hardness	Adhesiveness	Resilience	Cohesion	Springiness	Gumminess	Chewiness
D	966.3±20.2 b	0.5 ±0.0a	34.9±1.4b	0.7 ± 0.1a	87.2±2.4a	443.8±23.2 b	491.1±6.3a
F	1137.1±18. 3a	0.1±0.0b	44.8±3.5a	0.8 ±0.1a	87.9±1.9a	476.9±22.1 a	351.5±21.5 a
B	937.3±26.4 b	0.4±0.0a	37.8±0.6b	0.6 ±0.0a	76.7±6.3b	468.1±15.2 a	409.8±33.3 a
p-value	< 0.0001	< 0.0001	0.004	0.146	0.027	0.52	0.324

Composition of sourdough bread samples:

F- Composed of 9.6 log CFU/ml of *L. plantarum*, without dry yeast and was fermented for 24h

B- Composed of 8.3 log CFU/ml of *L. fermentum*, without dry yeast and fermented for 24h

D- Composed of 4.9 log CFU/ml of *L. fermentum*, without dry yeast and was fermented for 24h

7.3.4 Glycaemic index analysis

Glycaemic index was investigated for CWK sourdough bread samples B, D and F. The glycaemic index and the glycaemic load of the breads were determined and shown in Table 7.8. Glycaemic index was significantly highest for bread sample B with a value of 58.1 ± 2.1, $p<0.05$. Significantly lowest glycaemic index (GI) was obtained for bread sample D, which had a value of 56.2 ± 1.4, $p<0.05$. No significant results were obtained for glycaemic load for samples F, B and D.

The reason for the differences in GI may be caused by the different composition of the breads with respect to addition of isolate type and the concentration of the added isolate. Higher concentration of *L. fermentum* (8.3 log CFU/ml) added in bread sample B probably resulted in the production of higher amounts of lactic acid and acetic acid, as shown below in section 7.3.5. It is known that glycaemic response to bread is strongly affected by the presence of organic acids produced during sourdough fermentation (De Angelis et al., 2007; Liljeberg, Lönner, & Björck, 1995; Novotni et al., 2011). It is known that the production of high amounts of acids results in the acidification of the dough and thus, this could be the major factor behind the observed GI reduction. These results are consistent with Mettler et al. (2009) who found dose-dependent and additive effects of organic acids on glycaemic response to food. Further work should examine whether lactic acid or acetic acid is more effective at reducing GI, and whether their efficacy depends on the type of bread under consideration.

Table 7.8: Glycaemic index results for bread samples D, F and B. a–c: Different superscript letters in columns following means signify significant differences ($p < 0.05$). P-value greater than $\alpha = 0.05$ indicates no significant difference. Composition of Sample B, D and F is as shown in the footnote.

Bread samples	**Area under the curve- Test sample** (average for 5 participants for each bread sample)	**Area Under the Curve - reference 50g anhydrous glucose** (average for 5 participants for each bread sample)	**Glycaemic index**	**Glycaemic load**
Bread F	424.2 ± 8.8^{b}	738.1 ± 24.36^{b}	$57.5 \pm 2.7^{a,b}$	28.7^{a}
Bread D	419.5 ± 9.7^{c}	746.3 ± 16.30^{a}	56.2 ± 1.4^{a}	28.1^{a}
Bread B	426.4 ± 8.2^{a}	734.2 ± 19.55^{c}	58.1 ± 2.1^{b}	29.1^{a}
p-value	0.002	0.026	0.037	0.35

Composition of sourdough bread samples:

F- Composed of 9.6 log CFU/ml of *L. plantarum*, without dry yeast and was fermented for 24h

B- Composed of 8.3 log CFU/ml of *L. fermentum*, without dry yeast and fermented for 24h

D- Composed of 4.9 log CFU/ml of *L. fermentum*, without dry yeast and was fermented for 24h

7.3.5 Fermentation Quotient (FQ)

The fermentation quotient was determined for D, F and B CWK-sourdough bread formulations (Table 7.9). Sourdough bread B and F had significantly higher FQ of 5.8 and 5.6 respectively compared to that of bread D. The FQ values obtained in this study were between the range of 1.0-9.57. This range of FQ has been previously reported for wheat sourdoughs by Barber, Ortolá, Barber, & Fernandez, 1992; Barber, Báguena, de Barber, & Martínez-Anaya, 1991; Salovaara & Spicher, 1987). Spicher et al., (1981) and Spicher and Nierle (1984) have reported that FQ depends largely on the type and concentration of LAB added to the sourdough.

Martinez-Anaya, (1990) have reported that there was higher production of acids (TTA) such as lactic acid and acetic acid, which led to a decrease in pH, due to the addition of *L. plantarum* in the sourdough which improved the overall bread flavour, shape and crust of the bread.

These results agree with the current study as the breads B and F which were supplemented with higher concentration of *L. plantarum* and *L. fermentum* had higher FQ compared to Bread sample D, supplemented with low concentration (1.1 log CFU/ml) of *L. fermentum*.

Table 7.9: FQ for sourdough formulations D, F and B. Letters a–c: Different superscript letters in columns following means signify significant differences ($p < 0.05$). P-value greater than $\alpha = 0.05$ indicates no significant difference Composition of Sample B, D and F is as shown in the footnote.

Sourdoughs	Lactic acid (mg/L)	Acetic acid (mg/L)	FQ
B	6.22 ± 1.31a	0.71 ± 0.09a	5.8 ± 0.07a
F	6.31 ± 1.16a	0.75 ± 0.11a	5.6 ± 0.14a
D	5.88 ± 1.21b	0.74 ± 0.13a	5.2 ± 0.14b
p-value	0.011	0.1	0.012

Composition of sourdough bread samples:

F- Composed of 2.7 log CFU/ml of *L. plantarum*, without dry yeast and was fermented for 24 h

B- Composed of 2.2 log CFU/ml of *L. fermentum*, without dry yeast and fermented for 24 h

D- Composed of 1.1 log CFU/ml of *L. fermentum*, without dry yeast and was fermented for 24 h

7.3.6 Carboxylic acid analysis using LCMS

Carboxylic acids such as lactic acid, acetic acid, pyruvic acid and succinic acid were analysed for the B, D and F CWK fermented breads samples using LCMS (Table 7.10). A study carried out by Corsetti (2013) have reported that the fermentation quotient is associated with the production of acids such as lactic and acetic acid and other volatile compounds during the sourdough fermentation. The production of acids and other compounds depends on microbial composition and its activities.

According to Table 7.10, sample F had the significantly highest concentration of lactic acid, acetic acid, succinic acid and pyruvic acid (743.54 ± 5.8 mg/L, 63.39 ± 1.69 mg/L, 52.9 ± 2.63 mg/L and 242.94 ± 13.99 mg/L respectively), followed by sample B. Sample D had significantly the lowest concentrations of lactic acid, acetic acid, succinic acid and pyruvic acid (459.2 ± 11.99 mg/L, 42.78 ± 4.07 mg/L, 33.88 ± 2.47 mg/L and 130.5 ± 3.85 mg/L respectively) compared to samples B and F.

Sample F had a high concentration (9.6 log CFU/ml) of *L. plantarum* cells with no addition of baker's yeast, while sample B had a high concentration (8.3 log CFU/ml) of *L. fermentum* without the addition of baker's yeast. The high carboxylic acids in sample F and B could be explained by the presence of high concentrations of the LAB isolates. Hadaegh, Seyyedain Ardabili, Tajabadi Ebrahimi, Chamani, & Azizi Nezhad (2017) have also reported increased production in lactic and acetic acids in the sourdoughs incoculated with high concentration (30%) of starter cultures like

L. plantarum jQ 301799, *L. casei* jQ412732 and/or *L. brevis* IBRC-M10790, compared to sourdoughs inoculated with 20% inoculum. Sample D that had the lowest organic acids content had the lowest concentration (1.1 log CFU/ml) of *L. fermentum* without the addition of baker's yeast.

Table 7.10: Carboxylic acid analysis for breads D, B and F. a–c: Different superscript letters in columns signify significant differences ($p < 0.05$). Composition of Sample B, D and F is as shown in the footnote.

Bread sample	Pyruvic acid (mg/L)	Acetic acid (mg/L)	Succinic acid (mg/L)	Lactic acid (mg/L)
D	130.5 ± 3.85c	42.78 ± 4.07c	33.88 ± 2.47c	459.2 ± 11.99c
F	242.94 ± 13.99a	63.39 ± 1.69a	52.9 ± 2.63a	743.54 ± 5.8a
B	214.67 ± 14.12b	53.11 ± 2.38b	45.18 ± 1.16b	694.49 9.27b
p-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Composition of sourdough bread samples:

F- Composed of 9.6 log CFU/ml of *L. plantarum*, without dry yeast and was fermented for 24h

B- Composed of 8.3 log CFU/ml of *L. fermentum*, without dry yeast and fermented for 24h

D- Composed of 4.9 log CFU/ml of *L. fermentum*, without dry yeast and was fermented for 24h

7.3.7 Amino acid analysis using LCMS

Figure 7.2 shows the amino acid composition of sourdough bread samples B, F and D. Principal component analysis with bootstrapped confidence ellipses was carried out for better visualisation of the results. All the amino acid data points used for this graph which clearly distinguished the samples along Factor 1 (F1). F1 and F2 explained a total of 92.3% variance with F1 explaining 82.92% variance and F2 explaining 9.41% variance.

It can be observed that sample F was associated with the highest content of all amino acids except for arginine that had high positive scores along F1 (Table 7.11). Sample F had significantly high quantities of all amino acids. Sample B had lower positive scores along F1 and was associated less amino acids than sample F. Samples F and B contained high concentrations of *L. plantarum* (9.6 log CFU/ml) and *L. fermentum* (8.3 log CFU/ml) respectively. Sample D that had the highest negative score was associated with the least amino acids and in fact was inoculated with the lowest concentration of *L. fermentum* (4.9 log CFU/ml). The extent of the protein degradation together with the proportion of LAB in the bread are important parameters to consider, since they will impact on the final bread properties. A study carried out by Marco Gobbetti et al. (1994) have reported that LAB starter culture (containing *Lactobacillus plantarum* DC400) added to

sourdough at a high concentration of 10^7 CFU/ml, had higher proteolytic activity and production of amino acids such as glutamic acids and alanine compared to other starter used for sourdough fermentation. This supports the results obtained in the present study where sample F containing *L. plantarum* at a concentration of 9.6 log CFU/ml had the significantly highest quantities of glutamic acid and alanine (166.6 ± 1.8 µMoles/L and 783.1 ± 6.3 µMoles/L respectively, $p<0.05$). The increase in amino acid concentration was probably due to the greater inoculum concentration, which enhanced amino acid production (Challinor and Rose, 1954; Lyons and Rose, 1977) due to amino acid excretion by cell autolysis during the 24 h period (Selby Smith et al., 1975). The increased proteolytic activity of *Lactobacillus plantarum* CRL 759 and 778 was reported in the sourdough, producing significant levels of basic, aliphatic, and aromatic amino acids. Therefore, the results obtained in this study agree with that of Rollán, De Angelis, Gobbetti, & De Valdez, (2005).

Table 7.11: Amino acid analysis for breads D, B and F. a–c: Different superscript letters in columns signify significant differences ($p < 0.05$). Composition of Sample B, D and F is as shown in the footnote.

Bread samples	Histidine (µMoles/L)	Hydroxy Proline(µMoles/L)	Arginine(µMoles/L)	Serine(µMoles/L)	Glycine(µMoles/L)	Aspartic acid(µMoles/L)	Threonine (µMoles/L)	Glutamic acid(µMoles/L)	Alanine(µMoles/L)	Proline(µMoles/L)	Lysine(µMoles/L)	Methionine(µMoles/L)	Valine(µMoles/L)	Tyrosine(µMoles/L)	Isoleucine (µMoles/L)	Leucine(µMoles/L)	Phenylalanine(µMoles/L)	Tryptophan(µMoles/L)
D	10.4 ± 0.1c	8 ± 0.2b	53.8 ± 0.4a	298.2 ± 2.4c	354.1 ± 7.9c	295.9 ± 45.1b	88 ± 2.2c	121.4 ± 1.5c	531.2 ± 13.7c	251.3 ± 5.1c	31.3 ± 1.4c	8.3 ± 0.2c	5.2 ± 0.1c	4.2 ± 0.1c	3.2 ± 0c	4.6 ± 0.1c	2.3 ± 0.1c	8.3 ± 0.2c
F	11.8 ± 0.3a	9.5 ± 0.2a	54 ± 1.4a	373.1 ± 1.9a	413.9 ± 2.1a	350.2 ± 5a	184.3 ± 2.4a	166.6 ± 1.8a	783.1 ± 6.3a	333.1 ± 2a	46.1 ± 1.1a	12.1 ± 0.4a	10 ± 0.3a	6.3 ± 0a	7.6 ± 0.1a	8.8 ± 0.1a	5.3 ± 0.1a	15 ± 0.2a
B	10.8 ± 0.1b	7.2 ± 0.6c	54.5 ± 0.9a	358.6 ± 3.8b	403.1 ± 1.4b	297.3 ± 6.1b	160.2 ± 5.7b	152.8 ± 1.2b	755.2 ± 4.5b	312.7 ± 4.8b	37.4 ± 1.1b	11.1 ± 0.1b	8.4 ± 0.1b	5.3 ± 0.1b	5.7 ± 0.1b	6.6 ± 0.2b	3.6 ± 0.1b	11.4 ± 0.2b
p-value	< 0.0001	< 0.0001	0.537	< 0.0001	< 0.0001	0.010	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

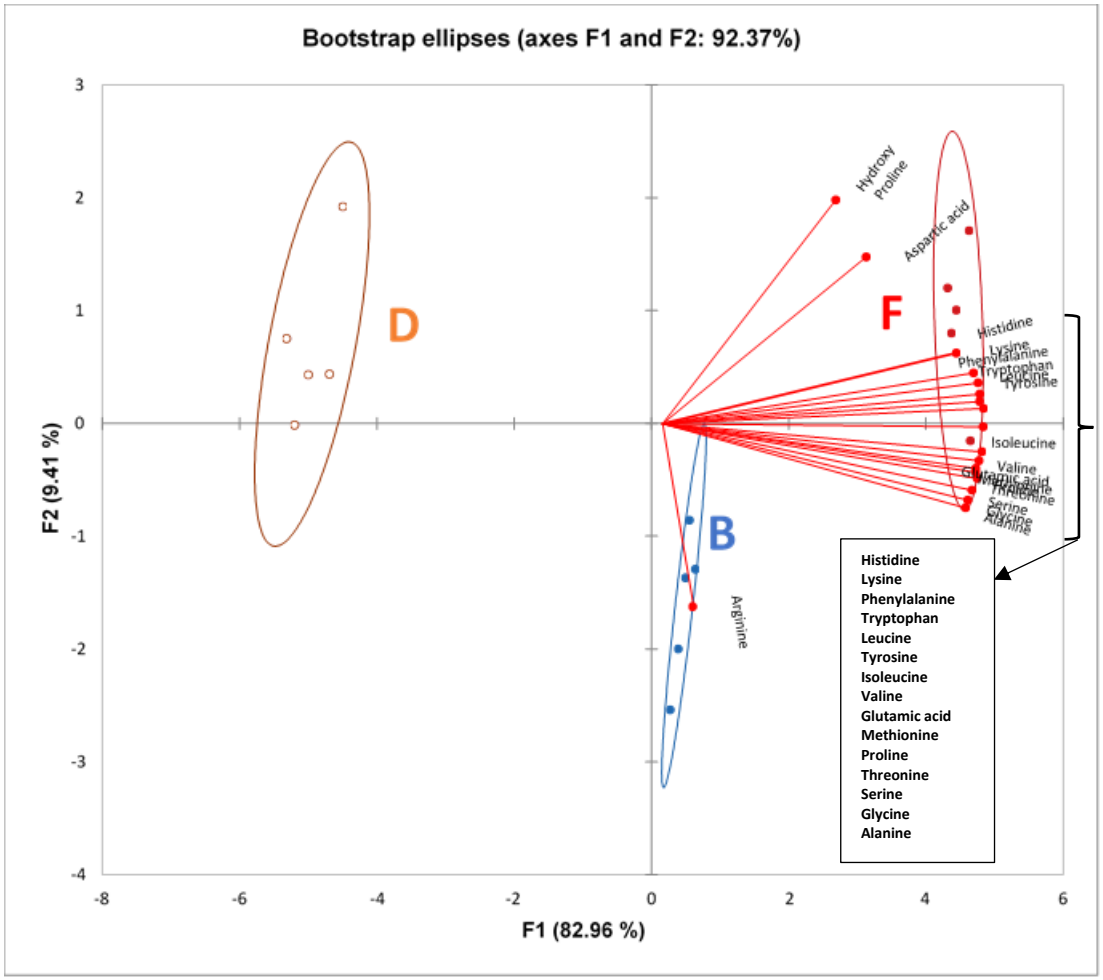
Composition of sourdough bread samples:

F- Composed of 9.6 log CFU/ml of *L. plantarum*, without dry yeast and was fermented for 24h

B- Composed of 8.3 log CFU/ml of *L. fermentum*, without dry yeast and fermented for 24h

D- Composed of 4.9 log CFU/ml of *L. fermentum*, without dry yeast and was fermented for 24h

Figure 7.2: Principal component analysis biplot with bootstrapped confidence ellipses at 95% confidence level for amino acid composition using LCMS for samples B, F and D. Composition of Sample B, D and F is as shown in the footnote.



Composition of sourdough bread samples:

F- Composed of 9.6 log CFU/ml of *L. plantarum*, without dry yeast and was fermented for 24h

B- Composed of 8.3 log CFU/ml of *L. fermentum*, without dry yeast and fermented for 24h

D- Composed of 4.9 log CFU/ml of *L. fermentum*, without dry yeast and was fermented for 24h

7.4. Summary

To summarise this chapter, bread samples B, D and F were selected on the basis of their sensory characteristics. Sample B and F were more desirable due to lower GI, higher carboxylic acid content and amino acid content in samples B and F, when compared to sample D.

The GI of the formulated CWK fermented sourdough bread prepared with varying concentrations of *L. plantarum* and *L. fermentum* was low compared to the traditional sourdoughs and commercial bread samples. Therefore, bread developed here is not likely to increase the blood glucose level in consumers.

The results of the fermentation quotient are related to the carboxylic acid and the sensory profile of the sourdough bread. The fermentation quotient of bread samples B and F were high, when compared to bread sample D. Due to the increase in lactic acid production, the sourdough bread samples could be perceived as significantly sour.

There was a higher production of carboxylic acids and amino acids in samples F and B, compared to sample D, suggesting that the higher concentration of LAB was positively correlated to sourdough acidification and proteolysis generating free amino acids.

Although it is well known that glutamic acid is a precursor of Gamma aminobutyric acid (GABA), the analysis for determining the concentration of GABA (a bioactive compound) could not be carried out due to time constraints.

Chapter 8 Overall discussion, recommendations, conclusions and market prospects

8.1 Thesis overview

Sourdough bread is an important part of the diet in many parts of the world. Systematic efforts have been made to revisit sourdough technology using advanced scientific tools which could help understand the complex interplay between the process conditions applied, type of inoculum used, the type of flour used and the prevailing ecosystem of the sourdough. The role of different factors can help understand their influences on physico-chemical properties, shelf-life, flavour, nutritional and functional properties of the sourdough bread produced. Additionally, to address the constantly increasing consumers' and industry's demand for new health foods of probiotic origin such as sourdough, it is important to use the various technologies which can maximise the technological potential and the outcome of this product.

To enhance the nutritional and functional properties of both, sourdough and bread, scientists have incorporated different types of flours such as legume flours, wheat-free cereal flours, and other fibre-rich flours, and use of different substrates which can improve the production of bioactive compounds and functional compounds, during fermentation. Sourdough made with LAB, AAB and yeast impart neutraceutical and organoleptic properties to the sourdough bread which accounts for consumer acceptability. Thus, the type of starter used to ferment the sourdough plays an important role in the final flavour and functionality of the bread produced. The ability of sourdough microorganisms such as LAB, AAB, and/or yeasts, that produce exopolysaccharide (EPS), bring about sourdough acidification and proteolysis that can increase in amino acids. Other enzymes such as phytase, plays an important role in determining the overall sourdough quality. Sourdough LAB, which have the ability to tolerate highly acidic environments, are generally divided into two groups: homofermentative and heterofermentative. Homofermentative types produce lactic acid as the major or sole product of glucose fermentation, whereas the heterofermentative types produce equal molar amounts of lactic acid, carbon dioxide, and ethanol. LAB could also produce anti-fungal compounds, which could results in the longer shelf life of the sourdough bread. Sourdough yeast produces carbon dioxide which brings about leavening, and also plays an important role in flavour production during sourdough fermentation. Small quantities of acetic acid and succinic acids are produced by sourdough yeasts, which affect the flavour profile of the leavened dough and also aid in acidification. Sourdough yeasts also contribute to the dephosphorylation of phytate via the action of phytases. The AAB present in the

sourdough are well-known for the incomplete oxidation of sugars and alcohols into organic acids and improve the nutritional and overall quality of the sourdough bread.

Kefir, an important probiotic ingredient used for fermentation, has attracted the attention of scientists, food industry, health care and pharma companies. Its use may have a great potential to prevent cancer and may have some other properties such as antibacterial, anti-fungal and anti-inflammatory properties. Development of sourdough using milk kefir grains as a substrate has been explored. However, the use of various low carbohydrate and dairy-free substrates to ferment kefir grains should be further explored. Therefore, increasing the functional properties of sourdough prepared using milk-free and low-carbohydrate alternatives should be considered to formulate a more nutritious sourdough bread product.

Coconut water was used in this study due to its rich nutritional profile with high amounts of glutamic acid, vitamin C, magnesium, vitamin B, arginine, alanine, lysine, calcium, potassium, minerals and antioxidants. It has important health properties such as presence of high potassium content for maintaining the osmotic pressure, prevention of oxidative stress, increased antioxidant activities, lipid peroxidation, improved lipid profile, blood pressure, cardio-protection and antidiabetic and anti-inflammatory effects. To ensure that the coconut water used for the production of kefir grains and eventually the sourdough is sustainable, it is important to manage this resource properly. A constant source of coconut water from the tropical countries will have to be sourced in an ecologically sustainable manner preferably from farms that were not developed from deforested areas and those that cultivated organically. A completer utilisation of the fruit after collection of coconut water would be ideal.

In the experimental chapters of this thesis, the coconut water kefir fermentation profile was investigated, in order to be used as a starter to prepare a functional sourdough bread. The positive impact of kefir on consumer's health, its use as a non-dairy medium for fermenting the kefir grains, and to meet the consumer's demand for functional sourdough breads, were among a few reasons which suggested the need for developing a new sourdough bread. Additionally, other properties like increasing the concentration of amino acids (precursors of bioactive compounds), phytase enzyme, and exopolysaccharide, lowering the glycaemic index, improving texture and flavour, as well as increasing acidification and proteolysis in the sourdough, were investigated to improve sourdough bread. Hence, the main aim of this thesis, therefore, was to develop a functional sourdough bread using fermented coconut water kefir as an inoculum. Development of the functional sourdough bread product was performed stepwise through chapters 3, 4, 5, 6 and 7.

8.2 General discussion

8.2.1 Chapter two – Literature review

A critical step towards development of a functional and healthy sourdough bread product is to first identify the inoculum which could be used to ferment the sourdough such as fruit or vegetable juices and kefir grains. The literature review presented in Chapter 2 of this thesis, was carried out to introduce the potential of kefir grains and coconut water to be used as a fermentation medium for producing a functional sourdough bread. There have been considerable advances in the sourdough technology with improved production of bioactive compounds, production of enzymes, amino acid production, improving bread flavour and glycaemic index analysis. This literature review provided an overview of the microbial properties of kefir grains, potential use of coconut water as the fermentation medium and application of fermented CWK to develop a functional sourdough bread. Suggestions and recommendations on selecting the materials for fermentation and product development were provided, a comprehensive review of previous studies and practical considerations were discussed. The literature review identified a gap in scientific knowledge related to fermented coconut water kefir and its potential use to develop a functional and nutritious sourdough bread product with desirable physicochemical and sensory qualities.

8.2.2 Chapter three – Fermentation of coconut water kefir

Chapter 3 aimed to characterise the fermentation profile of the coconut water with kefir grains. Since there are no scientific data available on the fermentation of coconut water with kefir (CWK), this chapter aimed at studying the microbial profile, carboxylic and amino acid producing capability, utilization of sugars and analysis of the physical structure of the kefir grains grown in coconut water. The results confirmed that there was significant growth of microorganisms such as LAB, AAB and yeast, and significant consumption of sugars such as glucose and sucrose. There was increased acidity at the end of 96 h fermentation period of the coconut water kefir grains, that resulted in pH value reduction and significant production of carboxylic acids such as D-/L-lactic acid, acetic acid and pyruvic acid. Eighteen amino acids were identified, which were produced during fermentation, of which glutamic acid was present in higher quantities. The SEM results confirmed the presence of higher density of LAB and/or AAB, and fewer yeast cells. However, LAB and AAB could not be differentiated. Finally, the coconut water kefir set supplemented with 12g/L of sucrose was selected to produce a sourdough due to its ability to produce significantly highest cell counts compared to those of the rest of the sets. Fermentation time of 48 h was chosen as the time for CWK fermentation.

8.2.3 Chapter four – Microbial and physico-chemical characteristics of sourdough prepared with fermented coconut water kefir

In this chapter, sourdough was prepared using the 48 h fermented mixture of coconut water kefir supplemented with 12g/L of sucrose. The effect of both time and temperature was studied on CWK fermented sourdough and the results revealed that the longer incubation times of the sourdough at the temperature of 30°C resulted in higher cell counts for LAB, AAB and yeasts compared to shorter incubation times at 22°C. Due to the high cell growth, there was increased production of metabolites in the sourdough such as amino acids and organic acids. Glutamic acid was produced in notably high quantities and so were D-/L- lactic acid, pyruvic acid, succinic acid and malic acids produced after incubation at 30°C after 96 h incubation period. Due to this, the overall titratable acidity of the sourdough decreased with increase in fermentation time and this affected the overall texture of the sourdough that became more hard, gummy, chewy, resilient and springy after 96 h incubation. Fermentation quotient of the sourdough bread was found to be between 8-8.9 for all the sourdough sets incubated at 30°C for 96 h. Thus, a sourdough was developed using a 48 h fermented coconut water kefir culture. The presence of significantly high cell counts for sourdough sets fermented at 30°C made it an ideal incubation temperature for all the sourdough formulations used in this research.

8.2.4 Chapter five – Identification of the microbiota in coconut water, kefir, CWK and CWK fermented sourdough using culture-dependent techniques and DNA-based techniques.

The objective of Chapter 5 was to identify and characterise the microorganisms present in the kefir grains, fermented coconut water kefir (CWK) and sourdough fermented with coconut water kefir grains (CWKS). The results obtained in this study showed that the combined methods of phenotypic identification, biochemical tests, and Sanger sequencing were able to identify five LAB species, three AAB species and five yeast species from kefir, CWK and CWKS. The species identified were *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus fusant, Lactobacillus reuteri, Lactobacillus kunkeei, Acetobacter aceti, Acetobacter lovaniensis, Acetobacter pasteurianus, Candida kefyr, Rhodotorula mucilaginosa, Saccharomyces cerevisiae, Candida guilliermondii* and *Candida colliculosa*. Some of these are common sourdough microflora such as *Lactobacillus fermentum, Lactobacillus plantarum, L. reuteri, Acetobacter aceti* and *Saccharomyces cerevisiae* and the rest have been recently identified.

Next, the prevalent microbial ecology of the CWKS, CWK, kefir and coconut water were determined using the MiSeq Illumina sequencing method. The results provided great insights to the relative abundance of the microorganisms present in each of the sample at a given time. The results demonstrated that LAB are present at a higher abundance compared to the rest of the

microorganisms in each of the kefir, CWK and CWKS sample. The microorganisms present at higher incubation time between 48-96 h suggested that the microorganisms present in those samples were able to sustain highly acidic environment, compared to those present during the earlier fermentation periods between 0-48 h. Succession of strains and species during sourdough propagation could affect the functional attributes of sourdough and this, in turn, would influence the characteristics of the final product.

Additional LAB species were revealed to be present in kefir, CWK and CWKS samples using the Illumina sequencing method, which could not be identified using phenotypic or biochemical tests. Therefore, phylogenetic characterisation of these sample using Illumina sequencing may be more beneficial in determining the microbial species present in them.

Furthermore, it should be noted that lactococci were identified using SEM and illumina techniques, but were not identified using the phenotypic identification using microbial methods, which could be because of the disadvantages of using the technique. The most probable reasons could be that the lactococci could be lost during the process of transfer or storage, due to its complex nature and the use of multiple biochemical media.

8.2.5 Chapter six – Functional properties of microorganisms isolated from the formulated sourdough, CWK and kefir

The aim of this chapter was to characterise the isolates identified in the previous chapter, based on their functional properties such as production of phytase enzyme and its ability to hydrolyse phytic acid, production of glutamic acid and exopolysaccharide production. Significantly high quantities of glutamic acid, exopolysaccharide and phytase enzyme were detected for two LAB isolates, *Lactobacillus fermentum* and *Lactobacillus plantarum*. These microorganisms were then used in the next chapter for the development of the sourdough bread using fermented coconut water kefir.

8.2.6 Chapter seven – Sensory and physico-chemical profiles of sourdough bread prepared with coconut water kefir

The aim of this chapter was to integrate the functional properties of fermented coconut water kefir and the LAB species, *Lactobacillus fermentum* and *Lactobacillus plantarum* in the sourdough bread. The final sourdough breads were analysed for their the sensory profile, physico-chemical profile and proximate composition. Sourdough bread samples D (formulated using 4.9 log CFU/ml of *L. fermentum*, without dry yeast and fermented for 24h), B (formulated with 8.3 log CFU/ml of *L. fermentum*, without dry yeast and fermented for 24h) and F (formulated with 9.6

log CFU/ml of *L. plantarum*, without dry yeast and was fermented for 24h) with desirable sensory qualities were subjected to further physiochemical characterisation. Samples B and F had desirable qualities of lower GI, higher carboxylic acid content and amino acid content in samples B and F, compared to sample D. The results of the fermentation quotient were related to the carboxylic acid and the sensory profile of the sourdough bread, as there was an increased lactic acid production and contributed to sourness. There was a higher production of carboxylic acids and amino acids in samples F and B, compared to sample D, suggested that the higher concentration of LAB was positively correlated to sourdough acidification and proteolysis that generated free amino acids.

8.3 Study limitations and future recommendations

Some limitation of the study and recommendations for future work are made as below:

Chapter three:

The aim of this chapter was to study the fermentation profile of kefir fermented using coconut water as the substrate. Coconut water is a suitable fermentation substrate using a kefir starter. The kefir microorganisms such as LAB, AAB and yeast, were able to ferment the sugars in the medium and produce compounds such as organic acids and amino acids, which could positively influence the favour profile product.

It would also be interesting to compare the results of this study with other fruit juices using the same kefir grains and fermentation conditions used in this study. In addition, sensory characterization of the coconut water kefir formulations supplemented with various concentrations of glucose and/or sucrose could be carried out to further understand the consumers' perception of the drink.

The inter-microbial study of coconut water kefir could be further carried out to explore the interactions between the microorganisms (LAB-yeast, LAB-AAB and AAB yeast) and the details about metabolite cross-feeding. Determination of the presence of anti-microbial compounds produced during fermentation is another potential investigation that can be explored.

Chapter four:

The objective of this chapter was to create a sourdough using fermented coconut water kefir as the starter. This dough was characterised microbially and physico-chemically to understand the metabolite production profile of the dough as affected by the temperature and duration of fermentation. A study using CWK as a starter with different flour types for sourdough would be

a good comparative study in addition to the use of a high-grade flour that was utilized in the current study. The ability of the CWK microorganisms to degrade gluten could also be investigated in future work.

Chapter five:

An attempt was made to identify and characterise the microorganisms present in coconut water, kefir, CWK and CWKS using phenotypic, biochemical and DNA-based identification methods. More species were identified using the MiSeq Illumina gene sequencing method, demonstrating the high detection capacity of this method. It would be beneficial to identify all the strains of microorganisms present in kefir, CWK and CWKS to determine the sourdough ecology and the microorganisms prevalent at a given time. Therefore, further studies can be performed in order to identify the underlying microbial structure and stability of sourdough. This knowledge would be helpful to assess the most appropriate conditions that allow preserving the typical traits of traditional sourdough over time. It will also help to identify which organisms have a greater tendency to tolerate the critically low pH. The limitation of the phenotypic and biochemical identification methods was that only a few microorganisms could be identified in this way.

Chapter six:

The aim of this chapter was to determine the functional properties of the microorganisms identified and characterised from kefir, CWK and sourdough fermented with coconut water kefir (CWKS). In addition to identifying functional properties such as glutamic acid production, phytase production, phytic acid degradation and exopolysaccharide production, investigating the ability of the microorganisms to produce bioactive compound such as GABA (γ-aminobutyric acid) could also be carried out, as GABA is a bioactive compound with many functional properties.

Chapter seven:

Further targeted research could be carried out to improve the functionality of sourdough breads to increase the concentration of bioactive compounds in final bread product. This could be achieved with the use of different flour type and/or the type of cultures used to prepare the sourdough.

Flavour analysis of the bread using GCMS could also be carried out to determine the complete falvour profile of the sourdough bread. This could provide interesting insights to the overall flavour of the final sourdough bread product.

No study on mineral bioavailability analysis was carried out when analysing the phytase activity in the sourdough bread(s) due to time constraints. It would be interesting to quantitate the change in the concentration of free-minerals due to phytic acid degradation.

Although it is well known that glutamic acid is a precursor of gamma aminobutyric acid (GABA), the determination of GABA (a bioactive compound) should be carried out in future investigations.

Due to time constraints, these parameters could not be included in this study.

8.4 Thesis impact, conclusions and market prospects

To conclude, this thesis provides the first scientific research on the use of coconut water as a fermentation substrate in the fermentation of kefir grains, which could be used as a starter culture to prepare a sourdough bread with improved functional properties. The microorganisms from kefir, CWK and CWKS were identified and characterised and using culture-dependent and culture-independent (DNA) methods. The functional properties of all the identified microorganisms were determined in terms of phytase enzyme producing capability, glutamic acid production and exopolysaccharide production. The microorganisms which produced the highest quantities of all three (*L. fermentum* and *L. plantarum*) were identified and incorporated into the flour, along with fermented CWK, to produce a functional CWK sourdough bread with improved functional properties. The results from this research can be used to further investigate how fruit juice can be used in kefir fermentation and their potential integration to produce other functional products.

8.5 Market prospects

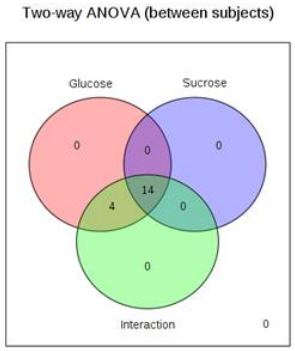
Sourdough can be formulated to improve bread quality without the use of additives. Development of a functional sourdough bread would improve the revenue of the baking industries by lowering additives like anti-staling, anti-fungal and other preservatives. The CWK fermented sourdough bread could be suitable for casein- and lactose-allergic patients who cannot consume dairy products, providing them with a nutritious and functional alternative. The increased level of phytase enzyme in the bread could increase the availability of iron and other essential minerals in consumer. Further studies investigating the shelf life studies of the sourdough bread should be carried out.

Chapter 9 Appendices

Appendix 1: Chapter 3: The ANOVA tables for Carboxylic acids produced during coconut water kefir fermentation.

	f.value	p.value	-$\log_{10}$(p)	FDR	Fisher's LSD
malic mg/L	1033.2	2.6681E-141	140.57	1.33E-140	0 - 24; 0 - 48; 0 - 72; 0 - 96
lactic mg/L	44.64	2.06E-27	26.687	4.16E-27	24 - 0; 48 - 0; 72 - 0; 96 - 0
acetic mg/L	44.463	2.50E-27	26.602	4.16E-27	24 - 0; 48 - 0; 72 - 0; 96 - 0; 48 - 72
pyruvic mg/L	41.65	5.66E-26	25.247	7.07E-26	24 - 0; 48 - 0; 72 - 0; 96 - 0; 72 - 24; 96 - 24
succinic mg/L	33.576	7.34E-22	21.134	7.34E-22	24 - 0; 48 - 0; 72 - 0; 96 - 0; 72 - 24; 96 - 24; 72 - 48; 96 - 48; 96 - 72

Appendix 1: Chapter 3: Venn diagrams for interaction of sucrose and glucose. Represents the interactions between glucose and sucrose and their effect on the different amino acids. That means, if the amino acids are affected by the glucose and sucrose interaction, it is determined by these Venn diagrams. Diagrams 1, 2, 3, 4 and 5 represent the interactions of glucose and sucrose for times, T=0, 24, 48, 72 and 96 h respectively.



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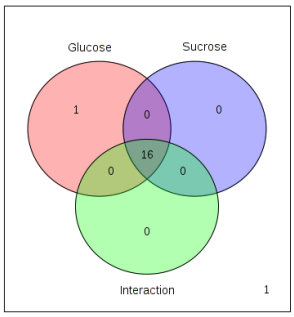
2.



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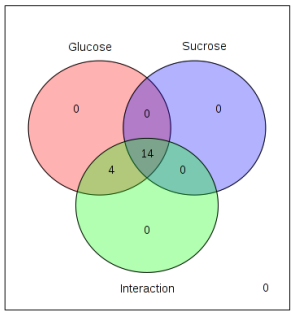
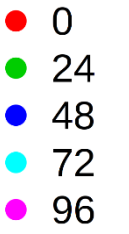


4.

5.

Appendix 2: Chapter 3: The Heat map and PCA (2D Score plots) represents the effect of time (0, 24, 48, 72 and 96 h) on the nine experimental sets. 1a, 2a, 3a, .., 9a: represent the principal component analysis for the individual sets with time and 1b, 2b, 3b,…,9b: represent the heat map plots for the individual sets with time.
Heatmaps and PCA 2D score plots for effect of time on the change in amino acid profile.

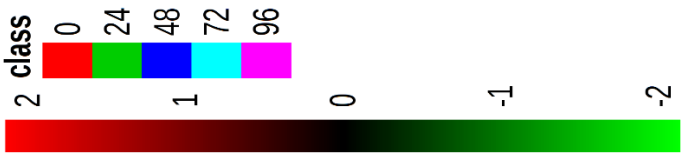
LEGEND: For PCA-2D Score plots **LEGEND:** For Heatmaps

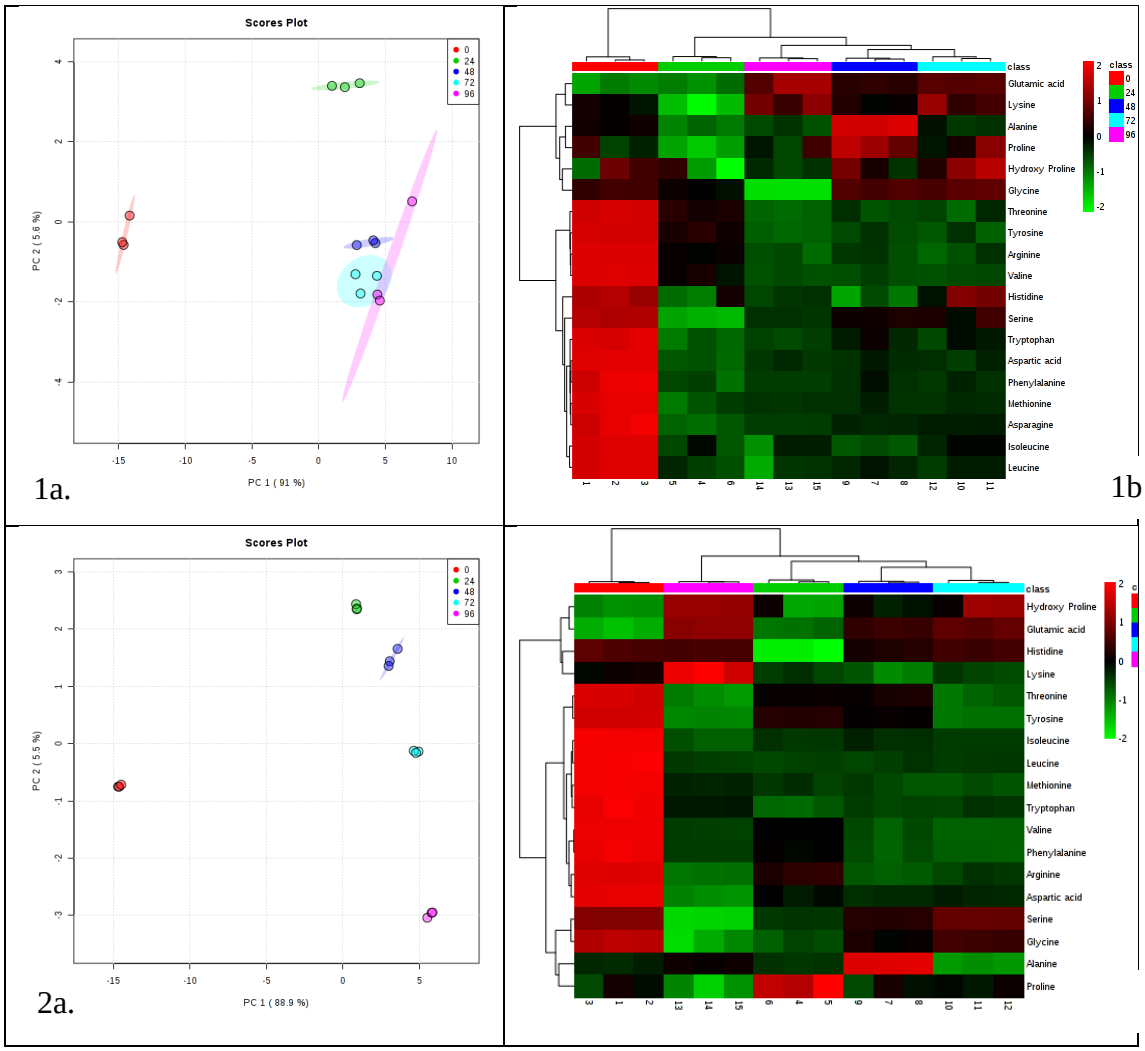


TIME IN H

TIME IN H

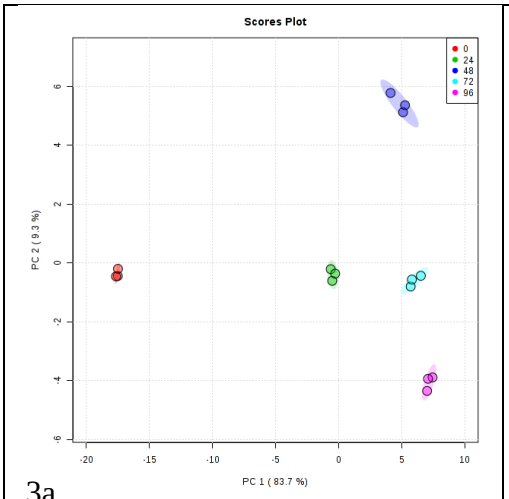
HIGH CONCENTRATION LOW CONCENTRATION





1a. 1b

2a. 2b



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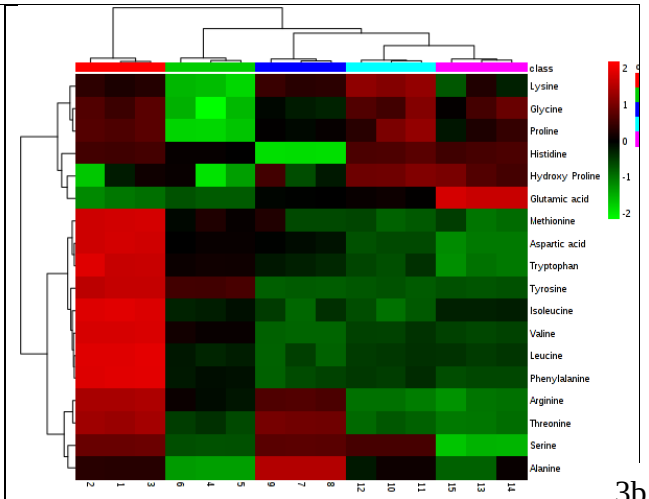

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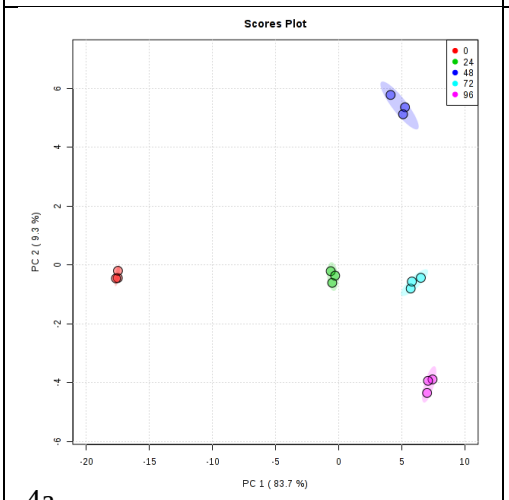

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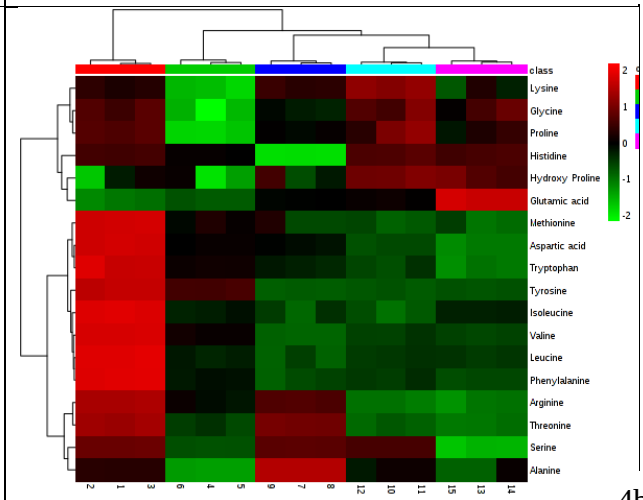
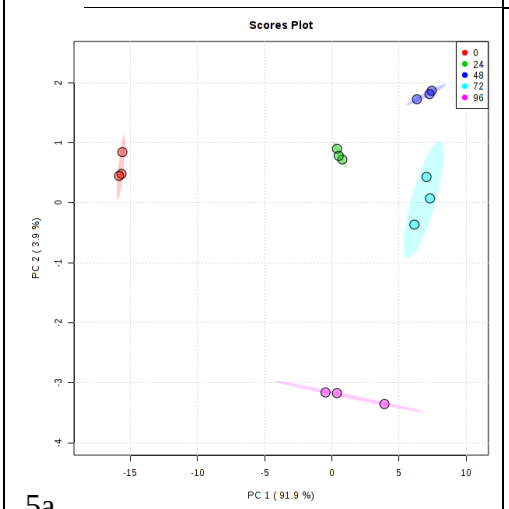


4b



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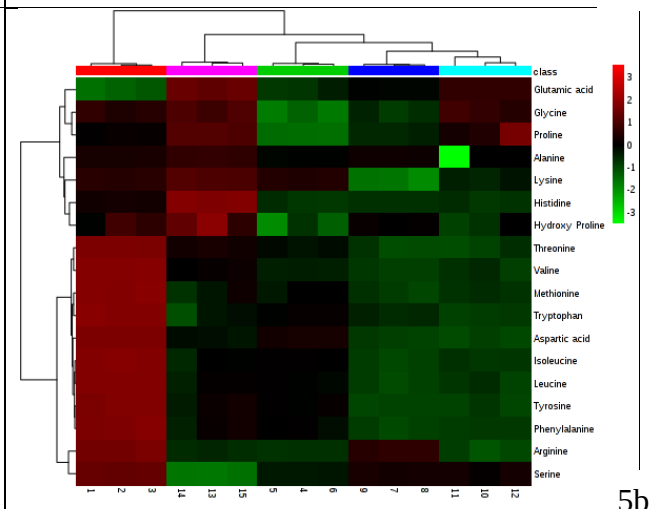

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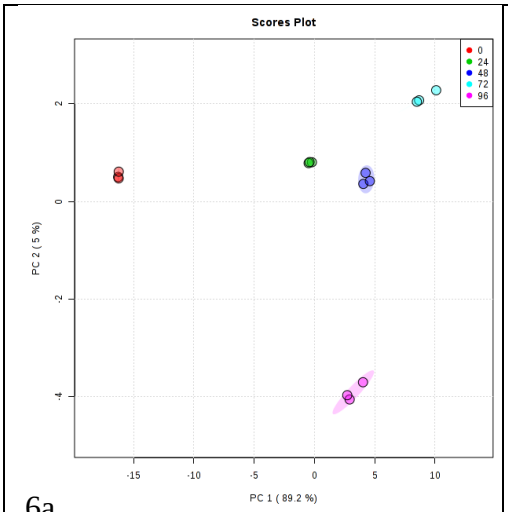

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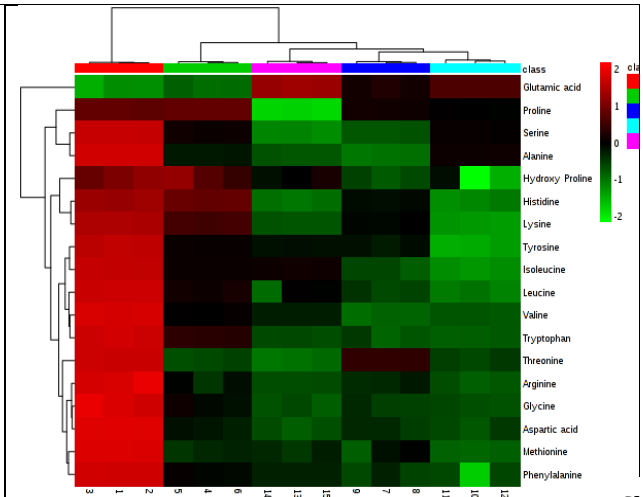

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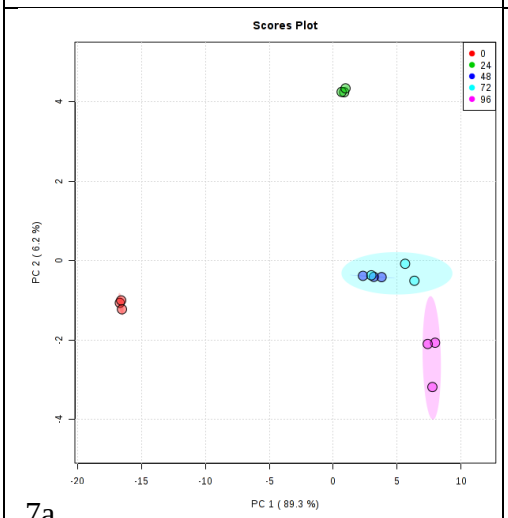

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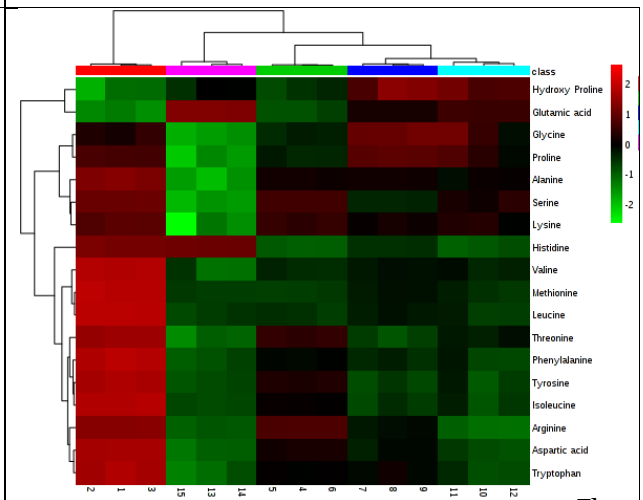
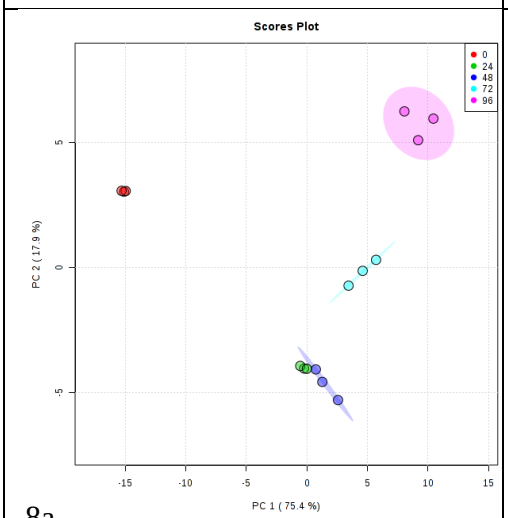


7b



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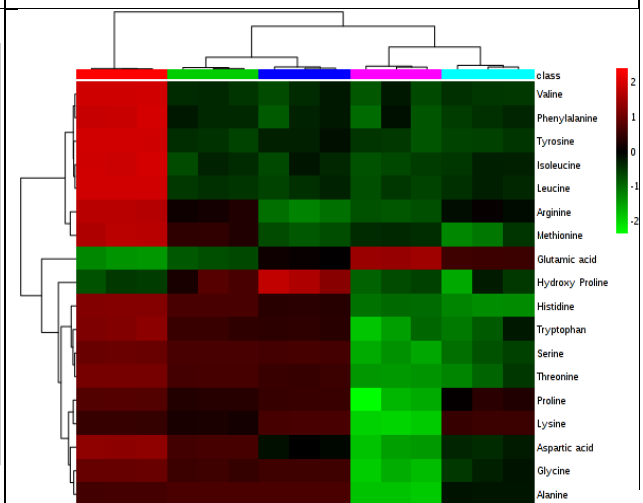

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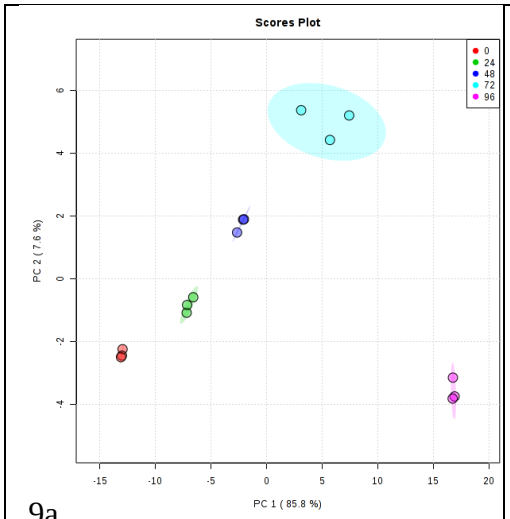
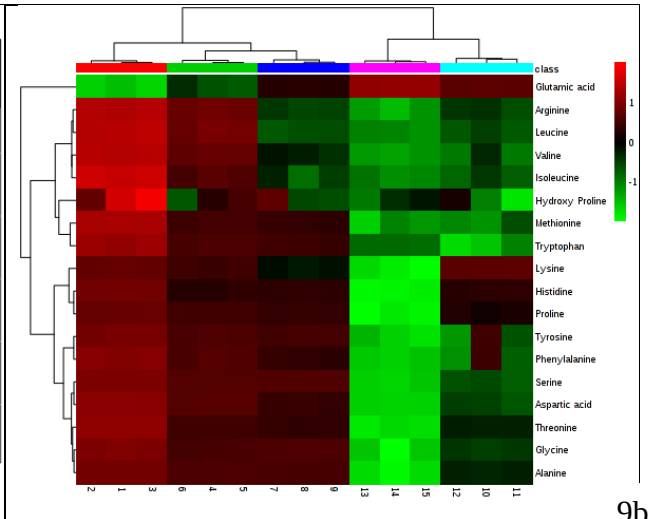

Scores Plot
0
24
48
72
96
PC 2 (7.6 %)
PC 1 (85.8 %)

9a.


class
Glutamic acid
Arginine
Leucine
Valine
Isoleucine
Hydroxy Proline
Methionine
Tryptophan
Lysine
Histidine
Proline
Tyrosine
Phenylalanine
Serine
Aspartic acid
Threonine
Glycine
Alanine

9b

Appendix 3: Chapter 3: Represent the amino acid data for all nine experimental sets sampled over time, expressed as mean and standard deviation.

	Set 1					Set 2					Set 3				
Amino acids in µM/L /time in h	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96
Histidine	108.49 ± 1.18	90.22 ± 5.71	86.72 ± 3.1	101.13 ± 6.31	91.05 ± 0.75	89.54 ± 3.61	28.06 ± 0.92	74.5 ± 2.06	84.25 ± 1.89	86.34 ± 0.72	51.35 ± 1.78	27.89 ± 0.9	2.91 ± 0.05	58.85 ± 4.48	53.44 ± 3.24
Hydroxy Proline	5.06 ± 0.89	4.06 ± 1.04	5.11 ± 0.7	5.88 ± 0.71	4.43 ± 0.11	3.51 ± 0.07	3.86 ± 0.97	4.69 ± 0.27	6.26 ± 1.16	6.83 ± 0.04	3.27 ± 0.81	2.89 ± 0.87	3.71 ± 0.65	4.98 ± 0.13	4.69 ± 0.3
Arginine	144.11 ± 2.29	11.56 ± 0.96	5.32 ± 0.92	4.52 ± 1.53	4.06 ± 0.84	134.03 ± 3.33	15.75 ± 2.1	3.37 ± 0.14	4.98 ± 0.67	2.56 ± 0.08	140.2 ± 4.92	10.18 ± 2.43	36.09 ± 2.46	1.94 ± 0.21	1.73 ± 0.44
Serine	561.44 ± 3.43	312.58 ± 5.79	431.9 ± 6.44	437.83 ± 27.68	384.95 ± 1.66	517.83 ± 6.58	95.87 ± 2.56	219.46 ± 4.93	390.72 ± 4.87	22.34 ± 1.02	653.11 ± 10.01	85.43 ± 2.1	546.33 ± 6.81	449.29 ± 2.14	27.43 ± 2.92
Glycine	71.49 ± 3.15	55.33 ± 3.23	76.62 ± 2.35	80 ± 3.98	21.64 ± 0.11	88 ± 1.36	45.1 ± 1.71	56.55 ± 2.28	64.33 ± 0.88	35.37 ± 3.71	105.64 ± 3.13	61.91 ± 4.97	87.42 ± 2.34	109.07 ± 7.25	102.59 ± 9.7
Aspartic acid	115.49 ± 1.51	10.01 ± 0.89	14.94 ± 1.61	14.09 ± 1.45	13.57 ± 0.88	122.5 ± 2.69	12.89 ± 1.51	9.7 ± 0.17	10.46 ± 0.68	3.96 ± 0.36	155.33 ± 4.26	22.27 ± 1.18	18.95 ± 1.39	10.16 ± 0.42	6.27 ± 0.61
Threonine	155.42 ± 2.12	89.29 ± 2.7	66.26 ± 4.19	65.63 ± 6.56	59.69 ± 0.83	146.63 ± 5.99	41.58 ± 0.64	44.39 ± 2.77	21.12 ± 2.13	16.83 ± 1.58	225.56 ± 12.6	24.78 ± 3.03	144.34 ± 6.64	17.34 ± 1.53	14.05 ± 0.87
Glutamic acid	5.3 ± 0.87	6.07 ± 0.95	23.03 ± 0.68	32.64 ± 0.24	52.25 ± 17.58	5.05 ± 0.46	8.85 ± 0.7	33.23 ± 2.01	45.82 ± 3.24	67.63 ± 2.03	8.57 ± 1.46	13.31 ± 0.8	41.25 ± 1.06	47.48 ± 2.9	634.54 ± 56.56
Alanine	681.74 ± 7.46	561.31 ± 12.84	917.14 ± 8.87	629.32 ± 20.8	602.96 ± 14.75	558.67 ± 6.8	545.35 ± 3.59	885.15 ± 4.66	459.64 ± 5.03	609.1 ± 5.07	793.49 ± 1.35	543.42 ± 2.64	1035.56 ± 1.11	735.52 ± 31.29	654.71 ± 79.24
Proline	280.59 ± 6.61	265.4 ± 2.21	296.46 ± 4.63	286.26 ± 8.1	280.88 ± 6.53	194.72 ± 3.98	215.26 ± 3.78	194.72 ± 4.16	195.94 ± 1.48	183.25 ± 3.16	343.56 ± 3.87	214.35 ± 3.33	300.35 ± 3.84	356.04 ± 31.21	310.28 ± 18.93
Lysine	84.82 ± 3.62	55.63 ± 4	86.69 ± 3.44	101.42 ± 11.26	104.1 ± 8.55	74.57 ± 2.18	64.68 ± 1.68	57.33 ± 3.5	63.77 ± 1.45	120.32 ± 5.89	186.4 ± 5.23	95.47 ± 4.47	192.45 ± 4.78	252.08 ± 6.08	156.08 ± 27.11
Methionine	22.79 ± 2.15	0.73 ± 0.24	1.16 ± 0.16	1.13 ± 0.07	1.06 ± 0.03	27.64 ± 0.8	0.77 ± 0.11	0.63 ± 0.11	0.56 ± 0.05	0.97 ± 0.02	35.55 ± 3.33	0.51 ± 0.21	0.31 ± 0.4	0.06 ± 0.02	0.06 ± 0.04
Valine	89.11 ± 0.37	1.94 ± 0.74	0.46 ± 0.09	0.45 ± 0.05	0.43 ± 0.05	75.5 ± 1.22	1.2 ± 0.02	115.19 ± 9.02	0.22 ± 0	0.41 ± 0.02	107.2 ± 4.41	1.39 ± 0.17	0.13 ± 0.01	0.29 ± 0.06	0.25 ± 0.02
Tyrosine	28.33 ± 0.76	0.73 ± 0.19	0.12 ± 0.04	0.1 ± 0.05	0.06 ± 0.01	25.31 ± 0.99	1.14 ± 0.03	0.65 ± 0.03	0.08 ± 0.01	0.05 ± 0	37.6 ± 4.18	2.27 ± 0.21	0.06 ± 0	0.07 ± 0.01	0.08 ± 0
Isoleucine	34.46 ± 4.08	0.24 ± 0.19	0.11 ± 0.02	0.42 ± 0.13	0.22 ± 0.16	31.98 ± 1.08	0.23 ± 0.02	0.28 ± 0.04	0.21 ± 0	0.13 ± 0.02	38.86 ± 2.3	0.26 ± 0.05	0.12 ± 0.06	0.07 ± 0.02	0.23 ± 0.01
leucine	56.23 ± 6.81	0.26 ± 0.11	0.42 ± 0.09	0.39 ± 0.12	0.19 ± 0.14	47.17 ± 3.16	0.25 ± 0.02	0.27 ± 0.04	0.28 ± 0.01	0.26 ± 0.02	68.34 ± 4.02	0.38 ± 0.05	0.13 ± 0.06	0.23 ± 0.02	0.23 ± 0.02
Phenylalanine	25.61 ± 6.11	0.39 ± 0.13	0.71 ± 0.21	0.61 ± 0.09	0.51 ± 0.01	27.06 ± 2.07	1.22 ± 0.1	0.42 ± 0.07	0.36 ± 0.01	0.57 ± 0.01	27.42 ± 1.45	0.21 ± 0.02	0.06 ± 0.02	0.1 ± 0.02	0.07 ± 0
Tryptophan	12.41 ± 0.59	1.67 ± 0.2	2.85 ± 0.5	2.57 ± 0.48	2.08 ± 0.07	10.5 ± 0.67	0.9 ± 0.07	1.15 ± 0.04	1.23 ± 0.09	1.55 ± 0.03	12.81 ± 2.59	0.81 ± 0.04	0.42 ± 0.05	0.26 ± 0.06	0.11 ± 0.02
Asparagine	2627.56 ± 69.87	239.19 ± 18.86	372.08 ± 10.04	397.66 ± 4.15	315.49 ± 1.27	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected

	Set 4	Set 5	Set 6

Amino acids in µM/L /time in h	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96
Histidine	8.12 ± 0.33	5.7 ± 0.79	6.25 ± 0.26	26.87 ± 1.86	34.31 ± 1.66	76.79 ± 1.23	46 ± 2.71	47.17 ± 0.22	46.57 ± 2.37	171.42 ± 4.09	119.03 ± 3.37	85.35 ± 1.14	42.22 ± 0.61	19.71 ± 1.31	22.37 ± 0.72
Hydroxy Proline	7.69 ± 1.6	7.48 ± 0.96	4.14 ± 0.71	2.18 ± 0.21	1.85 ± 0.41	5.53 ± 0.85	3.07 ± 0.61	4.78 ± 0.07	3.94 ± 0.63	7.26 ± 1.5	6.18 ± 0.6	5.49 ± 1.28	2.43 ± 0.14	1.95 ± 1.15	3.52 ± 0.34
Arginine	7.6 ± 1.4	8.17 ± 0.67	7.11 ± 1.02	5.18 ± 1.86	3.55 ± 0.28	64.67 ± 4.31	5.55 ± 0.19	21.69 ± 1.19	3.86 ± 0.65	6.06 ± 0.43	44.48 ± 4.3	6.55 ± 1.27	5.84 ± 0.45	3.92 ± 0.28	4.25 ± 0.06
Serine	326.18 ± 58.58	372.7 ± 52.07	340.95 ± 8.14	244.86 ± 11.58	159.19 ± 2.29	584.53 ± 9.25	198.44 ± 1.87	290.77 ± 8.62	276.75 ± 22.84	86.76 ± 2.44	861.43 ± 3.67	202.8 ± 4.02	92.47 ± 1.87	194.22 ± 3.24	63.28 ± 2.71
Glycine	78.5 ± 2.97	76.23 ± 4.17	74.64 ± 8.41	61.32 ± 2.76	54.57 ± 3.74	106.24 ± 3.58	63.47 ± 2.94	80.07 ± 3.13	110.63 ± 5.15	118.57 ± 4	190.17 ± 6.28	114.43 ± 4.33	101.65 ± 2.93	96.97 ± 1.58	95.93 ± 2.46
Aspartic acid	6.29 ± 1.3	6.83 ± 0.7	8.71 ± 0.48	4.27 ± 0.32	3.89 ± 0.27	186.44 ± 3.64	14.97 ± 0.67	1.99 ± 0.28	1.64 ± 0.23	6.25 ± 0.82	344.42 ± 5.45	9.07 ± 1.09	6.33 ± 1.08	4.44 ± 0.99	3.8 ± 0.52
Threonine	154.17 ± 9.87	166.29 ± 2.89	137.43 ± 1.96	73.86 ± 8.47	55.07 ± 9.66	250 ± 2.8	46 ± 2.81	23.59 ± 4.34	25.32 ± 5.18	69.69 ± 3.37	436.57 ± 6.12	46.22 ± 2.99	124.59 ± 0.95	49.4 ± 2.98	33.12 ± 1.87
Glutamic acid	7.69 ± 0.59	11.95 ± 2.12	30.74 ± 0.63	30.11 ± 0.04	73.77 ± 3.21	6.73 ± 1.06	13.06 ± 2.47	21.21 ± 0.3	41.26 ± 0.21	78.44 ± 4.65	8.72 ± 1.17	12.53 ± 0.66	32.45 ± 2.08	48.15 ± 0.06	86.11 ± 2.4
Alanine	626.07 ± 10.56	687.55 ± 5.5	966.09 ± 2.6	636.59 ± 4.56	505.07 ± 56.87	759.42 ± 4.56	601.3 ± 5.36	700.71 ± 7.46	435.79 ± 18.57	941.2 ± 14.97	1317.52 ± 1.39	667.32 ± 3.07	509.14 ± 5.24	743.13 ± 1.94	555.42 ± 3.83
Proline	286.8 ± 1.24	297.82 ± 7.24	296.59 ± 2.08	193.31 ± 15.58	147.85 ± 0.81	324.16 ± 2.61	228.18 ± 1.32	284.13 ± 3.76	381.71 ± 13.86	406.87 ± 4.63	507.92 ± 15.05	516.85 ± 2.34	247.71 ± 2.75	212.23 ± 6.57	31.46 ± 0.98
Lysine	103.45 ± 5.43	157.33 ± 2.99	121.19 ± 0.63	91.26 ± 10.51	60.54 ± 12.91	197.09 ± 1.3	194.25 ± 1.03	132.35 ± 4.41	166.74 ± 3.94	216.46 ± 1.88	430.48 ± 1.79	255.2 ± 4.05	179.27 ± 4.16	85.09 ± 1.7	120.21 ± 0.98
Methionine	26.49 ± 0.94	0.25 ± 0.06	0.73 ± 0.31	0.08 ± 0	0.08 ± 0.03	37.25 ± 3	0.46 ± 0.18	0.09 ± 0.03	0.12 ± 0.02	0.43 ± 0.39	65.32 ± 2.34	0.42 ± 0.08	0.58 ± 0.38	0.14 ± 0.01	0.43 ± 0.12

Valine	83.42 ± 3.52	0.12 ± 0.04	0.59 ± 0.08	0.14 ± 0.07	0.22 ± 0.07	113.99 ± 2.86	0.65 ± 0.02	0.24 ± 0.03	0.36 ± 0.14	2.09 ± 0.4	105.6 ± 3.44	2.6 ± 0.17	0.41 ± 0.04	0.54 ± 0.02	1.48 ± 0.02
Tyrosine	40.05 ± 0.01	0.06 ± 0.01	0.54 ± 0.4	0.09 ± 0.01	0.12 ± 0.03	35.94 ± 4.03	0.85 ± 0.14	0.1 ± 0	0.12 ± 0.03	0.96 ± 0.55	48.42 ± 3.12	1.03 ± 0.04	0.59 ± 0.1	0.02 ± 0.01	0.62 ± 0.03
Isoleucine	40.07 ± 0.05	0.08 ± 0.03	0.23 ± 0.11	0.19 ± 0.09	0.19 ± 0.13	44.21 ± 2.95	1.24 ± 0.05	0.2 ± 0.03	0.28 ± 0.02	0.88 ± 0.42	42.58 ± 2.03	1.05 ± 0.02	0.19 ± 0.05	0.05 ± 0.01	1.15 ± 0.05
leucine	40.09 ± 0.04	0.09 ± 0.02	0.44 ± 0.34	0.18 ± 0.06	0.27 ± 0.02	73.58 ± 0.22	1.31 ± 0.19	0.19 ± 0.05	0.29 ± 0.11	1.27 ± 0.65	62.95 ± 2.35	1.35 ± 0.15	0.25 ± 0.06	0.07 ± 0.01	0.64 ± 0.47
Phenylalanine	20.27 ± 0.07	0.38 ± 0.12	0.46 ± 0.24	0.11 ± 0.03	0.12 ± 0.01	23.06 ± 3.69	0.53 ± 0.11	0.09 ± 0.02	0.11 ± 0	0.64 ± 0.39	33.17 ± 0.7	0.74 ± 0.12	0.29 ± 0.12	0.15 ± 0.11	0.41 ± 0.01
Tryptophan	12.28 ± 0.68	2.01 ± 0.08	2.02 ± 0.77	0.35 ± 0.17	0.18 ± 0.03	17.18 ± 1.5	0.9 ± 0.12	0.34 ± 0.04	0.21 ± 0.02	0.42 ± 0.25	12.64 ± 0.9	1.43 ± 0.07	0.29 ± 0.09	0.24 ± 0.01	0.31 ± 0.01

	Set 7					Set 8					Set 9				
Amino acids in µM/L /time in h	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96
Histidine	125.11 ± 3.66	18.38 ± 0.53	28.12 ± 0.28	19.24 ± 1.85	113.59 ± 3.45	225.42 ± 2.14	129.21 ± 0.63	95.82 ± 2.28	18.2 ± 0.68	23.8 ± 1.1	347.15 ± 7.89	129.93 ± 12.87	137.04 ± 1.81	134.5 ± 7.4	2.72 ± 0.2
Hydroxy Proline	5.94 ± 1.22	8.97 ± 0.93	22.73 ± 4.25	19.66 ± 2.58	11.03 ± 1.51	9.4 ± 0.45	15.01 ± 1.71	22.06 ± 2.23	8.99 ± 2.26	8.94 ± 0.55	11.59 ± 1.12	9.2 ± 0.97	8.9 ± 1.18	7.92 ± 1.32	8.38 ± 0.56
Arginine	263.24 ± 2.17	94.25 ± 1.64	18.85 ± 2.31	3.88 ± 0.69	5.19 ± 0.52	145.49 ± 4.17	13.99 ± 1.96	1.79 ± 0.27	9.28 ± 1.59	2.94 ± 0.18	154.24 ± 6.91	65.1 ± 3.19	7.97 ± 0.74	8.51 ± 1.48	2.41 ± 0.57
Serine	980.83 ± 6.4	599.4 ± 2.63	175.89 ± 2.14	385.33 ± 15.51	39.86 ± 8.92	758.87 ± 10.92	462.78 ± 5.08	432.73 ± 7.13	40.4 ± 13.86	12.39 ± 2.98	972.49 ± 6.38	515.88 ± 3.59	494.59 ± 1.47	40.72 ± 6.37	6.19 ± 0.82
Glycine	163.74 ± 4.07	146.18 ± 1.98	184.59 ± 2	169.18 ± 17.48	118.61 ± 3.08	161.84 ± 2.27	99 ± 5.81	103.55 ± 1.05	35.04 ± 7.13	6.61 ± 1.17	183.91 ± 5.02	97.37 ± 3.38	106.19 ± 2.06	22.8 ± 1.44	3.78 ± 1.25

Aspartic acid	269.57 ± 0.96	37.35 ± 2.15	22.28 ± 4.16	10.46 ± 1.84	6.7 ± 1.26	329.23 ± 4.16	115.65 ± 3.94	37.26 ± 3.99	24.87 ± 2.76	3.73 ± 1.1	344.47 ± 4.48	155.55 ± 3.33	95.23 ± 3.86	14.15 ± 2.54	1.67 ± 0.03
Threonine	249.51 ± 8.15	111.87 ± 3.29	46.43 ± 4.88	65.49 ± 4.98	33.8 ± 5.97	646.52 ± 7.34	344.32 ± 4.17	288.42 ± 5.09	41.59 ± 21.48	18.84 ± 0.53	760.62 ± 2.47	228.95 ± 1.68	187.84 ± 6.69	56.08 ± 2.12	3.16 ± 0.4
Glutamic acid	5.81 ± 0.67	10.74 ± 1.27	30.12 ± 0.04	44.02 ± 1.14	90.95 ± 0.83	7.6 ± 0.57	13.25 ± 1	28.03 ± 1.49	43.57 ± 0.61	96.71 ± 3.07	4.04 ± 0.41	12.38 ± 2.76	30.44 ± 0.68	47.39 ± 0.74	75.44 ± 1.11
Alanine	1180.97 ± 8.18	1037.5 ± 3.53	1036.21 ± 1.12	1018.71 ± 15.63	840.83 ± 22.81	1168.16 ± 3.43	1271.46 ± 3.94	1253.74 ± 6.04	426.49 ± 5.1	56.48 ± 2.42	1239.64 ± 6.26	791.35 ± 4.33	750.96 ± 4.97	219.78 ± 8.19	23 ± 4.41
Proline	703.62 ± 3.62	590.91 ± 7.74	728.25 ± 4.24	667.02 ± 50.03	473.11 ± 27.44	664.35 ± 4.37	459.68 ± 6.36	534.75 ± 3.55	423.67 ± 64.11	67.84 ± 22.25	684.4 ± 15.37	438.73 ± 1.79	374.26 ± 2.51	275.8 ± 5.79	11.26 ± 1.52
Lysine	166.7 ± 3.57	145.46 ± 3.52	127.22 ± 3.82	131.62 ± 10.61	65.5 ± 16.58	92.62 ± 2.02	57.96 ± 3.12	123.24 ± 2.79	96.6 ± 3.43	1.46 ± 0.1	104.4 ± 1.78	57.31 ± 3.75	15.36 ± 1.87	92.94 ± 0.87	0.44 ± 0.13
Methionine	45.91 ± 3.19	0.08 ± 0.01	0.24 ± 0.04	0.13 ± 0.05	0.08 ± 0.01	36.86 ± 3.54	3.86 ± 0.49	0.52 ± 0.06	0.44 ± 0.33	0.96 ± 0.02	81.73 ± 2.41	10.24 ± 1.05	7.42 ± 0.72	0.28 ± 0.23	0.11 ± 0.07
Valine	135.92 ± 1.93	0.53 ± 0.07	0.95 ± 0.12	0.77 ± 0.3	0.19 ± 0.19	114.73 ± 1.37	0.65 ± 0.09	0.67 ± 0.32	0.54 ± 0.05	0.54 ± 0.38	105.73 ± 4.6	24.37 ± 1.84	2.39 ± 0.62	1 ± 0.89	0.27 ± 0.04
Tyrosine	49.18 ± 6.07	2.12 ± 0.12	0.25 ± 0.07	0.35 ± 0.25	0.2 ± 0.04	43.53 ± 1.38	0.26 ± 0.05	0.44 ± 0.09	0.22 ± 0.03	0.22 ± 0.07	45.9 ± 3.01	20.28 ± 1.26	17.42 ± 1.17	5.5 ± 8.55	0.08 ± 0.04
Isoleucine	50.98 ± 4.63	1.03 ± 0.06	0.25 ± 0.08	0.29 ± 0.17	0.18 ± 0.01	22.12 ± 1.92	0.24 ± 0.08	0.28 ± 0.11	0.29 ± 0.07	0.17 ± 0.03	17.43 ± 0.87	2.97 ± 0.44	0.39 ± 0.2	0.3 ± 0.11	0.15 ± 0.03
leucine	176.9 ± 5.5	0.72 ± 0.14	1.36 ± 0.2	0.77 ± 0.38	0.57 ± 0.13	77.09 ± 0.91	0.53 ± 0.05	0.58 ± 0.17	0.7 ± 0.11	0.42 ± 0.09	55.21 ± 3.78	18.87 ± 2.59	0.93 ± 0.09	0.97 ± 0.23	0.41 ± 0.06
Phenylalanine	47.11 ± 5.82	0.83 ± 0.04	0.41 ± 0.1	0.34 ± 0.22	0.17 ± 0.05	38.73 ± 5.82	0.44 ± 0.08	0.39 ± 0.2	0.35 ± 0.07	0.3 ± 0.27	35.76 ± 2.01	14.19 ± 1.41	8.31 ± 0.81	3.82 ± 5.76	0.11 ± 0.01
Tryptophan	18.1 ± 2.21	1.28 ± 0.08	1.27 ± 0.31	0.46 ± 0.17	0.25 ± 0.1	20.78 ± 2.53	5.81 ± 0.5	4.98 ± 0.22	0.85 ± 0.69	0.25 ± 0.19	23.96 ± 1.9	7.65 ± 0.36	6.12 ± 0.59	0.21 ± 0.15	0.53 ± 0.02

Appendix 4: Chapter 3: Shows all the 2-Way ANOVA tables for the different concentrations of glucose and sucrose and its effect on coconut water kefir fermentation with time, T=0h.

Time = 0h	Glucose(F. val)	Glucose(raw.p)	Glucose(adj.p)	Sucrose(F. val)	Sucrose(raw.p)	Sucrose(adj.p)	Interaction(F. val)	Interaction(raw.p)	Interaction(adj.p)
Alanine	10132	3.41E-28	6.15E-27	4049.9	1.30E-24	2.33E-23	1608.8	1.02E-22	9.18E-22
Histidine	7684.2	4.10E-27	3.69E-26	3348.3	7.15E-24	6.43E-23	3062.7	3.15E-25	5.66E-24
Proline	7100.6	8.35E-27	5.01E-26	984.93	4.09E-19	1.23E-18	393.81	2.97E-17	1.07E-16
Threonine	2135.5	4.04E-22	1.82E-21	1330.9	2.78E-20	1.67E-19	192.93	1.63E-14	3.66E-14
Arginine	1468.5	1.15E-20	4.16E-20	99.349	1.88E-10	3.77E-10	290.68	4.39E-16	1.32E-15
Lysine	1379.4	2.02E-20	6.06E-20	1252.9	4.77E-20	2.15E-19	886.5	2.13E-20	1.28E-19
Glycine	1076.3	1.85E-19	4.77E-19	536.13	9.11E-17	2.34E-16	129.01	5.44E-13	1.09E-12
Aspartic acid	886.92	1.04E-18	2.34E-18	1081.6	1.77E-19	6.39E-19	773.51	7.21E-20	3.24E-19
Valine	201.65	4.74E-13	9.49E-13	12.712	0.000361	0.000465	122.35	8.61E-13	1.55E-12
Leucine	193.74	6.70E-13	1.21E-12	21.155	1.88E-05	2.60E-05	204.74	9.65E-15	2.48E-14
Methionine	174.38	1.65E-12	2.70E-12	222.23	2.05E-13	4.61E-13	22.527	8.36E-07	1.16E-06
Serine	163.75	2.83E-12	4.24E-12	84.35	7.20E-10	1.30E-09	59.767	3.79E-10	5.69E-10
Isoleucine	93.348	3.14E-10	4.35E-10	49.133	5.11E-08	8.36E-08	75.907	5.11E-11	8.37E-11
Tyrosine	75.839	1.70E-09	2.19E-09	23.539	9.47E-06	1.42E-05	5.3278	0.005202	0.005202
Tryptophan	72.887	2.34E-09	2.81E-09	3.2591	0.061951	0.074341	7.7323	0.000825	0.000929
Hydroxy Proline	55.058	2.13E-08	2.40E-08	0.58654	0.56653	0.56653	10.92	0.000113	0.000136
Phenylalanine	38.997	2.87E-07	3.03E-07	2.0707	0.1551	0.17448	7.4464	0.001009	0.001069
Glutamic acid	13.967	0.000218	0.000218	0.88982	0.42804	0.45322	18.061	4.10E-06	5.27E-06

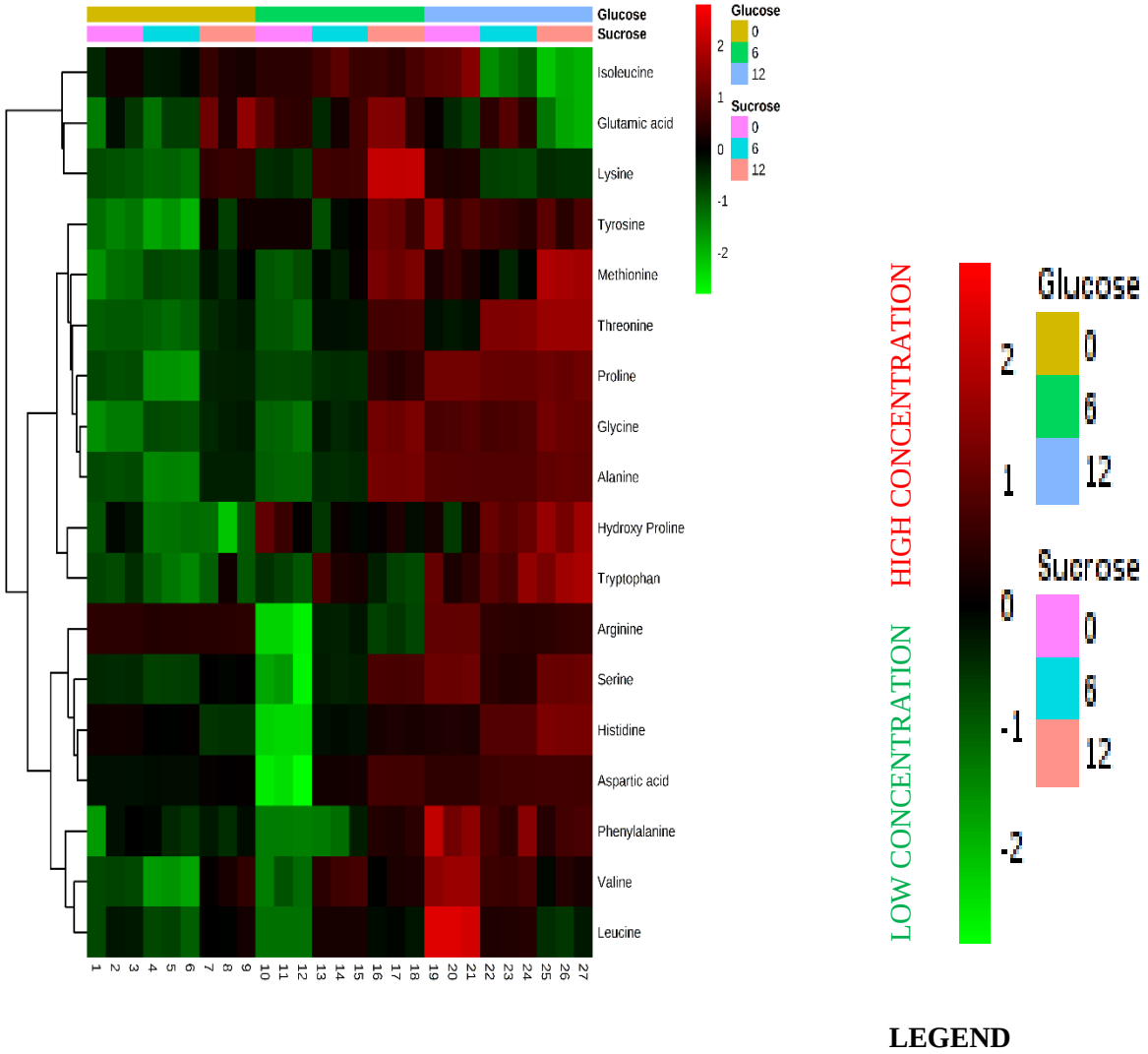
Time = 24h	Glucose(F. val)	Glucose(raw.p)	Glucose(adj.p)	Sucrose(F. val)	Sucrose(raw.p)	Sucrose(adj.p)	Interaction(F. val)	Interaction(raw.p)	Interaction(adj.p)
Alanine	10094	3.53E-28	6.36E-27	470.95	2.87E-16	1.03E-15	804.99	5.04E-20	3.03E-19
Proline	7154.9	7.80E-27	7.02E-26	1028	2.79E-19	5.03E-18	1261.2	9.05E-22	1.63E-20
Threonine	1951.7	9.05E-22	4.27E-21	303.74	1.35E-14	3.48E-14	534.98	1.94E-18	8.73E-18
Aspartic acid	1941.4	9.49E-22	4.27E-21	289.52	2.06E-14	4.63E-14	64.857	1.92E-10	2.47E-10
Serine	1776.8	2.10E-21	7.55E-21	616.07	2.66E-17	1.60E-16	122.89	8.29E-13	1.66E-12
Lysine	1560.3	6.71E-21	2.01E-20	65.669	5.37E-09	8.05E-09	304.09	2.94E-16	1.06E-15
Tryptophan	643.18	1.81E-17	4.67E-17	24.891	6.57E-06	7.39E-06	275.07	7.15E-16	1.84E-15
Arginine	498.65	1.73E-16	3.89E-16	60.263	1.06E-08	1.46E-08	69.684	1.05E-10	1.46E-10
Glycine	479.91	2.43E-16	4.85E-16	91.225	3.80E-10	6.21E-10	53.393	9.63E-10	1.16E-09
Histidine	399.9	1.21E-15	2.18E-15	800.83	2.59E-18	2.33E-17	894.67	1.96E-20	1.76E-19
Tyrosine	361.73	2.93E-15	4.79E-15	556.83	6.52E-17	2.93E-16	285.7	5.11E-16	1.53E-15
Leucine	210.88	3.23E-13	4.84E-13	266.11	4.29E-14	8.58E-14	109.18	2.30E-12	3.76E-12
Phenylalanine	108.93	8.78E-11	1.22E-10	50.096	4.41E-08	5.67E-08	119.69	1.04E-12	1.87E-12
Valine	102.58	1.45E-10	1.86E-10	335.76	5.63E-15	1.69E-14	125.8	6.77E-13	1.52E-12
Hydroxy Proline	76.684	1.56E-09	1.87E-09	2.7745	0.089067	0.089067	11.255	9.40E-05	9.95E-05
Methionine	64.026	6.56E-09	7.38E-09	105.36	1.16E-10	2.08E-10	87.867	1.48E-11	2.22E-11
Isoleucine	40.589	2.14E-07	2.26E-07	39.22	2.75E-07	3.30E-07	40.247	9.57E-09	1.08E-08
Glutamic acid	16.39	8.83E-05	8.83E-05	13.797	0.000233	0.000247	7.0868	0.001308	0.001308

Time=48 h	Glucose(F. val)	Glucose(raw.p)	Glucose(adj.p)	Sucrose(F. val)	Sucrose(raw.p)	Sucrose(adj.p)	Interaction(F. val)	Interaction(raw.p)	Interaction(adj.p)
Proline	9369.1	6.90E-28	1.24E-26	1452.2	1.28E-20	7.66E-20	1099.2	3.10E-21	1.40E-20
Alanine	6836.7	1.17E-26	1.06E-25	4283.9	7.82E-25	7.04E-24	3572.8	7.88E-26	4.73E-25
Histidine	5980.7	3.90E-26	2.34E-25	5410.2	9.61E-26	1.73E-24	12131	1.33E-30	2.39E-29
Serine	2413.3	1.35E-22	6.07E-22	7.2322	0.004952	0.005943	4458.4	1.08E-26	9.69E-26
Aspartic acid	781.48	3.22E-18	1.16E-17	137.73	1.23E-11	4.42E-11	68.753	1.18E-10	1.93E-10
Lysine	572.96	5.06E-17	1.52E-16	81.296	9.71E-10	1.94E-09	1010.6	6.59E-21	2.37E-20
Hydroxy Proline	363.24	2.82E-15	7.26E-15	65.404	5.54E-09	9.98E-09	6.7937	0.001624	0.001949
Glycine	345.18	4.42E-15	9.94E-15	88.376	4.92E-10	1.11E-09	82.25	2.59E-11	4.67E-11
Tryptophan	182.26	1.13E-12	2.26E-12	31.324	1.37E-06	2.25E-06	88.967	1.33E-11	2.66E-11
Threonine	179.07	1.32E-12	2.37E-12	283.31	2.49E-14	1.12E-13	418.08	1.74E-17	5.23E-17
Phenylalanine	48.147	5.96E-08	9.75E-08	12.816	0.000346	0.000479	53.581	9.36E-10	1.40E-09
Tyrosine	47.368	6.74E-08	1.01E-07	23.122	1.06E-05	1.60E-05	45.944	3.29E-09	4.56E-09
Leucine	38.147	3.37E-07	4.66E-07	9.8914	0.001264	0.001625	3.0489	0.044055	0.049561
Arginine	29.846	1.92E-06	2.47E-06	127.91	2.29E-11	6.88E-11	334.95	1.25E-16	3.21E-16
Glutamic acid	27.514	3.36E-06	4.03E-06	108.72	8.92E-11	2.29E-10	92.31	9.71E-12	2.18E-11
Methionine	8.7153	0.002254	0.002536	6.4871	0.007559	0.008504	19.939	2.03E-06	2.61E-06
Isoleucine	5.9231	0.010555	0.011176	1.7408	0.20364	0.21561	2.535	0.076072	0.080547

Time = 72h	Glucose(F. val)	Glucose(raw.p)	Glucose(adj.p)	Sucrose(F. val)	Sucrose(raw.p)	Sucrose(adj.p)	Interaction(F. val)	Interaction(raw.p)	Interaction(adj.p)
Histidine	641.2	1.87E-17	3.36E-16	80.963	1.00E-09	4.52E-09	557.71	1.34E-18	1.20E-17
Alanine	450.96	4.20E-16	3.78E-15	630.89	2.15E-17	3.88E-16	968.53	9.64E-21	1.73E-19
Aspartic acid	338.84	5.20E-15	3.12E-14	2.5686	0.10439	0.11759	41.491	7.50E-09	1.93E-08
Serine	272.56	3.48E-14	1.57E-13	84.431	7.14E-10	4.28E-09	76.286	4.90E-11	1.77E-10
Glycine	98.454	2.03E-10	7.30E-10	63.195	7.27E-09	2.62E-08	212.03	7.10E-15	3.19E-14
Hydroxy Proline	86.286	5.98E-10	1.80E-09	8.4303	0.002609	0.003913	6.7848	0.001635	0.002102
Proline	82.298	8.79E-10	2.26E-09	9.639	0.001427	0.002335	64.76	1.94E-10	5.83E-10
Leucine	76.674	1.56E-09	3.51E-09	5.5089	0.013598	0.017484	7.522	0.000957	0.001325
Glutamic acid	39.279	2.72E-07	5.44E-07	196.7	5.88E-13	5.29E-12	37.279	1.76E-08	3.96E-08
Tryptophan	23.133	1.06E-05	1.91E-05	19.187	3.45E-05	6.90E-05	6.5745	0.001916	0.002299
Arginine	22.559	1.25E-05	2.04E-05	6.0006	0.010074	0.013949	13.927	2.41E-05	4.34E-05
Threonine	21.52	1.69E-05	2.53E-05	50.163	4.36E-08	1.31E-07	8.7692	0.000412	0.000675
Methionine	18.017	5.05E-05	6.99E-05	11.512	0.000603	0.001085	21.324	1.25E-06	2.50E-06
Tyrosine	16.996	7.15E-05	9.19E-05	0.032605	0.96798	0.96798	4.3819	0.011966	0.01267
Valine	13.933	0.000221	0.000265	2.5669	0.10453	0.11759	5.7402	0.003694	0.004156
Isoleucine	9.1888	0.001778	0.002	22.25	1.36E-05	3.07E-05	8.2841	0.000567	0.00085
Phenylalanine	8.6306	0.002354	0.002492	0.09174	0.91277	0.96646	3.3809	0.031364	0.031364
Lysine	6.7871	0.00636	0.00636	29.772	1.96E-06	5.03E-06	232.16	3.19E-15	1.92E-14

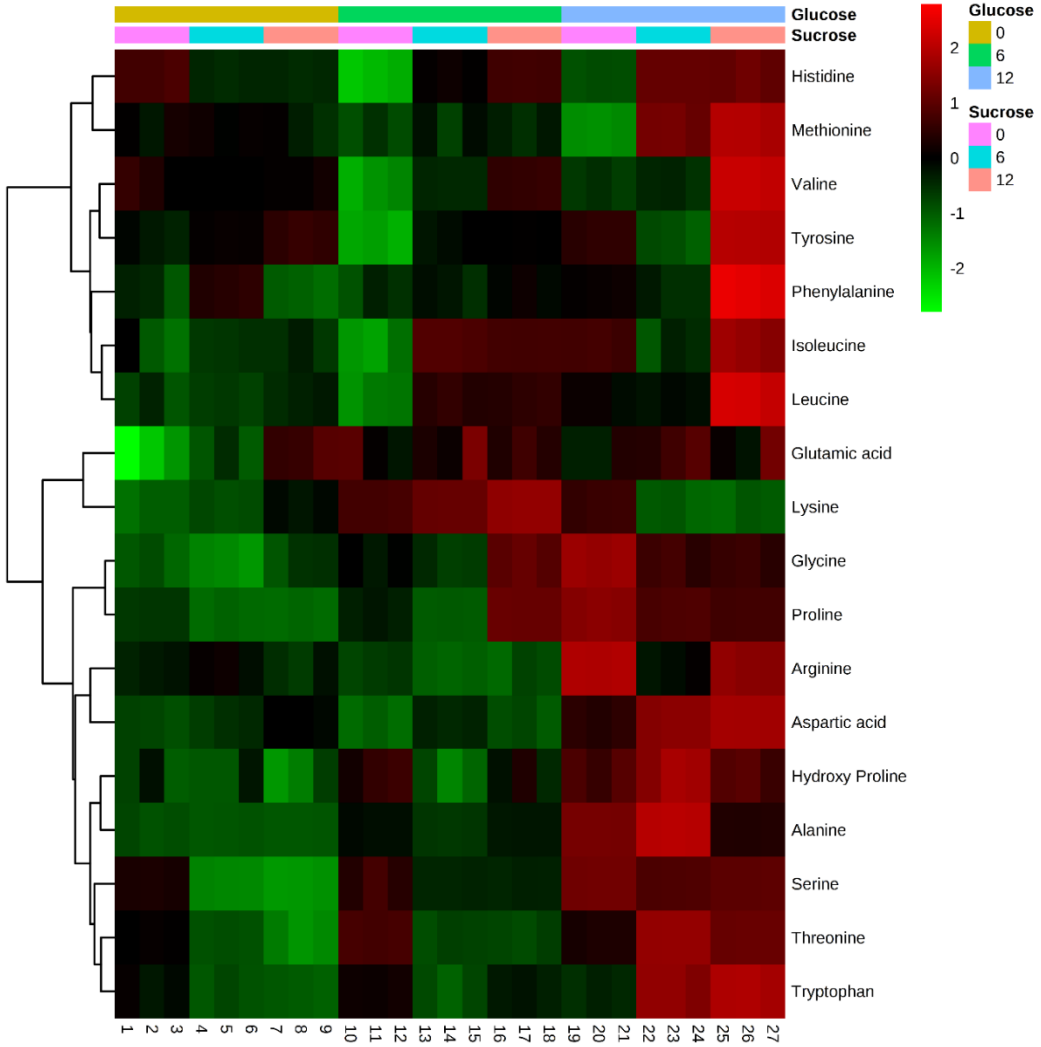
Time = 96h	Glucose(F. val)	Glucose(raw.p)	Glucose(adj.p)	Sucrose(F. val)	Sucrose(raw.p)	Sucrose(adj.p)	Interaction(F. val)	Interaction(raw.p)	Interaction(adj.p)
Histidine	2375	1.56E-22	2.80E-21	4012.5	1.41E-24	2.53E-23	1895.1	2.34E-23	4.22E-22
Lysine	1342.2	2.58E-20	2.32E-19	136.9	1.29E-11	3.88E-11	316.66	2.06E-16	1.23E-15
Alanine	1220.6	6.03E-20	3.62E-19	368.37	2.50E-15	8.99E-15	489.35	4.29E-18	3.87E-17
Serine	559	6.30E-17	2.83E-16	537.75	8.87E-17	7.99E-16	72.223	7.78E-11	1.75E-10
Threonine	372.8	2.25E-15	8.09E-15	410.81	9.57E-16	5.74E-15	91.393	1.06E-11	2.72E-11
Glycine	331.28	6.33E-15	1.90E-14	34.377	7.13E-07	1.28E-06	269.72	8.50E-16	3.83E-15
Proline	202.29	4.62E-13	1.19E-12	395.25	1.34E-15	6.05E-15	233.32	3.06E-15	1.10E-14
Hydroxy Proline	129.97	2.00E-11	4.51E-11	41.835	1.71E-07	3.42E-07	29.288	1.16E-07	2.09E-07
Tyrosine	66.002	5.16E-09	1.03E-08	8.4306	0.002608	0.00313	15.505	1.18E-05	1.63E-05
Aspartic acid	48.505	5.63E-08	1.01E-07	51.316	3.67E-08	8.25E-08	34.05	3.60E-08	7.20E-08
Glutamic acid	32.409	1.08E-06	1.77E-06	97.941	2.12E-10	5.45E-10	109.82	2.19E-12	6.57E-12
Arginine	28.661	2.54E-06	3.81E-06	23.059	1.08E-05	1.62E-05	14.803	1.61E-05	2.07E-05
Valine	21.288	1.81E-05	2.50E-05	18.013	5.06E-05	7.01E-05	8.7745	0.000411	0.000462
Tryptophan	15.137	0.000139	0.000169	5.7694	0.011586	0.013034	24.175	4.96E-07	8.11E-07
Isoleucine	15.112	0.000141	0.000169	4.2698	0.030366	0.032152	4.2049	0.014099	0.014928
Phenylalanine	5.6346	0.012582	0.014155	15.085	0.000142	0.000183	12.673	4.45E-05	5.34E-05
Leucine	5.3177	0.015322	0.016223	1.8854	0.18055	0.18055	1.4014	0.27343	0.27343
Methionine	4.1822	0.032233	0.032233	26.434	4.40E-06	7.20E-06	20.99	1.40E-06	2.10E-06

Appendix 5: Chapter 3: Effect of Sugars (glucose and sucrose) on the change in amino acid profile of CWK with time

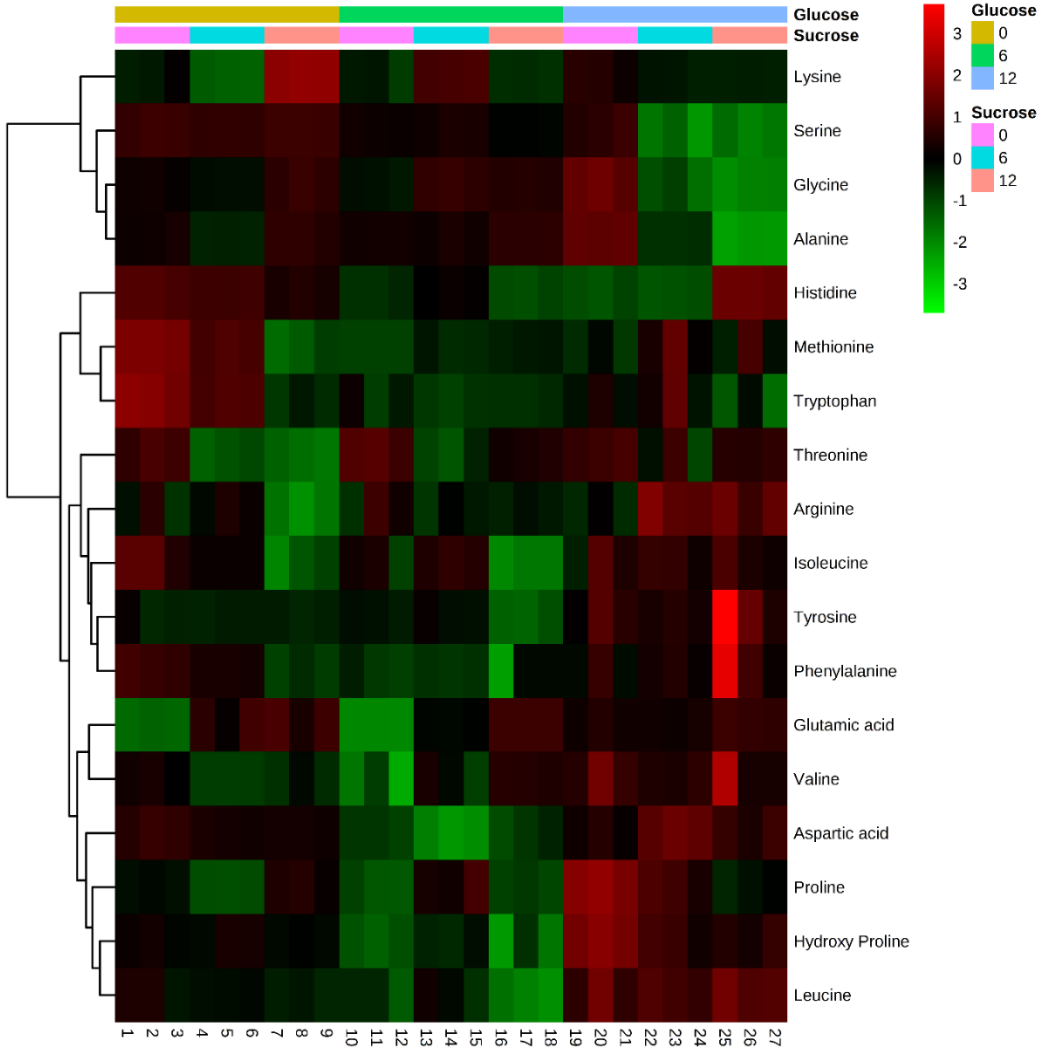


LEGEND

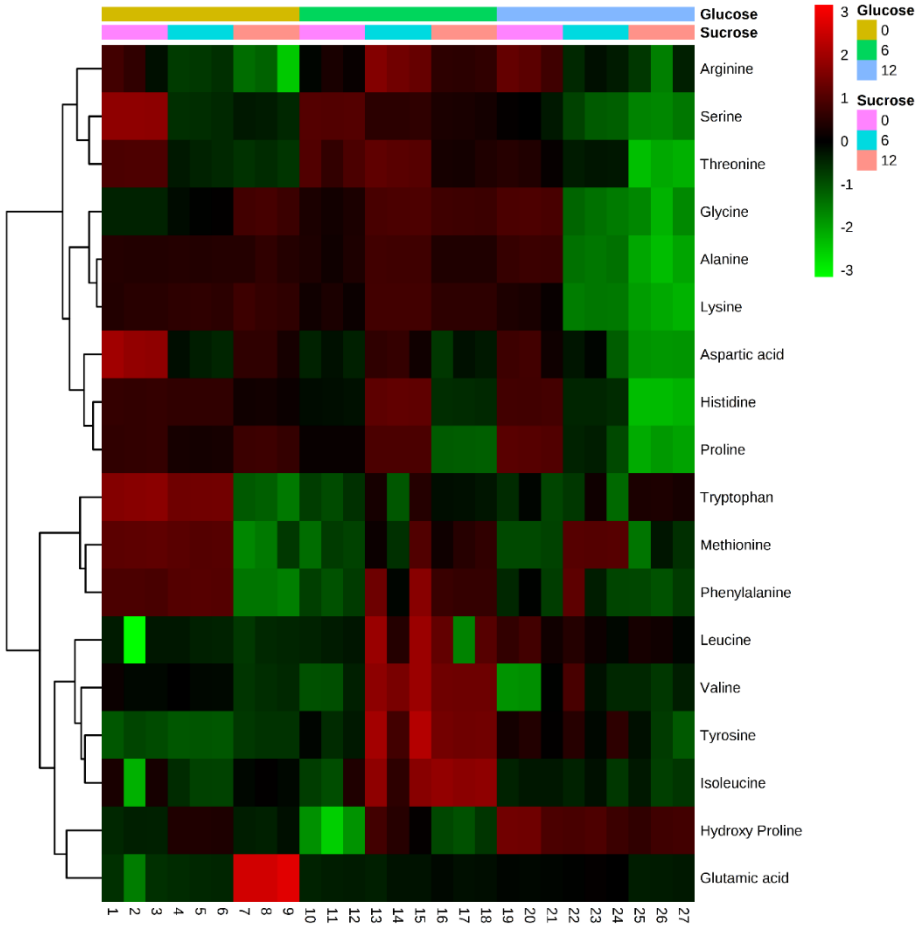
Represents heat map for Time (T=24 h) for all the amino acids detected in LCMS. Numbers 1,2,3: refer to set 1; 4,5,6- refer to set 2…., 25, 26, 27- refer to set 9.



Represents heat map for Time (T=72 h) for all the amino acids detected in LCMS. Numbers 1,2,3: refer to set 1; 4,5,6- refer to set 2…., 25, 26, 27- refer to set 9.



Represents heat map for Time (T=96 h) for all the amino acids detected in LCMS. Numbers 1,2,3: refer to set 1; 4,5,6- refer to set 2…., 25, 26, 27- refer to set 9.



Appendix 6: Chapter 4: Cell counts. The Log CFU/ml of LAB that were isolated from different dough sets (that were incubated at times, T=0, 24, 48, 72 and 96 h and at 22°C, 30°C and 4°C) on MRS agar incubated at 30°C with 5%CO_2. All the values for each experimental set is a mean of triplicate readings.

a, b, c= Alphabets a, b and c are different letters within the same column that demonstrate the effect of temperature (Temperature= 22°C, 30°C and 4°C) for each time period (T=0 h, 24 h, .., 96 h) on the Log CFU/ml of LAB and differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

A, B, C, D, E = Alphabets A,B,C,D,E are different letters within the same row that demonstrate the effect of a times (T= 0, 24, 48, 72 and 96 h) for each temperature (Temperature= 22°C, 30°C and 4°C) and differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Set 1						**Set 2**				
Temperature/ Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
22°C	3.51 ± 0.23a,E	4.69 ± 0.06b,D	5.36 ± 0.08b,C	7.28 ± 0.06b,B	9.1 ± 0.19b,A	3.47 ± 0.05a,b,E	4.75 ± 0.06b,D	5.85 ± 0.48b,C	7.19 ± 0.05b,B	8.58 ± 0.61b,A
30°C	3.36 ± 0.06a,D	6.96 ± 0.97a,C	8.9 ± 0.83a,B	9.59 ± 0.41a,B	12.39 ± 0.82a,A	3.52 ± 0.01a,D	6.31 ± 1.14a,C	8.69 ± 0.82a,B	10.37 ± 0.63a,A	11.35 ± 1.31a,A
4°C	3.27 ± 0.02a,D	3.8 ± 0.07b,C	3.85 ± 0.24c,C	4.65 ± 0.06c,B	4.98 ± 0.21c,A	3.43 ± 0.02b,C	3.91 ± 0.14b,B	3.88 ± 0.13c,B	5.09 ± 0.27c,A	5.06 ± 0.04c,A
Set 3						**Set 4**				
Temperature/ Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
22°C	3.31 ± 0.04b,E	4.81 ± 0.07b,D	5.91 ± 0.06b,C	6.7 ± 0.12b,B	9.03 ± 0.17b,A	3.36 ± 0.13a,E	5.04 ± 0.06b,D	6.06 ± 0.15b,C	7.04 ± 0.06b,B	9.14 ± 0.14b,A
30°C	3.36 ± 0.01a,D	7.68 ± 0.15a,C	9.28 ± 0.72a,B	9.62 ± 0.26a,B	13.82 ± 0.92a,A	3.2 ± 0.02a,b,E	6.75 ± 0.85a,D	8.47 ± 0.35a,C	10.19 ± 0.9a,B	13.37 ± 0.89a,A
4°C	3.27 ± 0.01b,C	3.86 ± 0.21c,B	3.85 ± 0.25c,B	4.91 ± 0.1c,A	4.85 ± 0.15c,A	3.12 ± 0.04b,D	4.17 ± 0.05b,B	3.77 ± 0.31c,C	5 ± 0.13c,A	4.85 ± 0.17c,A
Set 5						**Set 6**				
Temperature/ Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
22°C	3.38 ± 0.03a,E	4.86 ± 0.07b,D	6.01 ± 0.17b,C	6.96 ± 0.02b,B	8.93 ± 0.06b,A	3.38 ± 0.03c,E	4.86 ± 0.07b,D	6.01 ± 0.17b,C	6.96 ± 0.02b,B	8.93 ± 0.06b,A

30°C	3.07 ± 0.06b,E	6.75 ± 0.72a,D	9.28 ± 0.62a,C	10.36 ± 0.61a,B	12.66 ± 0.63a,A	3.56 ± 0.04a,E	6.08 ± 0.65a,D	8.23 ± 0.24a,C	9.86 ± 0.72a,B	12.2 ± 0.64a,A
4°C	3.12 ± 0.09b,C	3.82 ± 0.15c,B	3.89 ± 0.06c,B	5.15 ± 0.19c,A	5.16 ± 0.3c,A	3.44 ± 0.01b,E	4.08 ± 0.12c,C	3.67 ± 0.1c,D	5.08 ± 0.12c,A	4.61 ± 0.02c,B
Set 7						**Set 8**				
Temperature/ Time in h	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
22°C	3.13 ± 0.05b,E	5.16 ± 0.03b,D	6.09 ± 0.1b,C	7.24 ± 0.07b,B	9.32 ± 0.01b,A	3.1 ± 0.05b,E	5.05 ± 0.03b,D	6.06 ± 0.02b,C	7.11 ± 0.11b,B	9.07 ± 0.11b,A
30°C	3.41 ± 0.01a,E	6.49 ± 0.43a,D	8.63 ± 0.51a,C	10.19 ± 0.74a,B	12.49 ± 0.54a,A	3.34 ± 0.09a,D	6.14 ± 0.78a,C	8.74 ± 0.84a,B	10 ± 0.81a,B	12.93 ± 0.8a,A
4°C	3.23 ± 0.02b,C	4.06 ± 0.21c,B	3.94 ± 0.05c,B	5.02 ± 0.49c,A	5.05 ± 0.16c,A	3.17 ± 0.02b,C	3.75 ± 0.08c,B	3.68 ± 0.08c,B	5.07 ± 0.38c,A	5.11 ± 0.25c,A
Set 9						**F Value**				
Temperature/ Time in h	**0**	**24**	**48**	**72**	**96**	**Time**	**Temperature**	**Time*Temprature**		
22°C	3.35 ± 0.15b,E	5.15 ± 0.02b,D	6.32 ± 0.01b,C	7.48 ± 0.07b,B	9.22 ± 0.02b,A	213.3***	360.78***	33.31***		
30°C	3.25 ± 0.08b,D	7.19 ± 0.89a,C	9.42 ± 0.27a,B	10.14 ± 0.47a,B	12.43 ± 0.25a,A					
4°C	3.57 ± 0.02a,C	3.86 ± 0.2c,B,C	4.05 ± 0.22c,A	5.21 ± 0.28c,A	5.21 ± 0.34c,A					

The Log CFU/ml of yeast that were isolated from different dough sets (that were incubated at times, T=0, 24, 48, 72 and 96 h and at 22°C, 30°C and 4°C) on Malt extract agar incubated at 35°C. All the values for each experimental set is a mean of triplicate readings.

a, b, c= Alphabets a, b and c are different letters within the same column that demonstrate the effect of temperature (Temperature= 22°C, 30°C and 4°C) for each time period (T=0 h, 24 h, .., 96 h) on the Log CFU/ml of yeast and differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

A, B, C, D, E = Alphabets A,B,C,D,E are different letters within the same row that demonstrate the effect of a times (T= 0, 24, 48, 72 and 96 h) for each temperature (Temperature= 22°C, 30°C and 4°C) and differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Set 1						Set 2				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
22°C	3.12 ± 0.12a,E	4.93 ± 0.26b,D	5.72 ± 0.06b,C	6.5 ± 0.15b,B	7.57 ± 0.12b,A	3.2 ± 0.14a,E	4.74 ± 0.47b,D	5.49 ± 0.32b,C	6.85 ± 0.03b,B	7.73 ± 0.19b,A
30°C	3.11 ± 0.04a,E	5.83 ± 0.44a,D	7.14 ± 0.19a,C	8.31 ± 0.06a,B	9.11± 0.12a,A	3.14 ± 0.1a,E	5.93 ± 0.59a,D	7.17 ± 0.19a,C	8.21 ± 0.1a,B	9.69 ± 0.55a,A
4°C	2.96 ± 0.25a,C	3.75 ± 0.1c,B	3.86 ± 0.27c,B	4.05 ± 0.14c,B	4.48 ± 0.21c,A	3 ± 0.26a,D	3.67 ± 0.1c,C	3.83 ± 0.23c,B,C	4.13 ± 0.09c,B	4.59 ± 0.18c,A
Set 3						Set 4				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
22°C	3.12 ± 0.13a,E	4.66 ± 0.44b,D	5.54 ± 0.14b,C	6.73 ± 0.21b,B	7.64 ± 0.25b,A	3.14 ± 0.04a,E	4.68 ± 0.14b,D	5.34 ± 0.12b,C	6.57 ± 0.28b,B	7.76 ± 0.13b,A
30°C	3.19 ± 0.18a,E	6 ± 0.18a,D	7.16 ± 0.17a,C	8.16 ± 0.21a,B	11.38 ± 0.07a,A	3.23 ± 0.17a,E	6.1 ± 0.55a,D	7.18 ± 0.2a,C	8.06 ± 0.09a,B	9.9 ± 0.36a,A
4°C	2.75 ± 0.05b,C	3.73 ± 0.11c,B	3.69 ± 0.36c,B	3.9 ± 0.06c,B	4.62 ± 0.15c,A	3.14 ± 0.08a,D	3.76 ± 0.15c,C	3.75 ± 0.27c,B,C	4.07 ± 0.2c,B	4.51 ± 0.06c,A
Set 5						Set 6				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
22°C	3.06 ± 0.08a,D	5.15 ± 0.56a,C	5.46 ± 0.24b,C	6.43 ± 0.16b,B	7.56 ± 0.18b,A	3.13 ± 0.18a,E	4.59 ± 0.44b,D	5.64 ± 0.3b,C	6.65 ± 0.32b,B	7.66 ± 0.26b,A

30°C	3.01 ± 0.08a,E	5.82 ± 0.27a,D	7.21 ± 0.17a,C	8.2 ± 0.14a,B	10.02 ± 0.39a,A	3.26 ± 0.06a,E	6.08 ± 0.22a,D	7.15 ± 0.05a,C	8.28 ± 0.11a,B	9.53 ± 0.25a,A
4°C	2.87 ± 0.22a,D	3.72 ± 0.13b,C	3.51 ± 0.13c,C	4.11 ± 0.05c,B	4.52 ± 0.16c,A	2.89 ± 0.37a,C	3.63 ± 0.1c,B	3.78 ± 0.24c,B	3.95 ± 0.03c,B	4.55 ± 0.18c,A
Set 7						Set 8				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
22°C	3.1 ± 0.19a,E	4.72 ± 0.56b,D	5.46 ± 0.06b,C	6.65 ± 0.22b,B	7.45 ± 0.15b,A	3.08 ± 0.1a,E	4.95 ± 0.5b,D	5.58 ± 0.32b,C	6.55 ± 0.08b,B	7.54 ± 0.26b,A
30°C	3.31 ± 0.04a,E	6.17 ± 0.4a,D	7.04 ± 0.1a,C	8.1 ± 0.24a,B	9.9 ± 0.23a,A	3.24 ± 0.14a,E	5.82 ± 0.43a,D	7.06 ± 0.06a,C	8.23 ± 0.13a,B	9.33 ± 0.26a,A
4°C	3.01 ± 0.31a,D	3.7 ± 0.04c,C	3.7 ± 0.24c,C	4.11 ± 0.24c,B	4.6 ± 0.15c,A	3 ± 0.28a,C	3.85 ± 0.1c,B	3.61 ± 0.31c,B	3.92 ± 0.04c,B	4.57 ± 0.2c,A
Set 9						F Value				
Temperature/Time in h	0	24	48	72	96	Time	Temperature	Time*Tempratur e		
22°C	3.2 ± 0.05a,D	4.98 ± 0.56a,C	5.38 ± 0.06b,C	6.67 ± 0.15b,B	7.57 ± 0.29b,A					
30°C	3.27 ± 0.16a,E	5.41 ± 0.15a,D	7.09 ± 0.16a,C	8.09 ± 0.07a,B	9.79 ± 0.54a,A					
4°C	3.15 ± 0.16a,D	3.59 ± 0.07b,C	3.54 ± 0.19c,C	3.87 ± 0.08c,B	4.63 ± 0.03c,A	3529.699***	5461.852***	433.883***		

The Log CFU/ml of AAB that were isolated from different dough sets (that were incubated at times, T=0, 24, 48, 72 and 96 h and at 22°C, 30°C and 4°C) on Acetic acid bacteria agar incubated at 35°C. All the values for each experimental set is a mean of triplicate readings.

a, b, c= Alphabets a, b and c are different letters within the same column that demonstrate the effect of temperature (Temperature= 22°C, 30°C and 4°C) for each time period (T=0 h, 24 h, .., 96 h) on the Log CFU/ml of AAB and differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

A, B, C, D, E = Alphabets A,B,C,D,E are different letters within the same row that demonstrate the effect of a times (T= 0, 24, 48, 72 and 96 h) for each temperature (Temperature= 22°C, 30°C and 4°C) and differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Set 1				Set 2			Set 3		
Temperature/Time in h	48	72	96	48	72	96	48	72	96
22°C	1.92 ± 0.02b, C	2.19 ± 0.06a, B	2.65 ± 0.03b, A	1.92 ± 0.02b, C	2.22 ± 0.08a,B	2.66 ± 0.02b, A	1.84 ± 0.05b, C	2.33 ± 0.06a, B	2.73 ± 0.03b, A
30°C	2.07 ± 0.05a, C	2.25 ± 0.03a, B	2.93 ± 0.1a,A	2.09 ± 0.06a, C	2.29 ± 0.11a,B	2.93 ± 0.03a, B	2.11 ± 0.04a, C	2.32 ± 0.02a, C	3.22 ± 0.08a, A
4°C	1.12 ± 0.01c, B	1.3 ± 0.19b, B	1.61 ± 0.08c, A	1.23 ± 0.01c, B	1.23 ± 0.19b,B	1.55 ± 0.02c, A	1.04 ± 0.03c, C	1.31 ± 0.17b, B	1.52 ± 0.04c, A
Set 4				Set 5			Set 6		
Temperature/Time in h	48	72	96	48	72	96	48	72	96
22°C	1.85 ± 0.05b, C	2.32 ± 0.09a, B	2.75 ± 0.04b, A	1.8 ± 0.01b, C	2.22 ± 0.04b,B	2.68 ± 0.04b, A	1.94 ± 0.07b, C	2.21 ± 0.1a,B	2.68 ± 0.05b, A
30°C	2.1 ± 0.05a, B	2.23 ± 0.09a, B	2.93 ± 0.06a, A	2.06 ± 0.04a, C	2.37 ± 0.01a,B	2.94 ± 0.01a, A	2.15 ± 0.02a, B	2.14 ± 0.03a, B	2.93 ± 0.05a, A

4°C	1.23 ± 0.01c, B	1.39 ± 0.14b, B	1.62 ± 0.05c, A	1.17 ± 0.02c, C	1.28 ± 0.04c,B	1.61 ± 0.06c, A	1.13 ± 0.02c, B	1.26 ± 0.17b, B	1.57 ± 0.02c, A			
Set 7				Set 8			Set 9			F Value		
Temperature/Time in h	48	72	96	48	72	96	48	72	96	Time	Temperature	Time*Tempratu re
22°C	1.91 ± 0.01b, C	2.16 ± 0.06b, B	2.66 ± 0.04b, A	1.87 ± 0.01b, C	2.33 ± 0.07a,B	2.78 ± 0.01b, A	1.96 ± 0.05a, C	2.24 ± 0.06a, B	2.72 ± 0.06b, A	1650.525***	4609.461***	75.259***
30°C	2.12 ± 0.05a, C	2.32 ± 0.04a, B	2.92 ± 0.02a, A	2.05 ± 0.05a, C	2.22 ± 0.08a,B	2.92 ± 0.05a, A	2.05 ± 0.03a, C	2.22 ± 0.11a, B	2.95 ± 0.03a, A			
4°C	1 ± 0.03c, C	1.24 ± 0.09c, B	1.54 ± 0.03c, A	1.2 ± 0.11c, B	1.37 ± 0.14b,A, B	1.56 ± 0.03c, A	1.15 ± 0.05b, B	1.31 ± 0.19b, B	1.59 ± 0.06c, A			

Appendix 7: Chapter 4: pH, D/L-lactic acid and TTA data.

The D-Lactic acid concentration that was identified from different dough sets (that were incubated at times, T=0, 24, 48, 72 and 96 h and at 22°C, 30°C and 4°C). All the values for each experimental set is a mean of triplicate readings.

a, b, c= Alphabets a, b and c are different letters within the same column that demonstrate the effect of temperature (Temperature= 22°C, 30°C and 4°C) for each time period (T=0 h, 24 h, .., 96 h) on the D-Lactic acid and differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

A, B, C, D, E = Alphabets A,B,C,D,E are different letters within the same row that demonstrate the effect of a times (T= 0, 24, 48, 72 and 96 h) for each temperature (Temperature= 22°C, 30°C and 4°C) and differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Set 1						Set 2				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
22°C	0.77 ± 0.02a,E	1.55 ± 0.05b,D	1.78 ± 0.04b,C	2.03 ± 0.06b,B	2.58 ± 0.04b,A	0.75 ± 0.06a,E	1.53 ± 0.05b,D	1.78 ± 0.02b,C	2.01 ± 0.09b,B	2.68 ± 0.11b,A
30°C	0.75 ± 0.05a,E	1.79 ± 0.02a,D	2.1 ± 0.03a,C	2.66 ± 0.04a,B	3.66 ± 0.09a,A	0.77 ± 0.06a,E	1.76 ± 0.02a,D	2.07 ± 0.02a,C	2.66 ± 0.04a,B	3.74 ± 0.07a,A
4°C	0.76 ± 0.03a,E	1.17 ± 0.04c,D	1.44 ± 0.05c,C	1.64 ± 0.06c,B	1.97 ± 0.01c,A	0.73 ± 0.03a,E	1.14 ± 0.03c,D	1.44 ± 0.07c,C	1.59 ± 0.05c,B	1.95 ± 0.03c,A
Set 3						Set 4				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
22°C	0.76 ± 0.01a,E	1.54 ± 0.04b,D	1.79 ± 0.02b,C	1.98 ± 0.04b,B	2.62 ± 0.05b,A	0.73 ± 0.05a,E	1.53 ± 0.02b,D	1.79 ± 0.03b,C	2.1 ± 0.01b,B	2.65 ± 0.11b,A
30°C	0.78 ± 0.03a,E	1.75 ± 0.04a,D	2.05 ± 0.01a,C	2.61 ± 0.08a,B	3.63 ± 0.06a,A	0.78 ± 0.04a,E	1.8 ± 0.02a,D	2.07 ± 0.05a,C	2.67 ± 0.02a,B	3.7 ± 0.05a,A
4°C	0.73 ± 0.04a,E	1.21 ± 0.02c,D	1.41 ± 0.04c,C	1.63 ± 0.07c,B	1.94 ± 0.03c,A	0.73 ± 0.03a,E	1.13 ± 0.03c,D	1.44 ± 0.07c,C	1.61 ± 0.08c,B	1.92 ± 0.06c,A
Set 5						Set 6				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
22°C	0.76 ± 0.05a,E	1.53 ± 0.06b,D	1.78 ± 0.04b,C	2.02 ± 0.08b,B	2.69 ± 0.13b,A	0.77 ± 0.02a,E	1.55 ± 0.01b,D	1.78 ± 0.03b,C	2.09 ± 0.01b,B	2.65 ± 0.09b,A
30°C	0.75 ± 0.06a,E	1.76 ± 0.05a,D	2.04 ± 0a,C	2.61 ± 0.04a,B	3.64 ± 0.14a,A	0.75 ± 0.02a,E	1.73 ± 0.05a,D	2.09 ± 0.02a,C	2.62 ± 0.09a,B	3.68 ± 0.13a,A
4°C	0.74 ± 0.06a,E	1.17 ± 0.06c,D	1.41 ± 0.06c,C	1.59 ± 0.03c,B	1.87 ± 0.08c,A	0.75 ± 0.03a,E	1.15 ± 0.05c,D	1.43 ± 0.05cc,C	1.63 ± 0.04c,B	1.89 ± 0.05c,A
Set 7						Set 8				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
22°C	0.8 ± 0.02a,E	1.56 ± 0.06b,D	1.73 ± 0.04b,C	2.02 ± 0.04b,B	2.68 ± 0.07b,A	0.76 ± 0.03a,E	1.57 ± 0.02b,D	1.77 ± 0.04b,C	2.04 ± 0.06b,B	2.56 ± 0.03b,A
30°C	0.75 ± 0.05a,E	1.75 ± 0.04a,D	2.1 ± 0.01a,C	2.64 ± 0.04a,B	3.71 ± 0.07a,A	0.73 ± 0.06a,E	1.77 ± 0.03a,D	2.07 ± 0.02a,C	2.61 ± 0.03a,B	3.64 ± 0.07a,A
4°C	0.75 ± 0.04a,E	1.12 ± 0.04c,D	1.41 ± 0.05c,C	1.56 ± 0.03c,B	1.91 ± 0.05c,A	0.76 ± 0.04a,E	1.16 ± 0.03c,D	1.41 ± 0.09c,C	1.6 ± 0.07c,B	1.92 ± 0.06c,A

Set 9						F Value		
Temperature/Time in h	0	24	48	72	96	Time	Temperature	Time*Temprature
22°C	0.73 ± 0.02a,E	1.57 ± 0.01b,D	1.77 ± 0.03b,C	1.99 ± 0.05b,B	2.67 ± 0.12b,A	17106.73***	8650.79***	1115.13***
30°C	0.75 ± 0.03a,E	1.71 ± 0.03a,D	2.08 ± 0.04a,C	2.61 ± 0.01a,B	3.69 ± 0.01a,A			
4°C	0.73 ± 0.04a,E	1.1 ± 0.01c,D	1.39 ± 0.09c,C	1.62 ± 0.06c,B	1.89 ± 0.01c,A			

The L-Lactic acid concentration that was identified from different dough sets (that were incubated at times, T=0, 24, 48, 72 and 96 h and at 22°C, 30°C and 4°C). All the values for each experimental set is a mean of triplicate readings.

a, b, c= Alphabets a, b and c are different letters within the same column that demonstrate the effect of temperature (Temperature= 22°C, 30°C and 4°C) for each time period (T=0 h, 24 h, .., 96 h) on the L-Lactic acid and differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

A, B, C, D, E = Alphabets A,B,C,D,E are different letters within the same row that demonstrate the effect of a times (T= 0, 24, 48, 72 and 96 h) for each temperature (Temperature= 22°C, 30°C and 4°C) and differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Set 1						Set 2				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
22°C	2.1 ± 0.01a,E	3.92 ± 0.07b,D	4.34 ± 0.05b,C	6.25 ± 0.37b,B	8.08 ± 0.28b,A	2.09 ± 0.08a,E	3.92 ± 0.04b,D	4.33 ± 0.05b,C	5.92 ± 0.32b,B	7.97 ± 0.3b,A
30°C	2.14 ± 0.05a,E	4.18 ± 0.14a,D	6.6 ± 0.41a,C	7.79 ± 0.35a,B	10.26 ± 0.67a,A	2.04 ± 0.01a,E	4.16 ± 0.18a,D	6.46 ± 0.29a,C	7.68 ± 0.43a,B	10.29 ± 0.72a,A
4°C	2.07 ± 0.06a,E	3.1 ± 0.06c,D	3.29 ± 0.24c,C	3.6 ± 0.1c,B	3.8 ± 0.03c,A	2.06 ± 0.07a,E	3.05 ± 0.05c,D	3.37 ± 0.02c,C	3.6 ± 0.06c,B	3.8 ± 0.04c,A
Set 3						Set 4				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
22°C	2.17 ± 0.04a,E	3.91 ± 0.08b,D	4.48 ± 0.04b,C	5.99 ± 0.18b,B	8.31 ± 0.16b,A	2.15 ± 0.02a,E	3.96 ± 0.03b,D	4.5 ± 0.13b,C	6.25 ± 0.39b,B	8.15 ± 0.26b,A
30°C	2.07 ± 0.08a,E	4.12 ± 0.03a,D	6.73 ± 0.02a,C	8.04 ± 0.26a,B	9.85 ± 0.13a,A	2.14 ± 0.03a,E	4.15 ± 0.1a,D	6.86 ± 0.24a,C	7.7 ± 0.4a,B	10.12 ± 0.38a,A
4°C	2.16 ± 0.08a,E	3.13 ± 0.07c,D	3.59 ± 0.05c,C	3.66 ± 0.17c,B	3.81 ± 0.04c,A	2.17 ± 0.04a,E	3.1 ± 0.1c,D	3.55 ± 0.14c,C	3.61 ± 0.13c,B	3.82 ± 0.01c,A
Set 5						Set 6				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
22°C	2.14 ± 0.1a,E	3.96 ± 0.04b,D	4.45 ± 0.1b,C	5.8 ± 0.38b,B	8.33 ± 0.26b,A	2.05 ± 0.08a,E	3.91 ± 0.08b,D	4.45 ± 0.13b,C	6 ± 0.45b,B	8.1 ± 0.47b,A
30°C	2.06 ± 0.03a,E	4.21 ± 0.08a,D	6.8 ± 0.2a,C	7.52 ± 0.33a,B	10.23 ± 0.68a,A	2.07 ± 0.04a,E	4.19 ± 0.12a,D	6.55 ± 0.32a,C	7.42 ± 0.29a,B	10.44 ± 0.27a,A
4°C	2.16 ± 0.01a,E	3.04 ± 0.09c,D	3.21 ± 0.02c,C	3.6 ± 0.12c,B	3.78 ± 0.04c,A	2.06 ± 0.03a,E	3.11 ± 0.01c,D	3.33 ± 0.19c,C	3.61 ± 0.14c,B	3.81 ± 0.02c,A
Set 7						Set 8				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
22°C	2.07 ± 0.02a,E	3.87 ± 0.04b,D	4.38 ± 0.05b,C	6.37 ± 0.16b,B	7.91 ± 0.16b,A	2.09 ± 0.04a,E	3.91 ± 0.03b,D	4.47 ± 0.2b,C	5.41 ± 0.14b,B	8.1 ± 0.44b,A

30°C	2.08 ± 0.08a,E	4.19 ± 0.07a,D	6.57 ± 0.22a,C	7.44 ± 0.1a,B	10.69 ± 0.36a,A	2.11 ± 0.1a,E	4.1 ± 0.1a,D	6.57 ± 0.39a,C	7.49 ± 0.26a,B	10.34 ± 0.45a,A
4°C	2.14 ± 0.03a,E	3.09 ± 0.07c,D	3.4 ± 0.18c,C	3.54 ± 0.03c,B	3.77 ± 0.03c,A	2.16 ± 0.07a,E	3.12 ± 0.1c,D	3.51 ± 0.13c,C	3.6 ± 0.12c,B	3.76 ± 0.02c,A
Set 9						F Value				
Temperature/Time in h	0	24	48	72	96	Time	Temperature	Time*Temprature		
22°C	2.11 ± 0.06a,E	3.92 ± 0.08b,D	4.42 ± 0.13b,C	5.9 ± 0.53b,B	7.96 ± 0.28b,A	6726.45***	6155.8***	981.44***		
30°C	2.08 ± 0.09a,E	4.17 ± 0.07a,D	6.65 ± 0.06a,C	7.56 ± 0.36a,B	10.43 ± 0.45a,A					
4°C	2.05 ± 0.03a,E	3.09 ± 0.09c,D	3.34 ± 0.19c,C	3.79 ± 0.01c,B	3.79 ± 0.05c,A					

The change in pH detected for different dough sets (that were incubated at times, T=0, 24, 48, 72 and 96 h and at 22°C, 30°C and 4°C). All the values for each experimental set is a mean of triplicate readings.

a, b, c= Alphabets a, b and c are different letters within the same column that demonstrate the effect of temperature (Temperature= 22°C, 30°C and 4°C) for each time period (T=0 h, 24 h, .., 96 h) on the pH and differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

A, B, C, D, E = Alphabets A,B,C,D,E are different letters within the same row that demonstrate the effect of a times (T= 0, 24, 48, 72 and 96 h) for each temperature (Temperature= 22°C, 30°C and 4°C) and differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Set 1						Set 2				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
22°C	5.61 ± 0.01a,A	5.31 ± 0.08a,B	4.99 ± 0.07b,C	4.79 ± 0.1b,D	4.51 ± 0.16b,E	5.54 ± 0.08a,A	5.1 ± 0.08b,B	4.63 ± 0.11b,C	4.25 ± 0.18a,D	3.9 ± 0.16b,E
30°C	5.55 ± 0.09a,A	4.71 ± 0.33b,B	4.29 ± 0.22c,C	3.99 ± 0.14c,D	3.94 ± 0.14c,E	5.66 ± 0.1a,A	4.5 ± 0.1c,B	4.12 ± 0.11c,C	4.13 ± 0.16a,C	3.7 ± 0.13b,D
4°C	5.61 ± 0.01a,A	5.5 ± 0.04a,B	5.38 ± 0.04a,C	5.17 ± 0.09a,D	4.96 ± 0.07a,E	5.54 ± 0.08a,A	5.42 ± 0.06a,A	5.18 ± 0.07a,A,B	4.65 ± 0.62a,B,C	4.83 ± 0.07a,C
Set 3						Set 4				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
22°C	5.61 ± 0.03a,A	5.31 ± 0.08a,B	4.83 ± 0.08b,C	4.39 ± 0.2b,D	3.86 ± 0.13b,E	5.64 ± 0.05a,A	5.2 ± 0.03b,B	4.83 ± 0.09b,C	4.39 ± 0.03b,D	3.85 ± 0.19b,E
30°C	5.6 ± 0.03a,A	4.72 ± 0.16b,B	4.37 ± 0.17c,C	3.99 ± 0.14c,D	3.67 ± 0.27b,E	5.63 ± 0.01a,A	4.74 ± 0.14c,B	4.38 ± 0.08c,C	4.04 ± 0.12c,D	3.64 ± 0.23b,E
4°C	5.59 ± 0.1a,A	5.49 ± 0.08a,A,B	5.52 ± 0.01a,A,B	5.43 ± 0.09a,B	5.28 ± 0.01a,C	5.56 ± 0.08a,A	5.53 ± 0.07a,A	5.43 ± 0.03a,B	5.37 ± 0.02a,B	5.23 ± 0.03a,C
Set 5						Set 6				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
22°C	5.7 ± 0.09a,A	5.25 ± 0.09a,B	4.75 ± 0.11b,C	4.28 ± 0.14b,D	3.81 ± 0.1b,E	5.65 ± 0.09a,A	5.22 ± 0.14b,B	4.65 ± 0.16b,C	4.43 ± 0.12b,C	3.91 ± 0.15b,D
30°C	5.53 ± 0.05b,A	4.73 ± 0.24b,B	4.31 ± 0.06c,C	3.96 ± 0.02c,D	3.64 ± 0.3b,E	5.63 ± 0.01a,A	4.94 ± 0.03c,B	4.32 ± 0.12c,C	3.88 ± 0.09c,D	3.52 ± 0.24c,E
4°C	5.61 ± 0.11a,b,A	5.54 ± 0.03a,A	5.41 ± 0.02a,B	5.25 ± 0.04a,C	5.15 ± 0.02a,C	5.55 ± 0.02a,A	5.47 ± 0.02a,B	5.33 ± 0.03a,C	5.21 ± 0.03a,D	5.11 ± 0.02a,E
Set 7						Set 8				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
22°C	5.66 ± 0.02a,A	5.22 ± 0.12a,B	4.62 ± 0.1b,C	4.4 ± 0.18b,C	3.89 ± 0.15b,D	5.71 ± 0.08a,A	5.27 ± 0.06b,B	4.7 ± 0.2b,C	4.42 ± 0.22b,D	3.99 ± 0.05b,E

30°C	5.58 ± 0.09a,A	4.65 ± 0.21b,B	4.19 ± 0.11c,C	3.97 ± 0.11c,C,D	3.69 ± 0.25b,D	5.55 ± 0.08b,A	4.67 ± 0.09c,B	4.26 ± 0.21c,C	3.97 ± 0.1c,D	3.57 ± 0.19c,E
4°C	5.56 ± 0.1a,A	5.46 ± 0.04a,B	5.35 ± 0.03a,C	5.25 ± 0.03a,D	5.17 ± 0.02a,D	5.53 ± 0.06b,A	5.49 ± 0.04a,A	5.41 ± 0.02a,B	5.25 ± 0.01a,C	5.13 ± 0.03a,D
Set 9						F Value				
Temperature/Time in h	0	24	48	72	96	Time	Temperature	Time*Temprature		
22°C	5.63 ± 0.04a,A	5.18 ± 0.17b,B	4.62 ± 0.11b,C	4.35 ± 0.11b,D	3.94 ± 0.16b,E					
30°C	5.59 ± 0.03a,A	4.49 ± 0.09c,B	4.15 ± 0.06c,C	3.94 ± 0.09c,D	3.62 ± 0.2c,E					
4°C	5.57 ± 0.09a,A	5.48 ± 0.09a,A	5.35 ± 0.02a,B	5.21 ± 0.01a,C	5.18 ± 0.01a,C	908.23***	1146.86***	106.88***		

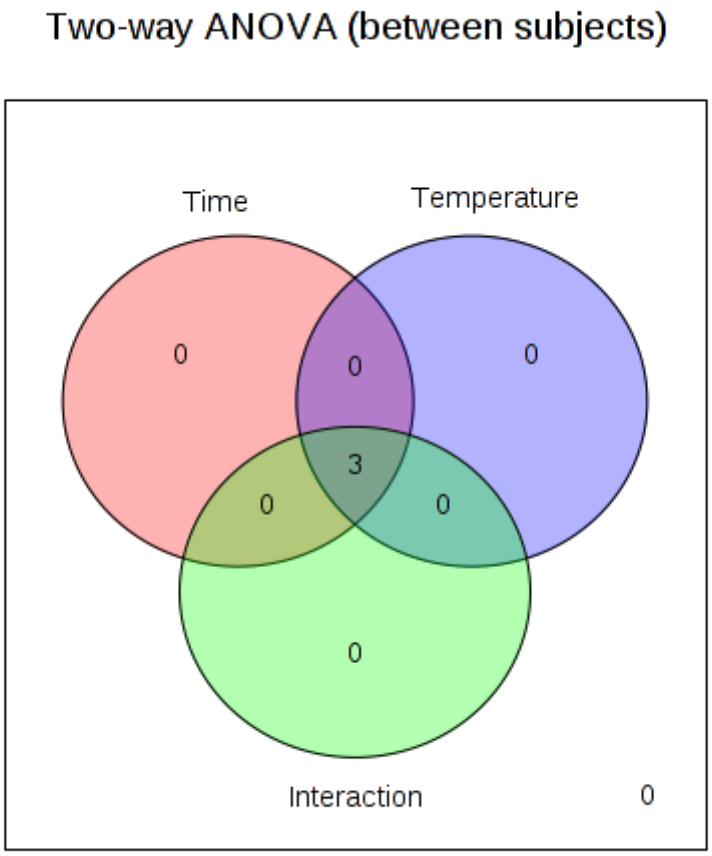
The change in TTA detected for different dough sets (that were incubated at times, T=0, 24, 48, 72 and 96 h and at 22°C, 30°C and 4°C). All the values for each experimental set is a mean of triplicate readings.

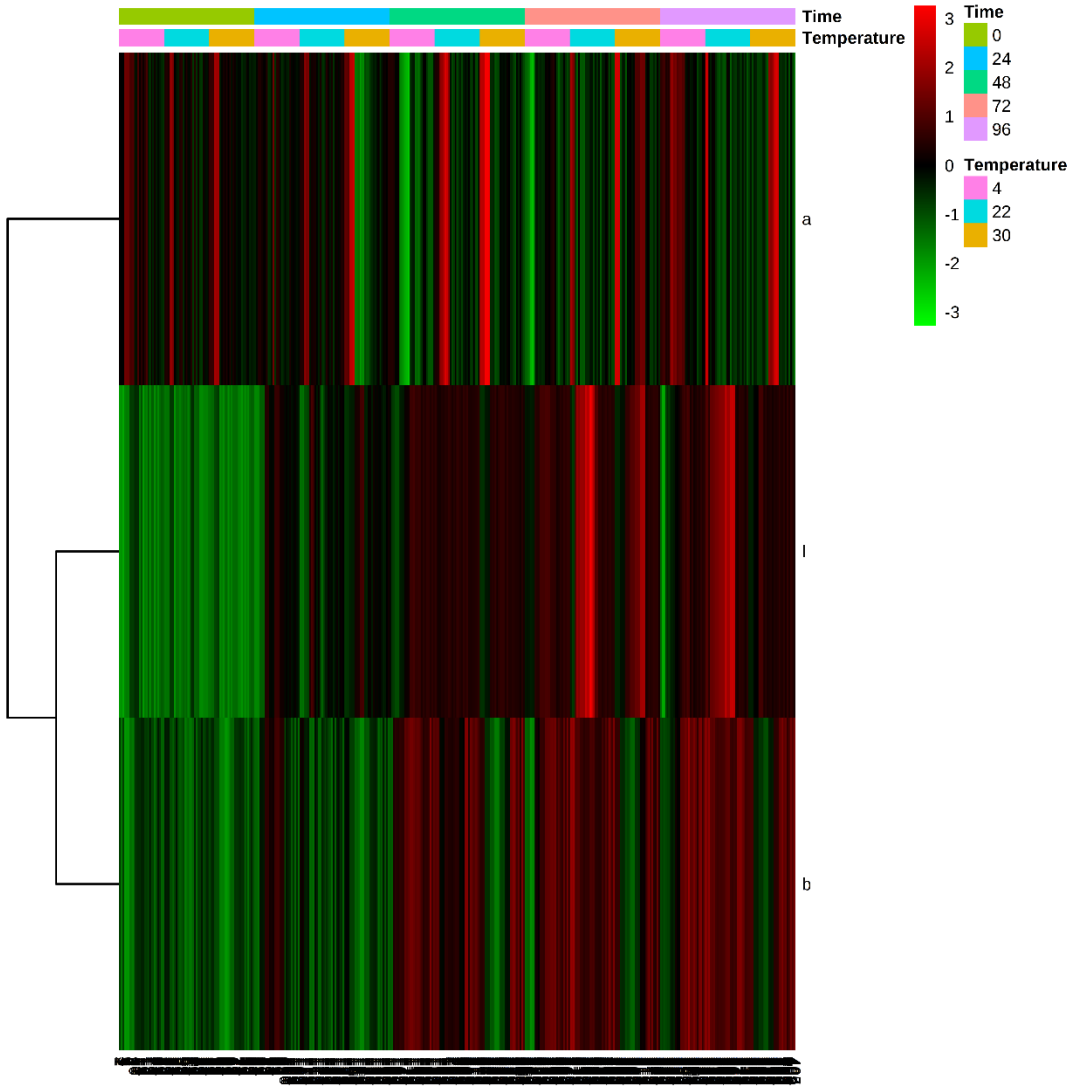
a, b, c= Alphabets a, b and c are different letters within the same column that demonstrate the effect of temperature (Temperature= 22°C, 30°C and 4°C) for each time period (T=0 h, 24 h, .., 96 h) on the TTA and differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

A, B, C, D, E = Alphabets A,B,C,D,E are different letters within the same row that demonstrate the effect of a times (T= 0, 24, 48, 72 and 96 h) for each temperature (Temperature= 22°C, 30°C and 4°C) and differ significantly using Fisher's least significant difference ($p < 0.05$). $*$ $p < 0.05$. $**$ $p < 0.01$. $***$ $p < 0.001$.

Set 1						Set 2				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
30°C	2.68 ± 0.04a,E	3.94 ± 0.06a,D	5.3 ± 0.93a,B	5.44 ± 0.26a,C	7.2 ± 1.51a,A	3.84 ± 0.07a,E	4.08 ± 0.13a,D	6.22 ± 0.28a,B	7.26 ± 0.1a,C	11.56 ± 0.11a,A
22°C	2.67 ± 0.06a,E	3.74 ± 0.14a,D	3.84 ± 0.05b,B	4.87 ± 0.1b,C	5.57 ± 0.09b,A	3.85 ± 0.11a,E	3.8 ± 0.16b,D	4.56 ± 0.08b,B	5.22 ± 0.1b,C	6.33 ± 0.08b,A
Set 3						Set 4				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
30°C	2.93 ± 0.07a,E	5.48 ± 0.11a,D	6.48 ± 0.12a,B	7.88 ± 0.09a,C	9.84 ± 1.6a,A	2.64 ± 0.05a,E	5.13 ± 0.42a,D	5.83 ± 0.08a,B	9.73 ± 0.12a,C	11.86 ± 0.48a,A
22°C	2.86 ± 0.07a,E	4.5 ± 0.21b,D	5.46 ± 0.11b,B	6.86 ± 0.1b,C	8.36 ± 0.1b,A	2.64 ± 0.1a,D	4.52 ± 0.6b,C	4.77 ± 0.11b,B	8.77 ± 0.01b,B	9.57 ± 0.11b,A
Set 5						Set 6				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
30°C	3.98 ± 0.05a,D	8.44 ± 0.17a,C	8.74 ± 0.11a,B	10.87 ± 0.06a,B	12.6 ± 0.21a,A	3.04 ± 0.05a,E	7.25 ± 0.11a,D	8.9 ± 0.06a,B	10.55 ± 0.11a,C	12.81 ± 0.15a,A
22°C	3.88 ± 0.12a,E	5.37 ± 0.12b,D	6.74 ± 0.1b,B	10.26 ± 0.6b,C	11.82 ± 0.11b,A	2.96 ± 0.04a,D	6.36 ± 0.22b,C	6.64 ± 0.19b,B	9.88 ± 0.09b,B	11.42 ± 0.33b,A
Set 7						Set 8				
Temperature/Time in h	0	24	48	72	96	0	24	48	72	96
30°C	3.19 ± 0.07a,E	6.16 ± 0.05a,D	7.22 ± 0.1a,B	8.22 ± 0.1a,C	10.3 ± 0.07a,A	3.91 ± 0.16a,D	7.13 ± 0.03a,C	7.94 ± 0.02a,B	9.13 ± 0.03a,B	10.92 ± 0.03a,A
22°C	3.16 ± 0.06a,E	5.44 ± 0.11b,D	5.73 ± 0.06b,B	6.02 ± 0.03b,C	8.18 ± 0.06b,A	3.89 ± 0.19a,D	6.14 ± 0.04,C	6.41 ± 0.02b,B	6.64 ± 0.08b,B	8.74 ± 0.02b,A
Set 9						F-value				
Temperature/Time in h	0	24	48	72	96	Time	Temperature	Time*Temperature		
30°C	3.57 ± 0.05a,E	6.27 ± 0.04a,D	7.26 ± 0.06a,C	10.4 ± 0.06a,B	11.32 ± 0.18a,A	11.11***	0	0.57		
22°C	3.47 ± 0.15a,E	5.88 ± 0.09b,D	6.1 ± 0.03b,C	8.43 ± 0.02b,B	10.33 ± 0.27b,A					

Appendix 8: Chapter 4: Analyses of colour of the coconut water kefir fermented sourdough





Heat map for all the sets at all temp and all time.. sig for l, a and b

	Time(F.val)	Time(raw.p)	Time(adj.p)	Temperature(F.val)	Temperature(raw.p)	Temperature(adj.p)	Interaction(F.val)	Interaction(raw.p)	Interaction(adj.p)
l	156.04	1.41E-79	4.22E-79	17.689	4.43E-08	6.64E-08	6.9702	1.34E-08	4.02E-08
b	84.043	2.07E-51	3.10E-51	21.962	9.16E-10	2.75E-09	3.7093	0.000341	0.000341
a	2.6144	0.034981	0.034981	3.3514	0.036047	0.036047	5.3597	2.09E-06	3.14E-06

Colour analysis of the coconut water kefir fermented sourdough. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$).

	l*					a*					b*				
	Set 1					Set 1					Set 1				
Temperature	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96
4	37.8 ± 0.2a,A	38.2 ± 0a,A	39.9 ± 0.5a,A	40.3 ± 0b,B	38 ± 0.6a,A	2.2 ± 0a,A	2.2 ± 0.1a,A	2.3 ± 0a,A	1.9 ± 0a,A	2.3 ± 0b,A	14.8 ± 0.3a,A	14.6 ± 0.1a,A	14.8 ± 6.9a,A	14.6 ± 0a,A	14.9 ± 0a,A
22	38.5 ± 0a,A	38.6 ± 0.1a,A	41.2 ± 0.3b,B	39.8 ± 0.3a,A	41.8 ± 0.5c,B	2.3 ± 0a,A	2.3 ± 0a,A	2.3 ± 0a,A	2.7 ± 0.1b,A	2.7 ± 0.4c,A	15.3 ± 0b,A	15.6 ± 0b,A	16.3 ± 0.1b,B	16.8 ± 0.1b,B	17.1 ± 0.1c,B
30	38.7 ± 0.1a,A	39.7 ± 0.1b,A	39.7 ± 0a,A	39.9 ± 0a,A	40.3 ± 0.2b,A	2.3 ± 0a,A	2.5 ± 0b,A	2.8 ± 0b,B	2.9 ± 0b,B	2 ± 0.1a,A	15.1 ± 0.2b,A	15.5 ± 0b,A	15.9 ± 0b,A	16 ± 0b,A	15.8 ± 0.5b,A
	l*					a*					b*				
	Set 2					Set 2					Set 2				
Temperature	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96
4	38.4 ± 0a,A	38.9 ± 0a,A	39.2 ± 0a,A	40.1 ± 0a,A	39.7 ± 0.2a,A	2.5 ± 0a,B	2.2 ± 0a,A	2.2 ± 0a,A	1.7 ± 0a,A	2.3 ± 0b,A	14.2 ± 0a,A	15.3 ± 0a,A	15.8 ± 0a,A	14.4 ± 0a,AA	15 ± 0a,
22	39.6 ± 0b,A	39.3 ± 0.1a,b,A	41.1 ± 0.1c,B	43 ± 0.7b,B	42.6 ± 0.1b,B	2.6 ± 0.1a,b, B	2.6 ± 0.1b,B	2.7 ± 0.1b,B	2 ± 0.1b,A	2.2 ± 0.1b,A	14.8 ± 0.1b,A	15.3 ± 0.1a,A	15.4 ± 0a,A	16.2 ± 0.1c,B	16.3 ± 0b,B
30	39.5 ± 0.1b,A	40.1 ± 0b,A	40.2 ± 0b,A	40.3 ± 0a,A	41.1 ± 0.7b,B	2.7 ± 0b,B	2.9 ± 0c,B	3.1 ± 0c,B	2 ± 0.1b,A	2 ± 0.1a,A	14.9 ± 0b,A	15 ± 0a,A	15.1 ± 0a,A	15.1 ± 0b,A	15.2 ± 0.1a,A
	l*					a*					b*				
	Set 3					Set 3					Set 3				
Temperature	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96
4	39.5 ± 0b,A	41.3 ± 0a,B	40.1 ± 0a,A	41.4 ± 0.1a,B	40.2 ± 0.1a,A	2.4 ± 0b,B	2.2 ± 0.1b,A	1.8 ± 0a,A	2.1 ± 0b,A	2.6 ± 0.1b,A	14.5 ± 0a,A	15.9 ± 0b,B	15.9 ± 0b,B	15.6 ± 0.1b,B	15.2 ± 0a,B
22	38.5 ± 0.4a,A	41.6 ± 0.4a,B	41.4 ± 0.1a,B	43.6 ± 0b,B	43 ± 0.1b,B	2.1 ± 0a,A	2.2 ± 0.1b,A	2.8 ± 0.2c,B	1.9 ± 0a,	2 ± 0.1a,A	15 ± 0.3b,A	14.6 ± 0a,A	15.8 ± 0.1b,B	15.9 ± 0.1b,B	16.1 ± 0b,B
30	38.2 ± 0.1a,A	41.1 ± 0a,B	41.3 ± 0.1a,B	41.6 ± 0a,B	41.6 ± 0.4a,B	2.2 ± 0.1a,A	1.8 ± 0a,A	2 ± 0b,A	2.1 ± 0.1b,A	2.1 ± 0.1a,A	14.4 ± 0a,A	14.6 ± 0a,A	14.7 ± 0a,A	14.9 ± 0a,A	15 ± 0.1b,A
	l*					a*					b*				
	Set 4					Set 4					Set 4				

Temperature	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96
4	39.9 ± 0b,A	40.5 ± 0a,A	40.9 ± 0a,A	41.9 ± 0a,B	40.8 ± 0.1a,A	2.2 ± 0.2a,A	2.2 ± 0.3b,A	1.7 ± 0a,A	2.1 ± 0a,A	2.5 ± 0.1b,A	15.1 ± 0.1b,A	15.6 ± 0b,A	16.3 ± 0b,B	16 ± 0b,B	15.6 ± 0.1a,b, A
22	38.6 ± 0.2a,A	40.6 ± 0.3a,A,B	41.3 ± 0.2a,B	44.3 ± 0.2c,D	43.2 ± 0b,C	2.2 ± 0.1a,A	2.2 ± 0.1b,A	2 ± 0.1b,A	2.1 ± 0a,A	2 ± 0a,A	14.8 ± 0.1a,b, A	15.1 ± 0.4a,A	15.8 ± 0a,b,B	15.9 ± 0b,B	16.1 ± 0b,B
30	38.5 ± 0.4a,A	41.9 ± 0a,B	41.3 ± 0.3a,B	42.2 ± 0b,B	41.2 ± 0.2a,B	2.2 ± 0.1a,A	1.8 ± 0a,A	1.9 ± 0a,b, A	2.1 ± 0a,A	2.2 ± 0.4a,b, A	14.3 ± 0.1a,A	14.4 ± 0a,A	14.5 ± 0a,A	14.7 ± 0a,A	15 ± 0.2a,A
	l*					a*					b*				
	Set 5					Set 5					Set 5				
Temperature	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96
4	38.5 ± 0.7a,A	41.7 ± 0b,B	41.8 ± 0.1a,B	42 ± 0a,B	41.6 ± 0.3a,B	2.2 ± 0.1a,A	2.2 ± 0.2b,A	2.1 ± 0a,A	2.2 ± 0b,A	2.5 ± 0a,A	15.2 ± 0.1b,A	16.1 ± 0b,B	16.5 ± 0.1b,B	16.4 ± 0b,B	16.4 ± 0b,B
22	38.6 ± 0.3a,A	39.2 ± 0.5a,A, B	41.4 ± 0.2a,B	44.8 ± 0.8b,C	44 ± 0.5b,C	2.2 ± 0.1a,A	2.1 ± 0.1a,A	2.1 ± 0.1a, A	2.1 ± 0.1a, A	2.1 ± 0.1a,A	14.6 ± 0.1a,A	15 ± 0.3a,A	15.9 ± 0a,b,B	16.1 ± 0b,B	16.3 ± 0b,B
30	38.6 ± 0.2a,A	40.3 ± 0.4b,A, B	41.6 ± 0a,A, B	42.6 ± 0a,B	41.2 ± 0.1a,A, B	2.2 ± 0.1a,A	1.9 ± 0a,A	2.1 ± 0a,A	2.4 ± 0c,A	2.7 ± 0.1b,A	14.6 ± 0a,A	14.8 ± 0a,A	14.9 ± 0a,A	15.1 ± 0a,B	15.5 ± 0.3a,B
	l*					a*					b*				
	Set 6					Set 6					Set 6				
Temperature	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96
4	38.7 ± 0.5a,A	41 ± 0a,B	41.6 ± 0.1a,B	41.5 ± 0a,B	41.8 ± 0.2a,B	2.3 ± 0.2a,A	2.1 ± 0.1a,A	1.9 ± 0a,A	2.3 ± 0b,A	2.1 ± 0a,A	15 ± 0.1a,	15.8 ± 0a,	16.4 ± 0b,	16.4 ± 0a,	16.6 ± 0.3b,
22	39.4 ± 0.6a,A	40.4 ± 0.4a,A, B	41.3 ± 0.2a,B	41.9 ± 0.4a,B	44.5 ± 0.1b,C	2.1 ± 0.1a,A	2.2 ± 0.1a,A	2 ± 0.1a, A	2.1 ± 0.1a, A	2 ± 0.1a,A	14.5 ± 0.1a,	15 ± 0.1a,	15.5 ± 0a,	15.8 ± 0a,	16 ± 0a,
30	38.6 ± 0.2a,A	40.6 ± 0.5a,A, B	41.3 ± 0.2a,B	43.8 ± 0.1b,C C	41.3 ± 0.2a,B	2.2 ± 0a,A	2.1 ± 0a,A	2.2 ± 0b,A	2.5 ± 0b,A	2.7 ± 0.5b,A	15.1 ± 0a,	15.2 ± 0a,	15.4 ± 0a,	15.6 ± 0a,	16.1 ± 0.2a,
	l*					a*					b*				
	Set 7					Set 7					Set 7				

Temperature	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96
4	38.6 ± 0.5a,A	40.5 ± 0.2a,A, B	41.4 ± 0.2a,B	41.2 ± 0.2a,B	41.2 ± 0.2a,B	2.1 ± 0.1a,A	2.1 ± 0a,A	2.1 ± 0.1 a,A	2 ± 0.1 a,A	2.1 ± 0 a,A	15 ± 0.2a,A	15.1 ± 0.3 a,A	16 ± 0.2a,B	16.3 ± 0.3a,B	16.4 ± 0.1a,B
22	38.9 ± 0.1a,A	40.3 ± 0.2a,A, B	41.4 ± 0.2a,B	41.3 ± 0.1a,B	41.2 ± 0.2a,B	2.2 ± 0.1a,A	2.1 ± 0.1 a,A	2.1 ± 0 a,A	2 ± 0.1 a,A	2.1 ± 0 a,A	15 ± 0.3 a,A	14.9 ± 0.4 a,A	16.4 ± 0.5a,B	16.2 ± 0.1a,B	16.4 ± 0.4a,B
30	38.3 ± 0.2a,	40.6 ± 0.2a,A, B	41.3 ± 0.1a,B	41.2 ± 0.2a,B	41.3 ± 0.1a,B	2.1 ± 0.1a,A	2.1 ± 0.1 a,A	2 ± 0 a,A	2.1 ± 0a,A	2 ± 0 a,A	15.1 ± 0a,A	15 ± 0.3a,A	16.6 ± 0.1a,B	16.3 ± 0.4a,B	16.5 ± 0.2a,B
	l*					a*					b*				
	Set 8					Set 8					Set 8				
Temperature	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96
4	38.3 ± 0.3a,A	40.6 ± 0.2a,A, B	41.4 ± 0.2a,B	41.4 ± 0.2a,B	41.2 ± 0.1a,B	2.1 ± 0.1a,A	2.2 ± 0.1a,A	2 ± 0.1a, A	2.1 ± 0.1a, A	2.1 ± 0.1a,A	14.9 ± 0.4a,A	15 ± 0.2a,A	15.9 ± 0a,B	16.1 ± 0.2a,B	16.3 ± 0.3a,B
22	38.2 ± 0.1a,A	40.5 ± 0.3a,A, B	41.2 ± 0a,B	41.4 ± 0.1a,B	41.3 ± 0.1a,B	2.1 ± 0.1a,A	2.2 ± 0a,A	2 ± 0.1a, A	2.1 ± 0.1a, A	2 ± 0.1a,A	15.1 ± 0.2a,A	15.1 ± 0.4a,A	16.3 ± 0.1a,B	16.3 ± 0.3a,B	16.5 ± 0.2a,B
30	38.3 ± 0.2a,A	40.4 ± 0.3a,A, B	41.2 ± 0a,B	41.4 ± 0.1a,B	41.3 ± 0.3a,B	2.1 ± 0.1a,A	2.1 ± 0.1a,A	2 ± 0.1a, A	2 ± 0a,A	2 ± 0.1a,A	14.7 ± 0.2a,A	15 ± 0.3a,A	16.3 ± 0.2a,B	16.2 ± 0.3a,B	16.2 ± 0.3a,B
	l*					a*					b*				
	Set 9					Set 9					Set 9				
Temperature	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96
4	38.9 ± 0.1a,A	40.5 ± 0.3a,A, B	41.3 ± 0.1a,B	41.5 ± 0.1a,B	41.3 ± 0.1a,B	2.1 ± 0a,A	2.1 ± 0a,A	2 ± 0.1a, A	2 ± 0a,A	2.1 ± 0.1a,A	14.8 ± 0.2a,A	14.8 ± 0.3a,A	16.3 ± 0.3a,B	16.2 ± 0.4a,B	16.3 ± 0.3a,B
22	38.4 ± 0.1a,A	40.7 ± 0.1a,A, B	41.3 ± 0.1a,B	41.4 ± 0.1a,B	40.9 ± 0.7a,A, B	2.2 ± 0a,A	2.3 ± 0b,A	2 ± 0.1a, A	2 ± 0.1a, A	2 ± 0.1a,A	15.1 ± 0.1a,A	15 ± 0.3a,A	16.3 ± 0.1a,B	16.4 ± 0.3a,B	16.1 ± 0a,B
30	38.8 ± 0.1a,A	40.6 ± 0.4a,A, B	41.1 ± 0.2a,B	41.3 ± 0.2a,B	41.3 ± 0.2a,B	2.1 ± 0a,A	2.2 ± 0.1a,b, A	2 ± 0.1a, A	2.1 ± 0.1a, A	2 ± 0.2a,A	15 ± 0.2a,A	14.9 ± 0.3a,B	16.2 ± 0.4a,C	16 ± 0.4a,D	16.4 ± 0.2a,E

Appendix 9: Chapter 4: Analyses of Texture and Viscosity (CVA score plot and loadings plot)

Canonical Variate Analysis bi-plot of texture and viscosity for CWK fermented sourdough at times 0, 24, 48, 96 and 96 h incubated at 22°C and 30°C. Hotelling-Lawley MANOVA test showed significant differences among the sets, based on the time and temperature of fermentation. This bi-plot represents the centroid variables of each environment which are correlated with the two factors on the factor axes.

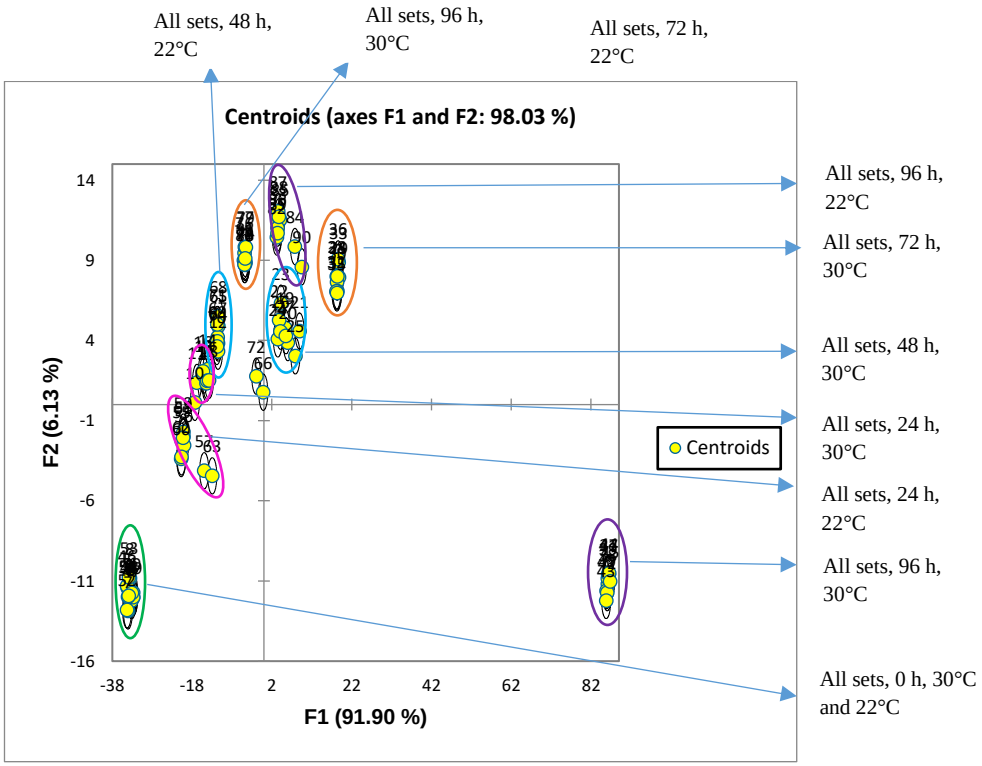


Figure :

Hotelling-Lawley trace:	
Trace	1764.358
F (Observed value)	488.026
F (Critical value)	1.123
DF1	623
DF2	1066
p-value	< 0.0001
alpha	0.05

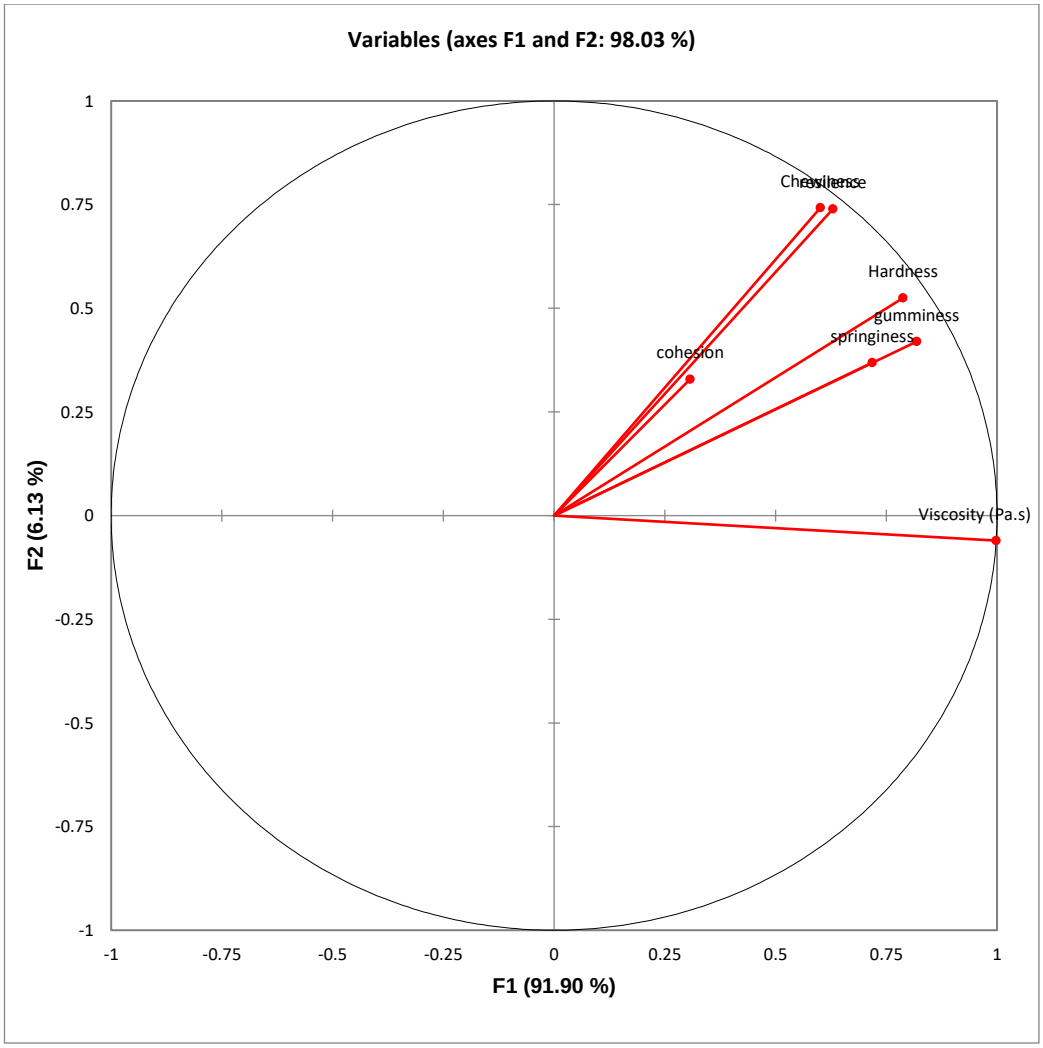


Figure: Canonical Variate Analysis bi-plot for texture and viscosity for the CWK fermented sourdough at 22°C and 30°C. Hotelling-Lawley MANOVA test showed significant differences ($F(63,343) = 10.682$, $p < 0.001$). This loadings plot shows all the above variables which are correlated with the two factors on the factor axes

Viscosity analysis of the coconut water kefir fermented sourdough. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$).

Viscosity										
	Set 1					**Set 2**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	612.07 ± 2.57E,a	681.6 ± 11.82D,a	781.53 ± 2.71C,a	844.67 ± 2.24B,a	1215.7 ± 2.1A,a	608.13 ± 3.54E,a	679.93 ± 5.73D,a	786.53 ± 10.75C,a	846.63 ± 4.28B,a	1215.4 ± 2.78A,a
22°C	611.87 ± 2.13E,a	661.89 ± 1.82D,b	702.02 ± 0.56C,b	732.07 ± 0.99B,b	770.61 ± 1.59A,b	610.34 ± 2.13E,a	664.32 ± 0.31D,b	701.95 ± 1.55C,b	731.95 ± 1B,b	771.13 ± 1.58A,b
	Set 3					**Set 4**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
,	606.53 ± 1.46E,a	685.17 ± 4.67D,a	800.07 ± 14.26C,a	844.93 ± 0.42B,a	1214.97 ± 4.27A,a	612.3 ± 2.41E,a	677.83 ± 4.31D,a	773.9 ± 15.27C,a	845.67 ± 1.8B,a	1214.07 ± 2.49A,a
,	607.5 ± 5.64E,a	691.94 ± 1.15D,b	763.83 ± 4.9C,b	731.06 ± 2.3B,b	794.15 ± 5.01A,b	612.15 ± 2.47E,a	662.61 ± 1.44D,b	701.07 ± 2.5C,b	730.82 ± 1.57B,b	773.39 ± 0.52A,b
	Set 5					**Set 6**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
,	610.3 ± 3.27E,a	682.4 ± 6.05D,a	775.47 ± 24.64C,a	844.63 ± 1.72B,a	1214.33 ± 4.09A,a	608.97 ± 5.75E,a	687.37 ± 5.58D,a	772.8 ± 3.29C,a	846.2 ± 1.47B,a	1214.43 ± 3.46A,a
,	609.97 ± 6.48E,a	662.33 ± 1.25D,b	701.11 ± 1.14C,b	730.52 ± 1.31B,b	770.67 ± 1.72A,b	605.69 ± 5.58E,a	661.38 ± 1.32D,b	702.64 ± 1.21C,b	731.9 ± 1.52B,b	770.43 ± 0.76A,b
	Set 7					**Set 8**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
,	606.67 ± 6.82E,a	682.07 ± 9.54D,a	797.07 ± 3.25C,a	845.6 ± 4.91B,a	1213.7 ± 5.05A,a	606.5 ± 3.24E,a	687.9 ± 2.86D,a	775.93 ± 8.56C,a	845.6 ± 2.17B,a	1215.5 ± 3.52A,a
,	605.36 ± 4.68E,a	662.64 ± 1.74D,b	701.46 ± 1.88C,b	731.93 ± 1.39B,b	771.36 ± 1.33A,b	605.52 ± 0.99E,a	661.67 ± 2.68D,b	701.25 ± 1.75C,b	731.26 ± 1.18B,b	770.59 ± 1.45A,b
	Set 9					**F Value**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**Time**	**Temperature**	**Time*Temprature**		
,	609.9 ± 5.91E,a	711.57 ± 4.6D,a	783.7 ± 15.11C,a	845.6 ± 4.52B,a	1217.73 ± 1.16A,a					
,	605.98 ± 4.58E,a	703.21 ± 0.75D,b	754.39 ± 4.83C,b	732.59 ± 0.58B,b	804.4 ± 4.4A,b	41097.03***	38203.20***	15264.26***		

Hardness analysis of the coconut water kefir fermented sourdough. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$).

Hardness										
Set 1						**Set 2**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	12.26 ± 0.55C,a	12.19 ± 0.13C,a	18.1 ± 0.16B,a	20.34 ± 0.24A,a	20.3 ± 0.38A,a	12.26 ± 0.55D,a	15.82 ± 0.19C,a	17.95 ± 0.19B,a	20.21 ± 0.28A,a	20.78 ± 0.34A,a
22°C	12.26 ± 0.55E,a	13.5 ± 0.46D,a	14.98 ± 0.36C,b	16.9 ± 0.45B,b	19.36 ± 0.13A,b	11.52 ± 0.18C,a	13.89 ± 0.33B,b	14.75 ± 0.26B,b	17.1 ± 0.3A,b	18.81 ± 0.14A,b
Set 3						**Set 4**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
,	11.52 ± 0.18D,a	15.72 ± 0.16C,a	18.01 ± 0.06B,a	20.1 ± 0.15A,a	20.15 ± 0.66A,a	12.11 ± 0.2C,a	13.18 ± 0.22C,a	17.81 ± 0.37B,a	20.1 ± 0.52A,a	20.28 ± 0.2A,a
,	12.11 ± 0.2D,a	14.03 ± 0.55C,a	15.19 ± 0.27B,C,b	16.71 ± 0.25B,b	18.96 ± 0.4A,b	11.93 ± 0.15E,a	13.15 ± 0.33D,a	15.51 ± 0.44C,b	17.13 ± 0.6B,b	19.32 ± 0.4A,a
Set 5						**Set 6**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
,	12.93 ± 0.15C,a	13.32 ± 0.1C,a	18.15 ± 0.4B,a	20.17 ± 0.44A,a	20.21 ± 0.34A,a	12.36 ± 0.62D,a	15.85 ± 0.24C,a	18.17 ± 0.23B,a	19.96 ± 0.21A,a	20.85 ± 0.28A,a
,	12.36 ± 0.62D,a	13.32 ± 0.39D,a	15.3 ± 0.39C,b	17.2 ± 0.23B,b	19.25 ± 0.3A,a	12.02 ± 0.52D,a	13.68 ± 0.09C,D,b	14.43 ± 0.15C,b	16.86 ± 0.2B,b	18.59 ± 0.37A,b
Set 7						**Set 8**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
,	12.02 ± 0.52D,a	15.64 ± 0.14C,a	17.8 ± 0.32B,a	19.87 ± 0.58A,a	20.17 ± 0.31A,a	12.13 ± 0.14D,a	15.89 ± 0.23C,a	17.78 ± 0.45B,a	20.04 ± 0.19A,a	20.11 ± 0.05A,a
,	12.13 ± 0.14D,a	13.92 ± 0.42D,b	15.11 ± 0.38C,b	17.19 ± 0.32B,b	19.56 ± 0.53A,a	12.43 ± 0.28C,a	14.02 ± 0.26B,a	14.65 ± 0.32B,b	17.36 ± 0.46A,b	18.94 ± 0.08A,b
Set 9						**F Value**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**Time**	**Temperature**	**Time*Temprature**		
,	12.43 ± 0.28D,a	15.68 ± 0.12C,a	18.46 ± 0.09B,a	20.2 ± 0.39A,a	20.22 ± 0.26A,a	1000.62***	339.65***	53.02***		
,	11.94 ± 0.17D,a	14.43 ± 0.38C,a	14.58 ± 0.3C,b	16.98 ± 0.4B,b	18.73 ± 0.53A,b					

Resilience analysis of the coconut water kefir fermented sourdough. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$).

Resilience										
Set 1						**Set 2**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	6.27 ± 2.57a,E	9.84 ± 11.82a,D	10.86 ± C2.71a,	11.62 ± 2.24a,B	12.01 ± 2.1a,A	6.44 ± 3.54a,D	9.78 ± 5.73a,C	10.55 ± 10.75b,B	12.16 ± 4.28a,A	11.66 ± 2.78b,A
22°C	6.27 ± 2.13a,E	9.99 ± 1.82a,D	10.96 ± 0.56a,C	11.93 ± 0.99a,B	12.5 ± 1.59a,A	6.44 ± 2.13a,D	9.94 ± 0.31a,C	11.18 ± 1.55a,B	12.06 ± 1a,A	12.62 ± 1.58a,A
Set 3						**Set 4**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	6.13 ± 1.46a,D	9.85 ± 4.67a,C	11.44 ± 14.26a,B	12.32 ± 0.42a,A	12.11 ± 4.27a,A	6.21 ± 2.41a,D	9.8 ± 4.31b,C	10.93 ± 15.27a,B	11.87 ± 1.8a,A	12.04 ± 2.49b,A
22°C	6.13 ± 5.64a,E	9.98 ± 1.15a,D	10.91 ± 4.9a,C	11.58 ± 2.3a,B	13 ± 5.01a,A	6.21 ± 2.47a,D	10.18 ± 1.44a,C	10.99 ± 2.5a,B	12.07 ± 1.57a,A	13.01 ± 0.52a,A
Set 5						**Set 6**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	6.44 ± 3.27a,D	9.9 ± 6.05a,C	10.96 ± 24.64a,B	11.89 ± 1.72a,A	12.04 ± 4.09a,A	6.01 ± 5.75a,E	9.7 ± 5.58a,D	10.92 ± 3.29a,C	11.91 ± 1.47a,B	12.08 ± 3.46b,A
22°C	6.44 ± 6.48a,D	10.08 ± 1.25a,C	11.17 ± 1.14a,B	12.3 ± 1.31a,A	12.77 ± 1.72a,A	6.01 ± 5.58a,E	9.55 ± 1.32a,D	10.5 ± 1.21a,C	11.72 ± 1.52a,B	13.28 ± 0.76a,A
Set 7						**Set 8**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	5.74 ± 6.82a,D	9.83 ± 9.54a,C	10.72 ± 3.25a,B	12.1 ± 4.91a,A	11.73 ± 5.05a,A	6.66 ± 3.24a,E	9.71 ± 2.86a,D	10.86 ± 8.56a,C	11.85 ± 2.17a,B	12.34 ± A3.52a,
22°C	5.74 ± 4.68a,D	9.98 ± 1.74a,C	10.62 ± 1.88a,C	12.05 ± 1.39a,B	12.91 ± 1.33a,A	6.66 ± 0.99a,E	9.51 ± 2.68a,D	10.89 ± 1.75a,C	11.93 ± 1.18a,B	12.67 ± 1.45a,A
Set 9						**F Value**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**Time**	**Temperature**	**Time*Temprature**		
30°C	6.48 ± 5.91a,D	9.73 ± 4.6a,C	10.48 ± 15.11a,B	12.15 ± 4.52a,A	11.96 ± 1.16b,A	2489.070	21.010	16.380		
22°C	6.48 ± 4.58a,E	9.82 ± 0.75a,D	10.92 ± 4.83a,C	11.87 ± 0.58a,B	12.87 ± 4.4a,A					

Cohesiveness analysis of the coconut water kefir fermented sourdough. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$).

Cohesiveness										
	Set 1					**Set 2**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	0.26 ± 0.04a,C	0.23 ± 0.03b,C	0.61 ± 0.08a,A	0.42 ± 0.15a,B	0.45 ± 0.19a,B	0.26 ± 0.04b,C	0.56 ± 0.01a,B	0.55 ± 0.23a,B	0.67 ± 0.09a,A	0.58 ± 0.15a,B
22°C	0.26 ± 0.04a,B	0.43 ± 0.05a,A	0.49 ± 0.19b,A	0.4 ± 0.16a,A	0.47 ± 0.1a,A	0.35 ± 0.03a,B	0.51 ± 0.14a,A	0.47 ± 0.23a,A	0.54 ± 0.24b,A	0.55 ± 0.09a,A
	Set 3					**Set 4**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	0.35 ± 0.03a,C	0.58 ± 0.1a,A	0.59 ± 0.05a,A	0.44 ± 0.14b,B	0.45 ± 0.21a,B	0.38 ± 0.04a,C	0.41 ± 0.02b,B	0.57 ± 0.06a,A	0.45 ± 0.15a,B	0.4 ± 0.12a,B
22°C	0.38 ± 0.04a,C	0.41 ± 0.13b,B	0.56 ± 0.11a,A	0.5 ± 0.13a,A	0.41 ± 0.1a,B	0.33 ± 0.03b,C	0.53 ± 0.19a,A	0.54 ± 0.17a,A	0.51 ± 0.11a,A	0.5 ± 0.11a,A
	Set 5					**Set 6**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	0.33 ± 0.03a,E	0.61 ± 0.1a,B	0.51 ± 0.09a,C	0.54 ± 0.14a,C	0.71 ± 0.01a,A	0.24 ± 0.02a,C	0.61 ± 0.14a,A	0.54 ± 0.16b,B	0.6 ± 0.13a,A	0.55 ± 0.16a,B
22°C	0.24 ± 0.02b,D	0.58 ± 0.26b,A	0.56 ± 0.17a,A	0.38 ± 0.08a,C	0.45 ± 0.11b,B	0.28 ± 0.02a,D	0.62 ± 0.08a,A	0.61 ± 0.02a,A	0.56 ± 0.21a,B	0.43 ± 0.24b,C
	Set 7					**Set 8**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	0.28 ± 0.02a,C	0.43 ± 0.18a,B	0.42 ± 0.03a,B	0.4 ± 0.25a,B	0.51 ± 0.12a,A	0.24 ± 0.03a,	0.52 ± 0.18a,	0.46 ± 0.09a,	0.44 ± 0.21a,	0.53 ± 0.2b,
22°C	0.24 ± 0.03a,B	0.52 ± 0.24a,A	0.5 ± 0.07a,A	0.5 ± 0.21a,A	0.54 ± 0.03a,A	0.23 ± 0.02a,D	0.54 ± 0.04a,B	0.45 ± 0.15a,C	0.45 ± 0.18a,C	0.66 ± 0.1a,A
	Set 9					**F Value**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**Time**	**Temperature**	**Time*Temprature**		
30°C	0.23 ± 0.02a,B	0.5 ± 0.17a,A	0.53 ± 0.1a,A	0.48 ± 0.21a,A	0.59 ± 0.26a,A	31.12***	1.98*	0.440		
22°C	0.23 ± 0.03a,B	0.33 ± 0.09b,A	0.35 ± 0.06b,A	0.35 ± 0.07b,A	0.37 ± 0.11b,A					

Gumminess analysis of the coconut water kefir fermented sourdough. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$).

Gumminess										
Set 1						**Set 2**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	3.54 ± 0.1a,D	5.76 ± 0.11a,C	7.14 ± 0.36a,B	7.85 ± 0.23a,A	7.78 ± 0.25a,A	3.34 ± 0.09a,D	5.74 ± 0.06a,C	6.88 ± 0.46a,B	7.33 ± 0.34a,A	7.85 ± 0.1a,
22°C	3.54 ± 0.1a,C	4.37 ± 0.02b,B	4.96 ± 0.05b,B	5.42 ± 0.05b,A	5.72 ± 0.05b,A	3.34 ± 0.09a,AC	4.39 ± 0.07b,B	5 ± 0.07b,A	5.37 ± 0.03b,A	5.68 ± 0.06b,A
Set 3						**Set 4**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	3.56 ± 0.06a,D	5.72 ± 0.08a,C	6.63 ± 0.41a,B	7.29 ± 0.36a,A	7.48 ± 0.35a,A	3.56 ± 0.1a,D	5.6 ± 0.1a,C	6.96 ± 0.28a,B	7.27 ± 0.16a,A	7.37 ± 0.49a,A
22°C	3.56 ± 0.06a,C	4.34 ± 0.05b,B	4.97 ± 0.03b,B	5.38 ± 0.04b,A	5.7 ± 0.03b,A	3.56 ± 0.1a,C	4.37 ± 0.05b,B	4.98 ± 0.05b,B	5.38 ± 0.06b,A	5.78 ± 0.01b,A
Set 5						**Set 6**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	3.35 ± 0.07a,C	5.83 ± 0.25a,B	7.11 ± 0.27a,A	7.33 ± 0.55a,A	7.61 ± 0.07a,A	3.5 ± 0.01a,D	5.71 ± 0.19a,C	6.62 ± 0.2a,B	7.81 ± 0.36a,A	7.29 ± 0.27a,A
22°C	3.35 ± 0.07a,C	4.4 ± 0.1b,B	5 ± 0.04b,A	5.37 ± 0.07b,A	5.73 ± 0.04b,A	3.5 ± 0.01a,D	4.43 ± 0.07b,C	4.97 ± 0.04b,B	5.45 ± 0.05b,A	5.73 ± 0.11b,A
Set 7						**Set 8**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	3.4 ± 0.15a,D	5.79 ± 0.18a,C	6.51 ± 0.1a,B	7.57 ± 0.41a,A	7.53 ± 0.45a,A	3.41 ± 0.13a,C	5.64 ± 0.12a,B	6.76 ± 0.25a,B	7.25 ± 0.23a,A	7.71 ± 0.34a,A
22°C	3.4 ± 0.15a,C	4.46 ± 0.02b,B	5.03 ± 0.05b,A	5.36 ± 0.07b,A	5.75 ± 0.02b,A	3.41 ± 0.13a,C	4.45 ± 0.03b,B	5 ± 0.09b,A	5.41 ± 0.07b,A	5.76 ± 0.05b,A
Set 9						**F Value**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**Time**	**Temperature**	**Time*Temprature**		
30°C	3.32 ± 0.08a,D	5.82 ± 0.1a,C	6.78 ± 0.45a,B	7.58 ± 0.12a,A	7.58 ± 0.45a,A	164.72***	339.79***	22.12***		
22°C	3.32 ± 0.08a,C	4.46 ± 0.06b,B	5.02 ± 0.06b,A	5.41 ± 0.03b,A	5.69 ± 0.04b,A					

Springiness analysis of the coconut water kefir fermented sourdough. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$).

Springiness										
	Set 1					**Set 2**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	28.24 ± 0.04a,B	29.81 ± 4.98a,B	41.08 ± 0.48a,A	42.73 ± 0.47a,A	42.61 ± 0.56a,A	28.27 ± 0.04a,C	29.87 ± 5.46a,C	40.98 ± 0.31a,B	42.81 ± 0.27a,A	42.59 ± 0.51a,A
22°C	28.24 ± 0.04a,B	29.08 ± 0.05a,B	33.91 ± 0.16b,A	34.41 ± 0.39b,A	35.3 ± 0.26b,A	28.27 ± 0.04a,C	31.57 ± 0.25a,B	33.59 ± 0.29b,A	34.73 ± 0.07b,A	35.57 ± 0.28b,A
	Set 3					**Set 4**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	28.26 ± 0.02a,C	33.22 ± 7.14a,B	41.17 ± 0.26a,A	42.45 ± 0.3a,A	42.79 ± 0.14a,A	28.26 ± 0.02a,B	42.9 ± 2.23a,A	42.82 ± 0.45a,A	42.83 ± 0.2a,A	42.95 ± 0.08a,A
22°C	28.26 ± 0.02a,C	31.8 ± 0.34a,B	33.63 ± 0.45b,A	34.65 ± 0.36b,A	35.65 ± 0.51b,A	28.26 ± 0.02b,C	31.73 ± 0.39b,B	33.83 ± 0.57b,A	34.43 ± 0.26b,A	35.53 ± 0.26b,A
	Set 5					**Set 6**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	28.24 ± 0.03a,C	42.99 ± 4.25a,A	40.74 ± 0.31a,B	42.37 ± 0.58a,A	42.81 ± 0.3a,A	28.25 ± 0.05a,D	35.84 ± 0.18a,C	40.62 ± 0.17a,B	42.64 ± 0.31a,A	42.6 ± 0.48a,A
22°C	28.24 ± 0.03a,B	31.69 ± 0.4b,A	33.67 ± 0.47b,A	34.66 ± 0.47b,A	35.64 ± 0.4b,A	28.25 ± 0.05a,B	31.88 ± 0.1a,A	33.26 ± 0.23b,A	34.82 ± 0.26b,A	35.43 ± 0.24b,A
	Set 7					**Set 8**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	28.26 ± 0.02a,D	35.75 ± 0.19a,C	40.99 ± 0.25a,B	42.42 ± 0.1a,A	42.58 ± 0.25a,A	28.23 ± 0.03a,C	35.84 ± 0.2a,B	41.22 ± 0.09a,A	42.55 ± 0.5a,A	42.88 ± 0.13a,A
22°C	28.26 ± 0.02a,C	31.95 ± 0.1b,B	33.39 ± 0.23b,A	34.71 ± 0.42b,A	35.8 ± 0.33b,A	28.23 ± 0.03a,B	31.58 ± 0.34a,A	33.57 ± 0.28b,A	34.7 ± 0.26b,A	35.5 ± 0.26b,A
	Set 9					**F Value**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**Time**	**Temperature**	**Time*Temprature**		
30°C	28.26 ± 0.02a,D	35.75 ± 0.02a,C	40.85 ± 0.52a,B	42.28 ± 0.35a,A	42.83 ± 0.23a,A					
22°C	28.26 ± 0.02a,C	31.55 ± 0.34a,B	33.77 ± 0.14b,A	34.47 ± 0.26b,A	35.58 ± 0.45b,A	2175.32***	3218.46***	225.45***		

Chewiness analysis of the coconut water kefir fermented sourdough. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$).

Chewiness										
Set 1						**Set 2**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	1.47 ± 0.14a,E	3.69 ± 0.1a,D	4.61 ± 0.1b,C	5.93 ± 0.25b,B	6.04 ± 0.22b,A	1.38 ± 0.15a,D	3.63 ± 0.15a,C	4.84 ± 0.22b,B	6.07 ± 0.55a,A	6.03 ± 0.14b,A
22°C	1.47 ± 0.14a,E	3.03 ± 0.21a,D	5.19 ± 0.37a,C	6.76 ± 0.27a,B	7.25 ± 0.34a,A	1.38 ± 0.15a,D	2.9 ± 0.34b,C	5.54 ± 0.34a,B	6.64 ± 0.39a,A	7.7 ± 0.19a,A
Set 3						**Set 4**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	1.34 ± 0.16a,E	3.68 ± 0.15a,D	5.05 ± 0.44a,C	5.84 ± 0.42b,B	6.23 ± 0.24b,A	1.42 ± 0.14a,D	3.79 ± 0.12a,C	4.83 ± 0.25b,B	5.96 ± 0.5b,A	6.31 ± 0.22b,A
22°C	1.34 ± 0.16a,D	2.83 ± 0.32b,C	5.27 ± 0.46a,B	7.12 ± 0.3a,A	7.22 ± 0.43a,A	1.42 ± 0.14a,E	2.9 ± 0.42b,D	5.22 ± 0.31a,C	6.89 ± 0.4a,B	7.36 ± 0.35a,A
Set 5						**Set 6**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	1.46 ± 0.21a,E	3.74 ± 0.18a,D	5.14 ± 0.32a,C	5.84 ± 0.46b,B	6.17 ± 0.2b,A	1.48 ± 0.04a,D	3.64 ± 0.14a,C	4.57 ± 0.31b,B	6.25 ± 0.19a,A	6.29 ± 0.21b,A
22°C	1.46 ± 0.21a,E	3.05 ± 0.42a,D	5.7 ± 0.58a,C	6.87 ± 0.12a,B	7.29 ± 0.47a,A	1.48 ± 0.04a,E	2.72 ± 0.33b,D	5.73 ± 0.32a,C	6.8 ± 0.4a,B	7.5 ± 0.39a,A
Set 7						**Set 8**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
30°C	1.38 ± 0.2a,D	3.71 ± 0.23a,C	4.99 ± 0.38b,B	5.95 ± 0.39b,A	5.72 ± 0.18b,A	1.57 ± 0.05a,E	3.78 ± 0.16a,D	4.81 ± 0.52b,C	5.95 ± 0.38b,B	5.9 ± 0.13b,A
22°C	1.38 ± 0.2a,D	2.97 ± 0.5b,C	5.27 ± 0.26a,B	7.1 ± 0.34a,A	7.46 ± 0.21a,A	1.57 ± 0.05a,E	2.75 ± 0.33b,D	5.81 ± 0.13a,C	6.58 ± 0.45a,B	7.24 ± 0.12a,A
Set 9						**F Value**				
Temperature/Time in hours	**0**	**24**	**48**	**72**	**96**	**Time**	**Temperature**	**Time*Temprature**		
30°C	1.34 ± 0.1a,D	3.69 ± 0.04a,C	5.05 ± 0.46a,B	6.36 ± 0.05a,A	6.16 ± 0.28a,A	3120.36***	113.89***	103.01***		
22°C	1.34 ± 0.1a,	2.91 ± 0.41a,CD	5.55 ± 0.3a,B	6.92 ± 0.49a,A	7.03 ± 0.27a,A					

Appendix 10: Chapter 4: Effect of time on all carboxylic acids

	f.value	p.value	#NAME?	FDR	Fisher's LSD
Glutamic acid mgL	2682.5	2.33E-185	184.63	1.17E-184	24 - 0; 48 - 0; 72 - 0; 96 - 0; 48 - 24; 72 - 24; 96 - 24; 72 - 48; 96 - 48; 96 - 72
lactic mgL	2545.3	6.66E-183	182.18	1.66E-182	24 - 0; 48 - 0; 72 - 0; 96 - 0; 48 - 24; 72 - 24; 96 - 24; 72 - 48; 96 - 48; 96 - 72
pyruvic mgL	1061.2	1.63E-142	141.79	2.71E-142	24 - 0; 48 - 0; 72 - 0; 96 - 0; 48 - 24; 72 - 24; 96 - 24; 72 - 48; 96 - 48; 96 - 72
succinic mgL	253.17	4.30E-81	80.367	5.38E-81	24 - 0; 48 - 0; 72 - 0; 96 - 0; 48 - 24; 72 - 24; 96 - 24; 72 - 48; 96 - 48; 96 - 72
acetic mgL	178.69	6.59E-68	67.181	6.59E-68	24 - 0; 48 - 0; 72 - 0; 96 - 0; 48 - 24; 72 - 24; 96 - 24; 72 - 48; 96 - 48; 96 - 72

Appendix 11: Chapter 4: Carboxylic acid data

Lactic acid analysis of the coconut water kefir fermented sourdough. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$).

Experimental sourdough sets	Lactic acid concentration (mg/L)				
	0h	24h	48h	72h	96h
Set 1	623.26 ± 12.04b,E	699.53 ± 8.75c,D	745.4 ± 9.62e,C	784.76 ± 6.29c,B	818.9 ± 8.9c,A
Set 2	627.09 ± 6.43a,E	698.82 ± 7.94c,D	768.59 ± 6.22b,C	795.53 ± 6.39a,B	820.9 ± 3.4b,A
Set 3	623.31 ± 5.55b,E	704.35 ± 3.03a,D	772.3 ± 5.76a,C	789.38 ± 4.33b,B	828.8 ± 4.8a,A
Set 4	608.61 ± 27.24f,E	699.22 ± 8.13c,D	751.2 ± 4.55d,C	778.6 ± 2.05e,B	812.3 ± 1.8d,A
Set 5	606.08 ± 13.73f,E	705.77 ± 5.08a,D	756.43 ± 7.54c,C	778.88 ± 1.73e,B	802.3 ± 0.8f,g,A
Set 6	627.09 ± 6.43a,E	705.41 ± 5.55a,D	753.01 ± 5.51d,C	779.05 ± 3.39e,B	802.7 ± 1.8f,A
Set 7	618.29 ± 16.7c,E	698.89 ± 6.42c,D	753.73 ± 7d,C	779.72 ± 2.7e,B	809.3 ± 1.4e,A
Set 8	613.44 ± 11.21d,E	697.98 ± 6.2c,D	755.71 ± 3.76c,C	781.62 ± 2.5d,B	801.4 ± 1.2g,A
Set 9	610.13 ± 3.19e,E	702.81 ± 5.17b,D	753.01 ± 4.33d,C	778.49 ± 3.72e,B	811.3 ± 1.8d,A

Acetic acid analysis of the coconut water kefir fermented sourdough. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$).

Experimental sourdough sets	Acetic acid concentration (mg/L)				
	0h	24h	48h	72h	96h
Set 1	26.38 ± 3.13d,E	43.69 ± 2.9b,D	47 ± 7.43b,C	55.57 ± 15.22b,B	62.5 ± 4.4d,A
Set 2	23.36 ± 2.74e,E	43.05 ± 1.38b,D	47.17 ± 0.76b,C	58.72 ± 2.02a,B	68.1 ± 0.7a,A
Set 3	24.15 ± 3.52d,E	45.11 ± 3.52a,D	54.34 ± 8.68a,C	56.65 ± 4.13b,B	62.6 ± 1.9d,A
Set 4	26.4 ± 2.07c,E	39.33 ± 1.03c,D	44.45 ± 1.24c,C	54.52 ± 1.94b,B	66.3 ± 2.2b,c,A
Set 5	28.6 ± 3.65b,E	39.7 ± 0.88c,D	44.11 ± 1.69c,C	54.23 ± 1.73b,B	66.1 ± 1.9b,c,A
Set 6	28 ± 5.2b,E	38.94 ± 0.61c,D	44.47 ± 2.05c,C	54.67 ± 1.51b,B	65.7 ± 1.8c,A
Set 7	30.48 ± 2.7a,E	39.22 ± 0.89c,D	43.5 ± 1.09c,C	54.99 ± 1.38b,B	65.6 ± 0.6c,A
Set 8	32.8 ± 1.99a,E	39.25 ± 0.76c,D	44.42 ± 1.42c,C	55.22 ± 1.83b,B	66.6 ± 1.8b,c,A
Set 9	31.2 ± 3.11a,E	39.95 ± 0.71c,D	44.55 ± 1.26c,C	55.41 ± 0.46b,B	66.5 ± 1.6b,c,A

Succinic acid analysis of the coconut water kefir fermented sourdough. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$).

Experimental sourdough sets	Succinic acid concentration (mg/L)				
	0h	24h	48h	72h	96h
Set 1	27.81 ± 2.65a,E	41.31 ± 1.77c,D	44.57 ± 0.81c,C	55.88 ± 1.24c,B	62.04 ± 1.7b,A
Set 2	26.23 ± 1.47a,E	41.95 ± 0.79c,D	49.03 ± 1.47b,C	59.5 ± 1.92b,B	69.93 ± 1.92b,A
Set 3	18.49 ± 1.62c,E	57.81 ± 1.03a,D	77.58 ± 8.82a,C	98.94 ± 7.1a,B	124.39 ± 6.89a,A
Set 4	16.64 ± 0.56c,E	44.46 ± 0.87b,D	50.08 ± 1b,C	54.89 ± 0.82c,B	58.03 ± 0.34c,A
Set 5	17.6 ± 8.96c,E	44.56 ± 1b,D	50.64 ± 0.88b,C	54.81 ± 0.9c,B	58.85 ± 0.6c,A
Set 6	27.26 ± 1.75a,E	44.02 ± 0.95b,D	49.93 ± 0.93b,C	54.5 ± 1.02c,B	58.4 ± 1.28c,A
Set 7	22.39 ± 0.14b,E	43.81 ± 0.45b,D	50.35 ± 1.19b,C	54.31 ± 1c,B	58.45 ± 1.21c,A
Set 8	23.27 ± 0.37b,E	44.4 ± 1.02b,D	50.19 ± 0.81b,C	54.35 ± 0.87cB,	57.92 ± 0.9c,A
Set 9	23.39 ± 0.91b,E	44.33 ± 0.83b,D	49.95 ± 0.88b,C	54.61 ± 0.92c,B	58.62 ± 0.74c,A

Pyruvic acid analysis of the coconut water kefir fermented sourdough. A, B, C, D, E,= Different letters within the same row (different fermentation times-0 h, 24 h, 48h, 72h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c: Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$).

Experimental sourdough sets	Pyruvic acid concentration (mg/L)				
	0h	24h	48h	72h	96h
Set 1	214.3 ± 9.1b,E	248.6 ± 3.2b,D	264.6 ± 4.5b,C	283.8 ± 4.0b,c,B	297.1 ± 5.9b,A
Set 2	209.5 ± 6.4d,E	247.5 ± 4.1b,c,D	261.3 ± 4.1c,C	281.1 ± 2.3c,B	293.7 ± 3.8c,A
Set 3	210.2 ± 8.6c,d,E	257.0 ± 2.4a,D	273.5 ± 4.8a,C	307.1 ± 2.9a,B	315.3 ± 3.9a,A
Set 4	217.4 ± 5.3a,E	245.1 ± 5.0c,D	258.8 ± 2.8d,C	282 ± 3.4c,B	292.2 ± 1.6c,A
Set 5	212.9 ± 8.0c,E	246.9 ± 4.6c,D	260.9 ± 5.2c,C	285.0 ± 3.4b,B	294.6 ± 2.1c,A
Set 6	209.5 ± 11.8d,E	248.5 ± 4.6b,D	264.6 ± 3.8b,C	280.4 ± 5.8,cB	294.6 ± 5.4c,A
Set 7	211.3 ± 6.1c,E	249.2 ± 4.9b,D	260.6 ± 4.1c,C	281.8 ± 2.5c,B	297.7 ± 3.4b,A
Set 8	209.5 ± 5.d,E	249.5 ± 4.9b,D	262.1 ± 3.9c,C	281.7 ± 4.1c,B	297.6 ± 4.4b,A
Set 9	215.4 ± 6.2b,E	248.9 ± 3.4b,D	260.2 ± 1.9c,C	277.8 ± 1.1d,B	294.8 ± 4.4c,A

Appendix 12: Chapter 5: Sanger sequencing output: 16s rDNA partial sequences for LAB and AAB

LAB Isolate 1: *Lactobacillus fermentum* strain CAU6479 16S ribosomal RNA gene

5’ACTGATTGATGGTGCTTGCACCTGATTGACGATGGATCACCAGTGAGTGGCGGAC
GGGTAGTAACACGTAGGTAACCTGCCCCGGAGCGGGGGATAACATTTGGAAACAG
ATGCTAATACCGCATAACAACAAAAGCCACATGGCTTTTGTTTGAAAGATGGCTTT
GGCTATCACTCTGGGATGGACCTGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCT
TACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGAACT
GAGACACGGTCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGC
GCAAGCCTGATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGC
TCTGTTGTTGGAGAAGAACGTGCGTGAGAGTAACTGTTCACGCAGTGACGGTATCC
AACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGC
AAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTGCTTAGGTCTG
ATGTGAAAGCCTTCGGCTTAACCGAAGAAGTGCATCGGAAACCGGGCGACTTGAGT
GCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGA
AGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGCAACTGACGCTGAGGCTCGAA
AGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAG
TGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGGAGCTAACGCATTAAGCACT
CCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGT
CTTGACATCTTGCGCAACCTTAGAGATAAGGCGTTCCCTTCGGGGACGCAATGACA
GGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
CGAGCGCAACCCTTGTTACTAGTTGCCAGCATTAAGTTGGGCACTCTAGTGAGACT
GCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAGATCATCATGCCCCTTAT
GACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGTCGCAAGCTCGCGA
GAGTAAGCTAATCTCTTAAAGCCGTTCTCAGTTCGGACTGTAGGCTGCAACTCGCCT
ACACGAAGTCGGAATCGCTAGAATCGCGGATCAGCATGCCGCGGTGAATACGTTCC

CGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTAACGCCCAAAGTCGG
TGGCCTAACCATTAGGAGGGAGCC 3’

LAB Isolate 2: *Lactobacillus plantarum* strain RS66X 16S ribosomal RNA gene, partial sequence

5’TTACCCCACCGACTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTA
CAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCC
GACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATGGCTTTAAGAGA
TTAGCTTACTCTCGCGAGTTCGCAACTCGTTGTACCATCCATTGTAGCACGTGTGTA
GCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGT
CACCGGCAGTCTCACCAGAGTGCCCAACTTAATGCTGGCAACTGATAATAAGGGTT
GCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATG
CACCACCTGTATCCATGTCCCCGAAGGGAACGTCTAATCTCTTAGATTTGCATAGTA
TGTCAAGACCTGGTAAGGTTCTTCGCGTAGCTTCGAATTAAACCACATGCTCCACCG
CTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAG
GCGGAATGCTTAATGCGTTAGCTGCAGCACTGAAGGGCGGAAACCCTCCAACACTT
AGCATTCATCGTTTACGGTATGGACTACCAGGGTATCTAATCCTGTTTGCTACCCAT
ACTTTCGAGCCTCAGCGTCAGTTACAGACCAGACAGCCGCCTTCGCCACTGGTGTTC
TTCCATATATCTACGCATTTCACCGCTACACATGGAGTTCCACTGTCCTCTTCTGCA
CTCAAGTTTCCCAGTTTCCGATGCACTTCTTCGGTTGAGCCGAAGGCTTTCACATCA
GACTTAAAAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGC
CACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAAAT
ACCGTCAATACCTGAACAGTTACTCTCAGATATGTTCTTCTTTAACAACAGAGTTTT
ACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAGACTTTCGTCCATT
GTGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAA
TGTGGCCGATTACCCTCTCAGGTCGGCTACGTATCATTGCCATGGTGAGCCGTTACC
CCACCATCTAGCTAATACGCCGCGGGACCATCCAAAAGTGATAGCCGAAGCCATCT
TTCAAACTCGGACCATGCGGTCCAAGTTGTTATGCGGTATTAGCATCTGTTTCCAGG
TGTTATCCCCCGCTTCTGGGCAGGTTTCCCACGTGTTACTCACCAGTTCGCCACTCA
CTCAAATGTAAATCATGATGCAAGCACCAATCAATACCAGA 3’

LAB Isolate 3: *Lactobacillus Fusant* XU1 16S ribosomal RNA gene, partial sequence

5’ATGCAGTCGAACGAGTTCTCGTTGATTGCATCGGTGCTTGCACCGAGATTCAACA
TGGAACGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCTTAAGTGGGG
GATAACATTTGGAAACAGATGCTAATACCGCATAGATCCAAGAACCGCATGGTTCT
TGGCTGAAAGATGGCGTAAGCTATCGCTTTTGGATGGACCCGCGGCGTATTGCTAG
TTGGTGAGGTAATGGCTCACCAAGGCGATGATACGTAGCCGAACTGAGAGGTTGAT
CGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGG
AATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG
CTTTCGGGTCGTAAAACTCTGTTGTTGGAGAAGAATGGTCGGCAGAGTAACTGTTG
TCGGCGTGACGGTATCCAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAAACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGC
GGTTTTTTAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAAGCGCATCGGAAA
CTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAAT
GCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGA
CGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATG

CCGTAAACGATGAATGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGCAGCTA
ACGCATTAAGCATTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA
GAACCTTACCAGGTCTTGACATCTTTTGATCACCTGAGAGATCAGGTTTCCCCTTCG
GGGGCAAAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCTTATGACTAGTTGCCAGCATTTAGTTGGGC
ACTCTAGTAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATC
ATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGT
TGCGAGACCGCGAGGTCAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGACTGTAG
GCTGCAACTCGCCTACACGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCG
CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGT
AACACCCGAAGCCGGTGGCGTAACTCCTTTAGGGAGCGAGCCGTCTAAGGTGACAA
ATT3’

LAB Isolate 4: *Lactobacillus reuteri* DSM 20016, 16S ribosomal RNA gene, partial sequence

5’AGAGTTTGATNNTGGCTCAGGATGAACGCCGGCGGTGTGCCTAATACATGCAAGT
CGTACGCACTGGCCCAACTGATTGATGGTGCTTGCACCTGATTGACGATGGATCAC
CAGTGAGTGGCGGACGGGTGAGTAACACGTAGGTAACCTGCCCCGGAGCGGGGGA
TAACATTTGGAAACAGATGCTAATACCGCATAACAACAAAAGCCGCATGGCTTTTG
TTTGAAAGATGGCTTTGGCTATCACTCTGGGATGGACCTGCGGTGCATTAGCTAGTT
GGTAAGGTAACGGCTTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATC
GGCCACAATGGAACTGAGACACGGTCCATACTCCTACGGGAGGCAGCAGTAGGGA
ATCTTCCACAATGGGCGCAAGCCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGG
TTTCGGCTCGTAAAGCTCTGTTGTTGGAGAAGAACGTGCGTGAGAGTAACTGTTCN
CGCAGTGACGGTATCCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGG
CGGTTGCTTAGGTCTGATGTGAAAGCCTTCGGCTTAACCGAAGAAGTGCATCGGAA
ACCGGGCGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGGAA
TGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGCAACTG
ACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCAT
GCCGTAAACGATGAGTGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGGAGCT
AACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGA
AGAACCTTACCAGGTCTTG1ACATCTTGCGCTAACCTTAGAGATAAGGCGTTNCCTT
CGGGGACGCAATGACAGGTGGTG1CATGGTCGTCGTCAGCTCGTGTCGTGAGATGT
TGGGTTAAGTCCTGCAACGAGCGCAACCCTTGTTACTAGTTGCCAGCATTAAGTTG
GGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAG
ATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACG
AGTCGCAAGCTCGCGAGATAAGCTAATCTCTTAAAGCCGTTCTCAGTTCGGACTGT
AGGCTGCAACTCGCCTACACGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGC
CGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTT
GTAACGCCCAAAGTCGGTGGCCTAACCTTTATGGAGGGAGCCGCCTAAGGCGGGAC
AGATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGAGAGCCTGCGGCTGGAT
CACCTCCTTTNT3’

LAB Isolate 5: *Lactobacillus kunkeei* strain H14_2_1 BCO2 16S ribosomal RNA gene, partial sequence

5’GACGAGCTCTCCTGAATTGATTTTATGCTTGCATAAATGATTTTTAGATTCGGAGC
GAGTGGCGAACTGGTGAGTAACACGTGGGTAACCTGCCCCGAAGCGGGGGATAAC
ATTTGGAAACAAATGCTAATACCGCATAATTAGGTGGAACCGCATGGTTCCAACTT
GAAAGATGGCTCTGCTATCACTTTGGGATGGACCCGCGCCGTATTAGTTAGTTGGT
GAGATAAAAGCCCACCAAGACCATGATACGTAGCCGACCTGAGAGGGTAATCGGC
CACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATC
TTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGATGAAGGTTTTC
GGATCGTAAAACTCTGTTGTTAAAGAAGAACAAGTGTTAGAGTAACTGTTAACACT
TTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAT
TTGTAAGTCTGCTGTGAAAGCCCTCAGCTCAACTGAGGAAGTGCAGTGGAAACTAC
AAAACTTGAGTACAGAAGAGGAAAGTGGAACTCCATGTGTAGCGGTGAAATGCGT
AGATATATGGAAGAACACCAGTGGCGAAGGCGGCTTTCTGGTCTGTTACTGACGCT
GAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGT
AAACGATGAATACTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGCAGCTAACGC
ATTAAGTATTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGAC
GGGGGCCCGCACAAGTGGTGGAGCATGTGGTTTAATTCGATGCTACGCGAAGAACC
TTACCAGCTCTTGACATCTTCTGCCAACCCAAGAGATTGGGCGTTCCCTTCGGGGAC
AGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTATTATTAGTTGCCAGCATTTAGTTGGGCACTCTA
GTGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGACGTCAAATCATCATG
CCCCTTATGAGCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTCGCGAA
ACCGCGAGGTCAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGCAGGCTGCA
ACTCGCCTGCATGAAGTTGGAATCACTAGTAATCGTGGATCAGCATGCCACGGTGA
ATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACC
CAAAGACGATGGGGTAA3’

AAB Isolate 1: *Acetobacter aceti* strain W1 16S ribosomal RNA gene, partial sequence

5’AGAGTTTGATTCTGGCTCAGAGCGAACGCTGGCGGCATGCTTAACACATGCAAGT
CGCACGAAGGCTTCGGCCTTAGTGGCGGACGGGTGAGTAACGCGTAGGAATCTATC
CATGGGTGGGGGATAACTCCGGGAACTGGAGCTAATACCGCATGATACCTGAGGGT
CAAAGGCGCAAGTCGCCTGTGGAGGAGTCTGCGTTTGATTAGCTTGTTGGTGGGGT
AAAGGCCTACCAAGGCGATGATCAATAGCTGGTCTGAGAGGATGATCAGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGAC
AATGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGTTTTCGGATTG
TAAAGCACTTTCGGCGGGGACGATGATGACGGTACCCGCAGAAGAAGCCCCGGCT
AACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATGAC
TGGGCGTAAAGGGCGTGTAGGCGGTTTGTACAGTCAGATGTGAAATCCCCGGGCTT
AACCTGGGAGCTGCATTTGATACGTGCAGACTAGAGTATGAGAGAGGGTTGTGGAA
TTCTCAGTGTAGAGGTGAAATTCGTAGATATTGGGAAGAACACCGGTGGCGAAGGC
GGCAACCTGGCTCATTACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCTGTAAACGATGTGTGCTGGATGTTGGGTAACTTA
GTTACTCAGTGTCGTAGCTAACGCGATAAGCACACCGCCTGGGGAGTACGGCCGCA
AGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGT

TTAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGTATGGAGAGGCTGTATTC
AGAGATGGATATTTCCCGCAAGGGACCTCTTGCACAGGTGCTGCATGGCTGTCGTC
AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCTTTA
GTTGCCAGCATGTTTGGGTGGGCACTCTAAAGAGACTGCCGGTGACAAGCCGGAGG
AAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGC
TACAATGGCGGTGACAGTGGGAAGCTAGATGGCGACATCGTGCCGATCTCTAAAAA
CCGTCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCTAG
TAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCC
CGTCACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCGAACCGCAAGGACGC
AGCCGACCACGGTCGGGTCAGCGACTGGGGTGAAGTCGTAACAAGGTAGCC3’

AAB Isolate 2: *Acetobacter lovaniensis* strain NBRC 13753 16S ribosomal RNA, partial sequence

5’TGAGTTTTGATCCTGGCTCAGAGCGAACGCTGGCGGCATGCTTAACACATGCAAG
TCGCACGAACCTTTCGGGGTTAGTGGCGGACGGGTGAGTAACGCGTAGGAATCTGT
CCACGGGTGGGGGATAACTCTGGGAAACTGGAGCTAATACCGCATGATACCTGAG
GGTCAAAGGCGCAAGTCGCCTGTGGAGGAGCCTGCGTTCGATTAGCTAGTTGGTGG
GGTAAAGGCCTACCAAGGCGATGATCGATAGCTGGTTTGAGAGGATGATCAGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
GACAATGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGTCTTCGGA
TTGTAAAGCACTTTCGACGGGGACGATGATGACGGTACCCGTAGAAGAAGCCCCGG
CTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATG
ACTGGGCGTAAAGGGCGTGTAGGCGGTTTACACAGTCAGATGTGAAATCCCCGGGC
TTAACCTGGGAGCTGCATTTGATACGTGTAGACTAGAGTGTGAGAGAGGGTTGTGG
AATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGGAAGAACACCGGTGGCGAA
GGCGGCAACCTGGCTCATGACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTGTGCTAGATGTTGGGTAA
CTTTGTTATTCAGTGTCGCAGTTAACGCGTTAAGCACACCGCCTGGGGAGTACGGC
CGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG
TGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGAATGTAGAGGCTGT
ATTCAGAGATGGATATTTCCCGCAAGGGACCTCTAACACAGGTGCTGCATGGCTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTAT
CTTTAGTTGCCAGCATGTTTGGGTGGGCACTCTAGAGAGACTGCCGGTGACAAGCC
GGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGTCCTGGGCTACACA
CGTGCTACAATGGCGGTGACAGTGGGAAGCTAGATGGTGACATCATGCTGATCTCT
AAAAGCCGTCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATC
GCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACA
CCGCCCGTCACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCGAACCCGCAA
GGGGCGCAGCCGACCACGGTCGGGTCCAGCGACTGGGGTGAAGTCGTAC3’

AAB Isolate 3: *Acetobacter pasteurianus* strain bh12 16S ribosomal RNA gene, partial sequence

5’CCAATGGCGGCAGCTTACACATGCAGTCGCACGAAGGTTTCGGCCTTAGTGGCGG
ACGGGTGAGTAACGCGTAGGTATCTATCCATGGGTGGGGGATAACACTGGGAAACT
GGTGCTAATACCGCATGACACCTGAGGGTCAAAGGCGCAAGTCGCCTGTGGAGGA
GCCTGCGTTTGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCAAT
AGCTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCT

ACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGCAAT
GCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTCGACGGGGACGATGA
TGACGGTACCCGTAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATA
CGAAGGGGGCTAGCGTTGCTCGGAATGACTGGGCGTAAAGGGCGTGTAGGCGGTTT
GTACAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAGCTGCATTTGATACGTGC
AGACTAGAGTGTGAGAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAG
ATATTGGGAAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCATTACTGACGCTGA
GGCGCGAAAGCGTGGGGAGCGGACAGGATTAGATACCCTGGTAGTCCACGCTGTA
AACGATGTGTGCTAGATGTTGGGTGACTTAGTCATTCAGTGTCGCAGTTAACGCGTT
AAGCACACCGCCTGGGGAGTACGGGCCGCGAGGTTGAAACTCAAAGGAATTGACG
GGGGGCGCCGCACAAGCGGTGGAGCATGTGGTTGAATTCGAAAGCAACGCGCAGA
ACCTTACCACGGCTTGGAGTGTAGAGGCTGCAAGCAGAGATGTTTGTTTCCCGCAA
GGGACCTCTAACACAGGTGCTGGCGTGGCTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCTCAGCGAGCGCAACCCGCTATCTTTAGTTGCCATCAAGTTTGGCC
TGGGCACTCTAGGAGAGACTGCCAGGTGACCGAGCCCCGCACAAGGTGGGAGAAT
GACGTGAAGTCCTCATGGCCGCTTAAGGGTGCCGTGGGACACGTGCTACAATGGCG
GTGACAGTGGGAAGCTAGGTGGTGACACCATGCTGATCTCTAAAAGCCGTCTCAGT
TCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCTAGTAATCGCGGA
TCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA
TGGGAGTTGGTTTGACCTTAAGCCGGTGAGCGAACCGCAAGGACGCAGCCGACCAC
GTCGTACGCGT3’

Appendix 13: Chapter 7: Ethics for Sensory and Glycaemic index studies

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12 October 2016

Noemi Gutierrez-Maddox
Faculty of Health and Environmental Sciences

Dear Noemi

Re Ethics Application: **16/340 Integration of the functional properties of coconut water kefir into sourdough bread**

Thank you for providing evidence as requested, which satisfies the points raised by the Auckland University of Technology Ethics Committee (AUTEC).

Your ethics application has been approved for three years until 12 October 2019.

As part of the ethics approval process, you are required to submit the following to AUTEC:

- A brief annual progress report using form EA2, which is available online through http://www.aut.ac.nz/researchethics. When necessary this form may also be used to request an extension of the approval at least one month prior to its expiry on 12 October 2019;
- A brief report on the status of the project using form EA3, which is available online through http://www.aut.ac.nz/researchethics. This report is to be submitted either when the approval expires on 12 October 2019 or on completion of the project.

It is a condition of approval that AUTEC is notified of any adverse events or if the research does not commence. AUTEC approval needs to be sought for any alteration to the research, including any alteration of or addition to any documents that are provided to participants. You are responsible for ensuring that research undertaken under this approval occurs within the parameters outlined in the approved application.

AUTEC grants ethical approval only. If you require management approval from an institution or organisation for your research, then you will need to obtain this.

To enable us to provide you with efficient service, please use the application number and study title in all correspondence with us. If you have any enquiries about this application, or anything else, please do contact us at ethics@aut.ac.nz.

All the very best with your research,

Kate O'Connor
Executive Secretary
Auckland University of Technology Ethics Committee

Cc: Mansi Jayantikumar Limbad, limbadmansi@gmail.com; Nazimah Hamid

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23 August 2018

Noemi Gutierrez-Maddox
Faculty of Health and Environmental Sciences
Dear Noemi

Re: Ethics Application: **16/340 Integration of the functional properties of coconut water kefir into sourdough bread**

Thank you for your request for approval of an amendment to your ethics application.

The amendment to the data collection protocols to allow for glucose testing is approved.

I remind you of the **Standard Conditions of Approval**.

1. A progress report is due annually on the anniversary of the approval date, using form EA2, which is available online through http://www.aut.ac.nz/research/researchethics.
2. A final report is due at the expiration of the approval period, or, upon completion of project, using form EA3, which is available online through http://www.aut.ac.nz/research/researchethics.
3. Any amendments to the project must be approved by AUTEC prior to being implemented. Amendments can be requested using the EA2 form: http://www.aut.ac.nz/research/researchethics.
4. Any serious or unexpected adverse events must be reported to AUTEC Secretariat as a matter of priority.
5. Any unforeseen events that might affect continued ethical acceptability of the project should also be reported to the AUTEC Secretariat as a matter of priority.

Please quote the application number and title on all future correspondence related to this project.

AUTEC grants ethical approval only. If you require management approval for access for your research from another institution or organisation then you are responsible for obtaining it. If the research is undertaken outside New Zealand, you need to meet all locality legal and ethical obligations and requirements.

For any enquiries please contact ethics@aut.ac.nz

Yours sincerely,

Kate O'Connor
Executive Manager
Auckland University of Technology Ethics Committee

Cc: limbadmansi@gmail.com; Nazimah Hamid

List of attributes for the sensory analysis of the breads

Please select all the attributes that apply.

Flavor (Taste and smell)	Appearance	Texture
Buttery	Airy crumb	Spongy
Nutty	Heterogenous	Crumbly
Fresh	Dry	Chewiness
Yeasty	Doughy	Hard
Sour	Dark	Soft
Roast	Dry	Elastic
Sweet	Blister	Firm
	Light	Coarse
	Moist	
	Big hole	
	Small holes	
	Overcooked	
	Porous	
	Compact	
	Shiny	
	Beige	
	Dense crumb	
	Greasy	
	Smooth	
	Developed	
	Supple	

Appendix 14: Chapter 7: One-way ANOVA for Amino acid analysis – between the samples (within column)

Bread sam	Histidine	HydroxyProline	Arginine	Serine	Glycine	Aspartic acid	Threonine	Glutamic acid	Alanine	Proline	Lysine	Methionine	Valine	Tyrosine	Isoleucine	Leucine	Phenylalanine	Tryptophan

ple																		
D	10.4 ± 0.1c	8 ± 0.2b	53.8 ± 0.4a	298 .2 ± 2.4 c	354. 1 ± 7.9c	295. 9 ± 45.1 b	88 ± 2.2c	121.4 ± 1.5c	531. 2 ± 13.7 c	251. 3 ± 5.1c	31.3 ± 1.4c	8.3 ± 0.2c	5.2 ± 0.1c	4.2 ± 0.1c	3.2 ± 0c	4.6 ± 0.1c	2.3 ± 0.1c	8.3 ± 0.2c
F	11.8 ± 0.3a	9.5 ± 0.2a	54 ± 1.4a	373 .1 ± 1.9 a	413. 9 ± 2.1a	350. 2 ± 5a	184.3 ± 2.4a	166.6 ± 1.8a	783. 1 ± 6.3a	333. 1 ± 2a	46.1 ± 1.1a	12.1 ± 0.4a	10 ± 0.3a	6.3 ± 0a	7.6 ± 0.1a	8.8 ± 0.1a	5.3 ± 0.1a	15 ± 0.2a
B	10.8 ± 0.1b	7.2 ± 0.6c	54.5 ± 0.9a	358 .6 ± 3.8 b	403. 1 ± 1.4b	297. 3 ± 6.1b	160.2 ± 5.7b	152.8 ± 1.2b	755. 2 ± 4.5b	312. 7 ± 4.8b	37.4 ± 1.1b	11.1 ± 0.1b	8.4 ± 0.1 b	5.3 ± 0.1b	5.7 ± 0.1b	6.6 ± 0.2b	3.6 ± 0.1b	11.4 ± 0.2b

Appendix 15: Chapter 3: Preliminary data for LAB, yeast and AAB counts

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Chapter 11 Publications and Conference presentation

1. Limbad, M., Gutierrez Maddox, N., Hamid, N., & Kantono, K. (2020). Sensory and Physicochemical Characterization of Sourdough Bread Prepared with a Coconut Water Kefir Starter. Foods, 9(9), 1165.
2. Mansi Jayantikumar Limbad, N. G.-M. a. N. H. (2015). *Coconut Water: An Essential Health Drink in both Natural and Fermented Forms.* In J. P. Owen (Ed.), Fruit and Pomace Extracts: Biological Activity, Potential Applications and Beneficial Health Effects (pp. 145--156)
3. Anand Mohan, Noemi Gutierrez-Maddox, Punit Chawda, Mansi Limbad, Yu Li, Ivanhoe K. H. Leung, Yihuai Gao, Quan Shu and Siew-Young Quek. (2020). Probiotic *Lactobacillus reuteri* growth and survival in fermented milk enhanced by UMF™ and MGO™ grade antibacterial Manuka honey. Journal of Functions Foods (manuscript submitted).
4. Limbad, M., Gutierrez-Maddox, N., Hamid, N., Higgins C. and Kevin Lee (2017). *Coconut water and Sourdough: Fermented with Kefir*. Oral presentation at NZ Microbiological Society Conference 2017, AUT, Auckland, New Zealand
5. Limbad, M., Gutierrez-Maddox, N., Hamid, N. and Kevin Kantono (2015). *Coconut water and Sourdough: Fermented with Kefir*. Presentation at the 50th Annual New Zealand Institute of Food Science and Technology Inc., Auckland, New Zealand
6. Limbad, M., Gutierrez-Maddox, N., Hamid, N. and Kevin Kantono (2015). *Coconut water and Sourdough: Fermented with Kefir*. Presentation at Post graduate research symposium, AUT, Auckland, New Zealand