Characterisation of acetic acid bacteria and yeast isolated from Kombucha produced in New Zealand

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Abstract

Background: Kombucha is a popular functional tea beverage commonly fermented by a complex symbiotic culture of acetic acid bacteria (AAB) and yeast (SCOBY) in a base of sugared tea infusion at ambient temperature for 7-14 days. Regular consumption of Kombucha confers potential health benefits due to the presence of live cultures and high concentrations of bioactive components such as vitamins, polyphenols, and organic acids. However, industrial production of Kombucha faces challenges due to the limited information on the dynamic changes in its microbial community composition and the lack of knowledge regarding their health-promoting characteristics. The impact of fermentation conditions and added substrates on starter cultures, physicochemical characteristics and functional activities is also not well understood.

Objectives: This study aimed to determine the microbiological characteristics of New Zealand Kombucha starter cultures and evaluate the probiotic potential of AAB and yeast isolated from commercial Kombucha products. The bioactive components, antioxidant activity, and antimicrobial activities of Kombucha fermented using a New Zealand starter culture under different fermentation conditions were also determined.

Methods: In phase I, the dominant AAB and yeast were isolated from Kombucha (tea broth and cellulosic pellicle) and screened for their biochemical characteristics. Dominant microorganisms were identified by sequencing of the ribosomal RNA gene (16S rRNA for AAB and ITS for yeast). In phase II, 62 yeast strains and 36 AAB strains were isolated from the Kombucha starter culture and commercial Kombucha. Representative yeast (n=15) and AAB (n=9) isolates were selected for evaluation using various techniques comprising tolerance to bile salt, NaCl, different temperatures, acidity, cell surface characteristics (auto-aggregation, co-aggregation with pathogens, and hydrophobicity with chloroform), and functional activities (antioxidant, antimicrobial and bile salt hydrolase activities). The safety of the strains was

evaluated by testing their susceptibility to antibiotics and enzymatic activities. In phase III, the Plackett-Burman design was applied to evaluate the impact of different fermentation conditions on the physicochemical and functional activities of black tea Kombucha. The following factors were tested: fermentation temperature (20-30°C), fermentation time (7-14 days), sugar levels (20-100 g/L), black tea levels (1.5-12 g/L), tea infusion time (5-15 min), inoculum volume (100-200 mL/L) and SCOBY (10-40 g/L). The total phenolic content (TPC) of black tea Kombucha prepared under different conditions was determined by the Folin-Ciocalteu assay, and the total flavonoid content (TFC) was measured using the modified aluminium chloride colorimetric assay. Seven individual phenolic components in the Kombucha broth were analysed by high performance liquid chromatography (HPLC). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ABTS+ assays were used to determine the *in vitro* antioxidant activity of Kombucha tea broth. Ethanol was determined by enzymatic assay and antimicrobial activity of the Kombucha was screened by agar well-diffusion assay.

Results: The dominant AAB species in the Kombucha starter culture were identified as *Komagataebacter rhaeticus*, which contributes to the cellulosic pellicle formation. The isolated yeast belonged to *Debaryomyces prosopidis* and *Zygosacchromyces lentus*. During fermentation, the acidity increased, while the total soluble solids decreased. Of the fifteen yeast strains tested, two strains yeast of *Pichia kudraivzevii* GBY1, *Saccharomyces cerevisiae* GBY2, three AAB strains of *Acetobacter musti* LOAAB1, and *Gluconobacter potus* (LOAAB2 and GBAAB3) isolated from New Zealand Kombucha showed promising probiotic characteristics.

Kombucha fermented under 17 different conditions generated by the Plackett-Burman design were all acidic (pH 2.55 - 3.16) with visible colour differences. Three phenolic components (gallic acid, theobromine, caffeine) were detected from all seventeen fermented Kombucha samples. All the Kombucha samples (seventeen) exhibited antioxidant activity for both DPPH (20.78 - 91.28%) and ABTS (22.63 - 551.10 µg TE/mL) relative to their total phenolic and

flavonoid content. High phenolic content and strong antioxidant capacity were detected in Kombucha samples prepared with high levels of tea (12 g/L). Kombucha samples with the highest titratable acidity (1.03%) showed the strongest antimicrobial activities against *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli*. The ethanol concentration of Kombucha prepared under the different conditions varied from 0.03% to 0.38% ABV and were all below the levels stipulated by Australia-New Zealand regulations for non-alcoholic beverages (1.15 % ABV).

Conclusion: The New Zealand Kombucha starter culture was composed of *K. rhaeticus*, *D. prosopidis*, and *Z. lentus*. Results indicated that the yeast and AAB strains isolated from commercial Kombucha produced in New Zealand may be probiotic. The antioxidant activity of Kombucha prepared under different fermentation conditions may be attributed to the phenolic components of the tea. Additionally, acidity and phenolic content may have contributed to the antimicrobial activity of the Kombucha tea against the three pathogens. Kombucha produced in this study may be considered as a functional product due to the presence of phenolic contents, potential probiotics, and antimicrobial activities against three pathogens. The results of this study showed the probiotic potential of AAB and yeast strains isolated from Kombucha based on some *in vitro* tests.

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List of peer-reviewed publications and poster presentations

Publications

1. **Wang, B.,** Rutherfurd-Markwick, K., Zhang, X.-X., & Mutukumira, A. N. (2024). Probiotic potential of acetic acid bacteria isolated from Kombucha in New Zealand *in vitro*. *The Microbe*. https://doi.org/10.1016/j.microb.2024.100130 (**Chapter 5, 1 citation**)
2. **Wang, B.**, Rutherfurd-Markwick, K., Zhang, X.-X., & Mutukumira, A. N. (2024). Evaluation of the probiotic potential of yeast isolated from kombucha in New Zealand, *Current Research in Food Science.* https://doi.org/10.1016/j.crfs.2024.100711 **(Chapter 4, JCRQ1, Impact Factor:6.2; 2 citations)**
3. **Wang, B**., Rutherfurd-Markwick, K., Naren, N., Zhang, X.-X., & Mutukumira, A. N. (2023). Microbiological and Physico-Chemical Characteristics of Black Tea Kombucha Fermented with a New Zealand Starter Culture. *Foods*, *12*(12), 2314. https://www.mdpi.com/2304-8158/12/12/2314 **(Chapter 3, JCR Q1, Impact factor: 4.7 ; 13 citations).**
4. **Wang, B.,** Rutherfurd-Markwick, K., Zhang, X.-X., & Mutukumira, A. N. (2022). Kombucha: Production and Microbiological Research. *Foods*, *11*(21), 3456. https://doi.org/10.3390/foods11213456. **(Chapter 2, JCR Q1, Impact Factor: 4.7; 42 citations, Editor's choice article).**
5. **Wang, B.,** Rutherfurd-Markwick, K., Zhang, X.-X., & Mutukumira, A. N. (2022). Isolation and characterisation of dominant acetic acid bacteria and yeast isolated from Kombucha samples at point of sales in New Zealand. *Current Research in Food Science*. 5, 835-844. https://doi.org/10.1016/j.crfs.2022.04.013 **(Master's work, JCR Q1, Impact Factor:6.2; 32 citations)**
6. Mutukumira, A. N., Rutherfurd-Markwick, K., **Wang, B**., Wang, X., Archer, R. (2020). What do you we know about Kombucha today? Microbiological, chemical and sensory analysis. Food New Zealand 20 (5), 28. (2 citations)

Conference poster presentations

- **Wang, B**., Rutherfurd-Markwick, K., Naren, N., Zhang, X.-X., & Mutukumira, A. N. (2021). *Characterization of dominant acetic acid bacteria and yeast associated with Kombucha sold in New Zealand*. [Poster presentation]. NZIFIST Annual Conference, Food with Care, 6-8th July, Palmerston North, New Zealand.
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List of abbreviations

AAB	Acetic acid bacteria
ABV	Alcohol by volume
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACE	Angiotensin converting enzyme
ALFP	Amplified length fragments polymorphism
AM	ampicillin
ANOVA	analysis of variance
BLAST	Basic local alignment search tool
BSH	Bile salt hydrolase
C	Chloramphenicol
CAGR	Compound annual growth rate
CFU	Colony forming unit
CFS	Cell free supernatants
CO	Colistin sulphate
CT	Cycle threshold
DHA	Dihydroxyacetone
DGGE	Denaturing gradient gel electrophoresis
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DPPH	1,1-diphenyl-2-picrylhydrazyl
DSL	D-saccharic acid 1,4-lactone
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ERIC-PCR	Enterobacterial repetitive intergenic consensus polymerase chain reaction
FAS	Fetal alcohol syndrome
FDA	Food and Drug Administration
GAE	Gallic acid equivalents
GIT	Gastrointestinal tract
GY	Glucose yeast extract
GYC	Glucose yeast extract calcium carbonate
GYMEA	Glucose yeast extract mannitol ethanol acetic acid agar
HPLC	High performance liquid chromatography
HS	Hestrin-Schramm
ITS	Internal transcribed spacer
K	Kanamycin
KCP	Kombucha cellulosic pellicle
LAB	Lactic acid bacteria

MATH	Modified adhesion ability to hydrocarbons
MHA	Muller-Hinton agar
MRS	De Man, Rogosa, and Sharpe
NA	Nalidixic acid
NCBI	National center for biotechnology information
NGS	Next generation sequencing
NI	Nitrofurantoin
NS	Not specified
OD	Optical density
OTU	Operational taxonomic unit
PCA	Principal component analysis
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction restriction fragment length polymorphism
PDA	Potato dextrose agar
QE	Quercetin equivalents
qPCR	Quantitative polymerase chain reaction
RAPD	Random amplification of polymorphic deoxyribonucleic acid
REP-PCR	Representative extragenic palindromic polymerase chain reaction
RNA	Ribonucleic acid
S	Streptomycin

SCOBY	Symbiotic culture of bacteria and yeast
SD	Standard deviation
T	Tetracycline
TA	Titratable acidity
TEAC	Trolox equivalent antioxidant capacity
TFs	Theaflavins
TFC	Total flavonoid content
TPC	Total phenolic content
TRs	Thearubigins
T- RFLP	Terminal restriction fragment length polymorphism
TSS	Total soluble solids
UDPGc	Cellulose precursor-uridine diphospho-glucose
UK	United Kingdom
USA	United state of America
WHC	Water holding capacity
WSR	Water swelling ratio
YPD	Yeast peptone broth
YGC	Yeast extract chloramphenicol

Chapter 1. Introduction

1.1 Background

Kombucha is an ancient functional beverage and has recently become one of the most popular beverages in the low-alcohol beverage category (Greenwalt et al., 2000; Vargas et al., 2021). Kombucha fermentation may have originated as early as 220 B.C. and is mostly prepared from a solution of sucrose-sweetened tea with a symbiotic culture of bacteria and yeast (SCOBY). The fermentation is conducted at ambient temperature for 7-14 days, resulting in a natural carbonated beverage with sweet to sour taste and pleasant aroma (Jayabalan et al., 2014; Liu et al., 2022). Regular consumption of Kombucha has been widely reported to be associated with a variety of beneficial effects, including antioxidant, antimicrobial, anticancer, antiproliferative and hepatoprotective activities *in vitro* (Chu & Chen, 2006; Jayabalan et al., 2011; Jayabalan et al., 2014; Nyiew et al., 2022). Due to the desirable sensory characteristics and potential health-promoting effects, the demand for Kombucha is increasing in the functional beverage market, and the global market value is predicted to reach 3.5 to 5 billion USD by 2025 (Kim & Adhikari, 2020).

The microbial composition of the Kombucha starter culture is diverse and complex, but the dominant microorganisms are acetic acid bacteria (AAB) and yeast (Greenwalt et al., 2000). The presence of lactic acid bacteria (LAB) has also been reported but their role in Kombucha fermentation is not clearly understood (Laureys et al., 2020). The most common genera isolated from Kombucha include *Acetobacter*, *Gluconobacter, Gluconacetobacter,* and *Komagataibacter,* whereas the most reported yeast belonged to *Saccharomyces, Brettanomyces, Candida, Dekkera, Pichia, Zygosaccharomyces* and *Hanseniaspora* (Barakat et al., 2023; Marsh et al., 2014; Mayser et al., 1995; Ramadani & Abulreesh, 2010; Villarreal-Soto et al., 2018). During Kombucha fermentation, sucrose is firstly metabolised by yeast cells into glucose and fructose, then converted into ethanol, carbon dioxide, and organic acids by AAB

(Coelho et al., 2020; Dufresne & Farnworth, 2000). The fermenting microorganisms in the broth and cellulose are responsible for the biotransformation process that occurs during fermentation and produce metabolites such as sugars, phenolic compounds, organic acids, ethanol, minerals, vitamins, and bacterial cellulose (Dutta & Paul, 2019; Kapp & Sumner, 2019; Leonarski et al., 2022). The metabolites and the components in Kombucha may contribute to the beneficial effects and sensory characteristics of the beverage (Liu et al., 2022; Tran et al., 2020).

The potential of large-scale production of kombucha is affected by several factors such as limited knowledge of the composition of starter cultures, and their fermentation characteristics (Reva et al., 2015; Shahbazi et al., 2018; Shi et al., 2023). The final chemical composition, sensory characteristics, perceived beneficial effects, and shelf-life stability of Kombucha are dependent on the types of tea and sugar, fermentation parameters (temperature and time), and microbial composition of the broth and SCOBY (Lončar et al., 2014; Malbasa et al., 2008). Thus, there is need to investigate the microbial characteristics of fermenting microorganisms and their probiotic potential, as well as the physicochemical and functional properties of Kombucha fermented with these microorganisms.

1.2 Aim, structure, and objective of this study

The overall aim of this study was to determine the microbiological characteristics of dominant microorganisms in a New Zealand starter culture and evaluate the impact of different fermentation conditions and formulations on bioactive components, physicochemical and functional characteristics of Kombucha prepared with the starter culture.

This thesis is presented as a thesis by publication contains seven chapters: **Chapter 1** introduces the background information on Kombucha, the current challenges of the industrial scale-up of commercial Kombucha, and the importance of this study. **Chapter 2** (published in

Foods 2022) provides a review of the current research on the microbiological characteristics, compositions of dominant fermenting microorganisms of Kombucha, as well as the recent advances in the phenotypic and molecular identification techniques. The methodologies used in this study are summarised in **Chapters 3-6**. The dynamic changes in the microbial compositions of Kombucha fermented with a New Zealand starter culture was determined by culture-depended methods and ribosomal genotyping in **Chapter 3** (published in Foods, 2023). The probiotic characteristics of AAB and yeast isolated from New Zealand Kombucha were evaluated through four main selection criteria: stress tolerance ability, cell-surface characteristics, potential beneficial effects, and their safety assessment in **Chapters 4** (published in Current Research in Food Science, 2024) **and 5** (to be submitted to Food Control, 2024). The impact of different fermentation parameters on the physicochemical, bioactive compounds, and functional characteristics of black tea Kombucha prepared with a New Zealand starter culture was investigated using the Plackett-Burman Design in **Chapter 6 (**to be submitted to Food Chemistry, 2024). The general discussion, conclusions of this study, and the recommendations for future work are discussed in **Chapter 7**.

The specific objectives of this study are as follows:

Objective 1: To determine the dominant microbiological and physicochemical characteristics of the black tea Kombucha prepared with a New Zealand starter culture (Chapter 3).

Objective 2: To evaluate the probiotic potential of fermenting microorganisms (AAB and yeast) isolated from commercial Kombucha beverages sold in New Zealand (Chapters 4 and 5).

Objective 3: To investigate the impact of different fermentation parameters on the physicochemical, bioactive compounds, and functional activities of black tea Kombucha prepared with a New Zealand starter culture (Chapter 6).

Chapter 2. Literature review

2.1 Abstract

Kombucha is a sparkling sugared tea commonly prepared using a sugared tea infusion and fermented at ambient temperature for several days using a cellulose pellicle also called tea fungus that is comprised of acetic acid bacteria and yeast. Consumption of Kombucha has been reported as early as 220 B.C. with various reported potential health benefits and appealing sensory properties. During Kombucha fermentation, sucrose is hydrolysed by yeast cells into fructose and glucose, which are then metabolised to ethanol. The ethanol is then oxidised by acetic acid bacteria (AAB) to produce acetic acid which is responsible for the reduction of the pH and also contributes to the sour taste of Kombucha. Characterisation of the AAB and yeast in the Kombucha starter culture can provide a better understanding of the fermentation process. This knowledge can potentially aid in the production of higher quality products as these microorganisms affect the production of metabolites such as organic acids which are associated with potential health benefits, as well as sensory properties. This review presents recent advances in the isolation, enumeration, biochemical characteristics, conventional phenotypic identification system, and modern genetic identification techniques of AAB and yeast present in Kombucha to gain a better under understanding of microbial diversity of the beverage.

2.2 Introduction

Kombucha is a traditional fermented sparkling tea beverage with a slightly sweet and acidic flavour that has been consumed in China since around 220 B.C. The beverage is consumed for its refreshing sensory characteristics and the perceived inherent health-promoting properties (Dufresne & Farnworth, 2000). It is reported that Dr Kombu introduced fermented tea to Japan around 414 A.D., where it was apparently used to alleviate digestive ailments (Dufresne & Farnworth, 2000; Jayabalan et al., 2014). The name, ‘Kombucha’ is documented to originate from “Dr Kombu” and “cha” refers to tea in Japanese. Kombucha was introduced to Russia as

"Tea Kvass" and then it spread to Eastern Europe during the 20th century. The popularity of Kombucha in Russia was attributed to its purported beneficial effects on healing metabolic diseases, haemorrhoids and rheumatism (Greenwalt et al., 2000; Kumar & Joshi, 2016). During World War II, Kombucha was introduced to Western Europe and North Africa. In recent decades, the production of Kombucha has become global, and it is now sold as a commercial beverage with different flavours. In addition, due to the high popularity of Kombucha products, small-scale home-brewed products are often made for personal use and can frequently be found for sale at farmer's markets and in communities.

The global market size for Kombucha has increased significantly in recent years. In 2018, Kombucha was worth USD 1.5 billion, and it is estimated to grow to around 5 billion by 2025 with an estimated compound annual growth rate (CAGR) of 23% (Kim & Adhikari, 2020). Most commercial Kombucha products sold in New Zealand are flavoured using fruits and/or herbs in single or mixed flavours. Information on commercial Kombucha products from different brands sold in New Zealand is shown in Table 2.1.

Table 2.1 Commercial Kombucha product sold in New Zealand.

Brands	**Manufacturing origin**	**Packaging**	**Flavours**	**Storage conditions**
Remedy	Australia	330 mL (glass bottles) 250 mL (cans) 1.25 L (plastic bottles)	Ginger lemon, Raspberry Lemonade, Cola, Wild berry, Mango Passion, Passionfruit, Apple Crisp, Peach	Chilled/ambient temperature
Batchwell	New Zealand	375 mL (glass bottles)	Braeburn apple, Pineapple & Ginger, Motueka hops, Ginger & Turmeric, Early grey	Chilled
Daily Organics	New Zealand	200 mL (glass bottles) 1 L (glass bottles)	Original Kombucha, Chai spices & ginger, Lemon and ginger	Chilled
LO BROS	New Zealand	330 mL (glass bottles) 250 mL (cans)	Feijoa, Raspberry & lemon, Orange & mango, Ginger &	Chilled/ ambient temperature

		750 mL (glass bottles)	lemon, Mango, Ginger and turmeric, Blueberry, Passionfruit, Cola, Ginger beer, Lemon lime & bitters, Pineapple & lime	
MAMA'S Brew Shop	New Zealand	375 mL (glass bottles) 330 mL (cans)	Lemongrass & ginger, Lavender & hibiscus	Chilled
Karma Drinks	New Zealand	330 mL (glass bottles)	Lemon & ginger, Raspberry & lemon, Mango & passionfruit, Cherry& berry	Chilled
Good Buzz	New Zealand	250 mL (cans) 328 mL (glass bottles) 375 mL (glass bottles) 888 mL (glass bottles)	Passionfruit & guava, Blueberry & peach, Pineapple & mango, Feijoa, Raspberry & lemon, Mango, Gisborne lemon & Manuka leaf, Hawkes Bay peach & kawakawa	Chilled/ ambient temperature

Kombucha is usually fermented from sweetened tea using a symbiotic culture of bacteria and yeast which is commonly abbreviated to SCOBY. The SCOBY is also known as tea fungus, cellulosic pellicle, or consortium. The microbial community in Kombucha is diverse and varies between fermentations, but it is mainly composed of AAB and yeast, although, the presence of small amounts of lactic acid bacteria (LAB) in Kombucha has also been reported (Marsh et al., 2014). These microorganisms (LAB, AAB, and yeast) have been suggested to be potential probiotics and therefore contribute to the health benefits of Kombucha (Coelho et al., 2020). However, the probiotic performance of the live cultures identified in Kombucha has rarely been studied (Vargas et al., 2021). Therefore, the probiotic potential of the microorganisms found in Kombucha should be determined using both *in vitro* and *in vivo* studies.

Many different substrates can be used for Kombucha, including green tea, oolong tea and black tea, as well as medicinal herbs including lemon balm, peppermint, thymes and sage or combinations thereof (Velićanski et al., 2013). Black tea is the most common tea substrate for

Kombucha fermentation (Reiss, 1994), and sucrose is the most popular source of carbon during fermentation (Jayabalan et al., 2014). For Kombucha fermentation, approximately 50 to 150 g/L (5% to 15% w/v) of sucrose is dissolved in boiled water, then tea leaves or tea bags are added to the sugar hot water and allow to infuse for around 5-10 min, followed by filtration to remove tea leaves (or removal of tea bags). Sugared tea is allowed to cool to room temperature (Dufresne & Farnworth, 2000). To prevent contamination from pathogenic and spoilage microorganisms, the production process is generally carried out under highly sanitary conditions with a small portion of previously fermented Kombucha broth added to reduce the pH (Greenwalt et al., 2000; Jayabalan et al., 2014). The cooled sugared tea is then transferred to a sterile wide-mouth container, and the "mushroom" (tea fungus) is placed onto the surface of the solution. The vessel is covered with a sterile cloth or paper towel to prevent insects and other undesirable cross-contamination from affecting the fermentation. The fermentation generally takes 7 to 10 days at room temperature (20 ℃ to 30 ℃), with fermentation temperatures ranging from 18 ℃ to 26 ℃ reported as optimal (Jayabalan et al., 2014). During fermentation, a newly formed jelly-like daughter tea fungus membrane is formed which floats on the surface of the broth. The tea fungus is removed and retained, together with a small amount of tea broth for the next fermentation. The daughter tea fungus can grow up to 2 cm thick and covers the surface of the mother tea fungus to gain better access to oxygen (Jayabalan et al., 2014; Steinkraus et al., 1996; Vijayaraghavan et al., 2000). After fermentation, the liquid broth is filtered through a clean cloth and stored in a sealed container at 4 ℃ for further processing such as packaging. With fermentation, the taste of Kombucha changes from pleasantly fruity at the beginning, to a vinegar-like flavour after longer fermentation (Jayabalan et al., 2014).

The consumption of Kombucha has been suggested to confer numerous health benefits for humans. However, the evidence for most of these benefits is based on *in vitro* studies. Therefore,

in vivo clinical trials are required to demonstrate any biological functions of Kombucha and to correlate them with active compounds (Kapp & Sumner, 2019). The fermented beverage is perceived to lower blood pressure (antihypertensive) by inhibiting the angiotensin- converting enzyme (ACE) and mediating blood sugar levels (antidiabetic) and cholesterol levels (Dutta & Paul, 2019). Furthermore, the consumption of Kombucha may be effective in weight management by controlling appetite due to its hypolipidemic effects associated with lipase inhibition (Dutta & Paul, 2019; Laureys et al., 2020). Kombucha has been reported to exhibit anticarcinogenic activity due to the presence of tea polyphenols and metabolites (Jayabalan et al., 2014). The antimicrobial activity of Kombucha against pathogenic microorganisms is mainly attributed to the action of acetic acid produced during fermentation (Dutta & Paul, 2019; Villarreal-Soto et al., 2018). The hepatoprotective activity of Kombucha is mainly due to the presence of the potential detoxifying agent, D-saccharic acid 1.4-lactone (DSL). However, there is scant information on the potential toxicity of Kombucha associated with metabolic acidosis and hyponatremia (Jayabalan et al., 2014; Kumar & Joshi, 2016). Thus, the recommended daily consumption of Kombucha should be no more than 118 mL (4 fluid ounces) for healthy people (Kapp & Sumner, 2019).

The objective of this review is to document the recent advances in the microbiological characteristics, composition, and phenotypical and molecular identification techniques of Kombucha. Systematic research is recommended as the identification of the microbial characteristics of Kombucha may allow producers to effectively control the fermentation process for the production of safer, consistently high-quality products.

2.3 Microbiological characteristics of kombucha

The Kombucha microbial community can be classified into two parts, that found in the floating cellulosic biofilm and that in the liquid broth (Chakravorty et al., 2016). An overview of the metabolic activities of the Kombucha fermentation process is summarised in Figure 2.1

(Markov et al., 2003). Sucrose is hydrolysed by yeast cells into fructose and glucose, which are metabolised by yeast to produce ethanol and carbon dioxide. Glycerol can also be produced by yeast due to the high osmotic pressure and further oxidised by AAB to dihydroxyacetone (DHA) (Laureys et al., 2020). Some esters are also produced during this process, which contribute to the aroma development of Kombucha. Fructose is preferably utilised as the substrate rather than glucose (Sievers et al., 1996), with the resulting ethanol produced being further metabolised by AAB to produce acetic acid, thereby reducing the ethanol content in the Kombucha. The low ethanol concentration facilitates the formation of the cellulosic pellicle (Laureys et al., 2020). Glucose is metabolised by AAB into gluconic and glucuronic acids. Autolysis of yeast provides vitamins and other nutrients to support the growth of the AAB (Villarreal-Soto et al., 2018). Yeast autolysis is commonly detected during the maturation of alcoholic beverages, which may impact the aroma and flavour of Kombucha products.

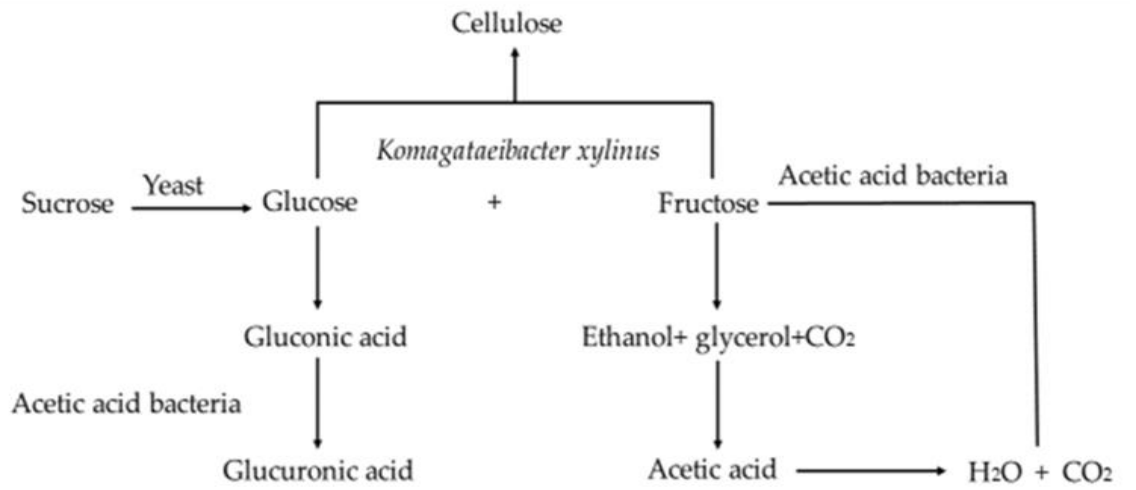

Figure 2.1 Metabolic activity of yeast and AAB during Kombucha fermentation. Adapted from Markov et al. (2003).

2.3.1 Presence of acetic acid bacteria in fermented foods

2.3.1.1 AAB used in the production of fermented products

Acetic acid bacteria play an imported role in the fermented food and beverage industry. These bacteria are widespread in the environment and can be found in sugary or acidic substances such as fruits and flowers (Ray, 2016). The genus *Gluconobacter* is easily isolated from environments abundant in sugar, while the genera *Acetobacter* and *Gluconacetobacter* are found in alcoholic environments (Laureys et al., 2020). AAB are widely used in the production of fermented food and beverage products such as vinegar, lambic beers, red wine, cocoa, and *nata de coco* (an edible bacterial cellulose formed from AAB in coconut water and kefir). The genera *Acetobacter* and *Gluconacetobacter* prefer to oxidise ethanol rather than glucose, while the genus *Gluconobacter* oxidises glycerol and glucose (Laureys et al., 2020).

2.3.1.2 Dominant acetic acid bacteria found in Kombucha

Acetic acid bacteria and gluconic acid-producing bacteria dominant prokaryotes found in Kombucha starter cultures. AAB belong to the family *Acetobacteraceae* and are classified into acetous or acidophilic groups. Currently, AAB are divided into 17 genera, of these, *Acetobacter*, *Gluconobacter*, *Gluconacetobacter* and *Komagataeibacter* are mainly used in the food industry (Lynch et al., 2019; Ray, 2016). The microbial community in Kombucha varies between products, which impacts the biochemical properties of the fermented beverage (Karabiyikli & Sengun, 2017). The production of the cellulosic biofilm (tea fungus) floating on the surface of the tea broth in Kombucha is associated with the presence of the AAB species *Komagataeibacter (K.) xylinus* (Dufresne & Farnworth, 2000; Jarrell et al., 2000; Jayabalan et al., 2014; Villarreal-Soto et al., 2018). Synthesis of the cellulosic pellicle involves the production of the cellulose precursor-uridine diphospho-glucose (UDPGc), which can be synthesised from several carbon sources including ethanol, sucrose, fructose, and glycerol (Villarreal-Soto et al., 2018). The presence of caffeine, theophylline and theobromine facilitates

the production of the cellulose network by *K. xylinus* (Fontana et al., 1991; Jayabalan et al., 2017), which is also affected by sugar types, sugar concentration, and pH (Laureys et al., 2020).

Other AAB isolated from Kombucha include *Bacterium (B.) gluconicum* (Jayabalan et al., 2011; Reiss, 1994), *Aceto- bacter (A.) aceti*, *Acetobacter (A.) pasteurianus*, *Glucobacter (G.) oxygendans* (Liu et al., 1996), *Acetobacter (A.) musti* and *Gluconobacter (G.) potus* (B. Wang et al., 2022a). Recently, species of *Komagataeibacter* and *Gluconoace- tobacter* have been reported in Kombucha including *Komagaeibacter (K.) kombuchae* (Dutta & Gachhui, 2007), *Komagataeibacter (K.) saccharivorans* (Mukadam et al., 2016), *Komagataeibacter (K.) rhaeticus* (Jacek et al., 2021), *Gluconaceto- bacter (G.) sacchari* (Trovatti et al., 2011) and *Gluconacetobacter* sp. A4 (G. sp. A4) are the main functional AAB isolated from Kombucha which synthesise D-saccharide acid-1,4 lactone (DSL), a promising detoxifying and antioxidant agent (Yang et al., 2010). Nitrogen-fixing *Acetobacter (A.) nitro- genifigens* sp. nov. and *Gluconactobacter (G.) kombucahe* sp.nov have also been isolated from Kombucha (Dutta & Gachhui, 2006).

2.3.2 Latic acid bacteria isolated from Kombucha

LAB have been utilised in different food products to achieve a unique flavour and deliver health benefits (Nguyen et al., 2015). The genera *Lactobacillus* and *Bifidobacterium* are commonly isolated from fermented foods (Diguta et al., 2020). LAB may be present in Kombucha; however, current information is not considered essential for its production. In certain Kombucha products, LAB have been reported to make up as much as 30% of the bacterial community. The addition of some LAB strains—*Lacticaseibacillus (L.) casei* and *Lactiplantibacillus (L.) plantarum* have been shown to enhance the antioxidant and antimicrobial activities of Kombucha (Nguyen et al., 2015). Moreover, the presence of LAB during Kombucha fermentation has been reported to enhance the production of D-saccharic acid, 1,4 lactone (DSL) and glucuronic acid (Yang et al., 2010). DSL has detoxifying properties

and may be an important factor contributing to the purported hepatoprotective activity of Kombucha (Wang et al., 2014). The LAB isolated from Kombucha are shown in Table 2.2.

Table 2.2 LAB isolated from Kombucha in different regions.

Species	Region	References
Lacticaseibacillus casei	NS	(Nguyen et al., 2015)
Lactiplantibacillius plantarum	China	(Pei et al., 2020)
Lactobacillus nagelii	United State of America (USA)	(Yang et al., 2022)
Lactobacillus rhamnosus	NS	(Kim & Adhikari, 2020)
Lactobacillus mali	USA	(Yang et al., 2022)
Pediococcus (P.) pentosaceus	Romania	(Bogdan et al., 2018)
P. acidiliactici	Romania	(Diguta et al., 2020)

NS= Not specified.

2.3.3 Yeast isolated from Kombucha

2.3.3.1 General characteristics of yeast

Yeast is a group of eukaryotic unicellular fungi which reproduced by budding or fission, with some capable of both (Deak & Beuchat, 1993). Yeast has been used for the fermentation of foods and beverages for thousands of years due to its ability to hydrolyse different substrates to produce valuable fermented final products such as beer, wine and bread (Buzzini et al., 2017). The first yeast used commercially was identified as the *Saccharomyces* (*S.*) *sensu stricto* complex (Buzzini et al., 2017). Yeast is currently classified into Ascomycetous and Basidiomycetous yeast, according to their molecular phylogenies (Deak & Beuchat, 1993). Yeasts are facultative anaerobes indicating that they can grow without oxygen. In the presence of oxygen, yeasts convert sugars to carbon dioxide and energy, whereas under anaerobic conditions, the sugars are converted to ethanol, glycerol and carbon dioxide (Bekatorou et al., 2006).

S. cerevisiae, also known as baker's yeast, is the most common food-grade yeast, widely used in bakery products, beer-brewing, and winemaking. Food-grade yeasts are also utilised as food additives, flavouring agents and as substrates for microbiological agar (Bekatorou et al., 2006).

Some non-*Saccharomyces* yeast such as the genera *Candida*, *Debaryomuces*, *Kluyveromyces*, *Yarrowia* and *Zygosaccharomyces* are gaining attention in industry for their possible commercial applications as starter cultures for food and non-food products due to their perceived probiotic properties and impact on the development of flavour and colour in some meat products (Buzzini et al., 2017; Fleet, 2006).

2.3.3.2 Dominant yeast present in Kombucha

Yeast species may vary between Kombucha products, particularly those from different regions due to the environment factors such as geographic and climatic conditions, and even contamination between starter cultures (Lachance et al., 2001; Teoh et al., 2004). Common yeasts isolated from Kombucha products produced in different regions and their characteristics (morphological and metabolic) are shown in Table 2.3 and Table 2.4 (Jayabalan et al., 2017). Generally, the yeast population outnumbers the bacteria present in Kombucha (Matei et al., 2018).

Table 2.3 Common yeast species isolated from Kombucha and their metabolic characteristics.

Species	Morphology	Characteristics
Zygosaccharomyces bailii (Dufresne & Farnworth, 2000)	White to cream colonies with brownish top, cylindrical or ellipsoidal shape, (3.5-6.0) x (4.5-11.5) μm in size	Tolerant to organic acids Forms acetic acid, heat tolerance < 75ºC Growth pH > 2 and < 7 (Thomas & Davenport, 1985)
Zygosaccharomyces rouxii	White to cream smooth colonies, round or oval shape	High osmotic stress and salt / sugar tolerant, grows under low oxygen and low water activity (Escott et al., 2018)
Schizosaccharomyces pombe	Cream to tan, butyrous colonies, rod shaped	Can convert malic acid to ethanol, high resistance to low water activity, low pH and wide range of temperature environments, highly sugar content tolerant (Loira et al., 2018)

Saccharomycodes ludwigi (Reiss, 1994)	Cream, butyrous colonies, elongated shape, and swelling in the center	Resistant to pressurized carbon dioxide, high sugar tolerant (Vejarano, 2018)
Saccharomyces cerevisiae (Liu et al., 1996)	White to cream, butyrous colonies, spherical or ovoid shape, 2.5-10.0 µm (diameter)	Can convert glucose to ethanol, high ethanol tolerance, rapid fermentation rate (Choonut et al., 2014)
Brettanomyces bruxellensis (Jayabalan et al., 2017; Matei et al., 2018)	Distinctive elongated shape, 2.5-10.0 µm (diameter)	Can produce high amount of acetic acid and ethanol under aerobic conditions, high ethanol concentration (up to 15%), able to grow under low pH and oxygen environment, high efficiency to utilize nitrogen source (Agnolucci et al., 2017)

Table 2.4 Yeast isolated from Kombucha in different regions.

Species	**Country**	**References**
Brettanomyces (B.) anamalus	New Zealand	(B. Wang et al., 2022b)
B. lambicus	Germany	(Mayser et al., 1995)
B. custerisii	Germany	(Mayser et al., 1995)
B. intermedius	NS	(Jayabalan et al., 2017)
B. claussenii	NS	(Jayabalan et al., 2017)
Candida (C.) albican	Japan	(Jayabalan et al., 2017)
C. colleculosa	Saudi Arabia	(Ramadani & Abulreesh, 2010)
C. kefir	Saudi Arabia	(Ramadani & Abulreesh, 2010)
C. krusei	Saudi Arabia	(Ramadani & Abulreesh, 2010)
C. guilliermondii	Japan/Saudi	(Kozaki et al., 1972)
C. obtuse	Arabia	(Teoh et al., 2004)
C. stellata	Formosa Australia	(Teoh et al., 2004)
C. famata	Indonesia	(Urbahillah et al., 2021)
C. magnoliae	Indonesia	(Urbahillah et al., 2021)
Dekkera bruxelensis	New Zealand	(B. Wang et al., 2022a)
Hanseniaspora valbyensis	New Zealand	(B. Wang et al., 2022a)
Lachancea fermentati	United State of America (USA)	(Bellut et al., 2020)
Kloeckera apiculata	NS	(Jayabalan et al., 2017)
Kluyceromyces africanus	NS	(Jayabalan et al., 2017)
Pichia (P.) fermentans	NS	(Kumar & Joshi, 2016)
P. membranefaciens	NS	(Jayabalan et al., 2011)
P. kudriavzevii	New Zealand	(B. Wang et al., 2022a)
Torulaspora delbrueckii	Australia	(Teoh et al., 2004)
Torulopsis famata	Japan	(Kozaki et al., 1972)

Zygosaccharomyces Kombucahensis sp.	Russia	(Kurtzman et al., 2001)

Note: NS = not specified.

2.4 Isolation of AAB and yeast from Kombucha

2.4.1 Isolation, enumeration, and preservation of AAB

AAB can be isolated from fermented foods and beverages including vinegar, cider and lambic beers (Yamada & Yukphan, 2008). However, it is difficult to isolate AAB using commercial media as AAB are nutritionally demanding (Gomes et al., 2018). Although there are many growth media designed for the isolation of AAB which are based on their metabolism and nutritional requirements, other microorganisms can also grow on these media (Sengun & Karabiyikli, 2011). The carbon sources utilised are mainly D-mannitol and D-glucose, with different concentrations of ethanol and acetic acid added to the medium. Nitrogen sources are mainly peptone and yeast extract with mineral salts including KH_2PO_4, Na_2PO_4 and $MgSO_4$ added to the medium to aid the recovery of the AAB cells (Gomes et al., 2018). The composition of culture media commonly used for the isolation of AAB are shown in Table A2.1 and the references are cited in the Appendices (Carr & JG, 1979; Cleenwerck et al., 2007; Cleenwerck et al., 2009; Entani et al., 1985; Fugelsang, 2007; Hestrin & Schramm, 1954; Mamlouk & Gullo, 2013; Ohmori et al., 1980; Sievers, 2005; B. Wang et al., 2022a; Wu et al., 2012; Yamada & Yukphan, 2008).

Some AAB species are not culturable, therefore, alternative techniques such as real time quantitative polymerase chain reaction (qPCR) are used to identify this population of AAB. Alternatively, epifluorescence staining techniques are also regarded as fast and simple methods for the enumeration of total viable/ non-viable AAB (Bartowsky & Henschke, 2008).

The preservation of AAB cultures is commonly achieved by sub-culturing, and storage under mineral oils, freeze-drying and cryopreservation, with frozen cultures stored at temperatures

between −70 ˚C and −150 ˚C. Cell damage caused by ultra-low temperatures can be prevented by adding cryoprotectants such as glycerol (10–25%) and dimethyl sulfoxide (DMSO) (5%). However, glycerol is not suitable for the AAB that form cellulose structures such as *K. xylinus*. In these instances, DMSO is preferable as it can maintain stability and high viability without affecting the cellulose structure (Wiegand & Klemm, 2006).

2.4.2 Isolation and enumeration of yeast

Conventional enumeration of yeast is carried out by spread plating as this technique allows microorganisms to be exposed to oxygen and avoids stress caused by hot or warm culture medium (Da Silva et al., 2018). There are also numerous commercial media available for the isolation and cultivation of yeast from foods including Kombucha (Table A2.2) (Atlas, 2004; Deak, 2007). These media provide the basic nutrients to support the growth of yeast and lower the pH to inhibit the growth of bacteria and moulds. Antibiotics such as penicillin can also be added to prevent the growth of bacteria and moulds (Deak, 2007).

2.5 Phenotypic characterisation and identification of acetic acid bacteria and yeast from Kombucha

2.5.1 Phenotypic characterisation of AAB

The traditional classification of AAB species includes differentiation by cellular morphology, flagellation, and physiological and biochemical properties (Kurtzman et al., 2011). Colony examination involves size, form, elevation, and colour. The cellular morphology of bacteria includes the cell shape, size and response to Gram reaction, motility, spore-forming and cellular arrangement (Schifferdecker et al., 2014).

AAB are commonly Gram-negative, aerobic rods or ellipsoidal non-spore forming cells, which can be single, in pairs, or clusters. Cell sizes range from 0.4 to 1.0 μm in width and 0.8 to 4.5 μm in length (Malimas et al., 2017). These bacteria are mostly catalase-positive and oxidase

negative. The optimum growth temperature varies from 25 ℃ to 30 ℃, however, some thermotolerant strains can grow at temperatures up to 42 ℃ (Lynch et al., 2019), and some AAB can grow under acidic conditions (e.g., pH 3.5) (Yamada & Yukphan, 2008).

The classification of AAB comprises five chemical properties which are: catalase positive, oxidation of ethanol to acetic acid, over-oxidation of ethanol to water and CO_2, oxidation of lactate to CO_2 and water, ketogenesis from glycerol and hydrolysis of D-glucose to different acids (Frateur, 1950). Initially, AAB were classified into *Acetobacter* and *Gluconobacter.* The genus *Acetobacter* has peritrichous flagella and can oxidise acetate and lactate. In contrast, the genus *Gluconobacter* lacks the ability to oxidise acetate and lactate, however, they can oxidise D-glucose to 2-ketogluconate and 5-ketogluconate. The main difference between the genera *Acetobacter* and *Gluconobacter* is the presence of ubiquinone 9 in the genus *Acetobacter*, while ubiquinone-10 is present in the genus *Gluconobacter* (Yamada et al., 1976). In 1997, a new genus, *Gluconacetobacter*, was reported with partial 16 ribosomal sequencing techniques and the species containing coenzyme Q10 (Cleenwerck et al., 2009). Later, another new genus, *Komagataeibacter*, was introduced based on 16S rRNA gene sequencing, phenotypic properties and different morphology to *Gluconacetobacter* (Yamada & Yukphan, 2008). The 11 species from the genus *Gluconacetobacter* were reclassified as *Komagataeibacter* based on their phenotypic and genotypic characteristics. Compared with *Gluconacetobacter*, the species of genus *Komagataeibacter* are not motile, and unable to produce a water-soluble brown pigment on glucose peptone yeast extract and calcium carbonate medium. The genus *Gluconacetobacter* can produce 2,5-diketo-D- gluconate but not *Komagataeibacter*. All AAB can oxidise sugars such as glucose, fructose, galactose, mannose, ribose, and xylose through the cytoplasmic hexose monophosphate pathway (Cleenwerck et al., 2009). Furthermore, AAB can oxidise sugar alcohols such as glycerol and convert them to dihydroxyacetone (DHA). Additionally, the ability to produce a cellulose structure is mainly found in the genera

Gluconacetobacter and *Komagataebacter*; these differential properties help to distinguish AAB at the genus or species level. The main biochemical characteristics of the genera AAB applied in the food industry are shown in Table 2.5.

Table 2.5 Differential characteristics of the genera *Acetobacter, Gluconacetobacter, Gluconobacter*, and *Komagataeibacter* commonly associated with food.

Characteristics	***Acetobacter***	***Gluconacetobacter***	***Gluconobacter***	***Komagataeibacter***
Cell shape	Ellipsoidal to rods	Ellipsoidal to rods	Ellipsoidal to Rods	Coccoid to rods
Cell size (μm)	0.4-1.0 x 1.0-3.0	0.5-0.9 x 1.0-2.0	0.6-1.0 x 1.0-3.0	0.6-0.8x1.0-3.0
Colonies appearance	Creamy to brown	Light brown to brownish	Smooth, entire, shinny, white, pink or brown	Raised, convex to umbonate, smooth to rough, entire to irregular
Catalase	+	+	+	+
Gram staining	Gram negative	Gram negative	Gram negative	Gram negative
Oxidase	-	-	-	-
Motility	Motile or non-motile	Motile or non-motile	Non-motile	No
Flagellation	Peritrichous	peritrichous	polar	No
Oxidation of ethanol to acetic acid	+	+	+	+
Oxidation of acetic acid to CO_2 and water	+	+	-	+
Oxidation of lactate/acetate to CO_2 and water	+	+	-	+
Production of cellulose	-	±	-	±
Production of water-soluble brown pigment	-	+	±	-
Nitrate reduction	±	-	-	ND
Gelatin liquefaction	-	-	+	ND
Indole formation	+	+	+	ND
Formation of H_2S	+	+	+	ND
Growth on 0.35% acetic acid containing medium	+	+	+	+
Growth in the presence of 1% potassium nitrate (KNO_3)	-	-	-	ND
Growth on methanol as carbon source	±	-	-	ND
Growth on the presence of 30% D-glucose	-	±	±	ND

Growth on 1% of glucose	+	+	+	+
Ketogenesis (dihydroxyacetone) from glycerol	+	+	+	+
Acid production from	±-	+	+	ND
1) Glycerol	-	-	+	-
2) D-Mannitol	-	-	-	ND
3) Raffinose		-	+	-
4) Sorbitol				
Production of from D-glucose of				
1) 2-keto-D-gluconic acid	±	±	+	±
	±	±	±	-
2) 5-keto-D-gluconic acid	±	±		
3) 2.5-Keto-D-gluconic acid				
Production of DHA from glycerol	±	±	+	+
Production of levan-like polysaccharide from sucrose	±	-	-	-
Ubiquinone type	Q9	Q10	Q10	Q10
G+C content (mol %)	50.5-60.3	55-67	52-64	56-64
Maintenance	2 weeks at 4ºC; frozen at -75ºC in the presence of 24% (v/v) glycerol	3-4 weeks at 4-5ºC; frozen at -75ºC in the presence of 24% (v/v) glycerol	3-4 weeks at 4-5ºC; Frozen at -75ºC in the presence of 24% (v/v) glycerol	3-4 weeks at 4-5ºC; Frozen at -75ºC in the presence of 24% (v/v) glycerol
Sources	Flowers, fruits, palm wine, vinegar, kefir	Rhizosphere of coffee plants, roots and stem of sugar cane	Strawberry, grape and spoiled jackfruit and sugar rich environments	Kombucha, vinegar, wine vinegar

Source: (Mamlouk & Gullo, 2013; Ray, 2016; Saichana et al., 2015; Yamada & Yukphan, 2008).
Note: "+" 90% or more strains shown positive results； "-" 90% or more strains shown negative results; ND: Not data; "±": strains are positive or negative.

2.5.2 Phenotypic characterisation and identification of yeast from Kombucha

The phenotypic identification of yeast from Kombucha is mainly achieved by morphological, and physiological tests and the use of rapid commercial yeast identification kits such as ID 32 C (Lin & Fung, 1987). Traditionally, it is time-consuming to conduct physiological (identification) tests at the species level. The morphological characteristics of yeast cells are important for their identification as they may be produced by different reproduction modes as shown in Table 2.6 (Boekhout & Robert, 2003). For example, the budding formation has been

observed from yeast species, *Z. kombuchaensis* isolated from a dried tea fungus of Russian Kombucha (Kurtzman et al., 2001). Cellular morphological tests are usually obtained using the wet-mount method, by suspending the culture in saline and mixing it with dyes such as India ink, lactophenol cotton blue, calcofluor white or methylene blue staining (Sangeetha & Thangadurai, 2013).

Table 2.6 Morphology of yeast isolated from Kombucha.

Different reproduction mode of yeast	Morphology characteristics of yeast
Vegetative or asexual reproduction	Budding: new cell is produced on the surface of parent cell and then separate Fission: an asexual cell is produced by a septum grown inward from cell wall to halve the long axis of the cell. Blastoconidiation: a mother cell of stalk-like tubular sterigmata produces a terminal conidium.
Sexual reproduction in ascomycetous yeast	Parent cell-bud conjugation Gametangial conjugation Heterothallism conjugation Conjugation between hyphae
Sexual reproduction in basdiomycetous yeast	Budding haplophase Dikaryotic hyphal phase or self-spore forming diplophase

Source: (Boekhout & Robert, 2003).

Physiological and biochemical tests (Table 2.7) can be conducted representative purified yeast to characterise the species present in food samples including Kombucha (Deak, 2007; Kurtzman et al., 2011). Kurtzman, Robnett and Basehoar-Powers conducted several nitrogen assimilation and carbohydrate fermentation tests in conjunction with other growth tests to determine the phenotypic characteristics of five new ascosporogeneous strains belonging to *Z. kombuchaensis*, which were isolated from Kombucha (Kurtzman et al., 2001).

Table 2.7 Physiological and biochemical tests used for the characterisation of yeast.

Physiological test	Biochemical test
Assimilation of carbon and nitrogen sources	Diazonium Blue B reaction

Fermentation of carbohydrates	Urease test
Growth at different temperature	
Growth in vitamin-free medium	
Growth in high osmotic pressure condition	
Starch hydrolysis activity	

Source: (Deak, 2007; Kurtzman et al., 2011).

Identification of yeast using commercial Kits

Several commercial identification systems based on conventional carbon fermentation or nitrogen assimilation reactions have been designed for rapid and accurate identification of food-related yeast in combination with morphological characterisation (Deak, 2007). The ability of yeast to grow on different carbon and nitrogen sources can be determined by the formation of turbidity or colour changes in the presence of a pH indicator (Lin & Fung, 1987). A description of common commercial systems such as API 20 C suitable for use in identifying yeast from Kombucha is summarised in Table 2.8.

Table 2.8 Summary of tests and accuracy of common commercial yeast identification systems.

Commercial system	**Description of tests and controls included in the kits**	**Incubation condition**	**Accuracy**	**References**
API 20 C	19 carbon assimilation test and 1 control test in 20 strips	30ºC for 72 h	98.9%	(St Germain & Beauchesne, 1991)
API Candida	5 carbohydrate and 7 enzyme colorimetric test in 10 strips	35ºC for 18-24 h	97.4%	(Fricker-Hidalgo et al., 1995)
API 32 C	29 assimilation tests (carbohydrate, organic acids, and amino acids); 1 negative control,1 susceptibility test (cycloheximide) and 1 colorimetric tests (esculin) in 32 wells. Includes 63 different species in database	30ºC for 48 h	92%	(Ramani et al., 1998)
Auxacolor system	13 carbohydrate tests with bromocresol purple, test for cycloheximide resistance and phenoloxidase production in 16 wells.	37ºC for 48 h	79.4%	(Milan et al., 1997)
RapID Yeast Plus system	5 carbon assimilation tests and 13 enzymatic hydrolysis substrate tests	30ºCfor 4h	96%	(Espinel-Ingroff et al., 1998)
The Uni-Yeast-Tek (UYT) system	7 carbon assimilation tests, urease, Nitrate and corn meal with Tween 80 agar	22-26ºC for 2-10 days.	99.8%	(Bowman & Ahearn, 1975)

MicroScan yeast identification panel	13 aminopeptidase, 3 carbohydrates, 9 glycosidase, phosphatase and urease tests.	37ºC for 4 h.	86.9%	(Land et al., 1975)
VITEK 2 YST	4 aminopetidase, 25 carbohydrate, esculin, 3 glycosidase, nitrate, 2 nitrogen, 9 organic acid, and urea tests	35ºC for 18 h	94.8%	(Aubertine et al., 2006)

Recently, several commercial kits have been successfully used to characterise yeast isolated from Kombucha. For example, the API 32 C system was used to characterise the carbohydrate assimilation pattern of the yeast strain, *Lanchacea fermentati* isolated from Kombucha (Bellut et al., 2020). Three different yeast species, *C. famata*, *C. krusei* and *C. magnoliae* from Kombucha SCOBY were identified using the API 20 C kit (Urbahillah et al., 2021). Commercial databases (Table 2.8) also contain yeast species isolated from Kombucha samples; hence, these commercial yeast identification systems may also be applied in the identification of yeast isolated from Kombucha by conducting different carbohydrate and nitrogen assimilation tests and comparing the results to the database.

2.6 Genotypic identification of AAB and yeast from Kombucha

2.6.1 Genotypic identification of AAB from Kombucha

It is difficult to identify AAB to species level using only phenotypic characteristics (Gomes et al., 2018). Compared with conventional biochemical and physiological identification, molecular techniques are generally more reliable and rapid (Abd El-Salam, 2012). Several DNA sequence-based techniques involving DNA extraction and polymerase chain reaction (PCR) have been widely applied to identify AAB to genera, species, or strain levels by comparing with reference strains (Andrés-Barrao et al., 2017). Commonly used DNA-based techniques applied for the identification of AAB are shown in Table 2.9.

Table 2.9 Commonly used molecular techniques applied in AAB identification and genotyping.

Techniques	Level	Advantages	Disadvantages
PCR-RFLP	Species	Rapid and convenient to setup	Difficult to identify small insertion and expensive.

DGGE	Species	Rapid and cost effective	Cannot to discriminate closely species.
RT-PCR	Species	Able to enumerate the specific PCR products, fast and reliable	Complex and expensive
RAPD	Strains	Not require designing primers, quick and simple.	The quality and concentration of template DNA influence the results
ALFP	Strains	Can be used for any DNA samples of any origins, reveal multiple polymorphic bands in one lane.	Complex and sensitive

Methods commonly used to discriminate AAB to species level include polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the 16S rRNA genes or 16–23S internal transcribed spacer (ITS), PCR amplification and direct sequencing of 16S rDNAs and 16S–23S ITS, denaturing gradient gel electrophoresis (DGGE) of partial 16S rRNA gene, and real-time PCR (qPCR) (Andrés-Barrao et al., 2017). The PCR-RFLP method involves amplification of the targeted regions of the 16S rRNA gene followed by digestion of the PCR products with restriction enzymes. The resultant DNA fragments are then separated by polyacrylamide or agarose gel electrophoresis. AAB isolates can be identified by comparing their restriction profiles with those of the reference strains (Rasmussen, 2012). Terminal restriction fragment length polymorphism (T-RFLP) is a modified version of PCR-RFLP, and it involves the use of fluorescent-labelled primers for detecting characteristic restriction fragments. T-RFLP has been used to determine the dynamic changes of AAB during the Kombucha fermentation process, and *Komagataeibacter* was revealed to be the dominant genus in both the tea fungus and the broth (Chakravorty et al., 2016). DGGE is commonly used to determine the diversity or species composition of AAB from fermented foods such as rice vinegar (Sengun & Karabiyikli, 2011). The principle of this technique is to separate DNA fragments of the same length on the basis of their differences in melting point, which is

determined by the nucleotide sequence of the DNA molecule (Andrés-Barrao et al., 2017). Thus, DGGE has a higher resolution than RFLP or T-RFLP in the discrimination of closely related AAB species.

Real-time PCR, also known as quantitative PCR (qPCR), is a well-established method that has been successfully used in the detection and quantification of AAB present in Kombucha. During each cycle of the qPCR reaction, fluorescence released by DNA-binding fluorescent dye or oligonucleotide probes is automatically measured as a proxy of the amplified DNA (Kubista et al., 2006). The amount of target gene (i.e., 16S rRNA) in the sample is then determined by calculating the cycle threshold (Ct), which is positively correlated with colony forming unit (CFU). qPCR is thus suitable for measuring AAB abundance in commercial Kombucha SCOBY (Harrison & Curtin, 2021).

To genetically validate the taxonomic identity of an AAB isolate, the full 16S rRNA gene is normally amplified by PCR and subsequently sequenced using the classic method of Sanger sequencing. However, along with the technical advancement of next-generation sequencing (NGS), the Illumina MiSeq has become a popular and powerful technique for determining microbial community structure and species composition. Briefly, total DNA is extracted from a Kombucha sample and then subjected to PCR amplification using general primers that target the V1–V3 (or V3–V4) region of the 16S rRNA gene (Coton et al., 2017; Reva et al., 2015; Savary et al., 2021). The resultant amplicons are then sequenced using the Illumina MiSeq platform. An operational taxonomic unit (OTU) is assigned at a given level of sequence similarity (e.g., 97%). Moreover, the NGS technology has recently been extended beyond the analysis of targeted rRNA genes, and shotgun metagenomic sequencing enables the analysis of whole genomic DNAs that are extracted from a particular sample (Pradhan et al., 2022). For example, Arikan and other researchers analysed the microbial communities of homemade Kombucha fermentations using a combination of metagenomic sequencing and 16S rRNA

amplicon sequencing (Arikan et al., 2020). Both methods consistently revealed that *Komagataeibacter* was the dominant bacterial genus. Interestingly, a search of secondary metabolite genes in the metagenome-assembled genomes identified novel gene clusters for bacteriocin production. Bacteriocins are a group of small antimicrobial peptides against closely related bacteria. The finding may partially explain the antimicrobial properties of Kombucha.

For identification to strain level, four PCR-based fingerprinting techniques are currently available: random amplification of polymorphic DNA (RAPD), amplified length fragments polymorphism (ALFP), enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), and repetitive extragenic palindromic PCR (REP-PCR) (Andrés-Barrao et al., 2017). The RAPD technique involves the amplification of random regions of genomic DNA with a short (~10 nucleotides) single arbitrary primer under low annealing temperatures (Kumar & Gurusubramanian, 2011). RAPD has been successfully used to identify the dominant bacterial composition of both tea fungus and the Kombucha broth (Gaggia et al., 2018). In contrast, the ALFP technique selectively amplifies a subset of digested DNA fragments to generate a unique restriction profile for each AAB genome. ALFP involves the use of a pair of specifically designed primers, which consists of three parts: a core sequence, a restriction site sequence, and a 3′ selective sequence (Andrés-Barrao et al., 2017). Both REP- and ERIC-PCR target repeat sequences that are commonly found in bacterial genomes. Both techniques can type AAB isolates to strain level, but ERI-PCR is more suitable for AAB due to its higher accuracy (González et al., 2004). Interestingly, REP- and ERIC-PCR can be used in combination to increase the sensitivity of these two fingerprinting techniques, as shown in a case study with cellulose-forming AAB isolates from Kombucha (La China et al., 2021). Molecular techniques commonly applied in the identification of the Kombucha bacterial community are summarised in Table 2.10. The specific organisms and primers are linked to the work in reference in Table 2.10.

Table 2.10 Molecular techniques commonly used for AAB identifications.

Method	Microorganism	Primer Sequence (5′–3′)	References
16S rRNA, Sanger sequencing	*Gluconacetobacter; Komagataeibacter;*	27F, AGAGTTTGATCMTGGCTCAG 1494R, TGACTGACTGAGGYTACCTTGTTACGACTT	(Gaggia et al., 2018)
	Gluconacetobacter; kombuchae sp. nov.;	fD1, CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG rD1, CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC	(Dutta & Gachhui, 2007)
	Acetobacter aceti;	16S d, GCTGGCGGCATGCTTAACACA 16S r, GCAGGTGATCCAGCCGCA	(Abd El-Salam, 2012)
16S rRNA V1-V3 region, Illumina MiSeq	Bacterial communities	Forward, TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG Reverse, GTVTVGTGGGCTCGGAGATGTGTATAAGAGACAG	(González et al., 2004)
16S rRNA V3-V4 region, Illumina MiSeq	Bacterial communities	Forward, CCTACGGGNGGCWGCAG Reverse, GACTACHVGGGTATCTAATCC	(Coton et al., 2017) (La China et al., 2021)
16S rRNA, Real-time PCR	Bacterial abundance	926f, AAACTCAAKGAATTGACGG 1062r, CTCACRRCACGAGCTGAC	(Harrison & Curtin, 2021)
16S rRNA, T-RFLP	Bacterial communities	27F, AGAGTTTGATCMTGGCTCAG 1525R, AAGGAGGTGATCCAGCC	(Chakravorty et al., 2016)
RAPD	*Komagataeibacter* spp.	M13, GAGGGTGGCGGTTCT	(Pradhan et al., 2022)
AFLP	*Komagataeibacter rhaeticus*	A03, GACTGCGTACAGGCCCCG T03, CGATGAGTCCTGACCGAG	(Semjonovs et al., 2017)
REP-PCR	*G. oxydans; A. aceti*	REPIR-I, IIICGICGICATCIGGC REP2-I, ICGICTTATCIGGCCTAC	(Arikan et al., 2020)
ERIC-PCR	*G. oxydans; A. aceti*	ERIC1R, ATGTAAGCTCCTGGGGATTCAC ERIC2, AAGTAAGTGACTGGGGTGAGCG	(Arikan et al., 2020)
Shotgun metagenomic sequencing	Bacterial and fungal communities		(Reva et al., 2015) (Coton et al., 2017)

2.6.2 Genotypic identification of yeast

Different rapid commercial kits such as API 20 C and API 32 C are convenient for yeast identification, however, they are associated with minor differences in biochemical profiles due

to variabilities in test conditions. Results from commercial kits need to correlate to morphological observations to identify the yeast at the species level. For instance, the species from *Dekkera* are the anamorphs of *Brettanomyces* and they are deficient in sexual characteristics. Thus, the biochemical profiles of these fungi with limited morphological features are not stable and therefore difficult to differentiate (Kurtzman & Robnett, 1998). Consequently, accurate identification of yeast strains often needs a combination of conventional biochemical methods and modern molecular biology techniques.

Genotypic identification of yeast is mostly focused on sequence variations in the ribosomal DNA (rDNA) region, which includes 18S, 5.8S, and 26S rRNA genes separated by two internal transcribed spacers (ITSI and ITS2). Several universal primers have been designed for genus- or species-specific identification and the D1/D2 domain of 26S rDNA is the most popular target (Table 2.11). Yeast strains that differ by more than 1% in the 26S rDNA D1/D2 region are generally considered distinct species (Pincus et al., 2007). As mentioned above, high-throughput sequencing techniques such as Illumina MiSeq have also been used to examine the yeast communities in Kombucha, and the analysis can target either specific rRNA genes or the whole metagenome (Coton et al., 2017). The relative abundance of yeast in Kombucha can be estimated using the real-time PCR method with one pair of specifically designed primers, which amplify a 124 bp region in the variable D1/D2 domain of the 26S rRNA gene (Mackay, 2004). Finally, it should be noted that other PCR-based techniques such as AFLP, RAPD and REP-PCR have been successfully developed for genetic identification of yeast at the sub-species or strains levels (Pincus et al., 2007).

Table 2.11 Molecular techniques used for the identification of yeast present in Kombucha.

Method	Microorganism	Primer Sequence (5′–3′)	References
26S rDNA, D1/D2 domain	*Brettanomyces/Dekkera*; *Pichia;* *B. bruxellensis*;	NL1, GCATATCAATAAGCGGAGGAAAAG NL4, GGTCCGTGTTTCAAGACGG	(Gaggia et al., 2018) (Pradhan et al., 2022)

	D. bruxellensis; *Hanseniaspora (H.) uvarum*		(La China et al., 2021)
26S rDNA, D1/D2 domain	*D. bruxellensis*; *Dekkera (D.) anaomala* *Z. bailii*; *H. valbyensis*	NL1, GCATATCAATAAGCGGAGGAAAAG NL4, GGTCCGTGTTTCAAGACGG	(González et al., 2004)
18S rDNA, D1/D2 domain	*Z. kombuchaensis* sp.	NS-1, GTAGTCATATGCTTGTCTC NS-8A, CCTTCCGCAGGTTCACCTACGGAAACC	(Kurtzman et al., 2011)
ITS, Illumina MiSeq	*Z. bailii*	ITS1F, CTTGGTCATTTAGAGGAAGTAA ITS2R, GCTGCGTTCTTCATCGATGC	(Coton et al., 2017)
Real-time PCR	*Brettanomyces*	Yeast-F, GAGTCGAGTTGTTTGGGAATGC Yeast-R, TCTCTTTCCAAAGTTCTTTTCATCTTT	(Harrison & Curtin, 2021)
PCR-ITS RFLP	*D. bruxellensis*;	ITS1, TCCGTAGGTGAACCTGCGG ITS4, TCCTCCGCTTATTGATATGC	(Matei et al., 2018)

2.7 Conclusion and future research on Kombucha

Kombucha is a refreshing “live” fermented beverage, and its popularity is partially derived from this characteristic. While previous studies have shown that phenotypic identification provides useful metabolic characteristics of the microbes in Kombucha, molecular techniques, mostly based on PCR, can provide more accurate, rapid and reliable identification of the microbiological composition of AAB and yeast at different phylogenetic classification levels. The microbial composition of the Kombucha starter culture is diverse and is largely undefined. Further, the kinetic growth pattern of dominant fermenting microbes during fermentation are poorly described. Detailed information on the dominant bacteria and yeast responsible for the fermentation of Kombucha would be useful for better control of commercial fermentation processes during the production of safe, high-quality products. This review has highlighted the need for more information on the systematic identification methods of dominant yeast and AAB in commercial Kombucha beverages. In addition, although the presence of live cultures in Kombucha has been associated with its probiotic potential, this has been scantly reported. Therefore, more studies are needed to fill this gap in the literature.

Chapter 3. Microbiological and Physico-Chemical Characteristics of Black Tea Kombucha Fermented with a New Zealand Starter Culture

3.1 Abstract

Kombucha is a popular sparkling sugared tea, fermented by a symbiotic culture of acetic acid bacteria (AAB) and yeast. The demand for Kombucha continues to increase worldwide mainly due to its perceived health benefits and appealing sensory properties. This study isolated and characterised the dominant AAB and yeast from starter culture and Kombucha broth after 0, 1, 3, 5, 7, 9, 11, and 14 days of fermentation at ambient temperature (22°C). Yeast and AAB were isolated from the Kombucha samples using glucose yeast extract mannitol ethanol acetic acid (GYMEA) and yeast extract glucose chloramphenicol (YGC) media respectively. The phenotypic and taxonomic identification of AAB and yeast were determined by morphological and biochemical characterisation, followed by a sequence analysis of the ribosomal RNA gene (16S rRNA for AAB and ITS for yeast). The changes in the microbial composition were associated with variations in the physico-chemical characteristics of Kombucha tea, such as pH, titratable acidity, and total soluble solids (TSS). During fermentation, the acidity increased, and TSS decreased. The yield, moisture content and water activity of the cellulosic pellicles which had developed at the end of fermentation were attributed to the presence of AAB. The dominant AAB species in the cellulosic pellicles and Kombucha broth were identified as *Komagataeibacter rhaeticus*. The yeast isolates belonged to *Debaryomyces prosopidis* and *Zygosaccharomyces lentus*.

3.2 Introduction

Kombucha is a popular functional sparkling fermented tea beverage that has been consumed for more than 5000 years (Dufresne & Farnworth, 2000). The taste of Kombucha varies from slightly fruity, sour and fizzy to vinegary, depending on the fermentation conditions. For

example, a longer fermentation time of Kombucha can result in a sour taste (B. Wang et al., 2022a). The regular consumption of Kombucha is perceived to confer numerous health-promoting benefits including antioxidants, antimicrobial, anti-carcinogenic, detoxification, antitumor and antihypertensive activities (Neffe-Skocińska et al., 2017; B. Wang et al., 2022a, 2022b). However, most of the perceived health benefits are based on *in vitro* models. Therefore, *in vivo* clinical trials are necessary to confirm the functional activities.

Kombucha is usually fermented from sucrose-sweetened black or green tea with an undefined symbiotic culture of bacteria and yeast (SCOBY) embedded in a cellulosic pellicle at ambient temperature for 7-14 days (Bishop et al., 2022; Laureys et al., 2020; Nyiew et al., 2022). Other tea varieties such as oolong tea, thyme, lemon balm, peppermint, rosemary, wheat grass, guava and oak leaves, fruit juices, milk and laver have also been used to make Kombucha (Aung & Eun, 2022; Dutta & Paul, 2019; Nyiew et al., 2022; Velićanski et al., 2013). During fermentation, sucrose is firstly hydrolysed by yeast to glucose and fructose. The glucose produced is then metabolised into gluconic and glucuronic acids by the AAB, whereas fructose is metabolised by yeast to produce ethanol and carbon dioxide. The resulting alcohol is further metabolised by the AAB into acetic acid. After fermentation, Kombucha contains a complex chemical composition and favourable bioactive components including organic acids (acetic, gluconic, lactic and glucuronic acids), polyphenols (epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate), vitamins (B_1, B_2, B_6, B_{12}, and C), minerals (Cu, Fe, Mn, Ni, Zn), ethanol and amino acids (Coelho et al., 2020; Emiljanowicz & Malinowska-Pańczyk, 2020; Neffe-Skocińska et al., 2017). The variations in the chemical and nutritional metabolites present in Kombucha may be attributed to the initial concentration of starter culture added, to its diverse microbial composition, as well as to the amount of sugar (mostly sucrose), the fermentation conditions (temperature and time), and the type of tea used (Savary et al., 2021; B. Wang et al., 2022a).

The microbial composition of Kombucha is diverse and varies between fermentations, but is mainly composed of AAB and yeast (Greenwalt et al., 2000). Previous studies indicate that gluconic acid-producing AAB are the dominant bacteria in the Kombucha culture (Jayabalan et al., 2014). The most common AAB include *Komagataibacter (K.) kombuchae, K. saccharivorans, K. rhaeticus, K. intermedius, K. europaeus, K. hansenii,* and *K. xylinus*; *Gluconobacter (G.) oxydans, G. potus; Gluconacetobacter (G.) sacchari, G.* sp A4; *Acetobacter (A.) musti*, *A. pasteurianus, A. aceti,* and *A. nitrogenifigens* sp. nov. (Coelho et al., 2020; Jayabalan et al., 2011; Mukadam et al., 2016; B. Wang et al., 2022a). Small amounts of lactic acid bacteria (LAB) have also been reported in Kombucha (Laureys et al., 2020), including *Lacticaseibacillus casei, Lactiplantibacillus plantarum, Lactobacillus rhamnosus, Lactobacillus mali, Pediococcus pentosaceus, P. acidiliactici, Liqourilactobacillus nagelii*, *Oenococcus oeni* and *Bifidobacterium* (Bogdan et al., 2018; Coton et al., 2017; Diguta et al., 2020; Nguyen et al., 2015; Pei et al., 2020; Yang et al., 2022). There is scant information on the role of LAB in Kombucha, thus, this is an area for future study. The most commonly reported yeast species in Kombucha are *Brettanomyces (B.) bruxellensis, B. lambicus, B. intermedius; Candida (C.) albican, C. kefir, C. zemplinina; Dekkera (D.) bruxelensis,* and *D. anomala; Hanseniaspora (H.) valbyensis; Pichia (P.) fermentans, P. occidentalis,* and *P. kudriavzevii; Saccharomyces (S.) cerevisiae; Schizosaccharomyces (S.) pombe;* and *Zygosaccharomyces (Z.) bailii* and *Z. rouxii* (Jayabalan et al., 2017; Mayser et al., 1995; Ramadani & Abulreesh, 2010; Savary et al., 2021; Teoh et al., 2004; Villarreal-Soto et al., 2020). The interactions between the bacteria and yeast produce Kombucha with different chemical compositions (Villarreal-Soto et al., 2018).

The unique cellulose forming characteristics of Kombucha may be useful for a variety of new applications in medicine and food production. The SCOBY has been used as artificial skin to treat wounds or burnt skin (Czaja et al., 2007). Dehydrated SCOBY has potential in the

production of Kombucha berry gummies and SCOBY hamburger due to its unique texture and flavour (Coelho et al., 2020). Therefore, it is important to determine and optimise the yield of the cellulosic pellicle from Kombucha fermentation for these innovative applications.

The current challenges for industrial production of Kombucha include the limited knowledge about the dynamics of its microbial community such as the distribution of the microbial composition and lack of standardised fermentation process (Laureys et al., 2020; Oliveira et al., 2022). The diversity in the microbial composition of Kombucha may be caused by differences in temperature, types of tea, climate, and also environmental conditions (Bishop et al., 2022). An improved understanding of the microbial composition of the SCOBY, and the changes in the levels of microorganisms during fermentation, will contribute to better control of Kombucha production.

This study determined the dominant microbial composition, and physico-chemical characteristics, of the Kombucha tea broth during fermentation and the cellulosic pellicle. The microbial communities of Kombucha and cellulosic pellicle during fermentation were determined by culture-dependent methods and ribosomal genotyping. The present study provides an insight into the microbiological distribution of dominant AAB and yeast in the starter culture, cellulosic pellicle, and broth during the fermentation of traditional black tea Kombucha.

3.3 Material and methods

3.3.1 Preparation of fermented Kombucha

The Kombucha starter culture (cellulosic pellicle and liquid broth) was supplied by Mamas Brewshop Ltd in Auckland, New Zealand in 2021. The samples were transported to the Food Technology Department at Massey University, Auckland, under cold chain maintained at 4 °C,

after which they were stored at refrigeration temperature (4°C) until required for further analysis (Figure 3.1).

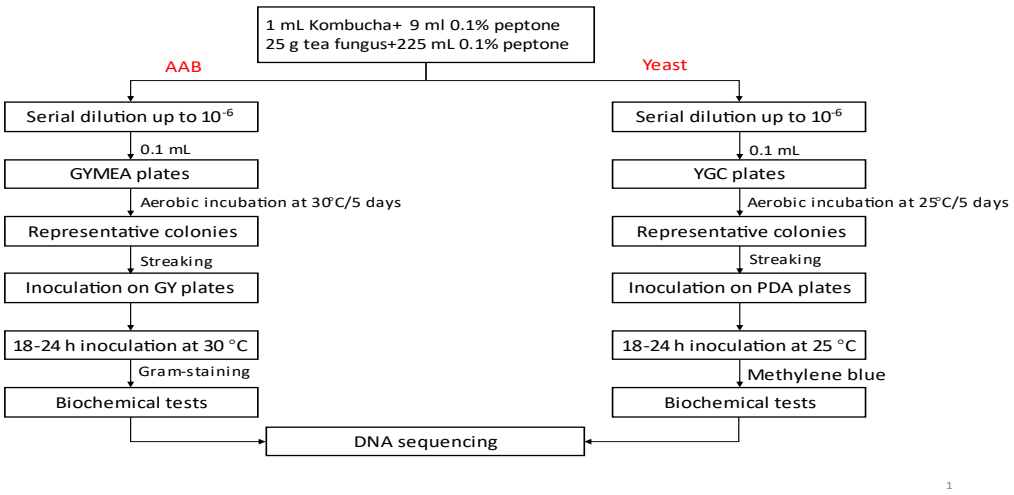

Figure 3.1 Various microbial analyses of Kombucha tea broth and cellulosic pellicle. Adapted from B. Wang et al. (2022b).

3.3.2 Kombucha fermentation progress

Laboratory-scale fermented Kombucha was prepared using the modified methods of Greenwalt et al. (2000) and Jayabalan et al. (2014). Sucrose (50 g/L) (white sugar, Chelsea, New Zealand) was added into boiling water and mixed until completely dissolved. Black tea bags (Bell®, New Zealand) (5 g/L) from a local supermarket were added to the mixture and allowed to infuse for 10 min then removed. The sugared tea was cooled to ambient temperature (~22 °C). The entire SCOBY (25 g/L) was gently placed on the surface of the sugared tea and a previous portion of fermented Kombucha tea broth (20 %, v/v) was added to reduce the pH to prevent growth of spoilage microorganisms, as well as promote the growth of fermenting microorganisms. The container was covered with a clean disposable cloth to keep out foreign matter. The fabric of the cloth still allowed adequate aeration of the broth which is essential for the growth of AAB. Fermentation was carried out at 22 °C for 14 days in a water bath (T100, Grant Optima, Cambridge, UK). Kombucha tea broth samples were collected for immediate physico-chemical

and microbiological analyses at day 0, then every 2 days until the end of fermentation. The newly formed cellulosic pellicle (25 g) and samples (20 mL) of the fermented Kombucha broth were collected at the end of fermentation. The two components (broth and cellulose) were maintained in fresh sugared tea broth at 4 °C for the next fermentation.

3.3.3 Physico-chemical characteristics of Kombucha broth samples during fermentation

3.3.3.1 Determination of pH, titratable acidity (TA), and total soluble solids (TSS) of Kombucha tea broth during fermentation

The pH of the Kombucha tea broth collected at different time points was measured using a Sartorius glass electrode pH meter (Model PH-11, Goettingen, Germany). Titratable acidity was determined by acid-base titration. Briefly, Kombucha tea broth samples (20 mL) from different stages of fermentation were titrated against standardised 0.1 M sodium hydroxide to pH 8.2. The calculated TA was expressed as a percentage (%) of acetic acid per gramme of sample, as it is the major organic acid in Kombucha (Equation 3.1) (Waisundara, 2018). The TSS of Kombucha tea broth samples were determined using a refractometer (Atago, pr-32 alpha, UK), according to Amarasinghe et al. (2018).

$$\% \text{ Titratable acidity of acetic acid} = \frac{V_{NaOH} * M_{NaOH} * 6.0053}{V_{sample}} \quad \text{----------[Equation 3.1]}$$

V_{NaOH} = volume of NaOH (mL)
M_{NaOH} = molarity of NaOH (M)
V_{sample} = volume of sample (mL)

3.3.3.2 Determination of wet yield, water holding capacity (WHC), moisture content and water activity of cellulosic pellicle at the end of the fermentation

The wet yield the fresh pellicle produced during fermentation was determined using the method of Aung and Eun (2021) with slight modifications. The weight of the initial mother pellicle

used in the fermentation was measured at day 0 and at the end of fermentation (day 14). The wet yield of tea fungus was calculated using Equation 3.2.

$$\text{Wet Yield (\%)} = \frac{\text{weight of total fungus at day14} - \text{weight of total fungus at day 0}}{\text{weight of total fungus at day 0}} \text{----------[Equation 3.2]}$$

The water holding capacity (WHC) and moisture content were determined using the methods of Avcioglu et al. (2021) with slight modifications. The fresh cellulosic pellicle was cut into small pieces and mixed by blending (BB25EP, Waring, New York, USA) for one minute. About 5-10 g of the pellicle were weighed into an aluminium pan and dried at 70 °C overnight. The WHC and moisture content were calculated using Equations 3.3 and 3.4, respectively.

$$\text{WHC} = \frac{\text{weight of water remove during drying (g)}}{\text{dry weigtht of tea fungus (g)}} \text{----------[Equation 3.3]}$$

$$\text{Moisture content} = \left(\frac{w2-w3}{w2-w1}\right) * 100\% \text{----------[Equation 3.4]}$$

W1: weight of empty container (g)
W2: weight of container and sample before drying (g)
W3: weight of container and sample after drying (g)

The water activity of the pellicle was measured using a water activity meter (AQUALAB 4TE, Pullman, WA, USA).

3.3.4 Isolation and enumeration of AAB, LAB, and yeast isolated from Kombucha

Samples of both the cellulosic pellicle and Kombucha tea collected separately during fermentation, were used for the isolation and identification of AAB, LAB, and yeast. The newly formed pellicle developed on the surface of mother pellicle at the end of the fermentation was gently separately by sanitised hands. Serial dilutions (10^{-6}) of the Kombucha tea broth and pellicle were plated on appropriate microbiological media. Isolation of AAB from the Kombucha tea/pellicle was performed on modified glucose yeast extract mannitol ethanol acetic acid agar (GYMEA) containing 5% D-glucose (w/v) (ThermoFisher, Waltham,

Massachusetts, USA), 2.5% mannitol (w/v) (ThermoFisher, Waltham, Massachusetts, USA), 1% yeast extract (w/v) (Sigma-Aldrich, St. Louis, Missouri, USA), bacteriological agar (ThermoFisher, Waltham, Massachusetts, USA) using a slightly modified method of B. Wang et al. (2022a). Absolute ethanol (2% v/v) (Sigma-Aldrich, St. Louis, Missouri, USA), glacial acetic acid (0.5% v/v) were added after autoclaving. The addition of cycloheximide (100 mg/L) and penicillin (1 mL/L) were intended to inhibit the growth of yeast and LAB respectively. Samples for isolation of LAB and yeast were plated on De Man, Rogosa and Sharpe (MRS) agar plates (ThermoFisher, Waltham, Massachusetts, USA) and yeast extract chloramphenicol (YGC) plates, respectively. The AAB were cultivated at 30 ℃ for 5-7 days; yeast was incubated at 25 ℃ for 5 days aerobically. The potential LAB were incubated anaerobically at 37 ℃ for 72 h. Colonies of the grown microorganisms were enumerated, and results were expressed as colony forming unit (CFU)/mL or CFU/g. Representative AAB, LAB and yeast isolates were purified by successive streaking on glucose yeast extract (GY), MRS and potato dextrose agar (PDA), respectively. The purified cultures were stored in 20% glycerol (w/w) at -80 °C for long term preservation, and on GY, MRS or PDA agar plates at 4 °C with monthly sub-culturing for routine use (Du Toit & Lambrechts, 2002).

3.3.5 Phenotypic characteristics of AAB and yeast isolated from Kombucha tea broth and cellulosic pellicle during fermentation

3.3.5.1 Phenotypic characteristics of AAB

Three to five presumptive AAB isolates were randomly selected from the highest dilution (10^{-6}) of Kombucha tea broth at each time interval during fermentation (B. Wang et al., 2022a). A loopful of freshly purified AAB culture was used to determine phenotypic characterisation. The purified AAB isolates were plated on GY agar plates at 30 °C for 5-7 days and inoculated in the GY broth overnight with shaking at 170 rpm (Orbital Shakers, Ohaus, Parsippany, USA). The AAB culture was Gram-stained to examine cell morphology under oil immersion using

the Carl Zeiss Transmission light microscope (Model HBO 50/AC, Oberkochen, Baden-Wurttebergy, Germany), and the cell length of AAB isolates was measured using the Carl Zeiss AvixVision microscope software 4.8.1. Biochemical tests were then conducted on the Gram-negative isolates. Catalase positive tests were determined by formation of air bubbles on young, purified colonies after the addition of 1-2 drops of 6% H_2O_2 (v/v) (Sigma-Aldrich, St, Louis, Missouri, USA) (Reiner, 2010; B. Wang et al., 2022a). Growth at different temperatures (25, 30, and 37 ℃) was conducted on GY agar plates that were incubated aerobically for 5-7 days. Growth at low pH was determined in GY broth at pH 2 and pH 3 and incubated aerobically at 30 ℃ for 24 h. The oxidase test was conducted with oxidase strips (Oxoid, Cheshire, UK), and colour change was observed for 30 s (Shields & Cathcart, 2010). The oxidation of ethanol to acetic acid and then oxidation acetic acid to water and CO_2 by AAB was carried out using Carr medium (Gomes et al., 2018). The ethanol tolerance test was performed on yeast extract agar plates containing 2%, 4%, 6%, 8% and 10% (v/v) absolute ethanol (Gullo & Giudici, 2008). Acid produced from sucrose, D-glucose, trehalose, and lactose was determined using the method of Arifuzzaman et al. (2014) with slight modifications. Testing for the formation of cellulose by the AAB isolates was determined using the modified methods of previous reports (Lavasani et al., 2017; Semjonovs et al., 2017; B. Wang et al., 2022a). The AAB isolates were inoculated in Hestrin-Schramm (HS) broth for 5-7 days at 30 ℃. A small portion of the cellulose developed on the surface of the broth was carefully transferred into a 2-mL micro centrifuge tube mixed with 1 mL of 0.1 M NaOH solution and heated at 90 °C on a hotplate (Benchmark Scientific, Sayreville, NJ, USA) for 45 min. The oxidation of lactate and acetate of AAB isolates were determined using published methods (Asai et al., 1964; B. Wang et al., 2022a). Ketogenesis of glycerol to dihydroxyacetone (DHA) was performed with the modified method of Swings (1992). The AAB isolates were inoculated in glycerol yeast extract agar

plates for 5-7 days at 30 °C, the surface of the agar plates was then flooded with Benedict's reagent and incubated at room temperature for another 3 h to observe any colour change.

3.3.5.2 Phenotypic characteristics of yeast

Three to five presumptive yeast isolates were randomly collected at each time interval during fermentation. The purified yeast isolates from Kombucha tea broth and cellulosic pellicle were plated on PDA plates and incubated at 25 °C for 5 days. The cell morphology of yeast isolates was observed using the methylene blue staining method and the cell length was determined using the Carl Zeiss Transmission light microscope (Model HBO 50/AC, Oberkochen, Baden-Wurttebergm, Germany) (Matthews, 1914; Painting & Kirsop, 1990; B. Wang et al., 2022a). The young, fresh, and representative budding yeast isolates were then characterised using biochemical tests. The cycloheximide resistance test was conducted using the modified method of Kurtzman et al. (2011). The yeast isolates were streaked on the PDA plates containing 0.01% and 0.1% cycloheximide (w/v) respectively and incubated at 25 °C for 5 days. The formation of colonies indicated resistance to the antibiotic. Growth at different temperatures was determined on PDA plates and yeast isolates were incubated at 25, 30, and 37 °C for 5 days, respectively. Resistance to high osmotic pressure was determined using the modified method of Kurtzman et al. (2003). Yeast isolates were inoculated on agar containing sodium chloride (10% w/v) and D-glucose (5% w/v) and incubated at 25 °C for 5 days. The growth of colonies suggested that the isolates had resistance to high osmotic pressure. The glacial acetic acid tolerance test was conducted on media containing (5% w/v) D-glucose, (1% w/v) tryptone, (1% w/v) yeast extract, (2% w/v) agar and (1% v/v) glacial acetic acid. Growth at low pH (2 and 3) and acid produced from sucrose, D-glucose, trehalose, and lactose were previously described in Section 3.3.5.1.

3.3.6 Sequence analysis of ribosomal RNA genes

Total DNA from representative AAB and yeast isolates were extracted using the Promega Wizard Genomic Purification Kit (ThermoFisher, Waltham, Massachusetts, USA) following the manufacturers' instructions. The full-length 16S rRNA gene of AAB isolates was amplified by the polymerase chain reaction (PCR) using universal primer 27F and 1492R (B. Wang et al., 2022a; Yuan et al., 2013) and Taq DNA polymerase from Invitrogen (ThermoFisher, Auckland, New Zealand).

For yeast isolates, the internal transcribed spacer (ITS) regions were amplified by PCR using primers ITS1 and ITS4 (Matei et al., 2018). The PCR products were purified with E.Z.N.A Cycle Pure Kit (Omega Bio-Tek InC., Norcross), and subsequent sequencing of the amplicons was performed by Massey Genome Service (Palmerston North, New Zealand), using primers 27F and 1492R, plus an internal primer 533F for 16S rRNA genes and primers ITS1 and ITS4 for the ITS region (Reller et al., 2007). DNA sequences were processed using Geneious 9.0.5 (Biomatters Ltd, Auckland, New Zealand).

3.3.7 Statistical analysis

The physicochemical and phenotypical characteristics experiments were performed in triplicate. Data on pH, titratable acidity, total soluble solids, yield, water activity, and moisture content were analysed by Microsoft Excel 2016 (Microsoft, USA) and represented as mean ± standard deviation. Data on pH, titratable acidity, total soluble solids, viable cell counts were analysed by one-way analysis of variance (ONEWAY-ANOVA) using the IBM SPSS version 26 (IBM, USA) to determine significant differences between the means ($p<0.05$). The DNA of the AAB and yeast was analysed by Geneious 9.1.8 (Biomatters, Ltd, Auckland). The closest homologues or best hits were identified by searching the EzBioCloud 16S rRNA gene database for AAB and ITS database of National Center for Biotechnology Information (NCBI) using Basic Local Alignment Tool (BLAST) (Yoon et al., 2017).

3.4 Results and discussion

3.4.1 Physico-chemical characteristics of Kombucha samples during fermentation

3.4.1.1 Acidity and TSS of Kombucha during fermentation

The changes in acidity of Kombucha tea broth during fermentation are shown in Figure 3.2. The pH of Kombucha tea broth decreased from 3.94±0.04 to 3.16±0.01, and the TA increased from 0.02±0.00 to 0.14±0.13%, during fermentation for 14 days at 22 °C ($p<0.05$). A sharp decrease in pH was observed during the first three days of fermentation and then it decreased slowly to the lowest pH observed at the end of fermentation (3.16±0.01). Meanwhile, TA increased steadily throughout fermentation as expected. During fermentation, sucrose is hydrolysed by the yeast into glucose and fructose. The AAB then convert the two simple sugars into various organic acids such as acetic, gluconic, glucuronic acids, which contribute to the acidity and functional activities such as the antimicrobial activity of Kombucha (Villarreal-Soto et al., 2018). Changes in the hydrogen ion concentration (pH) and TA during fermentation can affect the growth of fermenting microorganisms like AAB and yeast in Kombucha (Neffe-Skocińska et al., 2017). Low acid beverages (< pH 4) are considered to be microbiologically safe as they can prevent the growth of pathogenic microorganisms (Savary et al., 2021). Currently, there is no standardised pH range for Kombucha products. However, a very low pH may affect the overall sensory characteristics of Kombucha, conferring a very sour flavour (Chu & Chen, 2006). It is not recommended to produce Kombucha with a pH < 2.5 as it may be harmful to some consumers due to the high acidity (Nummer, 2013). A pH range between 2.5 and 4.2 is considered desirable and safe for Kombucha beverages (de Miranda et al., 2022).

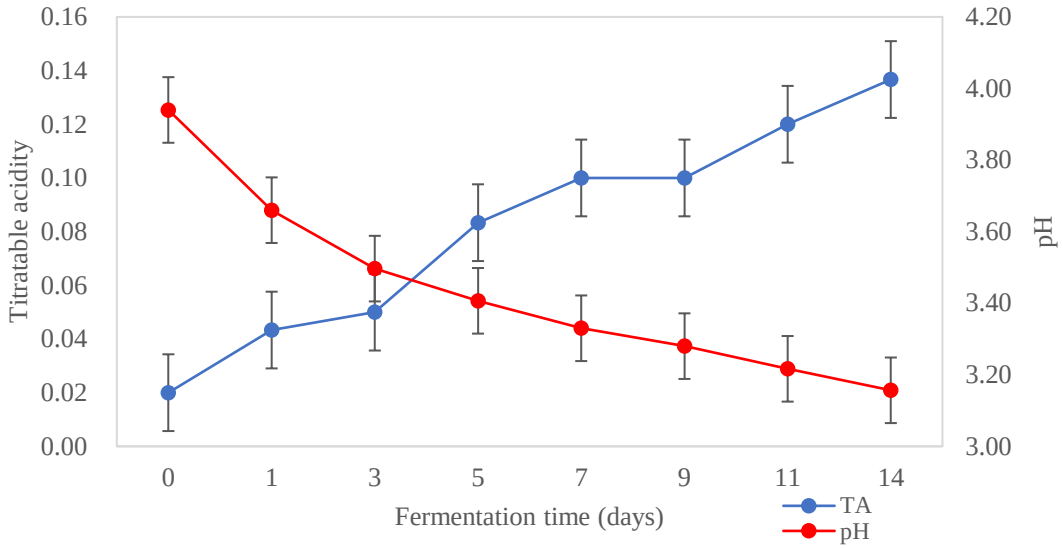

Figure 3.2 pH and titratable acidity of Kombucha tea broth during fermentation for 14 days at 22 °C (n=3).

The TSS of Kombucha steadily decreased from 4.87±0.06 to 3.13±0.21° Brix throughout fermentation for 14 days (22 °C) (Figure 3.3) ($p<0.05$). The reduction of TSS may be due to the microbial metabolism of sugar (sucrose) in the tea broth into a range of metabolites (Jayabalan et al., 2014).

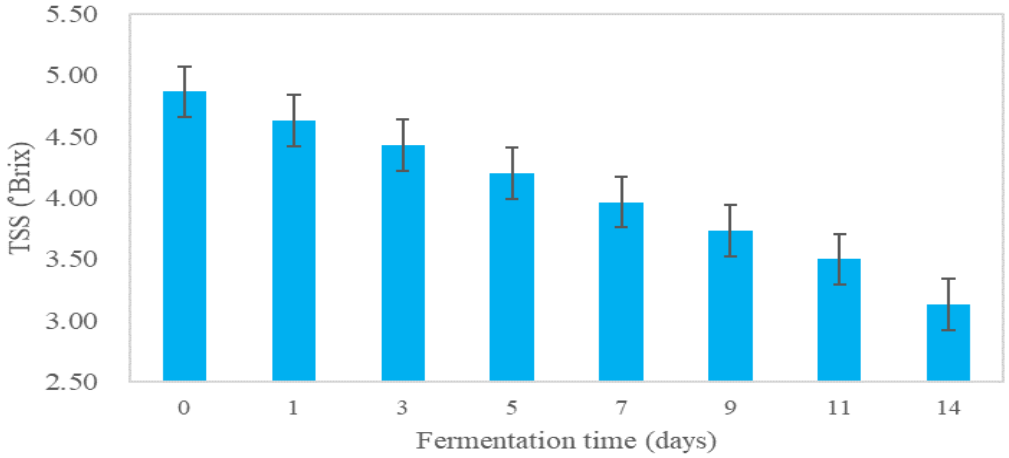

Figure 3.3 Total soluble solids (TSS) of Kombucha tea broth during fermentation for 14 days at 22°C (n=3)

3.4.2 Wet yield, WHC, moisture content and water activity of cellulosic pellicle at the end of fermentation

An approximately 2 cm thick transparent cellulosic layer was observed on the surface of the mother pellicle during Kombucha fermentation. The AAB, which produce the cellulosic pellicle, are aerobic and therefore the pellicle floats on top of the broth (Avcioglu et al., 2021). The size of the cellulose pellicle gives an indication of the success of the fermentation process (Aung & Eun, 2022). In this study, the wet yield of SCOBY was 27.75±0.05 % by the end of fermentation (Table 3.1). Bacterial cellulose has been widely used as an environmentally friendly nanomaterial in the cosmetic and food industries due to its high mechanical strength, high elasticity, high water-holding capacity, and chemical stability (Avcioglu et al., 2021; Jacek et al., 2021). The WHC was 17.82±0.16 g water/g dry pellicle which was lower than cellulose produced by *Gluconacetobacter xylinus* (98.5 g water/g dry pellicle) in a previous study (Wu & Liu, 2013). The WHC indicates the water retained by the cellulosic pellicle; a higher WHC results in a loose cellulosic structure which is desirable for nano-material applications (Cao et al., 2018).

Table 3.1 Wet yield water holding capacity (WHC) moisture content and water activity of cellulosic pellicle.

Characteristics	Cellulosic pellicle
Wet yield (%)	27.75±0.08
WHC (g water/g dry pellicle)	17.82±0.16
Moisture content (%)	94.68±0.05
Water activity at 21.45°C	0.996±0.002

3.4.3 Isolation and enumeration of AAB and yeast during fermentation of Kombucha

The changes ($p<0.05$) in the microbial population during fermentation of the Kombucha tea broth are shown in Figure 3.4. Yeast and AAB were found in the Kombucha tea broth during fermentation and the pellicle. No LAB were present in the Kombucha in this study. Kombucha was prepared using back-slopping fermentation, with the initial concentrations of yeast and

AAB being 3.08±0.42 log CFU/mL and 3.08±0.48 log CFU/mL, respectively, in the Kombucha tea broth. The microbial population of yeast and AAB in the Kombucha tea broth increased by nearly 2 log CFU/mL during fermentation for 14 days (Figure 3.4). Although the initial cell counts of AAB were lower, the final population was slightly higher than the yeast count. Both AAB and yeast counts increased markedly from day 0 to day 5 and then slowly to the end of fermentation; yeast and AAB counts remained relatively similar from day 5 to day 11, with small fluctuations visible between day 9 and day 11. The cell counts from the cellulosic pellicle was 7.44±0.31 log CFU/mL for AAB and 7.30±0.06 log CFU/mL for yeast, which were higher than the number of microorganisms present in the tea broth ($p<0.05$).

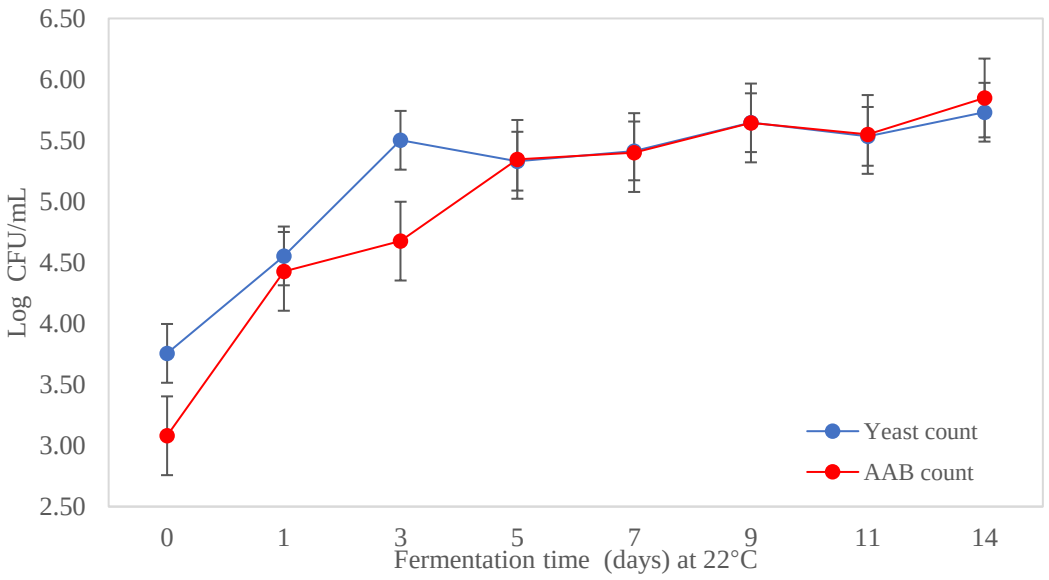

Figure 3.4 AAB and yeast count in Kombucha tea broth during fermentation.

3.4.4 Phenotypic characterisation of AAB and yeast isolated from the Kombucha broth and cellulosic pellicle

3.4.4.1 Morphology of AAB and yeast isolated from Kombucha broth and cellulosic pellicle

A total of sixty-eight isolates (n=29 AAB, n=39 yeast) were isolated from the black tea Kombucha during 14 days of fermentation. Based on the colony morphology, there was one group of AAB and two distinguishable groups of yeast isolated from the Kombucha tea broth and pellicle. The AAB colonies had a circular shape, and smooth surfaces of brownish-red colour with an entire margin. The diameter of the AAB colonies ranged from 1.0-1.5 mm; the morphology of yeast grown on YGC plates is shown in Table 3.2. The different morphologies of the yeast colonies indicated that the fungal isolates may belong to different species.

Table 3.2 Appearance of yeast colonies grown on YGC agar plates.

Colony group	Description of appearance
Group I	Circular and slightly umbonate in the center, white colour, smooth surface and entire margin
Group II	Circular, brownish cream colour, flat and smooth surface

Based on the Gram-staining reactions and cell morphology, 28 of the 29 AAB isolates from Kombucha tea broth and pellicle were Gram-negative (Gomes et al., 2018). All the presumptive AAB cells were rod-shaped with a similar cell length (~1.50 µm), and most occurred as singles. The cell morphology of AAB isolates in this study were similar to *Glucoacetobacter* or *Komagataeibacter* (Mamlouk & Gullo, 2013; Yamada & Yukphan, 2008). The formation of multipolar budding was observed from both groups (I and II) of yeast isolates. The average length of yeast cells ranged from 5.00 to 11.00 µm for the two groups of fungi, and were present singly, in pairs or in groups. The yeast cells were ovoidal to ellipsoidal shape in group I and had a spherical shape in group II. The presumptive AAB isolates (n=28) and yeast (n=39) from Kombucha tea broth and pellicle were subjected to further characterisation.

3.4.4.2 Phenotypic characteristics of AAB and yeast isolated from Kombucha tea broth and pellicle

The phenotypic and differential biochemical characteristics of the AAB isolates are shown in Table 3.3. Based on their biochemical profiles, the 28 AAB isolates were divided into six groups and, based on the data presented in Table 3.3, they were presumed to belong to the genus *Komagataeibacter*. All six groups of AAB isolates were oxidase-negative and catalase positive, and were able to grow at 25, 30, and 37 ℃ after 5-7 days incubation. The bacteria were able to grow at pH 3 but not pH 2. All the AAB isolates produced cellulose after 5-7 days incubation at 30 ℃ without shaking. The transparent cellulose was not soluble after heating in aqueous NaOH. The production of cellulose is a typical characteristic of the species of *Gluconacectobacter* and *Komagataeibacter* (Yamada & Yukphan, 2008). All six groups of AAB isolates oxidised both lactate and acetate as the agar plates turned to blue.

All six groups of AAB isolates were able to grow on agar plates containing 2% (v/v) ethanol (alcoholic tolerance test). However, the six groups of AAB exhibited different growth profiles in media containing 4% to 10% (v/v) ethanol. Group IV isolates were able to grow in the presence of 10% ethanol, while group II and III were not able to grow in the presence of 4% (v/v) ethanol. The ethanol concentration of non-alcoholic Kombucha from previous studies was reported to between 0.2% to 1.1% (v/v) at the end of fermentation (Laureys et al., 2020; Neffe-Skocińska et al., 2017). However, higher alcohol content above the tolerance level of AAB has been reported post-fermentation of Kombucha (B. Wang et al., 2022a). Therefore, it is important to monitor the fermentation process of Kombucha as high levels of ethanol are undesirable for the growth of AAB.

All six groups of isolates oxidised ethanol to acetic acid as the agar turned to yellow after three days incubation at 30 ℃. The results suggest that groups II, IV, and VI oxidised acetic acid to water and CO_2 after incubation for >5 days as the yellow became purple. The other three groups

were not able to oxidise acetic acid probably because they were deficient in succinate dehydrogenase and α-ketoglutarate dehydrogenase (Raspor & Goranovic, 2008). All six groups were also able to oxidise glycerol to dihydroxyacetone (DHA). In the food industry, AAB convert glycerol to produce DHA, which imparts a crust-like taste in winemaking (Drysdale & Fleet, 1988). The presence of DHA in Kombucha may confer a sweet, pleasant aroma and enhances the production of cellulose that can positively impact the sensory characteristics of Kombucha (B. Wang et al., 2022a).

Table 3.3 Phenotypic characteristic of AAB isolated from Kombucha broth and cellulosic pellicles.

Tests	Group I	Group II	Group III	Group IV	Group V	Group VI
Oxidase	-	-	-	-	-	-
Catalase	+	+	+	+	+	+
Growth at different temperature						
Growth at 25℃	+	+	+	+	+	+
Growth at 30℃	+	+	+	+	+	+
Growth at 37℃	+	+	+	+	+	+
Growth at different pH						
pH 2	-	-	-	-	-	-
pH 3	+	+	+	+	+	+
Cellulose formation	+	+	+	+	+	+
Growth without acetic acid	+	+	+	+	+	+
Oxidation of acetate	+	+	+	+	+	+
Oxidation of lactate	+	+	+	+	+	+
Alcoholic tolerance (v/v)						
2%	+	+	+	+	+	+
4%	+	-	-	+	+	+
6%	-	-	-	+	+	-
8%	-	-	-	+	-	-
10%	-	-	-	+	-	-
Acid produced from:						
D-glucose	+	+	+	+	+	+
Sucrose	-	-	-	-	-	-
Lactose	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-
Oxidation of ethanol	-	+	-	+	-	-
Oxidation of ethanol to water and CO_2	-	+	-	+	-	-
Ketogenesis of glycerol to DHA	+	+	+	+	+	+

Note: (+) indicated positive reaction (-) indicated negative reaction. Experiments were replicated 3 times. Group 1 (n=4), Group 2 (n=8), Group 3(n=6), Group 4 (n=2), Group 5 (n=5), Group 6 (n=3).

Table 3.4 shows the phenotypic characteristics of yeast isolated from Kombucha tea broth and cellulosic pellicle, with four isolates belonging to group II and 34 isolates to group I. The Group

II yeast isolates were presumed to be the dominant yeast during Kombucha fermentation. The Group I isolates were not able to grow at 30 ℃ and 37 ℃, while the group II isolates exhibited good growth at 30 ℃ and weak growth at 37 °C. Both the Group I and Group II isolates were able to grow on agar containing 0.01% cycloheximide. At higher cycloheximide concentrations, the Group I isolates showed weak growth. The Group I isolates had better growth on agar containing 1% glacial acetic acid than group II. The two groups of yeast isolates were able to produce acid from D-glucose and sucrose, but only Group II isolates produced acid from trehalose. These biochemical tests enable the differentiation of the yeast isolates into further groups (B. Wang et al., 2022a). However, it is difficult to identify yeast isolates to species or genus level based only on biochemical and physiological tests. Therefore, molecular methods were conducted for further identification.

Table 3.4 Phenotypic characteristics of yeast isolated from Kombucha broth and cellulosic pellicles.

Tests	**Group I**	**Group II**
Growth in broth at 37 ℃	-	w
Growth in broth at 30 ℃	-	+
Growth in broth at 25 ℃	+	+
Growth in medium containing 0.01% cycloheximide	+	+
Growth in medium containing 0.1% cycloheximide	w	+
Growth in medium containing 1% glacial acetic acid	+	w
Growth in medium containing 5% glucose and 10% NaCl	-	w
Growth in broth at pH 2	+	+
Growth in broth at pH 3	+	+
Acid produced from:		
D-glucose	+	+
Sucrose	+	+
Trehalose	-	+
Lactose	-	-

Note: (+) indicated positive reaction (-) indicated negative reaction, (w) indicated weakly growth. Experiments were replicated 3 times. Group I (n=35) and Group II (n=4).

3.4.5 Genetic identification of representative AAB and yeast isolates

Six representative AAB isolates from each group were subjected to sequence analysis of the full-length 16S rRNA gene. Similarly, three representative yeast isolates were subjected to ITS sequencing after biochemical testing. All six AAB isolates belonged to *Komagataeibacter*

rhaeticus, showing 99.92% sequence similarity with the reference strain (Table 3.5). Only one nucleotide (nt) mismatch was found within the 1287 nt aligned sequence region. Therefore, in this study the dominant species present in both tea broth and SCOBY was identified as *K. rhaeticus*.

Table 3.5 Genetic identification of representative AAB and yeast isolates.

Representative strains	Top hit taxon	Type strain	GenBank accession	% Similarity	Variation
AAB					
TFT1AAB26	*Komagataibacter rhaeticus*	DST GL02	AY180961	99.92	1/1292
Yeast					
GI: D3T3Y9	*Zygosaccharomyces lentus*	CBS 8574	NR_156001.1	99.84	1/634
GII: TFT1Y39	*Debaryomyces prosopidis*	JCM 9913	NR_077067.1	100.00	0/600

Note: the other five AAB strains genetically identified are D5T1AAB1, D7T2AAB10, D11T2AAB17, and TFT3AAB29.GI: yeast group I; GII: yeast group II. An additional GII strain TFT3Y38 was also included in the analysis.

All the 28 isolates obtained from different fermentation times in this study were isolated from New Zealand SCOBY in 2021, and there were minor differences in phenotypic characteristics compared with *K. rhzeticus* DST GL02^T (Dellaglio et al., 2005). This cellulose-producing strain was reported in Italian apple fruit, and its basonym was *Gluconacetobacter rhaeticus*. It was then re-classified into the genus *Komagataibacter* in 2012 (Yamada et al., 2012). Strains AF-1, P1463 and K3 were isolated from Kombucha on solid Hestrin-Schramm medium (Jacek et al., 2021; Machado et al., 2016; Semjonovs et al., 2017). For example, Groups I, III, IV were not able to oxidise ethanol to acetic acid, while reference strains can. Additionally, all six groups from our study exhibited different alcoholic tolerance in contrast to the reference strain (Dellaglio et al., 2005; Yamada et al., 2012). Although these six group isolates belong to the same species, they are different strains and thus exhibited different biochemical profiles. The

presence of *K. rhaeticus* contributes to the development of the cellulosic structure of Kombucha as shown by the results from the cellulose formation test (Table 3.3).

The demand for bacterial cellulose (BC) has been rapidly increasing for applications in food, biomedicine, and cosmetics (Jacek et al., 2021; Semjonovs et al., 2017). However, a low yield and high operational costs limit the production of BC (Jacek et al., 2021). Extensive research has been conducted to overcome these difficulties including isolating more productive strains, using lower cost carbon substrates, and optimising fermentation conditions (Thorat & Dastager, 2018). Various strains from the genus *Komagataibacter* have been reported to be potential producers of BC including *K. xylinus, K. saccharivorans, K. hansenii* and *K. rhaeticus* (Gopu & Govindan, 2018; Gupte et al., 2021; Thorat & Dastager, 2018). *K. rhaeticus* is a promising cellulose-producing resource, as it may have similar physicochemical characteristics to the BC produced by *K. xylinus* but with a higher yield under similar cultivation conditions (Machado et al., 2016). Therefore, more research should be carried out to optimise the yield of BC synthesised by the *K. rhaeticus* isolated from this study and their impact on Kombucha fermentation.

Based on the ITS sequence analysis, Group I yeast isolates belonged to *Zygosaccharomyces lentus* and showed a 99.84% sequence identity with the type strain CBS 8574. Only one mismatch was found with an ITS region of 634 nt in length. Group I isolates were obtained from both the cellulosic pellicle and Kombucha tea broth at each sampling point during fermentation (Figure 3.5). *Z. lentus* was firstly isolated from spoiled whole-orange drink and tentatively identified as *Zygosaccharomyces bailli* based on its carbohydrate assimilation profiles as analysed by API ID 32C system (Hazel Steels et al., 1999). This species has been isolated from Kombucha obtained in Germany and Ireland (Hopfe et al., 2017; Marsh et al., 2014). Strains of *Z. lentus* were distinguished from other strains of *Zygosaccharomyces* by their inability to grow above 25 ℃; however, they could grow at low temperatures (4 ℃) and low

pH (H Steels et al., 1999). The inability of *Z. lentus* to grow under aerobic conditions was due to their sensitivity to oxygen (Hazel Steels et al., 1999). Similarly, our yeast strains could grow at 25 ℃ but not 30 ℃ (Table 3.4). The higher cell counts of *Z. lentus* may be due to the favourable fermentation conditions used in this study (22 ℃ and low pH) under static conditions. As fermentation temperature can affect the balance of microorganisms in Kombucha (De Filippis et al., 2018), a fermentation above 25 ℃ with the SCOBY used in this study is not recommended, as the dominant yeast species *Z. lentus* is not able to grow at that temperature. The strains isolated in this study were most similar in phenotypic characteristics of reference strains IGC and 2406, especially their ability to grow in medium containing 1% (w/v) acetic acid at low pH (pH 2 and 3) (H Steels et al., 1999).

The Group II yeast isolates were present at the beginning (days 3, 5, and 7) of the fermentation in the Kombucha tea broth only (Figure 3.5). Previous research also found that yeast was more diverse in the broth around day 7 of fermentation (Chakravorty et al., 2016). Although the culture-based methods used in this study may provide a good understanding of the physiological characteristics of the isolates, they are only effective in identifying the culturable microorganisms (Moyer et al., 1994). There may be unculturable yeast present in the SCOBY that cannot be detected using culture-based methods.

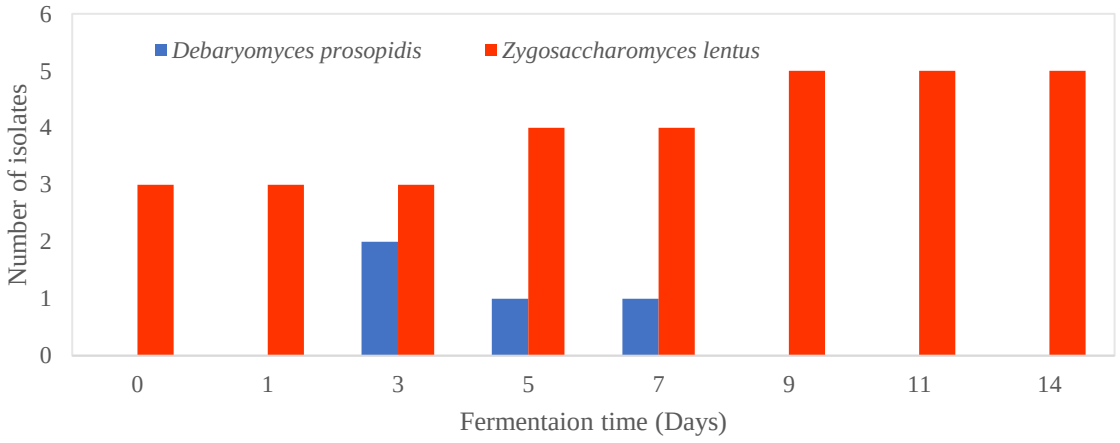

Figure 3.5 Distribution of yeast isolates in Kombucha tea broth during fermentation for 14 days.

The Group II yeast belonged to *Debaryomyces prosopidis* with 100% identity match to the type strain *D. prosopidis* JCM 9913. This species was considered a minor microbe from the Kombucha yeast community in this study due to its low proportion (10.3%). The phenotypic characteristics of the four strains isolated from this study agreed with the reference strains. The presence of *D. prosopidis* contributes positively to the texture and flavour characteristics of fermented meat products, possibly due to their high lipolytic activity and production of hexanal (Song et al., 2022). The presence of this species in Kombucha may therefore contribute to its sensory profile. Recently, this species has been used to produce arabitol, a potential polyols sweetener from glycerol (Filippousi et al., 2022). As glycerol is an intermediate metabolite during Kombucha fermentation, the reaction between glycerol and *D. prosopidis* to produce arabitol may impact the sensory characteristics of Kombucha to some extent. *Z. lentus* and *D. prosopidis* are rarely isolated from Kombucha samples and therefore there is scant information about their role in the fermentation. Hence, more research should be conducted to investigate the interactions between the two yeast species found in this SCOBY and their impact on Kombucha fermentation and metabolites.

3.5 Conclusions

All the AAB strains isolated from black tea Kombucha broth and pellicle during fermentation were identified as *Komagataeibacter rhaeticus*, which is known for cellulose production. The yeast species belonged to *Zygosacharomyces lentus* and *Debaryomyces prosopidis,* with the latter being dominant in the Kombucha tea broth and pellicle. This study contributes important information on the microbiological composition of the cellulosic pellicle and physico-chemical characteristics of Kombucha. Based on the findings of this study, it is recommended to ferment Kombucha using the SCOBY from this study at <25 ℃, as the growth of the dominant yeast species may be compromised. An improved knowledge of the microorganisms involved in

Kombucha fermentation can help manufacturers to better monitor the progress of fermentation, and aid in industrial scale-up to produce consistent high-quality products.

Chapter 4. Evaluation of the probiotic potential of yeasts isolated from Kombucha in New Zealand

4.1 Abstract

The current study investigated the *in vitro* probiotic potential of yeast isolated from Kombucha, a tea beverage fermented with a symbiotic culture of acetic acid bacteria and yeast. A total of 62 yeast strains were previously isolated from four different commercial Kombucha samples sold in New Zealand. Fifteen representative isolates belonging to eight different species were evaluated for their growth under different conditions (temperature, low pH, concentrations of bile salts, and NaCl). Cell surface characteristics, functional and enzymatic activities of the selected strains were also studied in triplicate experiments. Results showed that six strains (*Dekkera bruxellensis* LBY1, *Sachizosaccharomyces pombe* LBY5, *Hanseniaspora valbyensis* DOY1, *Brettanomyces anomalus* DOY8, *Pichia kudraivzevii* GBY1, and *Saccharomyces cerevisiae* GBY2) were able to grow under low-acid conditions (at pH 2 and pH 3) and in the presence of bile salts. This suggests their potential to survive passage through the human gut. All 15 strains exhibited negative enzymatic activity reactions (haemolytic, gelatinase, phospholipase, and protease activities), and thus, they can be considered safe to consume. Notably, two of the fifteen strains (*Pichia kudraivzevii* GBY1 and *Saccharomyces cerevisiae* GBY2) exhibited desirable cell surface hydrophobicity (64.60-83.87%), auto-aggregation (>98%), coaggregation, resistance to eight tested antibiotics (ampicillin, chloramphenicol, colistin sulphate, kanamycin, nalidixic acid, nitrofurantoin, streptomycin, and tetracycline), and high levels of antioxidant activities (>90%). Together, our data reveal the probiotic activities of two yeast strains GBY1 and GBY2 and their potential application in functional food production.

4.2 Introduction

Kombucha is a popular, slightly sweet, sparkling drink fermented for 7-10 days at ambient temperature in a base of sugared tea infusion, typically made with black or green tea (de Miranda et al., 2022; Jayabalan et al., 2014; Nyhan et al., 2022). The fermentation is conducted by a complex symbiotic culture of acetic acid bacteria (AAB) and yeast, commonly known as SCOBY. Some lactic acid bacteria (LAB) have been reported in fermented Kombucha, however, available information suggests that they may not be essential for the fermentation of the beverage (Laureys et al., 2020). Regular consumption of Kombucha may confer beneficial health-promoting effects, including antioxidant, anti-inflammatory, antibacterial, probiotic and anticarcinogenic activities (Coelho et al., 2020; Jayabalan et al., 2014; Jayabalan & Waisundara, 2019; Selvaraj & Gurumurthy, 2022). Due to the appealing sensory characteristics and beneficial effects, Kombucha has gained popularity around the world (Batista et al., 2022; Kapp & Sumner, 2019). Kombucha was reported to have a global market of about USD 1.5 billion around 2018, and the market value of fermented beverage is predicted to increase to 5 billion by 2025 (Kim & Adhikari, 2020).

The microbial composition of Kombucha starter cultures is diverse and complex, and it is largely determined by the original sources of the SCOBY and the cultivation conditions (Akhtar et al., 2021; Jayabalan et al., 2014; Mayser et al., 1995). Acetic acid bacteria in Kombucha are dominated by *Komagataeibacteri xylinus*, *K. rhaeticus, Acetobacter aceti, A. tropicalis, A. pasteurianus, Gluconobacter oxydans and Gluconacetobacter sacchari* (Akhtar et al., 2021; Barbosa et al., 2021; Jayabalan et al., 2014; Pradhan et al., 2022; Savary et al., 2021; Semjonovs et al., 2017; B. Wang et al., 2023; B. Wang et al., 2022b). The yeast community is generally more diverse than AAB, and it includes *Zygosaccharomyces bailii, Z. rouxii, Brettanomyces lambrcus, B. custerisii, B. intermedius, B. claussenii, B. bruxellensis Candida albican, C. kefir, C. obtuse, Schizosaccharomyces pombe, Pichia fermentans, P. membranefaciens, Torulaspora*

delbrueckii, T. famata and *Saccharomyces cerevisiae* (Arikan et al., 2020; Chakravorty et al., 2016; Gaggia et al., 2018; Jayabalan et al., 2014; Marsh et al., 2014; Mayser et al., 1995; Pradhan et al., 2022; Ramadani & Abulreesh, 2010; Teoh et al., 2004; B. Wang et al., 2022a). The presence of these microorganisms may be associated with reported probiotic characteristics of Kombucha.

Probiotics are defined as live microorganisms with appropriate concentrations of well-defined strains which exhibit health-promoting benefits to the host (Hill et al., 2014). To confer health benefits, probiotic microorganisms must be able to survive in the dynamic gastrointestinal environment, which includes low pH and pepsin in the stomach and bile salts and pancreatic enzymes in the intestine. Additionally, they must be capable of propagating at human body temperatures (Gil-Rodríguez et al., 2015; Hossain et al., 2020; Hsu & Chou, 2021; Suvarna et al., 2018). Therefore, several selection criteria are essential for microorganisms to be considered as probiotics, including being safe for consumption and producing non-toxic activities to the human body (Lahtinen et al., 2009). A safety assessment of any potential probiotic strain must be conducted before the culture can be considered for use in food or pharmaceutical products (FAO/WHO, 2006; Sanders et al., 2010). The ability of probiotics to adhere to epithelial cells is a desirable characteristic of beneficial microorganisms allowing them to manifest their beneficial effects by persisting longer in the host GI tract (Collado et al., 2008; García-Cayuela et al., 2014). Antioxidant and antimicrobial activities have also been used to identify potential probiotic strains (Binetti et al., 2013; Chen et al., 2010; Liu et al., 2021).

Most probiotics are lactic acid bacteria (LAB) with the majority belonging to the genera *Lactobacillus, Enterococcus* and *Bifidobacterium* (Alvarez et al., 2023; Y. Kim et al., 2022; Liu et al., 2021). However, to date only a few yeast strains have been identified as probiotics (Suvarna et al., 2018). *Sacchromyces cerevisiae* and *S. boulardii* are the only commercial

probiotic yeast species currently available for human consumption (Di Cagno et al., 2020; Suvarna et al., 2018). Yeast is commonly found in food and beverages and are utilised as starter cultures for fermentation of several food and beverages such as wine, Kombucha, lambic beer, kefir, sake, bread-making, and table olives (Alkalbani, Osaili, Al-Nabulsi, Obaid, et al., 2022). Their wide applications in the food industry indicate that most food-related fungi (yeast) are generally regarded as safe (Hsu & Chou, 2021). Recently, consideration of yeast as potential probiotic microorganisms has increased due to their advantages over bacteria (Alvarez et al., 2023), for example, the larger size of yeast cells (approximately 10 times larger) compared to bacteria allow them to exhibit stearic hindrance to bacteria (Czerucka et al., 2007). Yeast such as the genera *Pichia, Schizosaccharomyces, Kluyveromyces, Yarrow,* and *Torulaspora* have shown several beneficial characteristics for health, including excellent resistance to antibiotics which helps to restore gut microbiota after antibiotic administration. Yeast is also characterised by having a high mineral and vitamin B content, the presence of several immune response components in their cell wall such as mannose and glucan, and ability to grow under conditions similar to the harsh GI tract environment (Czerucka et al., 2007; Gil-Rodríguez et al., 2015; T. Li et al., 2020). Despite a large number of probiotics being used in commercial products, the rapid growth of the probiotic market indicates that demand exists for more strains possessing specific functional properties such as antioxidant and anti-inflammatory activities (Pereira et al., 2021). Therefore, it is necessary to determine the characteristics of other potential probiotic yeast species or strains from food or beverage sources (Alvarez et al., 2023; Hsu & Chou, 2021).

Kombucha is generally regarded as a probiotic drink due its inherent live microorganisms (Akhtar et al., 2021; Vargas et al., 2021). Although many commercial Kombucha products are labelled as containing “live cultures”, their probiotic properties have not been documented (Kim & Adhikari, 2020; Vargas et al., 2021). At present, there is scant information on the relative abundance of the viable microorganisms in Kombucha and their perceived health

promoting properties (Laureys et al., 2020; Vargas et al., 2021). Here we report the evaluation of the *in vitro* probiotic potential of 15 representative yeast strains previously isolated from commercial Kombucha samples produced in New Zealand.

4.3 Material and methods

4.3.1 Yeast strains and cultivation conditions

Sixty-two yeast strains from eight species were isolated from three branded Kombucha beverages purchased from local supermarkets in Auckland, New Zealand. The cultures were isolated from the Kombucha broth and bacterial cellulose (SCOBY). The identities of the yeast strains were reported earlier (B. Wang et al., 2023; B. Wang et al., 2022a). Of the 62 strains isolated, 15 representative yeast isolates were chosen and differentiated based on their phenotypic and biomedical characteristics and survival during fermentation (B. Wang et al., 2023; B. Wang et al., 2022a). All 15 representative yeast isolates (Table 4.1) were preserved in 20% glycerol (w/w) and stored at -80°C until required. Before use, the frozen cultures were activated on potato dextrose agar (PDA) plates for 5 days or yeast peptone broth (YPD) overnight.

Table 4.1 Identities of yeast strains and their origins.

Specie	Strain	Source
Debaryomyces prosopidis	[1]D3Y9	Kombucha tea broth
Debaryomyces prosopidis	[1]D5Y12	Kombucha tea broth
Debaryomyces prosopidis	[1]D7Y18	Kombucha tea broth
Zygosaccharomyces lentus	[1]D7T19	Kombucha tea broth
Zygosaccharomyces lentus	[1]D11Y30	Kombucha tea broth
Zygosaccharomyces lentus	[1]D14Y35	Kombucha tea broth
Zygosaccharomyces lentus	[1]TFY36	Cellulosic pellicle (MB)
Zygosaccharomyces lentus	[1]TFY38	Cellulosic pellicle (MB)
Zygosaccharomyces lentus	[1]TFY39	Cellulosic pellicle (MB)
Dekkera bruxellensis	[2]LBY1	Kombucha brand 1 (LB)
Sachizosaccharomyces pombe	[2]LBY5	Kombucha brand 1 (LB)
Hanseniaspora valbyensis	[3]DOY1	Kombucha brand 2 (DO)
Brettanomyces anomalus	[3]DOY8	Kombucha brand 2 (DO)
Pichia kudraivzevii	[4]GBY1	Kombucha brand 3 (GB)
Saccharomyces cerevisiae	[4]GBY2	Kombucha brand 3 (GB)

Note: [1]Strains were selected at the beginning, middle and end of fermentations from samples of SCOBY. [2,3,4] . Yeast isolates were selected based on representative strains of each species with the same phenotypic characteristics.

4.3.2 *In vitro* tolerance of yeast strains to simulated gastrointestinal tract (GIT) conditions

4.3.2.1 Tolerance to low pH

The tolerance of each yeast strain to low pH was determined using the method of Hsu and Chou (2021) with minor modifications. Briefly, overnight suspensions (1%, v/v) of each yeast strain were inoculated into YPD broth tubes adjusted to pH 2.0 and pH 3.0 with 0.1M HCl (Sigma Aldrich, St. Louis, MO, USA). The inoculated cultures were incubated at 25℃ for 24 h. Growth of yeast cultures was confirmed by measuring the absorbance of grown cultures at 600 nm using a spectrophotometer (Novaspec III, Amesham Biosciences, Buckinghamshire, United Kindom). The non-inoculated YPD broths (pH 2 and pH 3) were calibrated as the control blanks respectively and the growth index was calculated as shown in Equation 4.1.

$$\text{Growth index}(\%) = \left(\frac{A_{600}\,(24h)}{A_{600}(0h)}\right) * 100\% \text{ ----------[Equation 4.1]}$$

A_{600} (0h): $\text{Absorbance}_{600nm}$ at 0h
A_{600}(24h): $\text{Absorbance}_{600nm}$ at 24h

4.3.2.2 Tolerance to bile salts

Tolerance of cultures to bile salts was carried out using the method of Bogdan et al. (2018). Overnight suspensions (1%, v/v) of each yeast strain were inoculated into YPD broth supplemented with 0.5%, 1.0% and 1.5% (w/v) bile salts (Sigma Aldrich) and incubated at 25℃ for 24 h (Merchán et al., 2020). Absorbance of the incubated cultures were measured at 0 h and 24 h in a spectrophotometer at 600 nm to determine their growth. The non-inoculated YPD broth with different bile salt concentrations was calibrated as the control blank and the growth index was calculated using Equation 4.1.

4.3.2.3 Growth at different temperatures

The growth of yeast at different temperatures was examined using the method of Alvarez et al. (2023) with some modifications. Loopfuls of overnight yeast isolate were streaked on PDA plates and incubated at the selected temperatures: 25℃ and 30℃ (Kombucha fermentation temperature), 37℃ (human internal body temperature and anaerobic conditions), 39 and 42℃ (fever temperature) for 5 days. The development of colonies along the streaked lines was indicative of growth and therefore deemed a positive result.

4.3.2.4 Tolerance to $NaCl_{aqueous}$

The growth of yeast isolates under different salt (NaCl) concentrations was examined using the method of Zeng et al. (2019) with modifications. The PDA plates were supplemented with NaCl (2%, 4%, 6.5%, w/v). Loopfuls of each overnight cultured yeast strain were streaked on PDA plates with different salt concentrations and incubated at 25℃ for 5 days. The formation of colonies along the streaked lines indicated growth and tolerance to NaCl.

4.3.3 Cell surface characteristics

4.3.3.1 Auto-aggregation and co-aggregation assay

Determination of auto-aggregation was performed according to Gil-Rodríguez et al. (2015) with some modifications. Each yeast strain was separately cultured in YPD broth overnight. The cell pellets were harvested by centrifugation at 8000 *g* for 5 min at 4°C, washed twice with sterile 0.9% NaCl solution and resuspended in the same sterile saline solution to obtain ~10^8 CFU/mL (OD_{600nm} ~0.08-0.10). Each yeast cell suspension (4 mL) was mixed for 15 s using a vortex mixer (VM-10, WiseMix®, Germany) and incubated at 25℃ for 24 h without agitation. For measurement, 0.2 mL of the upper suspension was transferred to a new disposable plastic cuvette and mixed with 1.8 mL of sterile 0.9% NaCl solution and the $absorbance_{600nm}$ was measured at 0, 2,4 and 24 h. The auto-aggregation rate was calculated using Equation 4.2 from Gil-Rodríguez et al. (2015).

$$\text{Auto-aggregation (\%)} = \left[\frac{A_0 - A_T}{A_0}\right] * 100\% \quad \text{----------[Equation 4.2]}$$

A_T: $Absorbance_{600nm}$ at each time interval

A_0: $Absorbance_{600nm}$ at start

Co-aggregation of yeast strains with pathogenic bacteria was analysed using the method of Xu et al. (2009) and Lara-Hidalgo et al. (2019) with modifications. Yeast cells were cultured as previously described. Pathogenic strains comprising *Pseudomonas aeruginosa* MUA26*, Staphylococcus aureus* MCTIC 4163*, Escherichia coli* NCTIC 8196, *Bacillus cereus* MU-A44 were used in this study. *B. cereus* MU-A44 was cultured in Muller-Hinton broth (Thermofisher, Waltham, MA, USA) at 30℃ and all the other pathogens were cultured separately at 37°C overnight. Both yeast and the pathogens were centrifuged at 8000 *g* for 5 min at 4℃, washed

twice and resuspended in 0.9% NaCl solution to obtain ~10^8 CFU/mL. Equal volumes of yeast cell suspension (2 mL) and each pathogenic bacterium were mixed for 10 s using a vortex mixer and incubated at 25℃ for 4 h without agitation The $absorbance_{600nm}$ of the mixture was measured after incubation. Cell suspensions of individual strains were used as controls. The co-aggregation rate was calculated using Equation 4.3 developed by Handley et al. (1987).

$$\text{Co-aggregation (\%)} = \left(\frac{(A_{pro}+A_{path})/2-A_{mix}}{(A_{pro}+A_{path})/2}\right) * 100\% \text{----------[Equation 4.3]}$$

A_{pro}: absorbance of control tubes of probiotic strains
A_{path}: absorbance of control tubes of pathogenic bacteria
A_{mix}: absorbance of control tubes of mixture after incubation for 4 h

4.3.3.2 Cell surface hydrophobicity

The hydrophobicity properties of the yeast cells were determined according to Liu et al. (2021) and Xu et al. (2009). The yeast cells were cultured at 25℃ overnight and centrifuged at 8000 *g* for 5 min at 4℃. The cell pellet was washed twice with sterile 0.9% NaCl and resuspended in the same buffer to achieve an OD_{600nm} of approximately 0.4-0.6 (A_0). The adjusted yeast cell suspension (3 mL) was mixed thoroughly with chloroform (1 mL, Sigma Aldrich, St. Louis, MO, USA) using a vortex mixer for 30 s. The mixture was then incubated at ambient temperature (~22°C) for 30 min to separate the organic and aqueous phases. The aqueous phase was measured at OD_{600nm}. The hydrophobicity was calculated as shown in Equation 4.4 according to Xu et al. (2009).

$$\text{Hydrophobicity (\%)} = \left(1 - \frac{A_X}{A_0}\right) \times 100\% \text{ ----------[Equation 4.4]}$$

A_X: absorbance of the aqueous phase
A_0: absorbance of the initial probiotic strains

4.3.4 Potential health promoting properties of isolated yeast strains

4.3.4.1 Antioxidant activity

The reduction (%) of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was used to evaluate the antioxidant activity of the yeast isolates (Gil-Rodríguez et al., 2015; Hsu & Chou, 2021; Merchán et al., 2020). Yeast strains were cultured separately in YPD broth overnight at 25°C. The cell pellets were harvested by centrifugation (8000 *g,* 5 min, 4°C) and filtered through 0.22 µm syringe filters to prepare cell free supernatants (CFS). The initial concentration of the different yeast cultures (CFS) was standardised to OD_{600nm} =1.2. The cell suspension (0.8 mL) was then mixed with 1 mL of 0.2 mM methanolic DPPH solution (Sigma Aldrich, St. Louis, MO, USA) in 2 mL microcentrifuge tubes. The solution was mixed using a vortex mixer for 1 min and then incubated for 30 min at room temperature (~22 °C) in the dark. The reaction tubes were centrifuged at 12000 *g* for 5 min, and the absorbance of the supernatant was measured at 517 nm. The radical scavenging activity of DPPH was calculated using Equation 4.5 according to Hsu and Chou (2021).

$$\text{Scavenging ability } \% = \left[1 - \frac{A_{517\text{sample}}}{A_{517\text{ blank}}}\right] * 100\% \text{ ----------[Equation 4.5]}$$

$A_{517sample:}$ Absorbance of sample at 517 nm
A_{blank}: Absorbance of blank (methanol) at 517 nm

The Trolox equivalent antioxidant capacity (TEAC) of each yeast culture was determined using a solution of 6-hydroxy-2,5,7.8-tetramethylchroman-2-carboxylic acid (Trolox) (Sigma Aldrich, St. Louis, MO, USA) dissolved in methanol (1 mM). The Trolox was diluted with methanol to different concentrations (10-100 µM), and 0.8 mL of each concentration of Trolox was mixed with 1 mL of 0.2 mM DPPH solution and incubated at ambient temperature (~22°C) for 30 min in the dark. The absorbance of the mixture was measured at 517 nm using a spectrophotometer. The scavenging ability of the yeast cultures and Trolox on DPPH were

measured at the same time. Therefore, the linear regression Equation 4.6 of Trolox concentration and scavenging ability in the range of 10-100 µmol Trolox/mL was used to quantify TEAC.

$y = 0.9799x + 11.186$ $R^2 = 0.998$ ----------[Equation 4.6]

x: Trolox concentration (µM)
y: DPPH free radical scavenging activity (%)
R^2: Coefficient of determination

4.3.4.2 Bile salt hydrolase (BSH) activity

Bile salt hydrolase activity of the yeast isolates was assessed using the method of Gebre et al. (2023) with some modifications. The yeast isolates were cultured in YPD broth overnight and adjusted to 0.5 McFarland standard (~1.5-2.0 $x10^8$ CFU/mL) (Menezes et al., 2020). Ten (10) µL of standardised yeast culture were spotted on PDA plates containing 0.5% (w/v) bile salt and 0.37g/L calcium chloride. The plates were incubated at 25℃ for 5 days. The presence of an opaque precipitation indicated a positive reaction, and the diameter of the precipitation was measured. A larger diameter indicated stronger BSH activity by the yeast isolates.

4.3.4.3 Antimicrobial activity

The well-diffusion agar method was used to determine the antimicrobial activity of yeast isolates (Merchán et al., 2020; Srinivas et al., 2017). Four bacterial strains (*P. aeruginosa* MUA26*, S. aureus* MCTIC 4163*, E. coli* NCTIC 8196, *B. cereus* MU-A44) and two mycotoxigenic fungi (*Aspergillius brassilliensis* NZRM2578, and *Penicillium chrysogenum* NZRM2999) were used as the test microbial pathogens.

For the antibacterial activity, the yeast isolates were cultured overnight in YPD broth at 25℃. The yeast cell density was standardised to 0.5 McFarland standard. All the pathogens were cultured in tryptone soya broth (Thermofisher, Waltham, MA, USA) and incubated at 37℃ for

24 h and adjusted to a turbidity equivalent to 0.08-0.10 at OD_{600nm}. About 50 µL of pre-incubated pathogens (~ 0.5 McFarland standard) were spread onto solidified Muller-Hinton Agar (MHA) plates (Sigma Aldrich, St. Louis, MO, USA) with sterile cotton swab and wells (Ø ≈ 7 mm) were made with a sterile stainless steel cork borer. Standardised yeast culture (100 µL) was added to the wells of the MHA plates and incubated at 37℃ for 24 h. The diameter of clear zones was measured to determine the magnitude of antibacterial activity.

The antifungal activity of yeast isolates was evaluated with the method of Merchán et al. (2020); Rodríguez-Tudela et al. (2001) with slight modifications. The fungi were inoculated on PDA plates at 25℃ for 5 days until sporulation. The fungi culture suspension was adjusted to a 0.5 McFarland at OD_{530nm} (0.11-0.14) containing approximately 10^5 cfu/mL (Rodríguez-Tudela et al., 2001). About 50 µL of pre-incubated fungi were spread on the PDA plates with sterile cotton swab. Wells (Ø ≈ 7 mm) were made with a sterile cork borer. Standardised yeast culture (100 µL) was added to the wells in the PDA plates and incubated at 25℃ for five days. The diameter of clear zone around each disk was measured to determine antifungal activity.

4.3.5 Safety assessment

4.3.5.1 Susceptibility to antibiotics

Disk diffusion was used to determine the antibiotic susceptibility of yeast isolates (Fernández-Pacheco et al., 2021; Goktas et al., 2021; Liu et al., 2021). Antibiotic resistance was determined using the Mast-ring S® composed of eight clinical antibiotics (µg) as follows: ampicillin 25, chloramphenicol 50, colistin sulphate 100, kanamycin 30, nalidixic acid 30, nitrofurantoin 50, streptomycin 25, and tetracycline 100. The yeast strains were cultured in YPD broth overnight at 25℃. Then, 50 µL of each yeast suspension (0.08-0.10 at OD_{600nm}) was evenly spread on the PDA plates. A Mast-ring S ® disk was gently placed on the surface of each PDA plate with sterile forceps. The diameter of clear zone around each disk was measured after 5 days incubation at 25℃.

4.3.5.2 Haemolytic activity

The haemolytic activity of the yeast strains was performed using the method of Menezes et al. (2020) and Fernández-Pacheco et al. (2021). A loopful of fresh yeast culture was streaked on blood agar plates (5% sheep blood) and incubated at 25°C for 5 days. The presence of a green or brown zone around colonies indicated α-haemolysis. The development of a clear zone around colonies was referred to as β-haemolysis. If no clear zone developed around the colonies this was considered as γ-haemolysis. *S. aureus* was used as a positive control.

4.3.5.3 Proteolytic activity

The proteolytic activity of yeast isolates was determined according to the method of Moslehishad et al. (2013) and Gut et al. (2019). A suspension (~10 µL) of actived yeast culture was spot-inoculated on nutrient agar plates containing 5% (w/v) skim milk (Anchor, New Zealand). Plates were incubated at 25°C for 5 days. The presence of a clear zone around the colony indicated a positive reaction, and the diameter of the clear zone was measured. The size of the diameter of the clear zone around the colony was indicative of the strength of the proteolytic activity of the yeast isolates.

4.3.5.4 Phospholipase activity

Phospholipase production was evaluated on egg yolk medium (Price et al., 1982). The medium consisted of PDA supplemented with 5.85% (w/v) NaCl, 0.05% (w/v) $CaCl_2$ and 5% sterile egg yolk. A suspension (10 µL) of overnight yeast culture (~10^6 CFU/mL) was inoculated onto the agar plates and incubated at 25°C for 5 days. Enzymatic activity was visualised as areas of precipitation around each yeast colony. Phospholipase activity (P_z) was expressed as the ratio of the colony diameter to the total diameter of colony and precipitation. Hence, the P_z=1 indicated negative activity, lower P_z values indicated higher enzymatic activity.

4.3.5.5 Gelatinase activity

The gelatinase activity of yeast isolates was carried out using the stabbing method described by Pereira et al. (2021). Fresh yeast culture was stabbed into nutrient gelatine medium and incubated at 25°C for up to 14 days and placed at 4°C for 30 min to check for liquefaction. The presence of liquefaction was considered as a positive reaction.

4.3.6 Statistical analysis

All the experiments were performed in triplicate. Percentages, means, and standard error of means were analysed by Microsoft Excel 2019 (Microsoft, USA). Statistical analysis of data was carried out by analysis of variance-one way (ANOVA-ONEWAY) using Minitab 21 (Minitab, USA) to compare the means ($p<0.05$). Significant differences between the means were separated using Tukey's test. Correlation analysis of Pearson correlation coefficient and principal component analysis (PCA) of probiotic characteristics were performed using Origin® 2021b (OriginLab, Northampton, USA).

4.4 Results and discussion

Based on the information from the literature, several of the strains [*D. bruxellensis* (LBY1), *B. anomalus* (LBY5), *S. pombe* (LBY5) and *H. valbyensis* (DOY1)] investigated in the current study are likely to contribute to the appealing flavour, aroma, and taste of fermented beverages (Fadda et al., 2001; Mayser et al., 1995; B. Wang et al., 2022a). *P. kudriavzevii* (GBY1) is known to produce a biofilm and flavour compounds in Kombucha, while yeast strains of *D. prosopidis* (D3Y9, D5Y12, D7Y18) are associated with the sensory profile of Kombucha (Chelliah et al., 2016; B. Wang et al., 2023). Although strains of *Z. lentus* (D7T19, D11Y30, D14Y35, TFY36, TFY38, TFY39) have been reported as food spoilage yeast (Marsh et al., 2014; H Steels et al., 1999), they are likely to have a positive contribution on the flavour of the fermented beverage (B. Wang et al., 2023). *S. cerevisiae* (GBY2) has been widely used for fermentation and is well-recognised as being safe for human consumption (B. Wang et al.,

2022a). However, safety studies do not appear to have been conducted on the other strains. Therefore, both the probiotic characteristics and safety assessments of these fifteen isolates were investigated here.

4.4.1 In vitro tolerance to simulated gastrointestinal tract (GIT) conditions

4.4.1.1 Growth tolerance at different temperatures

Growth or tolerance at different temperatures, low pH, in the presence of bile salts and sodium chloride solutions are considered as a prerequisite for screening probiotic strains which will likely survive in the human gut (Menezes et al., 2020) (Table 4.2). All the yeast strains (n=15) were able to grow at 25°C. Of the strains, three isolates (20%) which belonged to *D. prosopidis* showed weak growth at 37°C, and six *Z. lentus* isolates (40%) were not able to grow at above 30°C. The remaining six strains were able to grow well at 37°C; of these, five strains which belonged to *D. bruxellensis, S. pombe, P. kudraivzevii,* or *S. cerevisiae* were also able to grow well at 39 and 42°C. However, the *H. valbyensis* DOY1, was unable to grow at either of the two highest temperatures tested (Table 4.2).

Table 4.2 Growth of yeast strains at different temperatures and in different NaCl concentrations.

Isolates and identities	**Growth temperature (°C)**					**Growth in $NaCl_{aq}$ (w/v)**		
	25	30	37	39	42	2%	4%	6.5%
Debaryomyces prosopidis D3Y9	+	+	w	-	-	+	+	+
Debaryomyces prosopidis D5Y12	+	+	w	-	-	+	+	+
Debaryomyces prosopidis D7Y18	+	+	w	-	-	+	+	+
Zygosaccharomyces lentus D7Y19	+	-	-	-	-	+	+	+
Zygosaccharomyces lentus D11Y30	+	-	-	-	-	+	+	+
Zygosaccharomyces lentus D14Y35	+	-	-	-	-	+	+	+
Zygosaccharomyces lentus TFY36	+	-	-	-	-	+	+	+
Zygosaccharomyces lentus TFY38	+	-	-	-	-	+	+	+
Zygosaccharomyces lentus TFY39	+	-	-	-	-	+	+	+
Dekkera bruxellensis LBY1	+	+	+	+	+	+	+	-
Sachizosaccharomyces pombe LBY5	+	+	+	+	+	+	+	w
Hanseniaspora valbyensis DOY1	+	+	+	-	-	+	+	+
Brettanomyces anomalus DOY8	+	+	+	+	+	+	+	+

Pichia kudraivzevii GBY1	+	+	+	+	+	+	+	+
Saccharomyces cerevisiae GBY2	+	+	+	+	+	+	+	+

Note: "+" indicated positive results; "-" indicated the negative results; "w" indicated weak growth'

Some non-*Saccharomyces* yeasts such as *Z. lentus* can grow at low temperatures (H Steels et al., 1999), possibly due to their lipid composition (Alvarez et al., 2023). Kombucha is commonly fermented between 25 and 30℃ for 7-14 days (Leonarski et al., 2022), hence, the ability to grow at temperatures below 30℃ is recommended for cultures intended for use in Kombucha production. Growth at 37°C (normal human body temperature) is an important criterion for probiotic selection (Alvarez et al., 2023; Gil-Rodríguez et al., 2015; Hsu & Chou, 2021; Menezes et al., 2020), however, it is also important to test the tolerance of potential probiotic yeast at higher temperatures of between 39℃ (febrile state) and 42℃ (hyperpyrexia-related to bacterial infection) (Alvarez et al., 2023; de Llanos et al., 2006). In the current study, five strains (LBY1, LBY5, DOY8, GBY1, and GBY2) showed good growth at five different temperatures (25, 30, 37,39 and 42°C) which indicated that the temperature tolerance of these strains may improve their survival during the heat treatment such as spray drying (Desmond et al., 2002).

4.4.1.2 Tolerance to different NaCl concentrations

All the yeast isolates showed good tolerance in 2.5% to 4% NaCl (w/v) (Table 4.2). Strains of *D. bruxellensis* were not able to grow at 6.5% NaCl (w/v) whereas *S. pombe* LBY5 showed weak growth along the streaked lines at this salt concentration. Some probiotics have been isolated from savoury fermented foods containing NaCl including fermented black olives, fish, and meat (Tamang & Lama, 2022). The addition of salt in fermented food products not only contributes to the aromatic profile but salt is also added as a preservative to inhibit the growth of pathogenic microorganisms (Alkalbani et al., 2022; Zeng et al., 2019). Therefore, good growth in the presence of salt is an important and desirable characteristic of probiotics in fermented food products (Szutowska & Gwiazdowska, 2021). Most yeast strains (86.67%) in

this study were able to grow in all the salt concentrations tested (2%-6.5% w/v), which may support their application in salty fermented food products (Diguță et al., 2023).

4.4.1.3 Tolerance to low pH

Another important challenge for probiotic yeast isolates is tolerance to extreme high acidic conditions (pH 2-3) in the stomach (Alkalbani et al., 2022; Pereira et al., 2012). Therefore, a fundamental selection property of probiotic yeast is that they tolerate low pH to survive in the gastric juice (Pereira et al., 2012). All 15 yeast isolates in this study exhibited excellent growth at pH 2 and 3 ($p<0.05$) (Figure 4.1A), indicating their ability to survive in simulated gastric juice *in vitro*. Most strains (73.33%) had a higher tolerance at pH 3 than pH 2 under similar incubation conditions. Four isolates (D7Y19, TFY36, TFY38, GBY1) exhibited better tolerance at pH 2 than pH 3, which was similar to the pH tolerance of *P. kudraivzevii* reported by Alvarez et al. (2023). Hsu and Chou (2021) reported that *D. bruxellensis, P. kudraivzevii* and *S. cerevisiae* yeast isolated from Kombucha, fermented vinegar and milk kefir produced in Taiwan also showed good growth at pH 3 which was consistent with our results. The tolerance to low pH conditions was not surprising as this level of acidity is commonly found in Kombucha and other fermented beverage products such as cider or kefir (Goktas et al., 2021; Tran et al., 2020). Based on the results, all 15 strains tested in the present study were considered as acid tolerant.

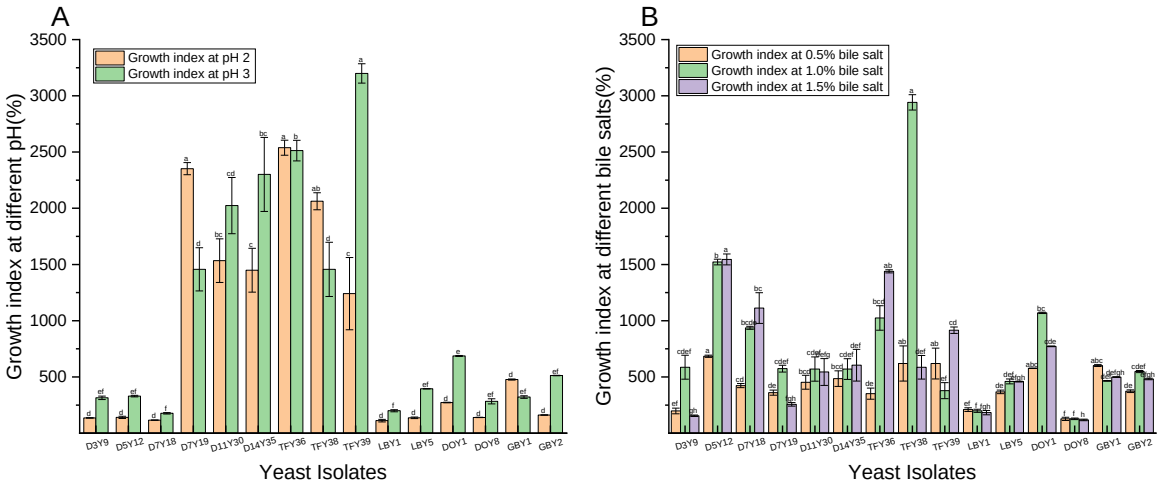

Figure 4.1 Growth index of yeast strains at low pH (A) and in the presence of bile salts (B). Different lowercase letters (a-f) above the chart bars indicate significant differences ($p<0.05$); n=3 triplicate experiments; error bars indicate SD of means.

4.4.1.4 Tolerance to bile salts

In addition to tolerance under high acid environments, another major barrier for probiotics to survive in the intestinal tract is the high bile salt concentration (Alkalbani et al., 2022). Bile salts are lipid emulsifying agents released in the duodenum after food ingestion, bile salts also have antimicrobial activity (Urdaneta & Casadesús, 2017). All 15 yeast strains tested here exhibited excellent growth after 24 h incubation in the presence of 0.5%-1.5% (w/v) bile salts (Figure 4.1B). However, the growth of the microorganisms in the presence of different bile salts concentrations was strain-dependent ($p<0.05$), with the highest growth rate observed for D5Y12 in 1.0% (w/v) bile salts, while the lowest growth was for DOY8 in 1.5% (w/v) bile salts. Although others have shown the growth rate of certain probiotic strains increase at lower bile salt concentrations, no such correlations were observed in the current study (Topçu et al., 2020).

4.4.2 Cell surface characteristics

The ability of potential probiotics to adhere to epithelial cells and mucosal surfaces is an important cell surface characteristic because it allows probiotics to persist longer in the GI tract, thus allowing more time for interactions to occur with host epithelial cells and hence greater

potential to confer their health benefits to the host (García-Cayuela et al., 2014; Pereira et al., 2012; Suvarna et al., 2018). The adhesion properties of probiotics are complex, involving electrostatic and Van der Waals forces between the cell surface of the probiotic and intestinal cells, especially the interaction between the physical and chemical composition of the probiotic cell surface and intestinal cell (Alvarez et al., 2023; Menezes et al., 2020). The auto-aggregation and hydrophobicity properties of probiotic yeast are related to their ability to adhere to the intestinal mucosa (Hsu & Chou, 2021; Pereira et al., 2012). In addition, the ability of probiotics to co-aggregate with pathogenic bacteria is a defense mechanism to inhibit the colonisation of pathogens in the intestine (Liu et al., 2021; Menezes et al., 2020).

4.4.2.1 Auto-aggregation activity

Auto-aggregation is the reversible adherence between identical cells resulting in a precipitate (Hsu & Chou, 2021; Trunk et al., 2018). This characteristic protects probiotic yeast from extreme conditions such as oxidative stress and nutrient deficiency (Fekri et al., 2020; Trunk et al., 2018). Auto-aggregation can also improve the adherence of probiotics to intestinal cells and prevent pathogens from colonising the GI tract (García-Cayuela et al., 2014). Figure 4.2 shows the auto-aggregation of the 15 yeast isolates. The yeast strains exhibited marked variability in their level of auto-aggregation after incubation for 2 h ($p<0.05$), ranging from 28.39±3.81% (TF39) to 92.35±0.87% (LBY5). Six strains (LBY1, LBY5, DOY1, DOY8, GBY1 and GBY2) showed a high percentage (>60%) of auto-aggregation after 2 h incubation. Of these six yeast strains, *S. pombe* (LBY5) and *S. cerevisiae* (GBY2) showed very high auto-aggregation (>90%), forming an obvious precipitate at the bottom of the tubes after 2 h incubation. The remaining strains showed auto-aggregation levels of 20% - 60% after 2 h. Most strains (80%) showed increased auto-aggregation (62.44% to 97.61%), between 2 and 4 h incubation (Figure 4.2). Except for TFY36 and TF39 (both belonging to *Z. lentus*), all yeast tested in this study showed greater than 80% auto-aggregation after 24 h incubation. In this

study, *S. pombe* LBY5 showed the fasted and best auto-aggregation performance of the 15 strains tested. Alvarez et al. (2023) evaluated auto-aggregation of five *P. kudraivzevii* yeast strains and showed much lower auto-aggregation percentages at 4 h inoculation than our study. However, after 24 h incubation, their results were similar to ours (98.54±0.25 %). As expected, *P. kudraivzevii* exhibited better auto-aggregation performance than bacteria, as yeast cells are usually heavier and larger. Accordingly, they easily precipitate in higher proportions (García-Cayuela et al., 2014; Hsu & Chou, 2021). The auto-aggregation capacity of yeast isolates can be affected by their different cell wall constituents, appendages (fimbriae), adhesins, and macromolecules (Nwoko & Okeke, 2021; Touhami et al., 2003). Hence, this may explain the variation of auto-aggregation capacity of yeast strains in this study.

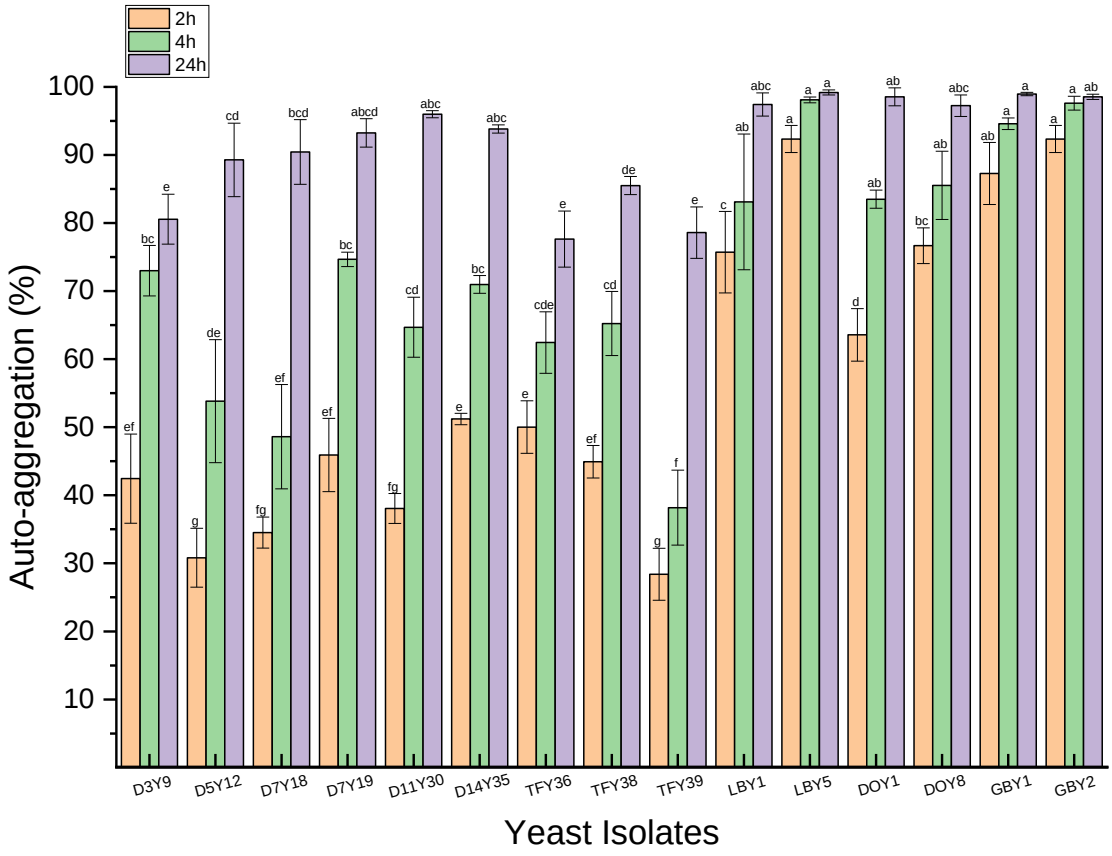

Figure 4.2 Auto-aggregation (%) of yeast strains after 2, 4 and 24 h incubation. Different lowercase letters (a-g) above the chart bars indicate significant difference ($p<0.05$); n=3 triplicate experiments; error bars indicate SD of means.

4.4.2.2 Co-aggregation activity

In contrast to auto-aggregation, co-aggregation occurs between genetically different microbial strains (Collado et al., 2008). All the yeast strains tested here exhibited different co-aggregation capacities against four pathogenic bacteria (two Gram-positive and two Gram-negative). Among the yeast strains, *S. cerevisiae* GBY2 showed the highest co-aggregation (%) against *S. aureus*. High co-aggregation rates (>80%) were also recorded for LBY5, GBY2, and DOY8 against *B. cereus*. These three yeast strains also showed high co-aggregation which is likely to be linked to auto-aggregation activity (Vlková et al., 2008). The lowest co-aggregation capacity was recorded for *Z. lentus* D11Y30 against *E. coli* (Figure 4.3). All the yeast strains tested exhibited higher co-aggregation rates against Gram-positive (*S. aureus* and *B. cereus*) than against Gram-negative pathogens (*P. aeruginosa* and *E. coli*). Results obtained in this study suggest that the co-aggregation capacity was dependent on the incubation conditions, specific combinations of pathogenic bacteria and yeast strains which is consistent with results from previous studies (Lara-Hidalgo et al., 2019). The high co-aggregation rate against the four pathogens tested allows the yeast cells to develop a competitive microenvironment around the pathogens, displacing them and preventing pathogens from adhering to host epithelial cells. The displacement of pathogens protects the host against pathogenic infection (Burns et al., 2011). Therefore, the high co-aggregation capacity of yeast isolates in the present study with the four tested pathogens indicated that these yeast strains may be beneficial for host health.

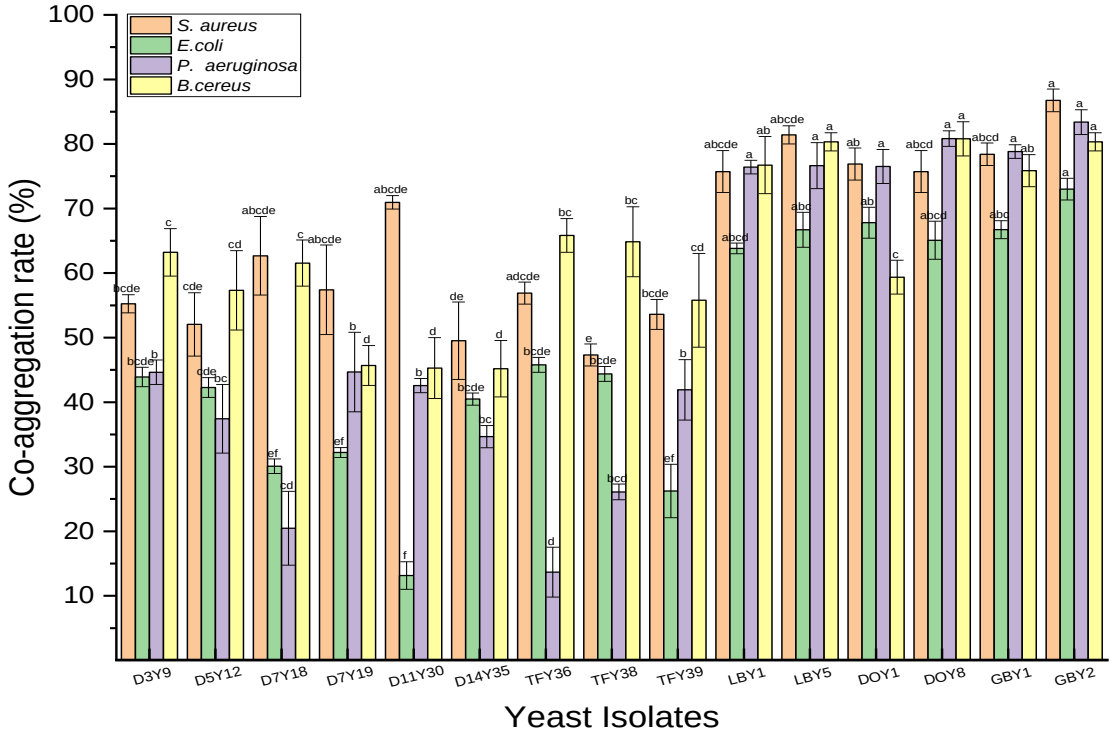

Figure 4.3 Co-aggregation ability of yeast strains with pathogenic bacteria. Different lowercase letters (a-f) above the chart bars indicate significant difference ($p<0.05$); n=3 triplicate experiments; error bars indicate SD of means.

4.4.2.3 Hydrophobicity activity

Modified adhesion ability to hydrocarbons (MATH) has been widely used for evaluating cell surface hydrophobicity of probiotic microbes (Chelliah et al., 2016; García-Cayuela et al., 2014; Suvarna et al., 2018). In this study, cell surface hydrophobicity was evaluated by affinity to chloroform (polar acid solvent) (Liu et al., 2021). According to Lara-Hidalgo et al. (2019), hydrophobicity rates higher than 40% indicate the yeast isolates are hydrophobic. The yeast strains tested exhibited moderate to high hydrophobicity in chloroform, varying from 42.63±0.91% to 83.87±0.98% (Figure 4.4) with a significant difference between the 15 strains ($p<0.05$). Of the 15 yeast strains in the present study, *P. kudraivzevii* GBY1 exhibited the highest hydrophobicity (83.87±0.98%) to chloroform which was consistent with the results

obtained by Helmy et al. (2019). This strain also had the highest auto-aggregation percentage. Results showed that hydrophobicity properties varied between strains and species probably due to the different composition of growth media, age of microorganisms, or surface-associated proteins between the strains (García-Cayuela et al., 2014; Nwanyanwu et al., 2012). Cell surface hydrophobicity is important in both proliferation and adhesion of probiotics (Sourabh et al., 2011). Having a high cell surface hydrophobicity capacity is vital as it may facilitate yeast strains to strongly attach (adhere) to hydrophobic surfaces such as the human gastric mucosa and colon through which they may confer their health-promoting effects to the host (Lugea et al., 2000).

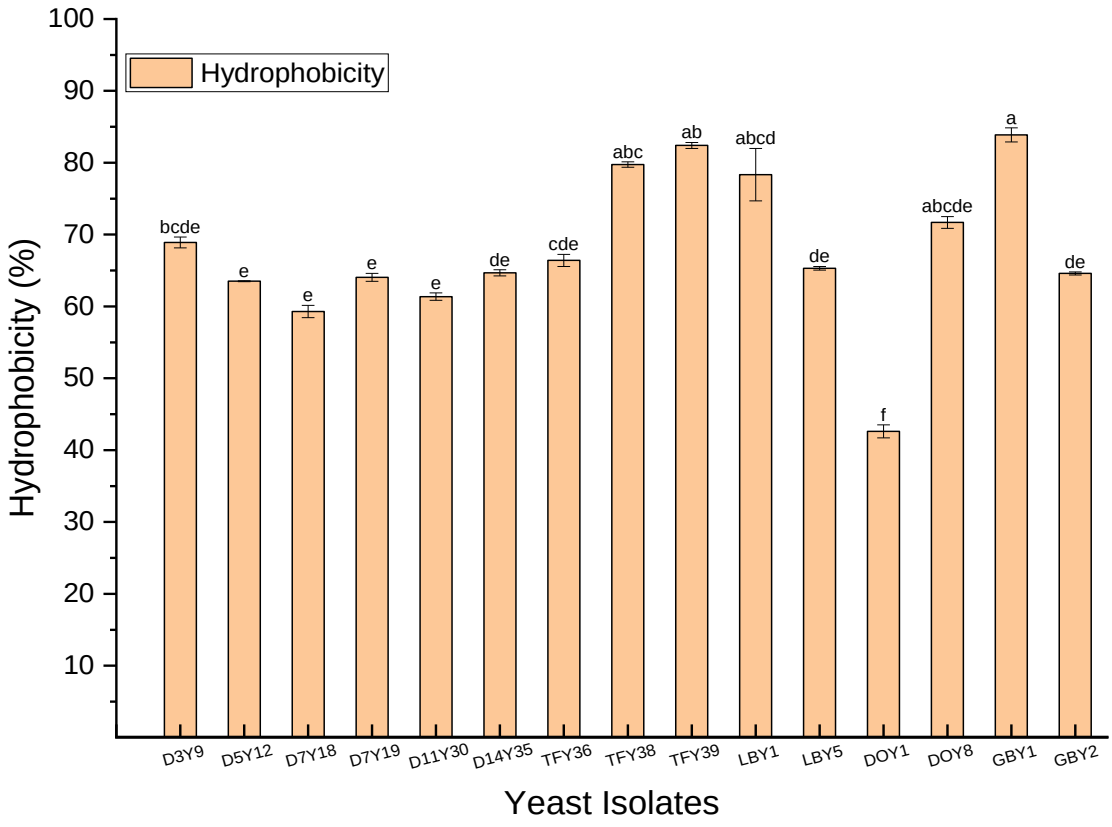

Figure 4.4 Hydrophobicity (%) of yeast strains. Different lowercase letters (a-e) above the chart bars indicate the significant difference ($p<0.05$); n=3 triplicate experiments; error bars indicate SD of means.

Correlation coefficients (r) between auto-aggregation, hydrophobicity and co-aggregation with pathogenic bacteria are shown in Figure 4.5. The auto-aggregation capacity of yeast isolates was positively correlated with their respective co-aggregation with all four pathogens, although co-aggregation was highly dependent on the combination with specific pathogenic strain of bacteria (r=0.31-0.75). All the yeast strains in this study were positive for hydrophobicity, auto-aggregation, and co-aggregation to different extents. For example, the highest auto-aggregation (99.19±0.38%) was shown by LBY5, which also showed a high co-aggregation (81.41±1.40%) with *S. aureus*. Other yeast strains (DOY1, DOY8, GBY1, GBY2) also showed high auto-aggregation rates and high co-aggregation with *S. aureus, E. coli,* and *P. aeruginosa*. However, a low correlation was found between auto-aggregation and the hydrophobicity (r = -0.26) of the tested yeast strains although GBY1 showed high values for both hydrophobicity and auto-aggregation. Similarly, hydrophobicity (affinity to chloroform) showed negative or no correlation with co-aggregation and pathogens excluding *B. cereus* (Figure 4.5).

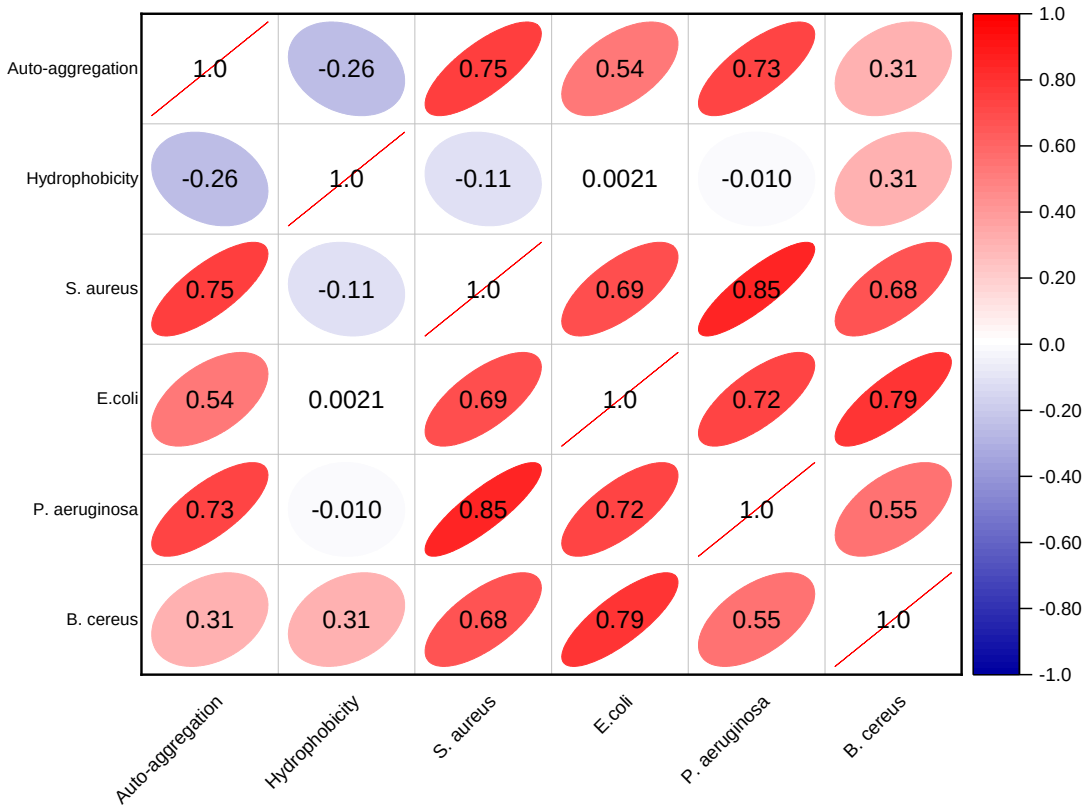

Figure 4.5 Pearson's correlation coefficient matrices between auto-aggregation, hydrophobicity, and co-aggregation (with pathogenic bacteria strains) of 15 yeast strains. *S. aureus:* yeast strains co-aggregated with *S. aureus*; *E. coli*: yeast strains co-aggregated with *E. coli*; *P. aeruginosa*: yeast strains co-aggregated with *P. aeruginosa*; Co-*B. cereus*: yeast strains co-aggregated with *B. cereus.*

Few studies have shown a high correlation between hydrophobicity and aggregation, most likely because these properties are generally strain-based (Alvarez et al., 2023; García-Cayuela et al., 2014). One possible reason for the low correlation is that adhesion to intestinal cells involves complex multifactorial interactions initiated by contact with host tissues followed by multiple cell surface interactions (García-Cayuela et al., 2014; Reuben et al., 2020). Moreover, other factors including surface associated proteins, polysaccharides, and surface charge may also affect the adhesion of probiotics to human epithelial cells. Cell surface hydrophobicity, auto-aggregation and coaggregation with pathogens can still be used as a tool for the

preliminary selection of probiotics suitable for administration to humans and animals as they may contribute to colonization of probiotics to some extent (Collado et al., 2008). However, the impact of other factors such as cell surface charge and composition may also need to be evaluated in further studies.

4.4.3 Potential beneficial effects of yeast isolates

4.4.3.1 Antimicrobial activity

In addition to tolerance to the GI tract environment and cell surface characteristics, probiotics are also expected to confer host-associated health benefits (Kechagia et al., 2013; Pereira et al., 2012). None of the yeast isolates tested in this study inhibited the growth of pathogenic bacteria and moulds tested as no clear inhibition halos were observed. Antimicrobial activity against pathogenic bacteria and fungi is considered as an important characteristic of probiotic strains. The antagonistic activity of yeast isolates against pathogens has been related to their ability to compete for nutrients, formation of organic acids, production of high levels of ethanol, and secretion of antimicrobial compounds such as killer toxins or mycotoxins (Golubev, 2006). However, the absence of antimicrobial activity in this study was still consistent with previous studies which also found that potential probiotic yeast isolated from cheese (*P. kudriavzevii, S. cerevisiae*) exhibited no antimicrobial activity against *E. coli* and *S. aureus* (Binetti et al., 2013). The ability to co-aggregate with pathogens is generally considered a beneficial feature for probiotics, as aggregation represents a physical barrier that can prevent surface colonization on human issues. However, our current data revealed no antimicrobial activities against the four tested bacterial pathogens. Consequently, co-aggregation may have a potentially negative effect of promoting pathogen adhesion to human tissues (Shruthi et al., 2022). Further study is thus required to determine the precise consequences of co-aggregation and the potential antimicrobial properties of the yeast isolates, which are normally dependent on the environmental conditions.

Kombucha has been reported to have considerable antimicrobial activity against different pathogenic microorganisms such as *E. coli, S. aureus,* and *Listeria monocytogenes* (Al-Mohammadi et al., 2021; Battikh et al., 2012; Battikh et al., 2013). The desirable antimicrobial activity of Kombucha may be due to the formation of acetic or other organic acids, or other bioactive components such as bacteriocins (produced from probiotics like LAB), proteins, ethanol, and enzymes or the phenolic components from tea (Nyiew et al., 2022; Sreeramulu et al., 2000). The yeast isolates tested in this study may not contribute to the antimicrobial activity of Kombucha.

4.4.3.2 Bile salt hydrolase (BSH) activity

The yeast isolates were inoculated on PDA plates containing differing concentrations of bile salts to determine their potential to deconjugate the bile salts. In this study, all 15 yeast strains exhibited BSH activity to different levels as indicated by the formation of a white precipitate around colonies ($p<0.05$) (Figure 4.6). The strongest BSH activity was observed from GBY1 with a diameter of 23.33 mm, which was consistent with *S. cerevisiae* AM18 isolated from a cereal-based fermented beverage (Gebre et al., 2023). While the weakest BSH activity was obtained from D14Y35 and TFY36 (12.00 mm). The deconjugation of bile salts by probiotic strains through bile salt hydrolase may be important for probiotic strains to adhere in the GI tract and survive in the intestine containing bile salts (Tsigkrimani et al., 2022). According to Kumar et al. (2012), a stronger BSH activity of probiotic strains may contribute to better bile salt tolerance, resulting in a higher survival rate and better colonization in the host gut. Bile salt hydrolase (BSH) activity could partially contribute to the excellent bile salt tolerance (0.5% ,1.0% and 1.5%) of the 15 yeast strains in this study (Figure 4.1B).

BSH is also able to break down bile salts into free primary acids (deoxycholic acid) and facilitate the intake and digestion of dietary fat (Gebre et al., 2023; Tsigkrimani et al., 2022). This characteristic may lower the risk of colon cancer and toxicity of conjugated bile salts

(Noriega et al., 2006). Furthermore, BSH activity of probiotic strains has often been associated with the reduction of serum cholesterol levels, homeostasis of GI tract, and detoxification of bile, which is beneficial to the host health (Begley et al., 2005; Bommasamudram et al., 2023; Kumar et al., 2012). Hence, BSH has been considered as an alternative clinical therapy to lower blood cholesterol levels of patients with hypercholesterolemia (Chand et al., 2017). The presence of active BSH has been widely used as a primary selection criterion for potential probiotic strains in conjunction with other criteria such as safety assessments (Bommasamudram et al., 2023; Farid et al., 2021). Therefore, the BHS activity present in this study may be a useful characteristic of the yeast isolates (Begley et al., 2006).

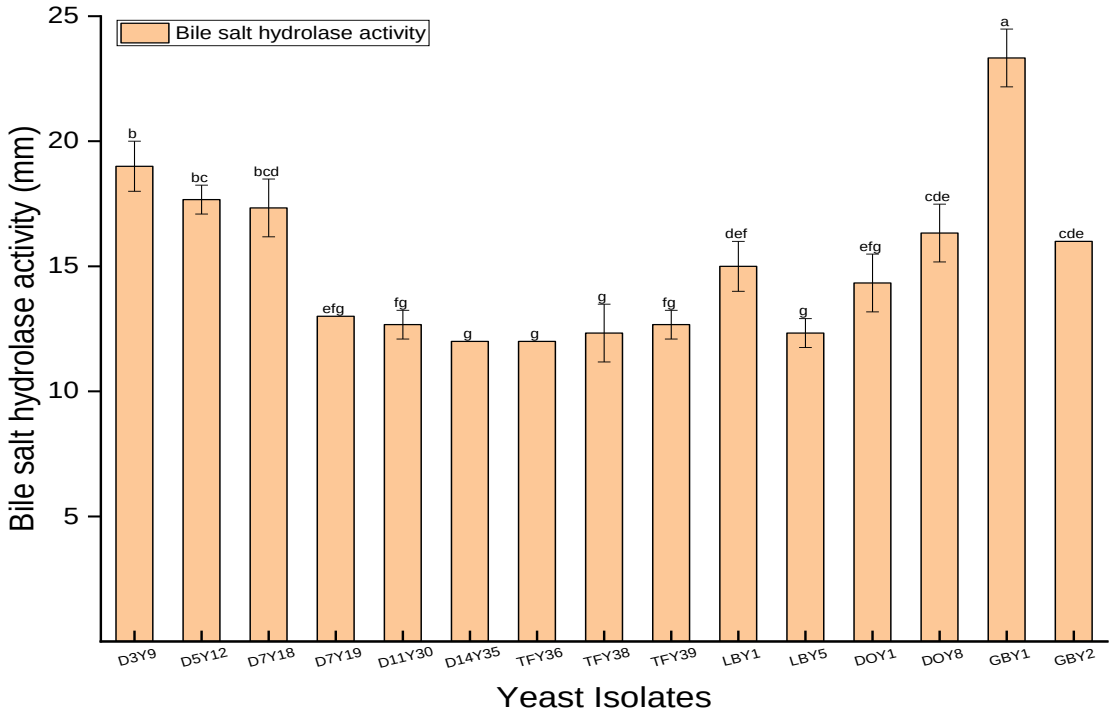

Figure 4.6 Bile salt hydrolase activity of yeast isolates. Different lowercase letters (a-g) above the chart bars indicate significant difference ($p<0.05$); n=3 triplicate experiments; error bars indicate SD of means.

4.4.3.3 Antioxidant activity

All the yeast strains exhibited excellent antioxidant activity with a high percentage reduction of DPPH (>80%) (Figure 4.7). Six strains displayed DPPH free radical scavenging rates of between 80 and 90 % and the remainder presented DPPH rates exceeding 90%. The lowest antioxidant activity was detected from *D. prosopidis* D3Y9 (82.85±0.47 %) and the highest value was recorded from *Hanseniaspora valbyensis* DOY1 (93.13±0.79%). The antioxidant activity quantified as Trolox equivalent antioxidant capacity (TEAC μM), ranged from 73.13±0.48 to 83.39±0.01 μM. Several studies have evaluated the antioxidant activity of yeast strains from different food samples. For example, one strain of *D. bruxellnesis* JYC2537 isolated from Taiwanese Kombucha reported an antioxidant activity (>60%) which is lower than *D. bruxellensis* LBY1 (Figure 4.7) in this study (Hsu & Chou, 2021). The variation in antioxidant activities of the yeast strains may be due to other active enzymes including superoxide dismutase, glutathione peroxidase and catalase, as well as exopolysaccharides, and lipids (Chen et al., 2010; Pereira et al., 2012). One study found that intact cells had higher antioxidant activity than a yeast extract, possibly due to the high concentrations of β-glucans in the intact cell walls (Chen et al., 2010). These results may explain why the antioxidant capacity of yeast isolates in this study were much higher than those reported in other studies, as our study used intact cells while other studies did not (Gil-Rodríguez et al., 2015; Hsu & Chou, 2021; Lara-Hidalgo et al., 2019; Menezes et al., 2020).

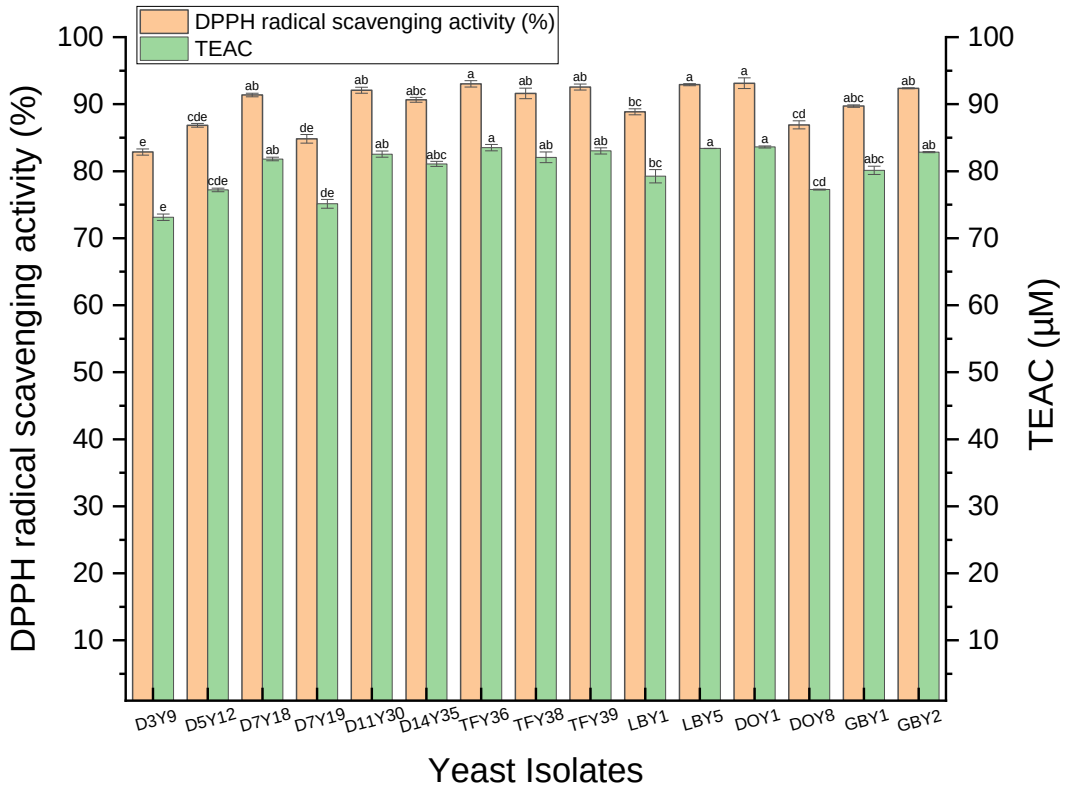

Figure 4.7 DPPH scavenging activity and TEAC of yeast strains. Different lowercase letter (a-f) above the chart bars indicated the significant difference ($p<0.05$): n=3 triplicate experiments; error bars indicate SD of means.

4.4.4 Principal component analysis (PCA)

Multivariate principal component analysis (PCA) which focuses on dimensionality reduction, has been widely used to screen promising probiotic strains (Gao et al., 2021; Mallappa et al., 2019; Solieri et al., 2014). In this study, PCA was used to analyse the correlations between thirteen probiotic characteristics (low pH tolerance at 2 and 3, bile salt tolerance at concentration of 0.5%, 1.0%, and 1.5%, auto-aggregation, co-aggregation with four pathogens, cell surface hydrophobicity, bile salt hydrolase, antioxidant activity) and yeast strains isolated from Kombucha produced in New Zealand. The results from PCA bi-plots indicated that the first two principal components explained 57.5% of the total variance in the probiotic characteristics of the fifteen yeast strains tested (Figure 4.8). The principal component (PC) 1

accounted for 42.7%, and PC2 accounted for 14.8% of total thirteen variances for the yeast isolates, respectively. The PC1 revealed the maximum variations of the data and the PC2 presented the rest of variations. The fifteen yeast isolates were distributed into each of the four different quadrants. Two yeast isolates (LBY5 and GBY2) present in quadrant I (top right) showed a high correlation in auto-aggregation and co-aggregation with the four different pathogens. Six yeast strains (D5Y12, D7Y18, D11Y30, D14Y35, TFY36, TFY38) distributed in quadrant II (top left) were related to antioxidant activity and tolerance to bile salts and low pH. Auto aggregation, co-aggregation with *S. aureus*, *B. cereus, P. aeruginosa*, and *E. coli* had similar scores along PC1, which could be important characteristics to differentiate and select the most potential probiotic strains. The highest weight was obtained from antioxidant activity for the PC2. Most variables contributed positively to the PC2 except hydrophobicity, tolerance to low pH at 2, and bile salt hydrolase activity. Based on the PCA biplots and overall tolerances to temperature and NaCl, two yeast isolates (GBY1, GBY2) appear to be the most promising probiotic strains of those tested as they exhibited the highest probiotic performance and best temperature and salt tolerances.

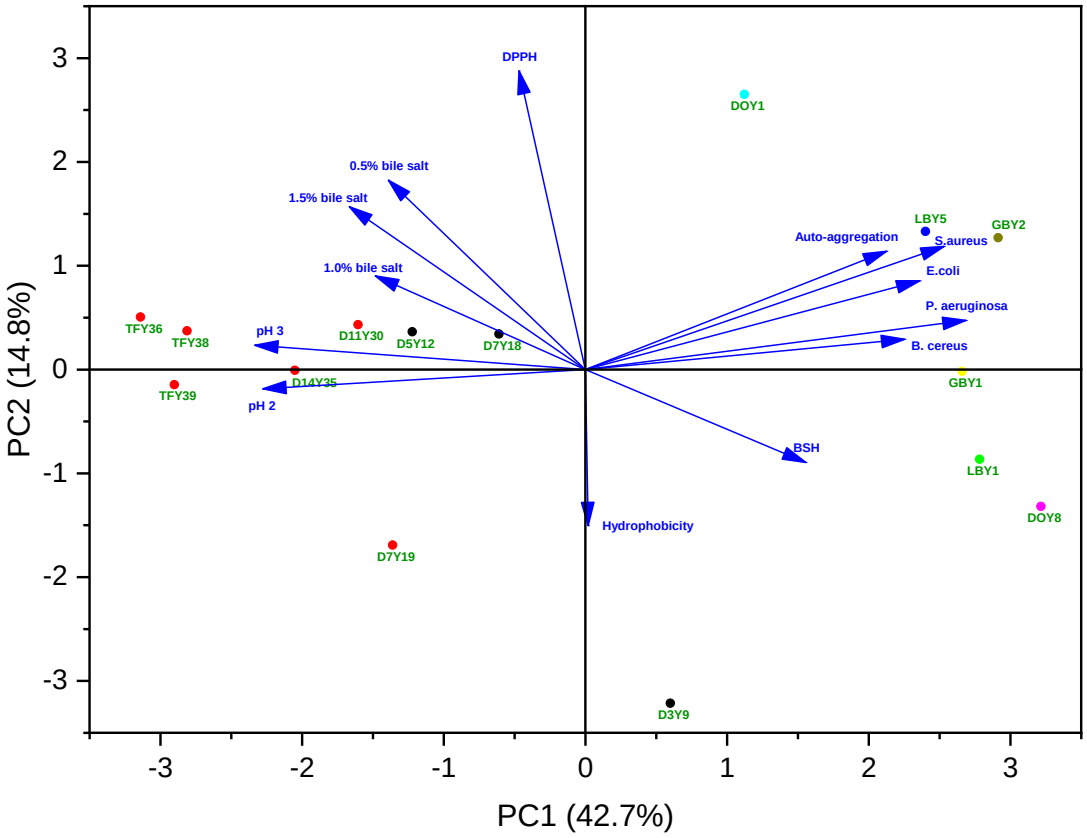

Figure 4.8 Principal component analysis (PCA) biplot of probiotic characteristics of fifteen yeast strains isolated from Kombucha. Blue arrows represent probiotic characteristics, and dots in different colours represent the yeast isolates.

4.4.5 Biosafety assessment

4.4.5.1 Susceptibility to antibiotics

The safety assessment of potential probiotic strains is important because some yeast isolates may be pathogenic (Fleet & Balia, 2006). Therefore, safety assessment must be carefully evaluated for all new potential probiotic strains before application for human use (Sanders et al., 2010). A key criterion for *in vitro* safety assessment of potential probiotic strains is antibiotic resistance (Lahtinen et al., 2009). Of the strains tested, two (GBY1 and GBY5) showed resistance to all eight antibiotics tested (Table 4.3). Yeast strain DOY8 showed resistance to most antibiotics but was sensitive to colistin sulphate. The remaining 12 yeast

strains were resistant to all the antibiotics except colistin sulphate. Resistance to ampicillin, chloramphenicol and nalidixic acid by all 15 yeast strains in this study agrees with Diguță et al. (2023). The increase in antibiotic resistance of pathogenic bacteria associated with probiotic treatment is a serious safety concern to public health (Czerucka et al., 2007). Horizontal gene transfer between bacteria and pathogenic bacteria in the GI tract can make pathogens resistant to antibiotics (Pereira et al., 2012). However, antibiotic resistance genes do not transfer from yeast to bacteria, making probiotic yeast isolates safe for administration during antibiotic treatment (Czerucka et al., 2007). Therefore, the resistance of yeast isolates to antibiotics in the present study could be a beneficial characteristic as the probiotics may survive during the antibiotic treatment and confer their beneficial effects to the host (Bacha et al., 2010). However, the yeast isolates used in this study do need to be tested against a larger number of traditional antibiotics.

Table 4.3 Susceptibility of yeast strains to antibiotics.

	Diameter of clear inhibition zone (mm)							
Yeast isolates	**AM**	**C**	**K**	**CO**	**NA**	**NI**	**S**	**T**
D3Y9	0	0	0	22.37±0.55	0	0	0	0
D5Y12	0	0	0	14.50±0.50	0	0	0	0
D7Y18	0	0	0	15.03±0.61	0	0	0	0
D7Y19	0	0	0	15.50±0.50	0	0	0	0
D11Y30	0	0	0	16.33±0.58	0	0	0	0
D14Y35	0	0	0	15.33±0.58	0	0	0	0
TFY36	0	0	0	17.83±0.76	0	0	0	0
TFY38	0	0	0	18.50±0.50	0	0	0	0
TFY39	0	0	0	16.07±0.12	0	0	0	0
LBY1	0	0	0	23.00±0.50	0	0	0	0
LBY5	0	0	0	9.60±0.36	0	0	0	0
DOY1	0	0	0	14.00±1.00	0	0	0	0
DOY8	0	0	0	27.00±0.65	0	0	0	10.00±1.00
GBY1	0	0	0	0	0	0	0	0
GBY5	0	0	0	0	0	0	0	0

AM: Ampicillin; C: Chloramphenicol; CO: Colistin Sulphate; K: Kanamycin; NA: Nalidixic Acid; NI: Nitrofurantoin; S: Streptomycin; T: Tetracycline. 0 = no observed inhibition zone. The results were expressed as mean±standard deviation (SD). The antibiotic susceptibility tests were done in triplicate. R: Resistant zone diameter (<12.4 mm); I: intermediate zone diameter (12.4-17.4 mm); S: susceptible (>17.5 mm).

4.4.5.2 Enzymatic activities

Safety aspects such as origin and possible harmful activities were also evaluated in screening for potential probiotic yeast in this study (Fernández-Pacheco et al., 2021; Menezes et al., 2020). The presence of haemolytic activity may cause the development of anaemia in the host by damaging host red blood cells (Fernández-Pacheco et al., 2021). The presence of gelatinase is another virulence factor because it degrades an appreciable number of host substrates such as collagens and fibrins. Additionally, *Enterococcus faecalis* is the main causative agent of infective endocarditis, resulting in urinary tract infection, damage to the heart valves, and high death rates of up to 20% (Baldassarri et al., 2004). The infective endocarditis is initialized by formation of vegetations on heart valves (Thurlow et al., 2010). The presence of gelatinase facilitates the development of vegetations and can lead to high death rate in endocarditis (Baldassarri et al., 2004; Johansson & Rasmussen, 2013). In addition to virulence factors, high amounts of extracellular phospholipase and proteases can promote colonisation by certain pathogens and cause tissue damage to host epithelial cells (D'Eça Júnior et al., 2011). Therefore, haemolytic, protease, gelatinase, and phospholipase activities should be included as part of safety studies when investigating potential probiotic strains. None of the 15 yeast strains secreted proteases, gelatinase, or phospholipase, and no positive results were obtained for the haemolytic activity. The *in vitro* tests carried out indicated no harmful properties, but *in vivo* safety testing is required to ensure the safety of probiotics for human administration.

4.5 Conclusion

In this study, the probiotic potential of yeast strains isolated from commercial Kombucha produced in New Zealand were evaluated *in vitro*. Four yeast strains of *S. pombe* LBY5, *B. anomalus* DOY8, *P. Kudraivzevii* GBY1, *S. cerevisiae* GBY2 exhibited excellent tolerance to simulated GI conditions (low pH, human temperatures, and presence of bile salts), cell surface, hydrophobicity, auto-aggregation, co-aggregation with pathogenic bacteria, and antioxidant

activity. Results showed that all the 15 strains tested in this study can be regarded as safe for consumption based on the absence of hemolysis, proteolytic, phospholipase and gelatinase activities. Strains of *P. kudraivzevii* GBY1 and *S. cerevisiae* GBY2 showed resistance to all tested antibiotics. Thus, these two yeast strains, (*P. kudraivzevii* GBY1 and *S. cerevisiae* GBY2) may be promising novel probiotic yeast for the food industry due to their excellent antioxidant activity and cell surface characteristics. However, *in vivo* testing, to analyse the health-promoting benefits of these probiotic strains as well as their stability during storage are recommended.

Chapter 5. Probiotic potential of acetic acid bacterial from Kombucha in New Zealand

5.1 Abstract

Kombucha is a fermented tea beverage containing live microorganisms, mostly beneficial strains of yeast and acetic acid bacteria (AAB), but empirical evidence is limited supporting the probiotic potential of Kombucha. This study reports the *in vitro* probiotic potential of 36 AAB strains isolated from three Kombucha samples commercially available in New Zealand. Nine representative AAB strains, belonging to three species (*Komagataeibacter rhaeticus, Acetobacter musti* and *Gluconobacter potus*), were examined for their primary probiotic characteristics such as tolerance to bile salts, NaCl, and low pH, and temperature. Three non-cellulose forming strains (*A. musti* LOAAB1, *G. potus* LOAAB2 and *G. potus* GBAAB3) were assayed for their cell surface characteristics such as auto-aggregation, co-aggregation with pathogenic bacteria, and hydrophobicity. Antimicrobial, antioxidant activities and enzymatic activities were also investigated for the strains of interest. Results indicated that nine strains were able to grow under low pH in the presence of bile salts, suggesting their potential to survive in the human gut. Six *K. rhaeticus* strains produced cellulosic pellicles, a potential source of prebiotics for beneficial bacteria. The three AAB strains LOAAB1, LOAAB2 and GBAAB3 showed promising cell surface characteristics, such as auto-aggregation rates (>80%), co-aggregation with four pathogenic bacteria (13.24% to 43.47%), hydrophobicity (42.12 % to 50.20 %), and antioxidant activities (>90%). All nine strains tested negative for enzymatic activities (haemolytic, proteolytic, phospholipase, and gelatinase), suggesting that they are safe to consume. Together, these data indicate the potential for the three AAB strains to be further investigated as probiotic sources with more *in vivo* tests for applications in the food and beverages industry.

5.2 Introduction

Kombucha is a traditional sparkling, slightly acidic and sweet tea beverage fermented by a complex symbiotic culture of bacteria and yeast called SCOBY (Jayabalan et al., 2014; Laureys et al., 2020; Phetxumphou et al., 2023). It is commonly fermented in a base of sugared tea (black, green or oolong tea) and a floating cellulosic pellicle for 7 to 14 days fermentation at ambient temperature (Greenwalt et al., 2000; Vargas et al., 2021). The consumption of kombucha dates to the Tsin Dynasty around 220 B.C. (de Miranda et al., 2022; Laureys et al., 2020). It is perceived that regular consumption of Kombucha may contribute to a variety of functional characteristics, including antioxidant, antimicrobial, antidiabetic, anticancer, anti-inflammatory, and hepatoprotective activities (Jayabalan et al., 2014; Jayabalan & Waisundara, 2019; Murugesan et al., 2009). Moreover, consumption of the beverage is associated with lowering cholesterol levels and blood pressure, prevention of bladder infection, and reduction in stress, nervous disorders and insomnia, likely due to the presence of bioactive ingredients such as organic acids (acetic, gluconic, glucuronic acids), vitamins (E and C), phenolic components (theaflavins, catechins, quercetin), diverse probiotics (AAB and yeast), and dietary fibre (Bortolomedi et al., 2022; Coelho et al., 2020; Ivanisova et al., 2020; Selvaraj & Gurumurthy, 2023; S. Wang et al., 2023). Due to the growing interest in natural and health foods, Kombucha has become one of the most popular fermented beverages worldwide (Batista et al., 2022; Freitas et al., 2022). In 2019, the global market size of Kombucha increased to about 2 billion dollars, and it is expected to reach around 10 billion dollars in 2027 (Freitas et al., 2022).

The composition of the microbial communities in Kombucha varies based on geographic locations, sources of raw materials, and fermentation conditions (Akhtar et al., 2021; Jayabalan et al., 2014). The most abundant prokaryotes are gluconic/acetic acid-producing bacteria: *Acetobacter aceti, A. pasteurianus, A. tropicalis, A okinawensis, A, musti*; *Gluconobacter*

oxydans, Gluconobacter potus; and cellulose-forming species such as *Komagataeibacter xylinus, K. rhaeticus, K. europaeus, K. intermedius, K. hansenii* (Barbosa et al., 2021; Gaggia et al., 2018; Pradhan et al., 2022; Savary et al., 2021). The microbiology of Kombucha is also characterised by various yeast species, including *Saccharomyces cerevisiae, S. ludwigii; Candida stellata, C. famata, C. guilliermondii, C. tropicalis; Zygosaccharomyces bailli, Z. rouxii, Z. lentus; Pichia kudraivzevii, P. fermentans, P. membranefaciens; Brettanomyces bruxellensis, B. lambrcus, B. intermediums, B. claussenii; Schizosaccharomyces pombe,* and *Torulaspora delbruckii* (Chakravorty et al., 2016; Gaggia et al., 2018; Jayabalan et al., 2014; Pradhan et al., 2022; B. Wang et al., 2023; B. Wang et al., 2022a). Lactic acid bacteria (LAB) have also been reported in Kombucha, and the common species are *Oenococcus oeni, Pediococcus acidiliactici, P. pentosaceus, Lacticaseibacillus casei,* and *liquorilactobacillus nagelii* (Bogdan et al., 2018; Nguyen et al., 2015; Savary et al., 2021). However, LAB strains are not often present, and their role in Kombucha fermentation is not well understood (Laureys et al., 2020). Nonetheless, the probiotic potential of the live microbial fermenters remains one of the most attractive factors for Kombucha consumers.

Probiotics are live microorganisms that occur singly or in mixed cultures to confer desirable health beneficial effects to the host when administrated in appropriate doses (FAO/WHO, 2006; Hill et al., 2014). Regular consumption of probiotics helps improve digestion, protects against pathogens, reduces cholesterol levels, boosts the immune system, and balances gut microbiota (Kim et al., 2022; Vargas et al., 2021). Microorganisms used as probiotics are mostly LAB strains belonging to *Lactobacillus, Bifidobacterium*, and *Enterococcus* (Kim et al., 2022; Pereira et al., 2021). Some yeast strains and endospore-forming bacteria such as *Bacillus subtillis* are also exploited as probiotics in the food industry (Pereira et al., 2021). Although diverse microorganisms have already been used in commercial probiotic products, there is a continuously increasing demand for multifunctional products. This requires the selection of

new potential probiotics from different sources with diverse functional activities. AAB strains represent a new source of probiotics due to their unique fermentation and acidification activities (Karabiyikli & Sengun, 2017). For example, some strains of *A. aceti, A. syzygii* and *A. indonesiensis* isolated from dairy products (yogurt, curd, and tarkhineh) were shown to possess probiotic characteristics such as tolerance to low pH and bile salts (Haghshenas et al., 2015). It has been reported that certain AAB strains exhibit anticancer and antimicrobial activities, as well as vitamin C production (Gomes et al., 2018; Haghshenas et al., 2015; Saichana et al., 2015).

Probiotics are commonly added to dairy products including yoghurt, cheese, milk, and kefir, and they can also be consumed as dietary supplements (Diguta et al., 2020; Farid et al., 2021). However, non-dairy probiotic products such as fermented juice, vegetables and beverages have recently gained increasing popularity due to their low risk of eliciting lactose intolerance symptoms and overall health properties (Oak & Jha, 2019). Unpasteurized kombucha is widely recognised as a probiotic beverage because consumers believe that it contains inherent live cultures which can confer beneficial health properties (Laureys et al., 2020; Vargas et al., 2021). However, there is limited evidence regarding the probiotic potential of microorganisms of Kombucha due to the low concentration of viable potential probiotics and a lack of empirical evidence supporting their health benefits (Vargas et al., 2021). The present study investigates the *in vitro* probiotic potential of nine AAB strains isolated from industrial Kombucha produced in New Zealand. The results led to the identification of three strains with promising probiotic activity.

5.3 Materials and methods

5.3.1 Bacterial growth conditions

A total of 36 AAB strains, belonging to three species, were previously isolated from two branded Kombucha beverages and bacterial cellulose (SCOBY) supplied by an artisan

kombucha producer in Auckland, New Zealand (B. Wang et al., 2023; B. Wang et al., 2022a). The strains were isolated using the standard dilution method, and their identities were determined by sequencing their 16S rRNA genes. Of the 36 strains isolated, 9 representative AAB strains were screened based on their different phenotypic characteristics and presence during fermentation. The AAB isolates (Table 5.1) were stored in 20% glycerol (w/w) at -80°C for long term preservation and propagated on glucose yeast extract (GY) agar plates by monthly subculturing before experimental analysis.

Table 5.1 Identities of AAB strains and their origins.

Species	Strain	Origins
Komagataeibacter rhaeticus	[1]D5AAB1	Kombucha tea broth
Komagataeibacter rhaeticus	[1]D7AAB10	Kombucha tea broth
Komagataeibacter rhaeticus	[1]D11AAB16	Kombucha tea broth
Komagataeibacter rhaeticus	[1]D14AAB17	Cellulosic pellicle (MB)
Komagataeibacter rhaeticus	[1]TFAAB26	Cellulosic pellicle (MB)
Komagataeibacter rhaeticus	[1]TFAAB29	Cellulosic pellicle (MB)
Acetobacter musti	[2]LOAAB1	Kombucha brand 1 (LB)
Gluconobacter potus	[2]LOAAB2	Kombucha brand 1 (LB)
Gluconobacter potus	[3]GBAAB3	Kombucha brand 2 (GB)

Note: [1]strains were selected from beginning, mid and end of fermentations from SCOBY. [2,3] AAB isolates were selected based on representative strains of each species with the same phenotypic characteristics.

5.3.2 *In vitro* tolerance of AAB strains to simulated gastrointestinal tract (GIT) conditions

5.3.2.1 Growth at different temperatures

The growth of AAB at different temperatures was assessed using the method of Alvarez et al. (2023). Loopfuls of overnight AAB suspension was streaked on GY agar plates and incubated at 25, 30, and 37°C for 5-7 days. The presence of colonies along the streaked lines indicated growth.

5.3.2.2 Cellulose production

Formation of cellulose was determined using the method of Semjonovs et al. (2017) with some modifications. Loopfuls of AAB cultures were inoculated in GY broth at 37°C for 5-7 days.

After incubation, the pellicle developed on the surface of the broth was mixed with 3-5 mL of 0.1 M NaOH solution and heated at 90℃ for 30 min in a hot water bath (T100, Grant Optima, Cambridge, UK). Insolubility of the pellicle indicated the production of cellulose.

5.3.2.3 Tolerance to low pH

The tolerance of each AAB strain to low pH was evaluated using the method of Haghshenas et al. (2015) with minor modifications. Overnight bacterial suspensions (1% v/v) of each AAB strain were inoculated into GY broth tubes adjusted to pH 3 with 0.1M HCl (Sigma, Aldrich, St. Louis, MO, USA). Strains were incubated at 37℃ for 24 h. Growth of AAB cultures was determined by measuring the absorbance at 600 nm using a spectrophotometer (Novaspec III, Amesham Biosciences, Buckinghamshire, United Kingdom) or the formation of cellulose on the surface of the broth. The non-inoculated GY broth (pH 3) was used as the blank control, and the growth index was calculated as shown in Equation 5.1.

$$\text{Growth index (\%)} = \left(\frac{OD_{600}\,(24h)}{OD_{600}(0h)}\right) * 100\% \text{ ----------[Equation 5.1]}$$

OD_{600} (0 h): $Absorbance_{600nm}$ at 0 h
OD_{600} (24 h): $Absorbance_{600nm}$ at 24 h

5.3.2.4 Tolerance to bile salts

The tolerance to bile salts was assessed using the method of Haghshenas et al., (2015) with some modifications. The overnight suspension (1% v/v) of each AAB strain was inoculated into GY broth supplemented with 0.5%, 1.0% and 1.5% (w/v) bile salts (Sigma Aldrich, St. Louis, MO, USA) and incubated at 37℃ for 24 h. The absorbance at 600 nm was measured at 0 h and 24 h to determine the growth of AAB isolates. The non-inoculated GY broth with different bile salt concentrations was used as the blank control and the growth index was calculated using Equation 5.1.

5.3.2.5 Tolerance to NaCl

The tolerance to different NaCl concentrations was determined using the method of Pitiwittayakul et al. (2016) with some modifications. The GY plates were supplemented with NaCl (1%, 2%, 4%, 6.5%) (w/v). Loopfuls of each overnight AAB culture were streaked on the GY plates containing the different salt concentrations and incubated at 37℃ for 5 days. The formation of colonies along the streaked lines indicated the growth and tolerance to NaCl.

5.3.3 Cell surface characteristics

5.3.3.1 Auto-aggregation and co-aggregation assay

The auto-aggregation was performed according to the method of Topçu et al. (2020) with modifications. Each non-cellulose forming AAB strain was separately cultured in GY broth for 18-24 h. After incubation, the AAB cultures were harvested by centrifuging (Heraeus Multifuge x1R, ThermoFisher, Germany) at 8000 *g* for 5 min. The cell pellet was washed with sterile 0.9% NaCl and resuspended to ~10^8 CFU/mL using the same saline solution. Each AAB cell suspension (4 mL) was vortexed for 10-15 s and incubated at 37℃ without shaking. For measurement, 0.2 mL of each AAB supernatant was carefully transferred to a new disposable plastic cuvette and mixed with 1.8 mL sterile 0.9% NaCl solution. The absorbance at 600 nm was measured at 0, 2, 4, 24 h. The auto-aggregation rate was calculated using Equation 5.2.

$$\text{Auto-aggregation (\%)} = \left[\frac{A_0 - A_T}{A_0}\right] * 100\% \text{----------[Equation 5.2]}$$

A_T: $\text{Absorbance}_{600nm}$ at each time interval (2,4, 24 h)
A0: Absorbance600nm at start (0h)

The co-aggregation of AAB isolates with pathogenic bacteria was analysed using the method of Kos et al. (2003) and Xu et al. (2009). The AAB cells were cultured as described above. *Peseudomonas aeruginosa* MUA26*, Staphylococcus aureus* MCTIC 4163*, Escherichia coli* NCTIC 8196, *Bacillus cereus* MU-A44 were used as pathogenic strains in this study. The

pathogens were cultured in tryptone soya broth (TSB) and incubated at 37°C overnight. Both AAB and pathogenic bacteria strains were centrifuged at 8000 *g* for 5 min at 4°C, washed with sterile 0.9% NaCl solution and resuspended in the same saline solution to ~ 10^8 CFU/mL. Equal volumes (2 mL) of AAB suspension (A_{pro}) and pathogenic bacteria (A_{path}) were mixed by vortexing for 10-15 s and incubated at 37°C for 4 h without shaking. The absorbance of each mixture (A_{mix}) at 600 nm was measured after 4 h incubation. The co-aggregation rate was calculated using Equation 5.3 developed by Handley et al. (1987).

$$\text{Co-aggregation (\%)} = \left(\frac{(Apro+Apath)/2-Amix)}{(Apro+Apath)/2}\right) * 100\% \text{ ----------[Equation 5.3]}$$

A_{pro}: absorbance of control tubes of AAB strains
A_{path}: absorbance of control tubes of pathogenic bacteria
A_{mix}: absorbance of control tubes of mixture after 4 h incubation

5.3.3.2 Cell surface hydrophobicity

The hydrophobicity properties of the AAB cells were determined using the method of Liu et al. (2021). The AAB cells were cultured at 30°C overnight and centrifuged at 8000 *g* for 5 min. at 4°C. The AAB cell pellet was washed with sterile 0.9% NaCl solution and resuspended to obtain an OD600nm value of approximately 0.4-0.6 (A_0) (Novaspec III, Amersham Biosciences). The adjusted AAB cell suspension (3 mL) was thoroughly mixed with chloroform (1 mL, Sigma Aldrich, St. Louis, MO, USA) using a vortex mixer for 1 min. The mixture was then kept at ambient temperature (~22°C) for 30 min to separate the organic and aqueous phases. The aqueous phase was carefully transferred to a clean disposal cuvette, and its absorbance was measured at 600 nm (A_x). The hydrophobicity was calculated using Equation 5.4:

$$\text{Hydrophobicity (\%)} = \left(1 - \frac{A_X}{A_0}\right) * 100\% \text{ ----------[Equation 5.4]}$$

A_X: absorbance of the aqueous phase
A_0: absorbance of the initial AAB strains

5.3.4 Assays for potential health benefits of the AAB strains

5.3.4.1 Antioxidant activity

The free radical scavenging activities of the AAB strains were determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (Diguta et al., 2020; S. Kim et al., 2022). The AAB strains were cultured separately in GY broth at 30°C overnight. The AAB cell suspensions were harvested by centrifugation (8000 *g*, 5 min, 4°C) and the concentration of AAB cultures was standardised to OD600 nm ~1.2 with sterile saline solution. The AAB cell suspension (0.8 mL) was mixed with 1mL of 0.2 mM methanolic DPPH solution (Sigma Aldrich) in 2 mL microcentrifuge tubes. The mixture was mixed using a vortex mixer for 1 min and then incubated for 30 min in the dark. The reaction tubes were centrifuged at 12000 *g* for 5 min, and the absorbance of the supernatant was measured at 517 nm. The control was prepared using absolute methanol (0.8 mL) with DPPH (1 mL) solution. The radical scavenging activity of DPPH was calculated using Equation 5.5:

$$\text{Scavenging ability } \% = \left[1 - \frac{A_{517\ AAB}}{A_{517\ control}}\right] x100\% \text{----------[Equation 5.5]}$$

A_{517AAB}: Absorbance of AAB isolates at 517 nm
$A_{517control}$: Absorbance of control at 517 nm
The results were also expressed as Trolox equivalent per mL of AAB cells (μM TE/mL).

5.3.4.2 Bile salt hydrolase (BSH) activity

Bile salt hydrolase activity was determined using a qualitative plate assay according to de Valdez and Taranto (2001) and Bommasamudram et al. (2023) with some modifications. The AAB strains were cultured in GY broth overnight and adjusted to a final concentration ~ 10^6 CFU/mL. Ten (10) μL of AAB culture (~10^6 CFU/mL) were spotted on GY plates supplemented with bile salts (0.5% w/v) and 0.37 g/L $CaCl_2$ (ThermoFisher, Waltham, MA, USA). The plates were incubated at 37°C for 5 days. The presence of precipitation around the

colonies indicated the hydrolysis of bile salt. The diameter of precipitation around each culture was measured using a calliper. The larger the diameter, the stronger the BSH activity.

5.3.4.3 Antimicrobial activity

Strain and propagation

Four bacteria (*P. aeruginosa* MUA26, *S. aureus* MCTIC 4163, *E. coli* NCTIC 8196, *B. cereus* MU-A44) and two mycotoxigenic moulds (*Aspergillius brassilliensis* NZRM2578, and *Penicillium chrysogenum* NZRM2999) were used as pathogenic microorganisms in this study. The four bacterial strains were inoculated on Muller Hinton agars (MHA) at 37℃ overnight and maintained at 4℃, monthly. The mould strains were inoculated on potato dextrose agar plates (PDA) at 25℃ for 5 days with monthly subculture and grown on yeast peptone dextrose (YPD) broth overnight.

Antimicrobial activity

The agar well diffusion method was used to determine the antimicrobial activity of AAB isolates (Haghshenas et al., 2015). For the antibacterial activity, the AAB isolates were cultured in GY broth at 30℃ for 18-24 h. The AAB cell density was adjusted to ~ 1.2 OD at 600nm. All the pathogens were cultured in TSB and incubated at 37℃ for 18-24 h and adjusted to 0.5 McFarland turbidity standard containing 1-2x10^8 CFU/mL (Balouiri et al., 2016). About 50 µL of each inoculum of pre-incubated pathogen (~0.5 McFarland standard) was evenly spread on MHA plates with sterile cotton swabs. Wells (Ø ≈ 7 mm) were made with a sterile cork borer in the MHA plates. The AAB cells (100 µL) were added to the wells in the MHA plates, and diameters of the inhibition zones were measured after incubation at 37℃ for 48 h.

The antifungal activity of AAB isolates was evaluated using the method of Haghshenas et al. (2015) with slight modifications. The moulds were inoculated on PDA plates at 25℃ for 5 days

until sporulation. The mould cultures were standardised to a 0.5 McFarland at OD_{530nm} (0.11-0.14). About 50 µL of pre-incubated moulds were evenly spread on PDA plates. Wells (Ø ≈ 7 mm) were made with sterile borer in the PDA plates. Standardised AAB cultures were added to the wells in the PDA plates and incubated at 25°C for five days. The diameter of clear zones around each well were measured to indicate the levels of antifungal activity. The antimicrobial activity of isolated AAB was classified into strong (diameter>20 mm), moderate (between 10 and 20 mm), and weak (less than 10 mm).

5.3.5 Assessment for safe consumption

5.3.5.1 Antibiotic susceptibility

Disk diffusion was used to determine the antibiotic susceptibility of AAB isolates (Liu et al., 2021; Szutowska & Gwiazdowska, 2021). Mast-ring S® (Mast Group, Liverpool, UK) composed of the following eight clinical antibiotics (µg): Ampicillin 25, Chloramphenicol 50, Colistin Sulphate 100, Kanamycin 30, Nalidixic Acid 30, Nitrofurantoin 50, Streptomycin 25, and Tetracycline 100 was used to assess the antibiotic resistance of AAB isolates. The AAB cultures were incubated in GY broth at 37°C overnight and adjusted to 0.5 McFarland standard. Then, 50 µL of AAB suspension was evenly and thoroughly spread on GY plates evenly and thoroughly. Mast-ring S ® disks were gently placed on the surface of the GY plates with using sterile forceps. The diameter of clear zones around each disk were measured after 5 days incubation at 37°C.

5.3.5.2 Haemolytic activity

The haemolytic activity of AAB isolates was performed using the method of Argyri et al. (2013) and Won et al. (2021). Fresh AAB suspensions were spot inoculated on blood agar plates (5% v/v sheep blood) and incubated at 37°C for 5 days. The presence of green or brown zones

around bacterial growth indicated α-haemolysis; clear zones around bacteria growth indicated β-haemolysis. Absence of clear zones around colonies was considered γ-haemolysis.

5.3.5.3 Proteolytic activity

The proteolytic activity of AAB isolates was assessed using the method of Moslehishad et al. (2013) and Padmavathi et al. (2018). AAB suspension (~10 µL) was spot inoculated on GY agar plates supplemented with 5% (w/v) skimmed milk (Anchor, New Zealand) and incubated at 37℃ for 5 days. The diameter of clear zones around colonies indicated proteolytic activity. The larger diameter of clear zones indicated stronger proteolytic activity.

5.3.5.4 Phospholipase activity

Phospholipase production was conducted on egg yolk medium (Price et al., 1982). The medium consisted of GY plates supplemented with 5.85% (w/v) NaCl, 0.05% (w/v) $CaCl_2$ and 5% sterile egg yolk (Sigma-Aldrich). A suspension (10 µL) of fresh AAB suspension (~ 0.08-0.10 at OD_{600nm}) was inoculated onto sterile paper disks and placed on agar plates, then incubated at 37℃ for 5 days. Enzymatic activity was visualized as areas of precipitation around each paper disk. Phospholipase activity (P_z) was expressed as the ratio of the colony diameter to the total diameter of colony and precipitation. Hence, P_z=1 indicated negative activity, lower P_z values indicated higher enzymatic activity.

5.3.5.5 Gelatinase activity

The gelatinase activity of AAB isolates was determined using the stabbing method described by Birri et al. (2013). Fresh AAB culture was stabbed into nutrient gelatine medium and incubated at 37℃ for 14 days and placed at 4℃ for 30 min to check for liquefaction. The presence of liquefaction was considered as a positive gelatinase reaction.

5.3.6 Statistical analysis

All the experiments were performed in triplicate. All the results were subjected to descriptive statistics using Microsoft Excel 2019 (Microsoft, USA). Further, data on tests for tolerance to bile salts, auto-aggregation and co-aggregation were analysed by analysis of variance-one way (ANOVA-ONEWAY) using Minitab 21 (Minitab, USA) ($p<0.05$). Significant differences between the means were separated by Tukey's test.

5.4 Results and discussion

5.4.1 *In vitro* tolerance to simulated gastrointestinal tract (GI) conditions

5.4.1.1 Growth tolerance to different temperatures

All AAB strains exhibited excellent growth on GY plates at different temperatures (25, 30, 37°C) as shown in Table 5.2. Growth at 25 and 30°C was not surprising as these are the most common fermentation temperatures used to produce Kombucha (Chu & Chen, 2006; Villarreal-Soto et al., 2019). Many previous studies have used 37°C as an essential selection criterion for probiotics as this is the average temperature of a healthy human body (Pereira et al., 2012; Pundir et al., 2013). The excellent growth of nine AAB isolates at these three temperatures in this study indicated their ability to survive in the human gut.

5.4.1.2 Production of cellulose

Six strains belonging to the species *K. rhaeticus* were able to produce a thin cellulosic pellicle floating on the surface of broth after 5-7 days incubation. AAB from the genus *Komagataibacter* commonly contribute to the production of the bacterial cellulosic pellicle (tea fungus) and cellulosic microfibres in the liquid portion of Kombucha (Jayabalan & Waisundara, 2019; Semjonovs et al., 2017). Bacterial cellulose produced from Kombucha has been widely used in the food industry to produce the traditional dessert called *nata de coco*, and as a fat replacer, and food additive (Azeredo et al., 2019; Leonarski et al., 2022). The Kombucha bacterial cellulose may also be a potential edible food packaging material due to its

antimicrobial activities (Doğan, 2023). The cellulosic pellicle found in this study may contain prebiotic components that promote the growth of probiotics present in the kombucha or human gut as well as stimulate probiotic strains to confer functional activities to humans (Vargas et al., 2021). Based on the evidence in the literature, consumption of the insoluble cellulose (dietary fibre) produced by the six strains may trap water in the colon, thereby, softening the stool and promoting digestion and indirectly helping to relieve constipation (Tungland & Meyer, 2002). Recently, Kombucha bacterial cellulose has been used as a protective barrier to immobilize probiotic strains such as *Lactobacillus plantarum* TISTR 541 to improve its survival and stability in simulated GIT (Charoenrak et al., 2023). Therefore, the formation of the cellulose pellicle from six strains in this study is a desirable characteristic, as cellulose can potentially benefit the survival of other beneficial microorganisms against extreme conditions in the gastric juice, as well as allowing Kombucha to be claimed as a promising "synbiotic" (mixture of prebiotics and probiotics) beverage source (Jayabalan & Waisundara, 2019).

5.4.1.3 Tolerance to low pH and bile salts

The tolerance to gastrointestinal tract conditions including the presence of bile salts and extreme acidic acid conditions is an important prerequisite characteristic for the selection of probiotics (Aswathy et al., 2008; Diguta et al., 2020; Topçu et al., 2020). Results presented in Figure 5.1 and Table 5.2 showed that the nine isolates exhibited promising growth in the presence of different levels of bile salts (0.5%, 1.0% and 1.5% w/v) and under pH 3. The growth rate of the three *K. rhaeticus* strains (D5AAB1, TFAAB26, and TFAAB29) and LOAAB2 decreased with the higher bile salt concentrations which was consistent with the results of probiotics isolated from pastirma, a type of air-dried cured beef commonly found in Turkish cuisine (Topçu et al., 2020). However, for the other five strains, the highest growth rate was detected with 1.0% bile salts. It is not surprising that the nine strains showed satisfactory growth under acidic environment conditions (pH 3), as this acidity is commonly found in

kombucha (2.5-4.0) (de Miranda et al., 2022). High tolerance against the presence of bile salts and acidic conditions have also been reported for other beneficial bacteria such as *Acetobacter cibinongensis* 34L, *Acetobacter syzygii* 38Lac, and *Acetobacter indonesiesis* 10HN (Haghshenas et al., 2015). All the AAB strains from the current study were viable at acidic and high bile salt conditions.

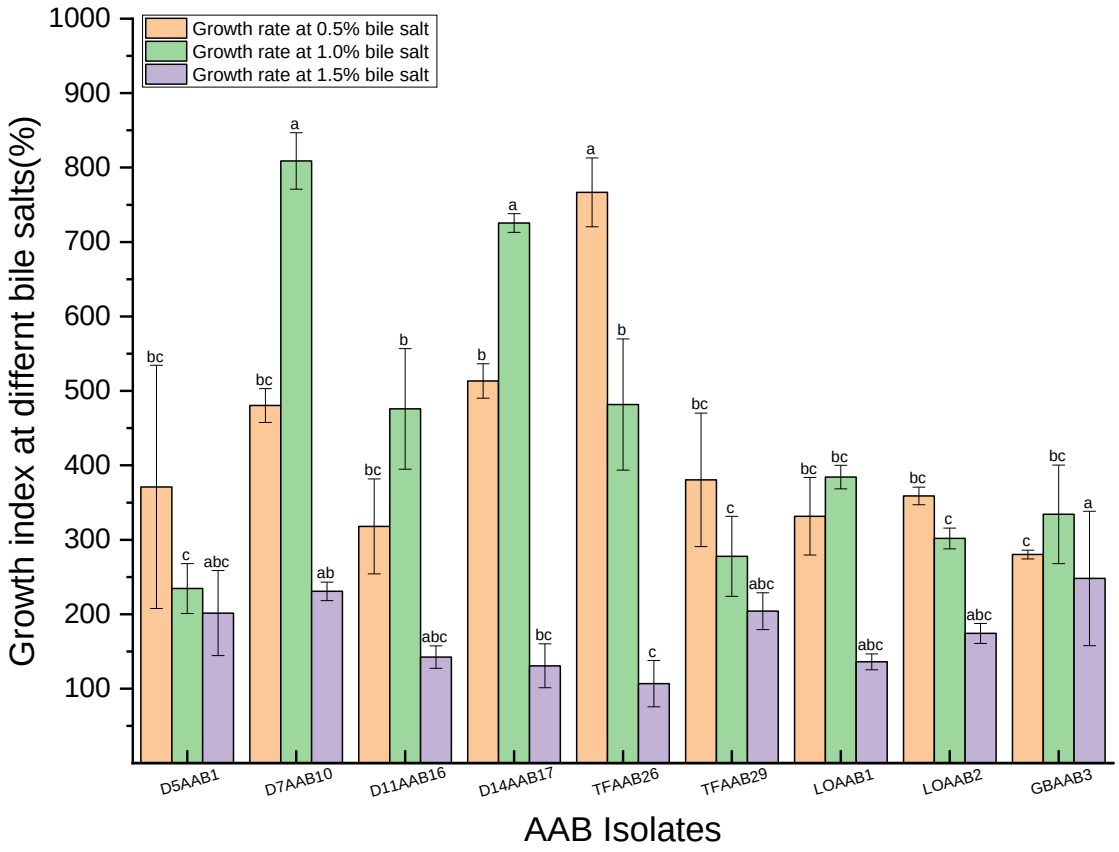

Figure 5.1 Growth index of AAB strains in the presence of bile salts. The results are expressed as mean ± standard deviation (SD) (n=3). Bars that do not have the same letter are significantly different (p<0.05). For the identifies of the AAB isolates, refer to Table 5.1.

5.4.1.4 Resistance to different NaCl concentrations

All nine AAB strains were able to grow in the presence of 1% (w/v) NaCl. This result is consistent with the known properties of *Acetobacter suratthanensis* sp. nov. (Pitiwittayakul et al., 2016). However, they were not able to grow under higher salt conditions with 2.5%, 4.0%,

or 6.5% (w/v) NaCl (Table 5.2). Some fermented foods including meat, kimchi, or black olives, which are considered as good sources of probiotics contain high salt concentrations (up to 10%) (Panagou et al., 2011; Tamang & Lama, 2022). The addition of salt in fermented food products may inhibit the growth of pathogenic microorganisms and improve the taste (Zeng et al., 2019). Therefore, the AAB strains in this study may be not suitable for producing certain salty food products due to their weak tolerance to NaCl.

Table 5.2 Growth at different temperatures, pH, salt concentrations and cellulose production.

Isolates	**Growth temperature (℃)**			**Growth in presence of NaCl (w/v)**					
	25	**30**	**37**	**1%**	**2.5%**	**4.0%**	**6.5%**	**Growth at pH 3**	**Cellulose production**
Komagataeibacter rhaeticus **D5AAB11**	+	+	+	+	-	-	-	+	+
Komagataeibacter rhaeticus **D7AAB10**	+	+	+	+	-	-	-	+	+
Komagataeibacter rhaeticus **D11AAB16**	+	+	+	+	-	-	-	+	+
Komagataeibacter rhaeticus **D14AAB17**	+	+	+	+	-	-	-	+	+
Komagataeibacter rhaeticus **TFAAB26**	+	+	+	+	-	-	-	+	+
Komagataeibacter rhaeticus **TFAAB29**	+	+	+	+	-	-	-	+	+
Acetobacter musti **LOAAB1**	+	+	+	+	-	-	-	865.46±11.50 %	-
Gluconobacter potus **LOAAB2**	+	+	+	+	-	-	-	417.03±46.03 %	-
Gluconobacter potus **GBAAB3**	+	+	+	+	-	-	-	159.04±6.56 %	-

Note: "+" = positive results; "-" = the negative results. All the experiments were done in triplicate. The growth index of three non cellulose producing AAB isolates was calculated as Equation 5.1.

5.4.2 Cell surface characteristics

5.4.2.1 Auto-aggregation activity

Bacterial auto-aggregation is a reversible physical interaction between the same strains resulting in the formation of a precipitate under resting conditions (Krausova et al., 2019; Nwoko & Okeke, 2021; Sorroche et al., 2012). Auto-aggregation is a desired feature for probiotics as it potentially causes competitive exclusion of pathogens (Nwoko & Okeke, 2021).

All three non-cellulose-forming strains showed a high (>80%) aggregation percentage after 24 h incubation (Figure 5.2). *G. potus* GBAAB3 showed the highest auto-aggregation ability at 37℃ after 24 h incubation (90.40%). *A. musti* LOAAB1 exhibited only low auto-aggregation ability after 2 h incubation but increased to levels similar to the other two strains after 4 and 24 h incubation. The auto-aggregation ability was also assessed by visual observation. All three strains showed a very clear supernatant and beige precipitate at the end of incubation, consistent with the probiotic *Lactobacilli* strains studied by others (Collado et al., 2008; García-Cayuela et al., 2014). The high auto-aggregation ability of three strains in the present study indicate binding characteristics specific to probiotics, which allows them to survive and grow in harsh environments such as the GIT (García-Cayuela et al., 2014; Xu et al., 2009). Auto-aggregation enhances bacterial adherence to intestinal cells, which can help prevent subsequent colonisation by pathogenic bacteria (Dlamini et al., 2019; M. Li et al., 2020).

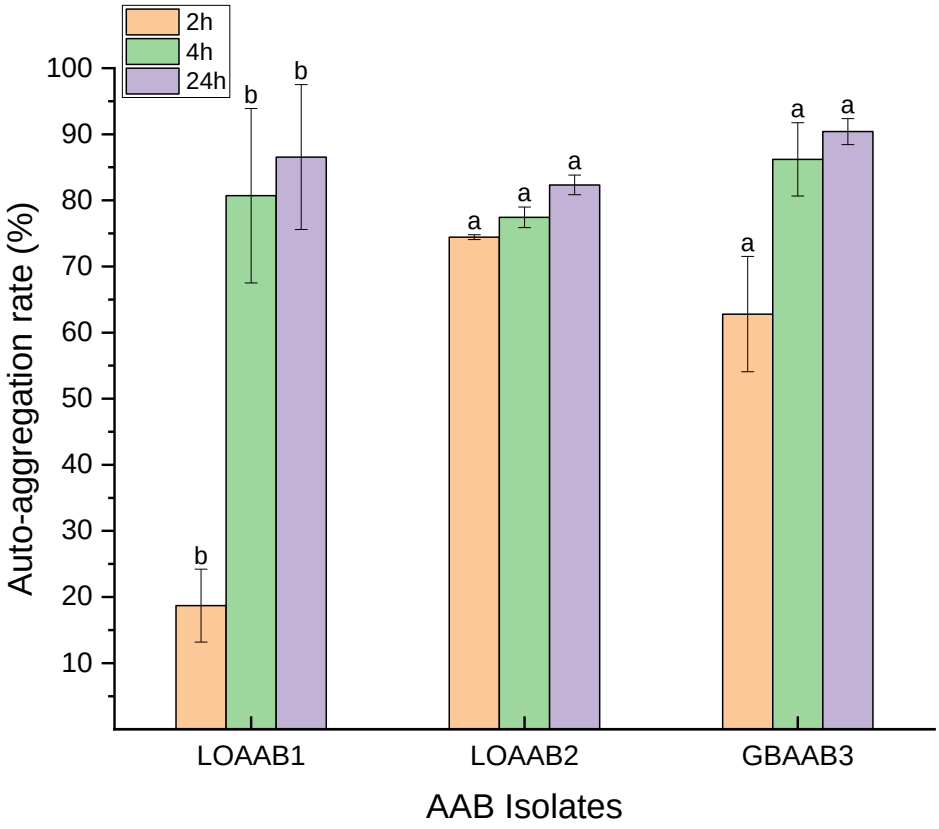

Figure 5.2 Auto-aggregation (%) of AAB strains after 2, 4, and 24 h incubation. The results are expressed as mean±standard deviation (SD), n=3. Bars that do not have the same letter are significantly different (p<0.05). For the identifies of the AAB isolates, refer to Table 5.1.

5.4.2.2 Co-aggregation activity

Co-aggregation occurs between genetically different bacterial strains (Collado et al., 2008; García-Cayuela et al., 2014). The co-aggregation activity between three AAB strains and four pathogens (*P. aeruginosa* MUA26, *S. aureus* MCTIC 4163, *E. coli* NCTIC 8196, *B. cereus* MU-A44) are shown in Figure 5.3. All strains showed significant co-aggregation with the four tested pathogens after 4 h (p<0.05). The highest co-aggregation ability of the four tested pathogens was exhibited by GBAAB3 with *S. aureus* (53.47±0.77%) after 24 h incubation. The highest co-aggregation percentage with *E. coli* NCTIC 8186 was obtained from LOAAB1. While the highest co-aggregation with *P. aeruginosa* MUA26 and *B. cereus* MU-A44 was

detected from LOAAB2 with 25.78% and 24.76%, respectively. Strain GBAAB3 exhibited the lowest co-aggregation with *E. coli* NCTIC 8186 (Figure 5.3). The results obtained in this study indicated that the co-aggregation ability of the three non-cellulose-forming AAB strains was possibly dependent on specific combination of probiotic strains and pathogenic strains, and co-incubation conditions (temperature and time), similar to the work reported by others (Collado et al., 2008; Dlamini et al., 2019; García-Cayuela et al., 2014; Vlková et al., 2008). Co-aggregation with different pathogens is an important characteristic for probiotic strains as this may contribute to host defence mechanisms by preventing infection by pathogens (Zhang et al., 2013). Co-aggregation also aids in the elimination of pathogens from the GI tract by balancing the micro-environment around pathogens and preventing their adherence to the host gut (Dlamini et al., 2019; M. Li et al., 2020). Strains with higher co-aggregation percentages may promote the adherence of these potential probiotic strains to the host gut (M. Li et al., 2020). Therefore, the ability of the AAB strains to co-aggregate with the four pathogens tested in this study indicates that these strains may be beneficial to host health.

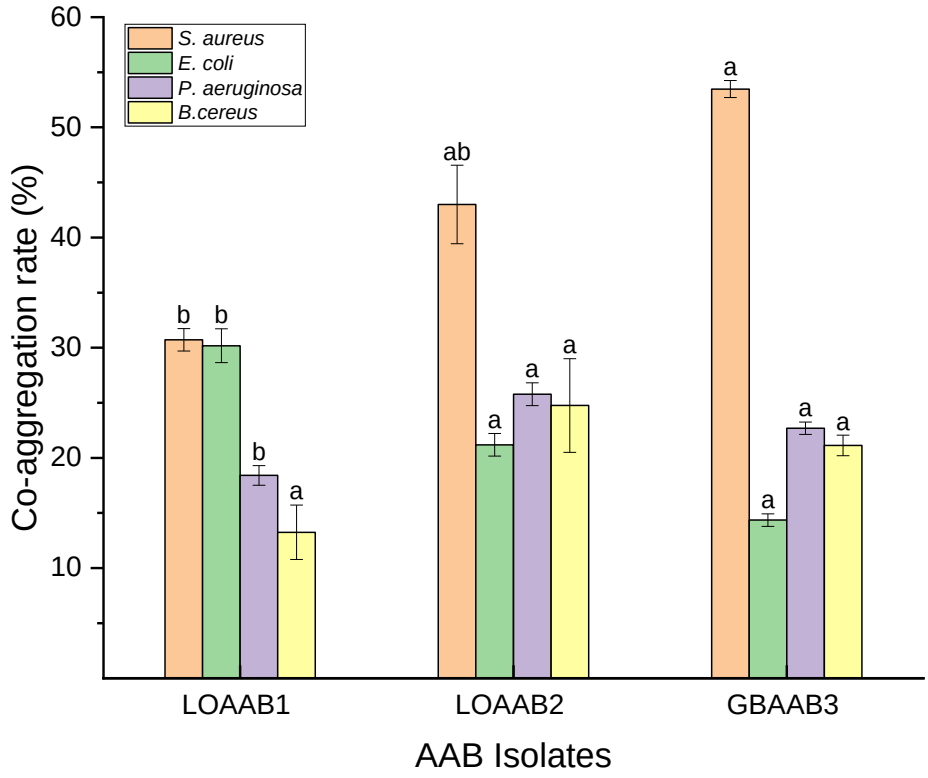

Figure 5.3 Co-aggregation ability of AAB strains with pathogenic bacteria. The results are expressed as mean with standard deviation (SD), n=3. Bars that do not have the same letter are significantly different (p<0.05). For the identifies of the AAB isolates, refer to Table 5.1.

5.4.2.3 Hydrophobicity activity

Bacterial adhesion to hydrocarbon like xylene (a polar solvent), chloroform (polar acidic solvent) and ethyl acetate (polar basic solvent) has been widely used to determine cell surface hydrophobicity of probiotic strains (Farid et al., 2021; Xu et al., 2009). Cell surface hydrophobicity may contribute to the adhesion of probiotic bacterial cells to the host intestine and colonisation of the GI tract, thus conferring health promoting effects to the host (Aswathy et al., 2008; Dlamini et al., 2019; Farid et al., 2021; Hashemi et al., 2014; Sadeghi et al., 2022; Silva et al., 2022). In this study, the cell surface hydrophobicity percentage of AAB strains to chloroform (polar acid) varied from 42.12% to 50.20% (Table 5.3). According to Tyfa et al.

(2015), the hydrophobicity ability to hydrocarbons (chloroform) can be divided into three levels: strongly hydrophobic (>50%), moderately hydrophobic (20-50%), and hydrophilic (<20%). Based on these criteria, strain GBAAB3 was strongly hydrophobic, while LOAAB1 and LOAAB2 were moderately hydrophobic. These results are slightly lower than those of probiotic strains belonging to *L. reuteri* (68%-75%) (Dlamini et al., 2019), but higher than most LAB strains tested with chloroform which were weakly hydrophobicity (<20%) (Xu et al., 2009). As well as AAB strain specific differences contributing to the observed bacterial cell hydrophobicity in this study, the incubation conditions, pH of media, and selection of hydrocarbons (chloroform), may also have affected the results as has been seen in previous studies (Liu et al., 2021; Silva et al., 2022).

Table 5.3 Hydrophobicity of AAB strains.

AAB strains	Hydrophobicity (%)
LOAAB1	47.22±0.38
LOAAB2	42.12±0.38
GBAAB3	50.20±0.67

The hydrophobicity tests were done in triplicate. The results were expressed as mean±standard deviation (SD). For the identifies of the AAB isolates, refer to Table 5.1.

5.4.3 Beneficial effects of AAB isolated from Kombucha

5.4.3.1 Antimicrobial activity

A large number of probiotic strains isolated from fermented foods or beverages have been reported to confer antimicrobial activities against pathogenic microorganisms including *E. coli, S. aureus, Salmonella enterica typhimurium, Listeria monocytogenes, Shigella sonnei*, *Shigella flexneri* and *B. cereus* (Diguta et al., 2020; M. Li et al., 2020; Liu et al., 2021; Zhang et al., 2011). This characteristic is one of the most important beneficial activities of probiotics (Diguta et al., 2020). Although none of the nine AAB strains tested in this study showed antimicrobial activities against food borne bacteria and moulds, these results are consistent with previous studies where neither *Acetobacter syzgii* 38 Lac or *Acetobacter indonesiensis* 10 HN isolated

from curd and yoghurt exhibited antimicrobial activity against four tested pathogenic bacteria (Haghshenas et al., 2015). However, strains of *Acetobacter cibinongesis* showed moderate (diameter of inhibition zone between 10-20 mm) to strong (diameter> 20 mm) antimicrobial activities against *E. coli, P. aeruginosa, S. aureus, S. saprophyticus* and *B. cereus* (Haghshenas et al., 2015).

The presence of antimicrobial activity by probiotic bacterial strains may be associated with antimicrobial substances such as organic acids, hydrogen peroxide, and bacteriocins (Arena et al., 2018; Sidooski et al., 2019). Co-aggregation also contributes to the antimicrobial activities to some extent, as the intimate contact occurring between probiotics and pathogenic microorganisms allows the antimicrobial substances produced by probiotics to inhibit the pathogens (Arena et al., 2018). The antimicrobial activities against other food borne pathogens should be tested in further studies.

5.4.3.2 Bile salt hydrolase activity

The deconjugation of bile salts through BSH activity has been associated with the reduction of serum cholesterol levels and improved survival of probiotics in the GI tract (Bommasamudram et al., 2023). Therefore, consumption of probiotic strains with BSH activity are beneficial to the host, especially those with hypercholesterolemia (Chand et al., 2017; Farid et al., 2021). None of AAB strains in this study exhibited BSH activity. The absence of BSH activity has also been found for other probiotic LAB strains including those isolated from dairy sources such as sheep milk and Feta cheese (Tsigkrimani et al., 2022).

5.4.3.3 Antioxidant activity

The DPPH free radical scavenging activity of AAB strains were all above 90% and the TAEC ranged between 81.46 and 84.92 μM/mL (Table 5.4). The highest antioxidant activity was observed for LOAAB1 (94.40%) followed by GBAAB3 (93.39%). Antioxidant activity is

another important functional activity of probiotics. Numerous studies have reported the antioxidant capacity of LAB isolates belonging to *Lactiplantibacillus acidophilus*, *L. plantarum, L. brevis,* and *P. pentosaceus* (Bommasamudram et al., 2023; Das & Goyal, 2015; Farid et al., 2021; S. Kim et al., 2022; Liu et al., 2021). High level of free radicals such as reactive oxygen species have been reported to induce a number of diseases such as cardiovascular diseases and cancer (Das & Goyal, 2015; Liu et al., 2021). The high antioxidant activity of the AAB isolates in this study could contribute to the relief of oxidative stress damage to host cells (Bommasamudram et al., 2023; Das & Goyal, 2015). The antioxidant activity of probiotic bacteria has been suggested to be due to the presence of bacterial exopolysaccharides on their cell surface, antioxidant enzymes, and lipoteichoic acids (Farid et al., 2021; S. Kim et al., 2022).

Table 5.4 Antioxidant activity of AAB strains.

AAB Strains	DPPH (%)	TAEC(μM/mL)
LOAAB1	94.40±0.55	84.92±0.56
LOAAB2	91.01±0.72	81.46±0.74
GBAAB3	93.39±0.64	83.88±0.66

The antioxidant activity tests were performed in triplicate. The results were expressed as mean±standard deviation (SD). For the identifies of the AAB isolates, refer to Table 5.1.

5.4.4 Safety assessment

5.4.4.1 Antibiotic susceptibility

Although AAB have been widely used in food production and they are generally considered safe, several safety criteria including susceptibility to antibiotics, haemolytic activity, and virulence factors (e.g., gelatinase, protease) must still be assessed to eliminate possible harmful effects of any new probiotic strains before their application in the food industry (Hashemi et al., 2014; Karabiyikli & Sengun, 2017; Pereira et al., 2012; Sanders et al., 2010). All nine AAB isolates were resistant to chloramphenicol, colistin sulphate and nitrofurantoin, but were sensitive to ampicillin, streptomycin, and tetracycline (Table 5.5). Strains of D5AAB1,

LOAAB1, LOAAB2 and GBAAB3 showed intermediate sensitivity to nalidixic acid. The sensitivity of AAB strains to ampicillin and tetracycline has been previously reported (Haghshenas et al., 2015; Haghshenas, Nami, Abdullah et al., 2015). About 70% of probiotics isolated from different sources have been reported to exhibit resistance to more than two antibiotics (Gueimonde et al., 2004). Resistance to streptomycin is commonly intrinsic and not transmissible (Ammor et al., 2007; Cao et al., 2019). The intrinsic resistance of beneficial bacteria to different antibiotics is a health promoting characteristic as the probiotics could remain viable during antibiotic treatment (Bacha et al., 2010; Das et al., 2020; Reuben et al., 2020). However, the horizontal antibiotic resistance gene transfer from probiotic bacteria such as LAB to pathogenic bacteria in the intestinal environment is a major safety concern (Lahtinen et al., 2009; Sanders et al., 2010). Therefore, probiotic products must not contain transferable antibiotic resistance genes (Diguta et al., 2020). The sensitivity (intrinsic resistance) of AAB strains to certain traditional antibiotics in this study may be a desirable beneficial characteristic as these strains are not reservoirs to transfer antibiotic resistance genes to pathogens (Haghshenas et al., 2015).

Table 5.5 Susceptibility of AAB strains to antibiotics.

AAB strains	AM	C	CO	K	NA	NI	S	T
D5AAB1	27.67±0.58	0	0	11.50±0.50	13.67±0.29	0	17.67±0.58	32.67±0.58
D7AAB10	58.67±0.58	0	0	12.00±0.00	8.67±0.58	0	19.33±0.58	33.67±0.58
D11AAB16	29.33±0.58	0	0	11.83±0.29	10.17±0.76	0	18.00±0.00	32.67±0.58
D14AAB17	29.33±0.58	0	0	11.50±0.50	8.17±0.29	0	18.00±0.00	32.67±0.58
TFAAB26	28.33±0.58	0	0	11.33±0.29	7.23±0.32	0	18.33±0.58	31.67±0.58
TFAAB29	28.50±0.50	0	0	13.33±0.58	8.27±0.21	0	24.33±0.57	35.17±0.29
LOAAB1	19.93±0.47	8.53±0.49	0	11.83±0.15	13.87±0.57	0	16.23±0.51	26.57±0.50
LOAAB2	21.80±0.61	0	0	11.50±0.50	16.13±0.77	0	19.57±0.50	29.43±0.67
GBAAB3	18.57±0.60	0	0	10.63±0.63	17.37±0.64	0	18.50±0.56	31.00±0.50

AM: Ampicillin; C: Chloramphenicol; CO: Colistin Sulphate; K: Kanamycin; NA: Nalidixic Acid; NI: Nitrofurantoin; S: Streptomycin; T: Tetracycline.0= no observed inhibition zone. The antibiotic susceptibility testwere done in triplicate. The results were expressed as mean±standard deviation (SD). R: Resistant zone diameter (<12.4mm); I: intermediate zone diameter (12.4-17.4 mm); S: susceptible (>17.5mm). For the identifies of the AAB isolates, refer to Table 5.1.

5.4.4.2 Enzymatic activities

Haemolysis is a virulence factor which destroys the red blood cells of the host, resulting in anaemia and oedema (Romero-Luna et al., 2020; Vesterlund et al., 2007). Gelatinase is another virulence factor that may facilitate infections and toxicity in the host by breaking down immunoproteins (collogen) and complement (C3a) (Y. Kim et al., 2022; Thurlow et al., 2010). The proteolytic activity of gelatinase also interferes with the host immune system and contributes to endocarditis caused by *Enterococcus faecalis* (Thurlow et al., 2010). Furthermore, proteolysis enhances the colonization of pathogens in host tissues and compromises the host immune system by destroying important proteins including immunoglobins (Mattei et al., 2013). Phospholipase activity makes it easier for pathogens to invade the host cells, resulting in epithelial cell damage (Gokce et al., 2007). Therefore, haemolytic, proteolytic, gelatinase, and phospholipase activities should be carefully assessed prior to the selection of new potential probiotic strains for use in food industry applications. None of the AAB isolates showed haemolytic activity as no colonies developed on the blood agar plates after incubation. None of the AAB isolates produced gelatinase, proteolytic activities, or phospholipase in this study. The absence of the above enzymatic activities is similar to other potential probiotic strains belonging to *Lactococcus lactis, Leuconostoc mesenteriodes* and *Weissella parammesenteroides* and suggests that these AAB strains are safe for consumption (Tsigkrimani et al., 2022).

5.5 Conclusion

This study is the first to evaluate the probiotic characteristics of *Acetobacter, Gluconobacter*, and *Komagataibacter* strains isolated from Kombucha produced in New Zealand. The results indicated that nine AAB strains (*K. rhaeticus* D5AAB1, D7AAB10, D11AAB16, D14AAB17, TFAAB26, TFAAB29, *A. musti* LOAAB1, and *G. potus* LOAAB2, GBAAB3) exhibited promising growth at acidic pH (3), different temperatures (25, 30, 37°C), and in the presence

of bile salts (0.5%, 1.0% and 1.5%, w/v). The six *K. rhaeticus* strains were capable of producing cellulose, which may act as a potential prebiotic. All nine strains can be considered safe for consumption because of their acceptable antibiotic susceptibility and absence of harmful activities (haemolytic, proteolytic, gelatinase, and phospholipase activities). Three of the nine AAB strains (LOAAB1, LOAAB2, and GBAAB3) exhibited excellent antioxidant activity and cell surface characteristics (auto-aggregation, co-aggregation with pathogens), suggesting that they may be potential probiotic candidates for use in production of functional foods.

Chapter 6. Effect of fermentation conditions on the physicochemical and functional characteristics of black tea Kombucha

6.1 Abstract

Kombucha is a popular fermented tea beverage, reported to have various beneficial effects. This study aimed to determine the effect of various fermentation factors on bioactive components, antioxidant, and antimicrobial activities of Kombucha fermented using a New Zealand starter culture. The Plackett-Burman design was used to identify the significant fermentation factors affecting the physicochemical and functional activities of black tea Kombucha. The total flavonoid and phenolic content of the Kombucha were analysed using the Folin-Ciocalteu and the modified aluminium chloride colorimetric assay, respectively. The antioxidant and antimicrobial activities were also determined. The results showed that Kombucha fermented using 17 different fermentation conditions had low acidity (pH 2.55-3.16) with visible colour differences. Of the seven measured phenolic compounds, caffeine, gallic acid and theobromine were the main phenolic compounds in the 17 fermented Kombucha samples. All the Kombucha samples showed antioxidant activities for DPPH (20.78-91.28%) and ABTS+ (22.63-551.10 μg TE/mL) assays. The antioxidant activities had positive correlations with total phenolic, total flavonoid content, and individual phenolic compounds. The phenolic content and antioxidant activities were mainly influenced by the concentrations of black tea used ($p<0.05$). Kombucha samples with the highest titratable acidity (1.03%) exhibited the strongest antimicrobial activities against *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli*. Therefore, the Kombucha produced in this study may be considered a potential functional beverage due to the presence of high levels of bioactive compounds, as well as its strong antioxidant and antimicrobial activities.

6.2 Introduction

Kombucha is a popular low-alcohol fermented tea beverage, commonly prepared in a base of sweetened tea with a symbiotic culture of acetic acid bacteria (AAB) and yeast abbreviated as SCOBY (Jayabalan et al., 2014; Leonarski et al., 2022; Nyhan et al., 2022). Kombucha has a sweet to sour taste, naturally sparkling characteristics, and a unique pleasant aroma (Phetxumphou et al., 2023). Generally, the preparation of Kombucha commences by dissolving sugar and tea substrate in boiling water, and then allowing the mixture to infuse for short periods (Dutta & Paul, 2019; Jayabalan et al., 2014). After cooling to ambient temperature, the sugared tea is inoculated with the appropriate amount of SCOBY or a previous Kombucha broth, and it is then fermented for 7- 14 days (Coton et al., 2017; Malbasa et al., 2008; B. Wang et al., 2023). A new thin layer of pellicle forms on the surface of the Kombucha and then the fermentation is considered complete. At this stage, the fermented Kombucha may be consumed (Chakravorty et al., 2016).

Regular consumption of Kombucha is reported to confer several potential health benefits, including antioxidant, antimicrobial, antivirus, antiproliferative, antidiabetic, anticarcinogenic, and hepatoprotective activities (Aung et al., 2022; Martínez Leal et al., 2018; Selvaraj & Gurumurthy, 2023; S. Wang et al., 2023). Kombucha is also believed to lower cholesterol and blood pressure, improve the immune system, relieve constipation, reduce the risk of coronary heart disease, as well as aid in weight management (Chakravorty et al., 2019; Jayabalan & Waisundara, 2019; Laureys et al., 2020). Increased acceptance of these desirable health-promoting effects by consumers and the appealing sensory characteristics of Kombucha have contributed to the growing market of Kombucha in many countries, especially in North America and Europe. Consequently, Kombucha has become one of the best-selling low-alcohol drinks with an estimated global market size of 1.8 billion USD in 2018 (Vargas et al., 2021), and it is expected to reach more than 5 billion USD by 2025 (Kim & Adhikari, 2020).

A wide range of organic and inorganic compounds are present in Kombucha at the end of fermentation. These include sugars (sucrose, fructose, and glucose), organic acids (acetic acid, gluconic acid, glucuronic acid), polyphenols (catechin) from tea, ethanol, trace elements (copper, manganese, and zinc), probiotic microorganisms (AAB and yeast), and vitamins (C, E, B group) (Coelho et al., 2020; de Miranda et al., 2022; de Noronha et al., 2022; Kapp & Sumner, 2019; Kumar & Joshi, 2016; S. Wang et al., 2023). The final chemical composition of the fermented beverage varies between products, which is largely influenced by fermentation conditions such as tea types, added sugar and tea, fermentation temperature and time, as well as the diverse microbial composition of the starter culture (Martínez Leal et al., 2018; Tran et al., 2020). Traditionally, black tea is the most common tea substrate used to prepare Kombucha, and it is known to be a rich source of polyphenols, thus contributing to the antioxidant capacities of Kombucha (Aung et al., 2022; Cardoso et al., 2020; Jakubczyk et al., 2020). Recent reports on Kombucha fermentation have used alternative substrates such as different types of tea (green tea, oolong tea, jasmine tea), fruit juice (grape, apple, goji berry, snake fruit, sour cherry, pomegranate), milk (soybean and raw cow's milk), as well as by-products and organic food waste (banana peel extract) (Ayed et al., 2017; Emiljanowicz & Malinowska-Pańczyk, 2020; Leonarski et al., 2022).

The microbial community in the starter culture is complex and diverse, but it is mainly composed of AAB and yeast (B. Wang et al., 2022b). These microorganisms play an important role in the biological transformation of the chemical components during Kombucha fermentation, producing metabolites such as organic acids, CO_2, ethanol, and the cellulosic pellicle (Jakubczyk et al., 2020; Kallel et al., 2012). The invertase released by the yeast converts sucrose to fructose and glucose, while AAB converts glucose to gluconic acid and ethanol. The ethanol is then utilised to produce organic acids particularly acetic acid, which contributes to the sour taste of Kombucha (Dufresne & Farnworth, 2000; Sievers et al., 1996).

Despite the production process of Kombucha having been widely reported in the literature, the industrial scale-up of Kombucha still faces challenges as the fermentation process has not been standardised. Variations in fermentation conditions and added substrates can result in inconsistent physicochemical, sensory, and nutritional characteristics, as well as a final product of undesirable quality (Emiljanowicz & Malinowska-Pańczyk, 2020; Villarreal-Soto et al., 2018). Therefore, a better understanding of the effect of fermentation factors on physicochemical characteristics, and functional activities at the end of fermentation is required to facilitate better control of production processes. This knowledge should aid in producing consistent, high quality Kombucha beverages. Therefore, this study investigated the effect of fermentation conditions on physicochemical characteristics, bioactive compounds, and functional activities of black tea Kombucha fermented using a defined New Zealand starter culture.

6.3 Materials and Methods

6.3.1 Kombucha starter culture

The Kombucha starter culture (SCOBY) was obtained from an artisan Kombucha producer in Auckland, New Zealand in 2021. The microbiological composition of this starter culture was previously identified as *Debaryomyces prosopidis* JCM 9913*, Zygosaccharomyces lentus* CBS 8574*, Komagataeibacter rhaeticus* DST GL02 (B. Wang et al., 2023). The SCOBY was propagated and maintained by successive cultivation in a black tea infusion (5 g/L) supplemented with table sugar (50 g/L) at 4°C until required for further use.

6.3.2 Experimental design

6.3.2.1 Fermentation factors

The effect of seven independent variables on the fermentation of Kombucha using a defined culture were conducted as follows: (Table 6.1): black tea, sugar, tea infusion time, fermentation temperature, fermentation time, amount of inoculum and amount of SCOBY. Two levels of

each of the seven variables were tested in this study. Maximum and minimum levels of independent variables are indicated by the use of positive (+1) and negative (-1).

Table 6.1 Independent variables and their levels developed through the Plackett-Burman design.

Independent variables (Factors)	Code	Minimum level (-1)	Maximum level (+1)
Black tea leaves (g/L)	A	1.5	12
Sugar (g/L)	B	20	100
Infusion time (min)	C	5	15
Temperature (°C)	D	20	30
Time (days)	E	7	14
Inoculum (mL/L)	F	100	200
SCOBY (g/L)	G	10	40

6.3.2.2 Plackett-Burman design

The Plackett-Burman design was used to generate different fermentation conditions for black tea Kombucha. The design comprised of 17 experiments containing 5 centre points (K5, K7, K12, K13, K16) based on the seven independent variables (Table 6.2). This matrix was designed using Minitab 21 statistical software (USA).

Table 6.2 The Plackett-Burman design.

Experiment code	A	B	C	D	E	F	G
K1	1.50	100	15	20	14.0	100	10
K2	1.50	100	5	20	7.0	200	40
K3	1.50	20	5	30	14.0	200	10
K4	12.00	20	5	20	14.0	200	40
K5	6.75	60	10	25	10.5	150	25
K6	12.00	100	5	30	7.0	100	10
K7	6.75	60	10	25	10.5	150	25
K8	12.00	20	15	20	7.0	100	40
K9	1.50	100	15	30	7.0	200	40
K10	1.50	20	5	20	7.0	100	10
K11	12.00	100	5	30	14.0	100	40
K12	6.75	60	10	25	10.5	150	25
K13	6.75	60	10	25	10.5	150	25
K14	1.50	20	15	30	14.0	100	40
K15	12.00	100	15	20	14.0	200	10
K16	6.75	60	10	25	10.5	150	25

K17	12.00	20	15	30	7.0	200	10

Notes: A: black tea leaves (g/L); B: Sugar (g/L); C: Infusion time (min); D: Temperature (°C); E: Time (days); F: Inoculum (mL/L); G: SCOBY (g/L).

6.3.3 Preparation of black tea Kombucha

Fermented Kombucha was prepared at the laboratory scale following the method of B. Wang et al. (2023) with some modifications and the fermentation parameters shown in Table 6.2. Refined sugar (white sugar; Chelsea, Auckland, New Zealand) was added into boiling water and mixed well using a sterile spatula. Black tea (Bell@, Auckland, New Zealand) was added to the sugar solution and allowed to infuse then removed after the appropriate time (Table 6.2). After the sugared tea solution had cooled to room temperature (~22°C), previous Kombucha was added as inoculum (Table 6.2) as well as to lower the pH, prevent the growth of pathogens, and enhance the growth of AAB and yeast (Jayabalan et al., 2014). An appropriate weight of starter culture (SCOBY) (Table 6.2) was gently placed on the surface of the cooled sugared tea solution. The container was tightly covered with a clean, porous cloth to keep out foreign matter such as insects. The pores of the cloth allowed adequate air to penetrate the broth which is required for the growth of AAB. The fermentation was carried out at a set temperature and time (Table 6.2). The newly formed cellulosic layer and fermented Kombucha broth were collected at the end of fermentation and stored at -20°C until required for analysis.

6.3.4 Characteristics of Kombucha cellulosic pellicle

6.3.4.1 Wet yield

The wet yield of the newly formed pellicle at the end of fermentation was determined using the modified methods of Aung and Eun (2021) and Amjadi et al. (2023). The weight of the pellicle was measured at the start of fermentation (day 0) and end of fermentation (day 7.0, 10.5 and 14.0). The wet yield of pellicle was calculated using Equation 6.1 and expressed as %.

$$\text{Wet yield (\%)} = \frac{\text{Microbial cellulose weight (g)}}{\text{Sugar concentration}} \times 100\% \text{----------[Equation 6.1]}$$

6.3.4.2 Moisture content and water holding capacity (WHC)

The moisture content and water holding capacity (WHC) were determined using the method of Avcioglu et al. (2021) and Lin et al. (2014) with some modifications. The wet cellulosic pellicle samples were cut into small pieces and about 1-5 g of the pellicle was dried overnight at 60℃ in a vacuum oven (Heratherm^{TM}, ThermoFisher Scientific, Massachusetts, USA) to remove all the water content until the weight of sample was constant. The WHC and moisture content were calculated as shown in Equations 6.2 and 6.3:

$$\text{WHC (g/g)} = \frac{\text{weight of water remove during drying (g)}}{\text{dry weigtht of tea fungus (g)}} \text{----------[Equation 6.2]}$$

$$\text{Moisture content (\%)} = \left(\frac{w2-w3}{w2-w1}\right)\text{x100\%----------[Equation 6.3]}$$

W1: weight of empty container (g)
W2: weight of container and sample before drying (g)
W3: weight of container and sample after drying (g)

6.3.4.3 Water swelling ratio (WSR)

The cellulosic pellicle was dried at 60℃ in a vacuum oven (Heratherm^{TM}, ThermoFisher Scientific) for 48 h to remove all the water in pellicle (Żywicka et al., 2018). The dried pellicle was then completely immersed in distilled water at ambient temperature until the weight of pellicle was constant. The WSR was calculated as:

$$\text{WSR (\%)} = \left(\frac{W1-W2}{W2}\right) \times 100\% \text{----------[Equation 6.4]}$$

W1: weight of swollen pellicle (g)
W2: dried weight of pellicle (g)

6.3.5 Physico-chemical characteristics of black tea Kombucha

6.3.5.1 Acidity of black tea Kombucha

The pH of the black tea Kombucha collected from different fermentation conditions was measured using a Sartorius glass electrode pH meter (Model PH-11, Sartorious AG, Gottingen,

Germany). Titratable acidity was determined by acid-base titration. Black tea Kombucha (10 mL) was titrated with standardised 0.1 M NaOH to an end point of pH 8.2. The TA was expressed as a percentage of acetic acid per gram, as it is the major acid in Kombucha (Equation 6.5).

Titratable acidity of acetic acid (%) $= \frac{V_{NaOH} \times M_{NaOH} \times 6.0053}{V_{kombucha}}$----------[Equation 6.5]

V_{NaoH}: volume of NaOH (mL)
M_{NaOH}: molarity of NaOH (M)
$V_{kombucha:}$ volume of Kombucha (mL)

6.3.5.2 Total soluble solids (TSS), colour and turbidity of black tea Kombucha

The total soluble solids (TSS) of black tea Kombucha were measured using a refractometer (pr-32 alpha, Atago co Ltd, Tokyo, Japan), according to Amarasinghe et al. (2018). The colour of the Kombucha was measured with a colorimeter (Minolta Chroma Meter CR-300, Konica Minolta Co Ltd, Tokyo, Japan) according to Zou et al. (2021) with slight modifications. In terms of the CIE L*a*b system, L stands for lightness, a* values represent redness, and b* values represent yellowness. The turbidity of the Kombucha tea broth was determined by measuring the optical density (OD) at 660 nm using a spectrophotometer (Genesys 150, ThermoFisher Scientific, USA) (Aharwar & Parihar, 2023). The turbidity was calculated using Equation 6.6 (Kitchener et al., 2017; Mountain & Keating, 2021):

Turbidity % = [100- antilog(2-$A_{kombucha}$)] %----------[Equation 6.6]

$A_{Kombucha}$: absorbance of Kombucha at 660nm

6.3.5.3 Ethanol concentration of black tea Kombucha

The ethanol concentration of black tea Kombucha was determined using an enzymatic assay kit (Megazyme, Bray, Ireland) (Ivory et al., 2021). Kombucha samples (2 mL) were filtered through 0.22 µm sterile syringe filters (Sigma-Aldrich, St. Louis, Missouri, USA) to remove the live microorganisms. Then, a 200-fold dilution of the Kombucha was prepared with distilled water. The diluted Kombucha sample (0.1 mL) and distilled water (2 mL) were mixed

well with 0.2 mL solution 1 (buffer), 0.2 mL solution (NAD+) and 0.05 mL solution 3 (aldehyde dehydrogenase). The absorbance of the mixture (A1) was recorded after 2 min at 340 nm using a spectrophotometer. Solution 4 (alcohol dehydrogenase) was then added to the mixture and the absorbance (A2) recorded at the end of the reaction (about 5-10 min). The blank sample was prepared by replacing the Kombucha samples with distilled water. The concentration of ethanol was calculated as shown in Equation 6.7:

$$ABV\% = \frac{V \times MW}{\varepsilon \times d \times v \times 2 \times 7.894} \times \Delta A_{Ethanol} \times F\ [mg/mL] \text{----------[Equation 6.7]}$$

V: final volume
MV: molecular weight of ethanol (g/mol)
E: extinction coefficient of NADH at 340 nm
V: sample volume (mL)
2= 2 mol of NADH produced for each mole of ethanol
F: dilution factor
7.489: factor used to convert g/L to % ABV

6.3.6 Total phenolic content of black tea Kombucha

The Folin-Ciocalteu assay was used to analyse the total phenolic content of Kombucha tea with slight modifications (Amjadi et al., 2023; Ebrahimi Pure, 2016). Diluted (up to 10-fold) Kombucha sample (0.2 mL) was mixed with 1 mL Folin-Cocalteu reagent (10% v/v). Sodium carbonate (0.8 mL, 7.5% w/v) was added after 3-5 min. The absorbance at 765 nm was measured after 1 h incubation at ambient temperature (~22℃) in the dark. The blank sample was prepared by replacing Kombucha with distilled water. Gallic acid (50 to 250 µg/mL) was used for preparation of a standard curve ($y=0.0096x+0.0585$ $R^2=0.9981$). The results were expressed as gallic acid equivalents (GAE) per mL of Kombucha (µg GAE/mL).

6.3.7 Total flavonoid content of black tea Kombucha

The modified aluminium chloride colorimetric assay was used to determine the total flavonoid content of Kombucha (Amjadi et al., 2023; Chakravorty et al., 2016; Chang et al., 2002; Shahbazi et al., 2018). Kombucha sample (0.5 mL) and absolute ethanol (Sigma-Aldrich) (1.5

mL) were thoroughly mixed with 0.1 mL of aluminium chloride (10% w/v, Sigma-Aldrich), 0.1 mL of 1 M potassium acetate (Sigma-Aldrich), and 2.8 mL distilled water. The mixture was incubated for 30 min in the dark at ambient temperature (~22℃) and the absorbance measured at 415 nm. A serial dilution of quercetin (25 to 150 µg/mL) was used for the preparation of a standard curve ($y=0.0063x+0.0222$ $R^2=0.9919$). The results were expressed as quercetin equivalents (QE) per mL of Kombucha (µg QE/mL).

6.3.8 Phenolic compounds of black tea Kombucha determined by HPLC

The phenolic compounds [gallic acid, theobromine, caffeine, epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin (EC), and epicatechin gallate (ECG)], in Kombucha prepared under different conditions were identified and quantified by the methods described by Yao et al. (2004). The Kombucha tea broth (1.5 mL) was passed through a syringe membrane filter (0.22 µm) into HPLC vials. A 20-µL aliquot of each Kombucha filtrate was injected into the HPLC system (Shimazu, Kyoto, Japan) equipped with an autosampler (SIL-20ACHT), single pump (LC-20 AD), and a photodiode array detector (SPD-M20A). Separation of phenolic compounds were carried out using a reversed phase column (Grace Smart RP18, 250 x 4.6 mm, 5 µm). Mobile phase A consisted of 0.1% trifluoroacetic acid in Milli-Q water, and mobile phase B was 0.1% trifluoroacetic acid in acetonitrile. The gradient elution was used as follows: 0-15 min, 8.5% solution B; 15-22 min, 10% solution B; 22-32 min, 11% solution B; 32-50 min, 14.8% solution B; 50-60 min, 31.8% solution B; 60-78 min, 100% solution B; 78-95 min, 0% solution B. The flow rate and column temperature were maintained at 0.75 mL/min and 18℃, respectively. Phenolic compounds were detected at 270 nm and 317 nm. The phenolic compounds in Kombucha were identified by comparisons of the retention time and spectra of pure reference standards (La Torre et al., 2021). Individual phenolic compounds were quantified by the external standard method. Standard mixtures (gallic acid, theobromine,

caffeine, EC, EGCG, EGC, and ECG) at different concentrations were used to prepare calibration curves.

6.3.9 Antioxidant activity of black tea Kombucha

Two *in vitro* assays: DPPH and ABTS+ free radical scavenging assays were used to determine the total antioxidant activities of Kombucha tea broth produced under different fermentation conditions. The Kombucha samples were filtered through a 0.22 µm sterile syringe filter to remove microbial cells prior to analysis.

6.3.9.1 DPPH free radical scavenging assay

The free radical scavenging activity of Kombucha was determined using the DPPH assay (Amjadi et al., 2023; Jafari et al., 2020; La Torre et al., 2021). One mL of appropriately diluted (10-40 folds) Kombucha was mixed with 0.1 M DPPH solution (1 mL) prepared with absolute ethanol. The mixture was then incubated at ambient temperature (~22℃) in the dark for 30 min. The absorbance of the mixture was measured at 517 nm using a spectrophotometer. The control sample was prepared with absolute ethanol instead of sample. The DPPH free radical scavenging activity of Kombucha was calculated as:

$$\% \text{ inhibition of radical DPPH} = \left(1 - \frac{A_{kombucha}}{A_{Control}}\right) \times 100\% \text{----------[Equation 6.8]}$$

$A_{kombucha}$: absorbance of kombucha at 517nm
$A_{control}$: absorbance of control at 517 nm

The Trolox equivalent antioxidant capacity (TEAC) of Kombucha sample was quantified using a solution of 1mM 6-hydroxyl-2,5,7.8-tetramethylchroman-2-carbxylic acid (Trolox) dissolved in methanol. A series of Trolox (20-100 µg/mL) was used for preparation of a standard curve (y=0.7278x-3.4414 R^2=0.9943). The results were expressed as µg Trolox Equivalent /mL of Kombucha (µg TE/ mL).

6.3.9.2 ABTS free radical scavenging assay

The ABTS radical cation (ABTS+) scavenging activity of Kombucha was conducted using the method of Kilic and Sengun (2023) and La Torre et al. (2021). ABTS+ was prepared by mixing ABTS (7 mM) with an equal volume of potassium persulphate (2.45 mM, Sigma-Aldrich). The mixture was incubated in the dark for 12-16 h and diluted with absolute ethanol to achieve an absorbance of 0.70±0.02 at 734 nm. Diluted ABTS+ solution (1.8 mL) was mixed with Kombucha sample (0.2 mL) and incubated at ambient temperature (~22℃) in the dark for 6-10 min. The absorbance of the mixture was measured at 734 nm. Absolute ethanol was used as the blank sample. The antioxidant activity was expressed as percentage of radical inhibition of ABTS+ and the results were calculated as shown in Equation 6.9.

$$\% \text{ inhibition of ABTS+} = \left(1 - \frac{A_{kombucha}}{A_{Control}}\right) \times 100\% \text{-----[Equation 6.9]}$$

A control: absorbance of ABTS+ solution at 734 nm
A kombucha: absorbance of Kombucha sample at 734 nm

The Trolox in a range of 20 to 100 µg/mL was used to prepare a standard curve (y=0.5388x+1.9797 R^2=0.9967). The results were expressed as µg Trolox Equivalent /mL of Kombucha (µg TE/ mL).

6.3.10 Antimicrobial activity of black tea Kombucha

6.3.10.1 Tested strains and cultivation

The pathogenic bacteria strains used to determine the antimicrobial activities were Gram-positive bacteria: *Staphylococcus aureus* MCTIC 4163, *Bacillus cereus* MU-A44; and Gram-negative bacteria: *Pseudomonas aeruginosa* MUA26, *Escherichia coli* NCTIC 8196. The bacterial strains were maintained on Muller Hinton agar (MHA) plates at 4°C and grown on tryptone soya broth (TSB) at 37℃ for 18-24 h.

6.3.10.2 Antimicrobial activity of black tea Kombucha

Antimicrobial activity of Kombucha was screened using the agar well-diffusion assay according to the method of Battikh et al. (2012) and Tan et al. (2020) with some modifications. The bacterial strains were inoculated in TSB at 37°C overnight and adjusted to 0.5 McFarland standard containing approximately 1-2 x10^8 CFU/mL. The standard suspension was diluted to a final concentration of ~10^6 CFU/mL in molten Muller Hinton agar (MHA), poured into plates and allowed to solidify. Wells (~ 7 mm diameters) in the inoculated MHA plates were prepared using a stainless steel sterile cork borer. Kombucha samples were centrifuged (Heraeus Multifuge x1R; ThermoFisher, Germany) at 8000 *g* for 5 min and the supernatants were filtered through sterile 0.22 µm microfilters to remove microbial cell debris. Filtered Kombucha (~100 µL) was transferred to wells and inoculated with appropriate bacterial strains. The plates were placed at 4°C for about 2 h to diffuse Kombucha samples and then incubated at 37°C for 24 h (Sreeramulu et al., 2000). Trigene (5%) solution and TSB were used as positive and negative controls, respectively. The diameter of clear inhibition zone was measured after incubation.

6.3.11 Statistical analysis

Moisture content, water holding capacity, water swelling ratio of Kombucha cellulosic pellicle; colour, total soluble solids, turbidity, pH, titratable acidity, total phenolic content, total flavonoid content, free radical scavenging activity, and antimicrobial activities of black tea Kombucha were performed in triplicate and results expressed as mean value ± standard deviation. The Plackett Burman design was carried out using Minitab 21 statistical software (USA). Statistical differences between samples were evaluated with variance-one way (ANOVA-ONEWAY) and Tukey test to compare the significant differences of the means ($p<0.05$). Heatmap of phenolic compounds, Pearson correlation and principal component analysis (PCA) of black tea Kombucha were evaluated using Origin 2021b (OriginLab, USA).

6.4 Results and discussion

6.4.1 Characteristics of Kombucha cellulosic pellicles

6.4.1.1 Wet yield of Kombucha cellulosic pellicles

A thin transparent cellulosic layer was observed on the surface of the black Kombucha tea broth at the end of fermentation (day 7,10.5, and 14). The formation of cellulosic pellicles was likely caused by the action of *K. raheticus* DST GL02 in the starter culture (Machado et al., 2016; B. Wang et al., 2023). The wet yield of Kombucha cellulosic pellicle (KCP) varied from 4.82 % to 131.15% (Figure 1A). Higher yields (>100%) were observed in K4 and K14, while K9 and K15 gave low yields (<15%). Of the seven key fermentation parameters, SCOBY concentrations, black tea infusion time, and Kombucha inoculum impacted on the wet yield of KCP ($p<0.05$).

KCP yield has been reported to be affected by complex parameters including the fermentation temperature, sugar types, nitrogen sources and fermentation time (Neera et al., 2015; Villarreal-Soto et al., 2019; Yim et al., 2017). In our study, black tea leaves were selected as a cost-effective nitrogen source to replace the traditional Hestrin and Schramm (HS) medium supplemented with expensive nitrogen sources (i.e., yeast extract and peptone) to reduce the cost of KCP production (Jacek et al., 2021). However, when added at very high concentrations the carbon source may inhibit or even halt the formation of the bacterial cellulose produced by *Komagataeibacter.* Similar results were obtained in the current study where low yields were observed in K1, K9 and 15, which were prepared with high sugar levels (100 g/L) (Avcioglu et al., 2021; Bae & Shoda, 2004). The abundant catechins and flavanols present in black tea and organic acids produced during Kombucha fermentation may also contribute to the cell growth in the KCP (da Silva Pinto, 2013). Avcioglu et al. (2021) indicated that higher black tea concentrations (1%) increased the KCP yield. However, there was no effect of black tea concentrations on the KCP wet yield in the present study ($p>0.05$).

Kombucha tea is commonly consumed as a functional fermented beverage (Selvaraj & Gurumurthy, 2023). However, the KCP by-product, present at the end of fermentation has received increasing attention due to its unique beneficial characteristics and numerous applications in food, biomedical and pharmaceutical industries (Doğan, 2023; Shi et al., 2014). KCP has been recognised as a good source of dietary fibre to lower the risk of diabetes and obesity due to its appealing taste and low calories (Azeredo et al., 2019). The cellulose can also be used as a raw material to make traditional desserts such as *nata de coco* from the Philippines, SCOBY hamburger, and gummy candies due to their simple production and desirable mouthfeel (Coelho et al., 2020). Recently, KCP has been shown to have strong antioxidant characteristics (DPPH 74.22%) and antimicrobial activities against several pathogens (*E. coli, S. aureus*, and *B. cereus*), which make it suitable as a potential food packaging material (Azeredo et al., 2019; Doğan, 2023; Shi et al., 2014). Despite KCP having several potential applications in the food industry, its commercialization faces many challenges, such as high operation costs and low yield (Azeredo et al., 2019; Żywicka et al., 2018). Several studies have been conducted to maximize KCP yield and using lower-cost culture media composed of by-products produced during food manufacturing (Bodea et al., 2021; Treviño-Garza et al., 2020). The high yield of KCP prepared with K4 and K14 formulation and fermentation conditions in this study may be a promising process to further optimize the yield of bacterial cellulose.

6.4.1.2 Water swelling ratio

The dried Kombucha cellulosic pellicle (KCP) was able to rapidly absorb large amounts of distilled water to saturation after one hour of soaking (Figure 6.1B). Black tea and sugar concentrations were identified as significant factors affecting the water swelling ratio (WSR) of the 17 KCP samples in this study ($p<0.05$). The highest WSR (1024.25%) in K4 was similar to the swelling ratio of the bacterial cellulose film in wet state (1027%) from a *nata de coco* consortium (Sanchavanakit et al., 2006). A very high water swelling ratio (2121%) was

reported for bacterial cellulose soaked in media composed of D-glucose, yeast extract, and calcium carbonate (Bodea et al., 2021). Wei et al. (2011) reported that bacterial cellulose developed by *K. xylinus* had a much higher swelling ratio (26.2 times dry weight) in distilled water and 37.3 times in saline solution. Therefore, based on previous studies, variations in WSR by the Kombucha cellulosic pellicle could also be attributed to the fermentation conditions, bacterial strains, composition of inoculum media and soaking solutions (Bodea et al., 2021; Sanchavanakit et al., 2006; Wei et al., 2011). A high-water swelling ratio is a desirable characteristic for KCP used as a wound dressing to absorb blood and wound exudate from severe traumas, and maintaining an appropriate moisture level during wound healing, which facilitates oxygenation of the wound, prevents contamination, and permits painless wound dressing changes (Lin et al., 2013; Wei et al., 2011). Therefore, the high-water swelling ratio (>1000%) of KCP observed in this study (K4) indicates that the Kombucha cellulosic pellicle could be a promising material for applications in the biomedical industry.

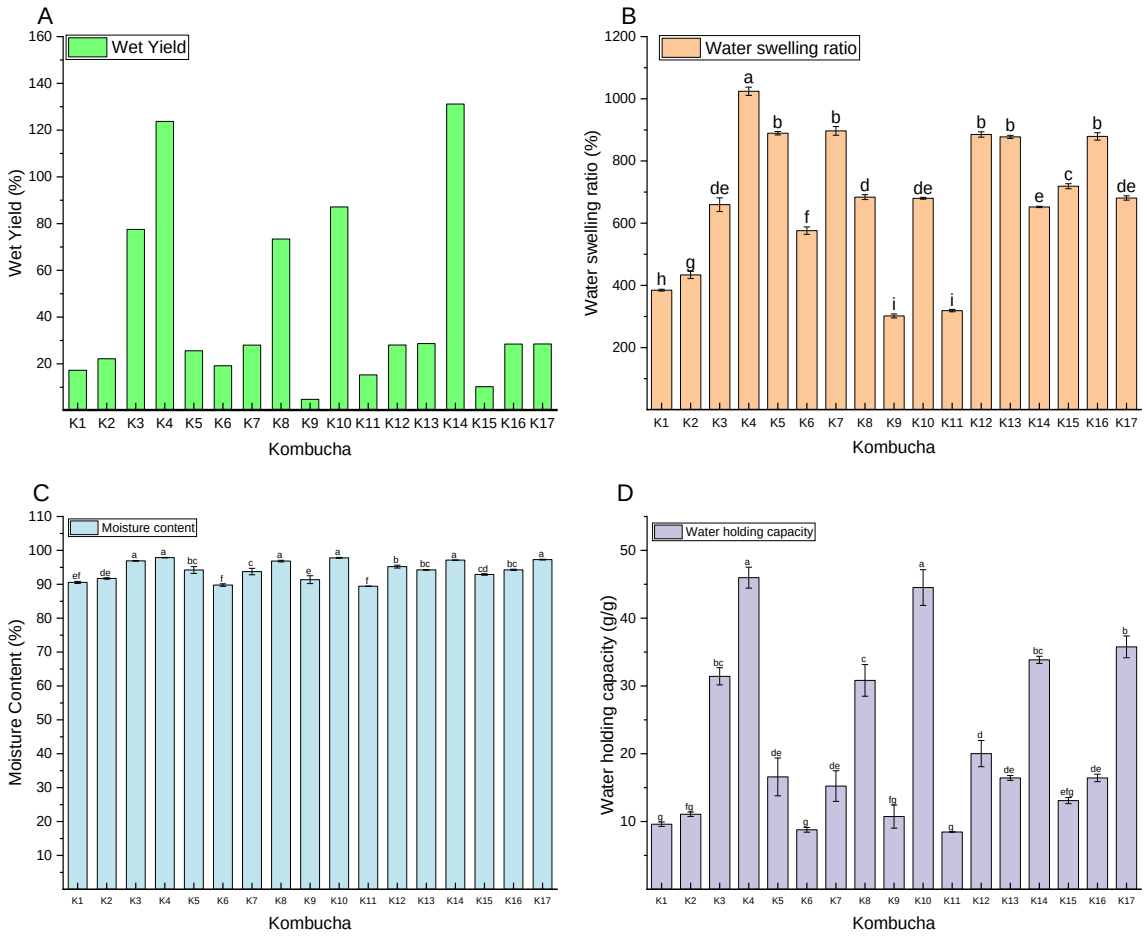

Figure 6.1 Wet yield (A), water swelling ratio (B), Moisture content (C), and water holding capacity (D) of Kombucha samples prepared under different fermentation conditions. Different lowercase letters (a-g) on the chart bars indicate significant differences between samples ($p<0.05$). For the composition of the samples K1-K17, refer to Table 6.2.

6.4.1.3 Moisture content and water holding capacity

The moisture content of KCP was between 89.76% to 97.87% (Figure 6.1C) ($p<0.05$). The KCP is hydrophilic with a high moisture content that can be removed and then rehydrated depending on the intended use, for example as a wound dressing (Choi et al., 2022; Sederavičiūtė et al., 2019). Moisture content is influenced by the microstructure of the KCP and is indirectly correlated with mechanical characteristics such as tensile strength, thickness of the pellicle and plasticity (Bodea et al., 2021; Sederavičiūtė et al., 2019). Water holding capacity (WHC) indicates the water retained by the cellulosic pellicle per unit (Avcioglu et al., 2021). A loose cellulosic structure results in a higher WHC (Avcioglu et al., 2021; B. Wang et

al., 2023). In this study, the WHC of the cellulose pellicle prepared using the different fermentation conditions varied from 8.45 to 45.98 g water/g dry KCP (Figure 6.1D). The WHC was mainly affected by sugar concentrations ($p<0.05$), increasing with higher sugar concentrations. The results of the WHC of the Kombucha cellulosic pellicle in this study were much lower than for the bacterial cellulose composed of *Gluconacetobacter hansenii* (84.36-106.43 g water/g dry bacterial cellulose) and *K. saccharivorans* (114.01 g water/g dry bacterial cellulose), respectively (Avcioglu et al., 2021; Ul-Islam et al., 2012). Variations in the WHC of the cellulosic pellicles between different fermentations in this study may have been due to their respective microstructures, especially porosity and surface area (Ul-Islam et al., 2012). Water molecules are embedded in the cellulosic matrix; having greater available space between the cellulosic fibres leads to more water being absorbed, creating a larger surface area and more porous structure, and hence a higher WHC (Guo & Catchmark, 2012).

6.4.2 Physico-chemical characteristics of black tea Kombucha

6.4.2.1 Acidity of black tea Kombucha

Kombucha was recovered on the final day of fermentation for each of the different fermentation treatments for immediate determination of acidity (pH and titratable acidity). Acidity is the most important parameter during Kombucha fermentation and at the point of sale, as it can affect the sensory sourness and overall quality of the beverage (Jafari et al., 2020; B. Wang et al., 2022a). The acidity of the Kombucha is determined by the metabolism of AAB, yeast in the culture and their metabolites produced during the fermentation process (e.g. acetic, gluconic and glucuronic acids) (Andreson et al., 2022; Villarreal-Soto et al., 2018). In this study, the pH of all the Kombucha samples were less than 4.0, placing the beverages in the acidic foods category (pH 2.55-3.17) (Figure 6.2). The titratable acidity was between 0.03% and 1.03% at the end of fermentation ($p<0.05$). The highest titratable acidity (1.03%) and lowest pH (2.55) were observed in sample K11 (prepared with 12g/L black tea, fermented at 30°C for 14 days).

The lowest titratable acidity (0.03%) was found in K10 along with the second-highest pH of 3.16. Results indicated that the titratable acidity (TA) was affected by black tea leaf concentrations, fermentation temperature, and fermentation time ($p<0.05$). According to the US Food and Drug Administration (FDA), commercial Kombucha products must fall into the pH range of 2.5 to 4.2 (de Miranda et al., 2022; FDA, 2009; Nummer, 2013). Low pH (<4.2) after the first week of Kombucha fermentation is expected to lower the risk of contamination from pathogens (Andreson et al., 2022). However, Kombucha with a very low pH (<2.5) may not be acceptable and can also be harmful to the consumer due to the high acidity, resulting in a very sour taste and decreasing the overall sensory performance of the Kombucha (Chu & Chen, 2006; Nummer, 2013). Therefore, additional fresh tea infusion should be added after fermentation to increase the pH to acceptable levels (Nummer, 2013). The Kombucha samples in this study were all in the pH range of 2.5-4.2, which is safe for consumption. However, samples K3 and K11 had a low pH (2.55) at day 14 of fermentation which is at the lower end of the regulatory limit of the FDA (pH 2.50). Kombucha prepared under these two conditions (K3 and K11) would need to be stored properly (cold chain ~4°C) to prevent high acidity caused by overfermentation.

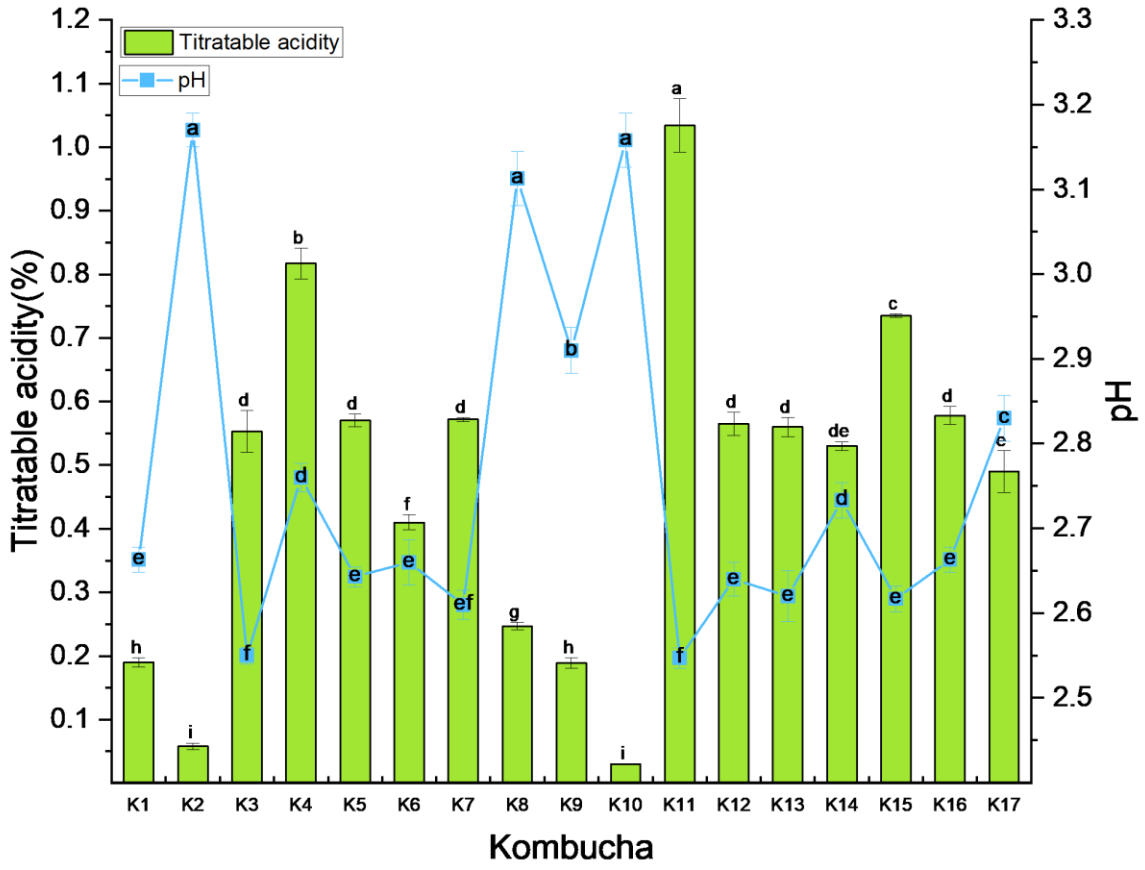

Figure 6.2 pH and titratable acidity of Kombucha samples prepared under different fermentation conditions. The results are expressed as mean ± standard deviation (SD) (n=3). Different lowercase letters (a-g) above the chart bars indicate the significant differences between samples($p<0.05$). For the composition of the samples K1-K17, refer to Table 6.2.

6.4.2.2 TSS, colour and turbidity of black tea Kombucha

The highest TSS were observed in K6 with 9.17±0.06, followed by K11, K2 and K9 (Table 6.3). The lowest levels of TSS (°Brix <2.5) were found in K4, K3, K8, K14 and K17. The TSS of black tea Kombucha in this study were higher than black tea Kombucha fermented for 8 days (< 3.0) by Amarasinghe et al. (2018) and laver Kombucha (°Brix 3.83-4.17) fermented by Aung and Eun (2021). The TSS of Kombucha commonly indicates the sugar dissolved in the drink as well as content of trace amounts of minerals and vitamins (Zubaidah et al., 2019). The TSS levels of Kombucha usually decreases during fermentation, due to the sucrose and nutrients being metabolised by the fermenting microorganisms in Kombucha to support their

growth and metabolite production (Jayabalan et al., 2014; Muhialdin et al., 2019; B. Wang et al., 2023). The significant differences of TSS between the 17 samples observed in this study ($p<0.05$) may be attributable to the different initial concentrations of sugar and tea utilised.

Table 6.3 Total soluble solids (TSS), turbidity and colour of Kombucha tea prepared under different fermentation conditions.

Sample	TSS (°Brix)	Turbidity (%)	Colour		
			L*	a*	b*
K1	6.40±0.00^{d}	5.81±0.57^{o}	96.60±0.75^{ab}	0.55±0.06^{abcd}	1.81±0.21^{i}
K2	8.33±0.12^{b}	12.29±0.40^{n}	98.17±0.60^{a}	-0.92±0.33^{fg}	2.67±0.57^{hi}
K3	2.47±0.06^{f}	31.03±0.09^{k}	94.28±0.49^{c}	0.28±0.08^{bcde}	4.98±0.66^{g}
K4	2.06±0.06^{g}	67.24±0.12^{d}	89.52±0.61^{fgh}	1.12±0.23^{a}	10.45±0.14^{e}
K5	5.33±0.06^{e}	51.84±0.17^{g}	89.48±0.33^{gh}	0.88±0.06^{ab}	12.31±0.04^{cd}
K6	9.17±0.06^{a}	76.36±0.06^{c}	95.13±1.28^{bc}	-1.39±0.33^{g}	7.82±0.26^{f}
K7	5.43±0.06^{e}	59.57±0.39^{e}	89.09±0.45^{h}	-0.84±0.17^{ab}	14.66±0.14^{ab}
K8	2.07±0.12^{g}	91.90±1.09^{a}	92.07±1.23^{de}	-0.20±0.55^{e}	14.94±1.82^{a}
K9	8.33±0.06^{b}	16.76±0.22^{m}	98.15±0.59^{a}	-1.19±0.28^{g}	4.46±1.00^{gh}
K10	2.53±0.06^{f}	36.61±0.64^{j}	95.55±1.03^{bc}	-0.34±0.37^{ef}	2.63±0.11^{i}
K11	9.07±0.12^{a}	41.34±0.62^{i}	90.68±0.13^{efgh}	-0.18±0.04^{de}	11.74±0.22^{de}
K12	5.30±0.00^{e}	57.67±0.39^{f}	88.74±0.76^{h}	0.91±0.04^{ab}	13.51±0.48^{abcd}
K13	5.47±0.12^{e}	58.88±0.33^{ef}	90.20±0.70^{efgh}	0.82±0.04^{ab}	13.59±0.62^{abc}
K14	2.50±0.00^{f}	43.29±0.46^{h}	94.50±0.33^{bc}	0.67±0.25^{abc}	4.91±0.16^{g}
K15	7.87±0.06^{c}	28.77±0.34^{l}	93.65±0.17^{cd}	-0.02±0.04^{cde}	10.31±0.21^{e}
K16	5.50±0.00^{e}	57.47±0.31^{f}	91.38±0.39^{efg}	0.91±0.03^{ab}	12.88±0.03^{bcd}
K17	2.40±0.00^{f}	82.44±1.10^{b}	91.60±0.65^{def}	0.30±0.19^{bcde}	13.50±0.33^{abcd}

The results are expressed as mean±SD (n=3). Different lowercase letters (a-h) in the same column indicate the significant differences between samples ($p<0.05$). For the composition of the samples K1-K17, refer to Table 6.2.

The colour of the beverage is an important visual parameter, which may affect consumers' acceptability and therefore intention to purchase (Amjadi et al., 2023; Aung & Eun, 2021; Ulusoy & Tamer, 2019). The colour differences between the black tea Kombucha samples in this study were easily differentiated by the naked eye and had diverging values (Table 6.3). The brown colour of black tea Kombucha is mainly attributed to the presence of thearubigin (Chu & Chen, 2006). However, the polyphenols in black tea such as thearubigin are converted to theaflavin catalysed by the enzymes secreted from AAB and yeast, resulting in a colour change from dark to light during the Kombucha fermentation (Jakubczyk et al., 2020). This microbial

transformation process may be associated with the high lightness L* values (>85) in all Kombucha samples in the current study. Additionally, a brownish colour was found in Kombucha samples with higher initial tea concentrations (12 g/L) and longer fermentation time (14 days). Therefore, the fermentation time and tea substrates influence the total colour of the Kombucha samples ($p<0.05$).

Turbidity of black tea Kombucha varied significantly between samples (5.81% to 91.90%; $p<0.05$) (Table 6.3). A high turbidity (cloudiness) in Kombucha is not visually appealing for some consumers and decreases their acceptance of fermented beverages (Daneluz et al., 2023). The turbidity of Kombucha samples in this study may be attributed to biofilm formation and the presence of microorganisms such as cellulose-forming bacteria *K. rhaeticus* and other fibrous components in the Kombucha broth that hinder the transmission of light (Amarasinghe et al., 2018; Dartora et al., 2023).

6.4.2.3 Concentration of ethanol of black tea Kombucha

The alcohol content regulations for non-alcoholic beverages vary between different countries: in the United States of America (USA), Australia and New Zealand (FSANZ, November 2014), the ethanol content of Kombucha must not exceed 0.5 % alcohol by volume (ABV) to comply with the non-alcoholic beverage regulations. The ethanol concentration of the 17 lab-scale Kombucha samples collected at the end of fermentation in this study varied from 0.03% ABV to 0.38% ABV (Figure 6.3; $p<0.05$), which complied with the legal standard (<0.5% ABV) in New Zealand and Australia. Of the 17 samples, K2, K9 and K15 had a similar ethanol content (~0.35% ABV), while K3 and K14 had low ethanol concentrations (< 0.05% ABV) similar to the Kombucha prepared in the Netherlands (Sreeramulu et al., 2001). The different ethanol concentrations of Kombucha samples in this study were mainly influenced by fermentation temperatures and fermentation time ($p<0.05$). Reported ethanol levels of commercial Kombucha products ranged between 0.07% and 2.03% ABV in Canada, whereas ethanol

concentrations of America Kombucha products (1.12-2.00 % ABV) were all above local standards (0.5 % ABV) (Jang et al., 2021; Liu et al., 2019; Talebi et al., 2017). Only about one third (35.2 %) of Australian Kombucha products had ethanol concentrations under 0.5% ABV (FSANZ, 2019). Excess ethanol concentrations in these commercial Kombucha beverages can result in the recall of the products due to non-compliance with the local regulations (Kim & Adhikari, 2020; Murphy et al., 2018).

Consumption of Kombucha with excess ethanol levels could be harmful to pregnant women and their babies due to the potential risks of fetal alcohol syndrome (FAS) (Meschke et al., 2003; Vogel et al., 1995). The excess ethanol concentration may be caused by residual sugar (sucrose) in the unpasteurised Kombucha samples contributing to the overgrowth of yeast, accelerating the synthesis of ethanol during the secondary fermentation (Jang et al., 2021; Talebi et al., 2017; Villarreal-Soto et al., 2018). Although the ethanol concentrations of Kombucha samples in this study did not exceed the local regulation (0.5 % ABV) at the end of fermentation, the high residual sugar concentration in unpasteurised Kombucha samples such as K9 and K15 may contribute to the overgrowth of yeast if the product is not stored appropriately, resulting in post-fermentation and elevated ethanol levels during distribution and storage (Jang et al., 2021; Talebi et al., 2017; Villarreal-Soto et al., 2018). According to Talebi et al. (2017), Kombucha stored at ambient temperature and longer periods of storage also increased in ethanol content. Therefore, precise fermentation processes, cold-chain transportation and storage at refrigerated temperature are essential to lower the risks of excess ethanol concentrations in industrial scale-up Kombucha (Jang et al., 2021).

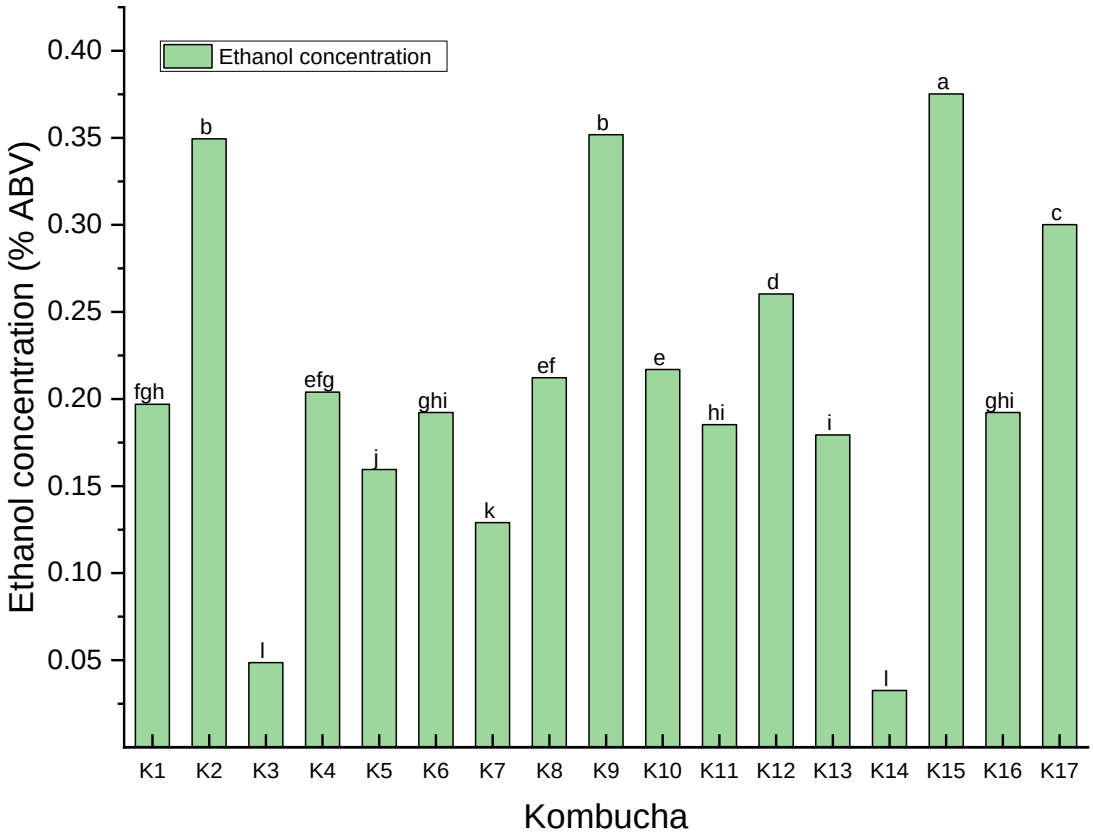

Figure 6.3 Ethanol concentrations of Kombucha samples prepared under different fermentation conditions. The results are expressed as mean± SD (n=2). Different lowercases (a-i) above the chart bars indicate the significant differences between samples ($p<0.05$). For the composition of the samples K1-K17, refer to Table 6.2.

6.4.3 Total phenolic content of black tea Kombucha

Phenolic compounds are secondary metabolites, commonly derived from plant-based products such as vegetables, fruits, and tea (Kilic & Sengun, 2023; Sun et al., 2015). The presence of phenolic compounds influences the sensory characteristics (taste, colour, and aroma) of Kombucha (Alara et al., 2021). Moreover, a high phenolic content in the diet may inhibit oxidative damage and lower the risk of chronic diseases such as diabetes, cancer, and Parkinson's disease (Haminiuk et al., 2012). Therefore, the presence of bioactive phenolic compounds in Kombucha may be associated with therapeutic effects (Leonarski et al., 2022).

The total phenolic content of black tea Kombucha samples prepared under 17 different conditions are presented in Figure 6.4. In this study, the TPC of Kombucha ranged from 50.21±3.85 to 777.94±13.46 µg GAE/mL ($p<0.05$) with the black tea concentration being the most significant factor affecting the TPC ($p<0.05$). The highest TPC value was detected from K15 which was produced using the highest tea concentration (12 g/L), followed by K6 (425.50±18.30 µg GAE/mL). High TPC values have also been detected in previous studies, the TPC of black tea Kombucha sweetened with honey (75 g/L) was reported as 2083.17 GAE µg/L, which is much higher (almost three times) than Kombucha produced in the current study (Kilic & Sengun, 2023). Therefore, selection of carbon sources may also affect the TPC of black tea Kombucha.

Phenolic compounds in black tea Kombucha increased during fermentation possibly due to the complex microbial biotransformation of phenolic compounds (Cardoso et al., 2020; Chakravorty et al., 2016; Kallel et al., 2012; Kilic & Sengun, 2023). This occurs as during fermentation and in an acidic environment, hydrolytic enzymes released by AAB and yeast in the Kombucha starter culture may degrade complex phenolic compounds (such as lignans, stilbenes and phenolic acids) into smaller molecular weight phenolic compounds, resulting in an increase in TPC (La Torre et al., 2021; Markov et al., 2003; Sun et al., 2015). The depolymerization of thearubigin in black tea Kombucha may be another possible reason for the increased TPC that occurred during fermentation (Chu & Chen, 2006; Ulusoy & Tamer, 2019). Several researchers have shown that the phenolic compounds may increase with longer fermentation times (Aung & Eun, 2021; Chakravorty et al., 2016; Jafari et al., 2020; Jakubczyk et al., 2020; Tanticharakunsiri et al., 2021). However, here the initial black tea concentration was identified as the only significant factor that influenced the TPC of Kombucha prepared under the different conditions ($p<0.05$). Results from the current study confirmed that black tea Kombucha can be a good source of phenolic compounds and the concentration of phenolic

compounds increased with a higher concentration of black tea leaves. This observation may help to develop high quality functional fermented beverages (Tanticharakunsiri et al., 2021).

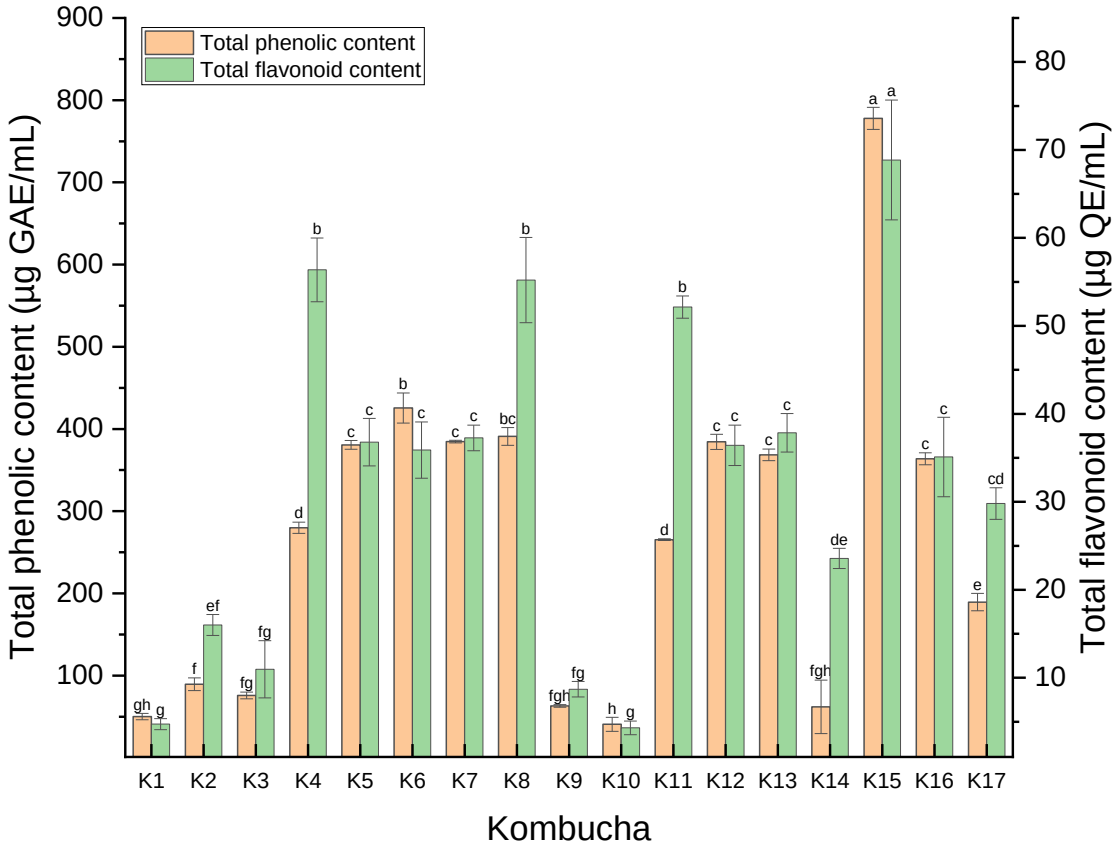

Figure 6.4 Total phenolic content (µg GAE/mL) and total flavonoid content (µg QE/mL) of Kombucha samples prepared under different fermentation conditions. The results were expressed as mean ± SD (n=3). Different lowercases (a-g) above the chart bars indicated the significant differences between samples ($p<0.05$). For the composition of the samples K1-K17, refer to Table 6.2.

6.4.4 Total flavonoid content of black tea Kombucha

Black tea Kombucha is a rich source of dietary flavonoids due to oxidation occurring during the fermentation process of producing black tea from tea leaves (Łuczaj & Skrzydlewska, 2005). Flavonoids present in black tea Kombucha are mainly dimeric theaflavins and polymeric thearubigin which, when consumed regularly have been shown to lower the risk of death due to cardiovascular disease and inhibit neuropathy (Peterson et al., 2004; Ullah et al., 2020).

The total flavonoid content (TFC) of Kombucha prepared under 17 different conditions varied from 4.31±0.78 μg QE/mL to 68.86±6.81 μg QE/mL ($p<0.05$). Similar to the results of total phenolic content, the highest TFC were observed in K15 prepared with 12 g/L black tea leaves and the lowest TFC were found in K10 produced with 1.5 g/L black tea leaves (Figure 6.4). However, a high total flavonoid content was also found in K4, K8 and K11, with values of 56.73 μg QE/mL, 55.21 μg QE/mL and 52.14 μg QE/mL, respectively. The results indicated that in the present study the fermentation time and black tea concentrations were the most significant factors ($p<0.05$) affecting the total flavonoid content of Kombucha, similar to previous studies (Amjadi et al., 2023; de Noronha et al., 2022). The higher black tea concentrations and longer fermentation time resulted in increased flavonoid content of Kombucha at the end of fermentation. The further increase in TFC after fermentation may be due to the following reasons: firstly, several enzymes such as glucosidase, pectinase, and xylanase are secreted by fermenting microorganisms (AAB and yeast) during Kombucha fermentation, these enzymes may break large molecular polyphenols into smaller polyphenol monomers under acidic conditions, thus increasing the overall flavonoid concentrations of black tea Kombucha (Aung & Eun, 2021; Chu & Chen, 2006; Jayabalan et al., 2008; Shahbazi et al., 2018; Tu et al., 2019). Secondly, there are insoluble, bound flavonoid compounds in black tea leaf cell walls, which are difficult to liberate directly into Kombucha (Zhang et al., 2020). During fermentation, the Kombucha starter culture (SCOBY) transfers sugars to a large number of organic acids, esters and other metabolites, and these metabolites may promote the liberation of insoluble bound flavonoid compounds (X. Wang et al., 2022).

The total flavonoid content of black tea Kombucha has been previously shown to vary greatly with different standard equivalent concentration utilised (quercetin, catechin and rutin). For example, the TFC of black tea Kombucha with different ratios of black tea (3 g/L-8 g/L) fermented for 15 days were between 15.56 to 36.65 μg QE/mL (Amjadi et al., 2023), which is

very similar to the current findings. However, another study found that the TFC of black tea Kombucha (3 g/L) fermented for 30 days was 2.3 µg QE/mL (La Torre et al., 2021), which was much lower than the Kombucha prepared in the current study. Higher TFC (90.50 -126.70 µg QE/mL) than the current study was detected in black tea Kombucha (8 g/L) fermented for 14 days (Jakubczyk et al., 2020). These studies consistently indicated that fermentation time and the levels of tea substrate influenced the final TFC of the Kombucha, but to varying degrees.

6.4.5 Phenolic components of black tea Kombucha

The seven most common phenolic compounds (gallic acid, theobromine, caffeine, EGC, EC, EGCG and ECG) were identified and quantified in black tea Kombucha based on retention time and peak area compared with standard solutions (Table 6.4). Results indicated that only three compounds (gallic acid, theobromine, and caffeine) were detected in all seventeen Kombucha samples, with caffeine being the most abundant phenolic acid in all Kombucha samples. Caffeine content ranged from 20.33 µg/mL (K1) to 362.52 µg/mL (K8), with gallic acid being the next most abundant (1.99-69.30 µg/mL) followed by theobromine (0.96- 28.67 µg/mL). The highest concentration of these three phenolic compounds was detected in K8 followed by K6 and K4. The ANOVA analysis showed that fermentation time and black tea concentrations were the most important factors affecting the levels of these three bioactive phenolic compounds ($p<0.05$), with the content increasing as black tea concentrations increased and decreasing with prolonged fermentation. No EGC was found in any of the 17 samples, while EGCG was only detected in K8. Epicatechin (EC) was detected in K4, K6, K7, K8, K10, K12, K16 and K17, and varied from 0.01 to 0.21 µg/mL, while ECG was between 2.12 (K4) and 11.49 µg/mL (K17).

Black tea Kombucha contains diverse phenolic compounds including theaflavins (TFs), thearubigins (TRs), catechins, theobromine, caffeine, caffeic acid, gallic acid, chlorogenic acid, cinnamic acid, and ferulic acid (Cardoso et al., 2020; Villarreal-Soto et al., 2019). The presence

of these phenolic compounds in Kombucha has been associated with various health-promoting benefits, particularly its antioxidant properties (de Noronha et al., 2022; Shi et al., 2023). Caffeine has been previously identified as the main bioactive compound in black tea Kombucha followed by gallic acid which agrees with our results for all seventeen samples (Barbosa et al., 2020; Shi et al., 2023). Furthermore, the presence of these phenolic compounds in black tea Kombucha may contribute to the bitterness, and astringency of Kombucha (Jayabalan et al., 2007; Łuczaj & Skrzydlewska, 2005; Santos et al., 2018). The low catechin and epicatechin content found in this study and other previous studies may be due to the biodegradation of catechin during Kombucha fermentation (Jayabalan et al., 2007; Villarreal-Soto et al., 2019; Villarreal-Soto et al., 2020). Additionally, the metabolic activity of specific fermenting microorganisms also influences the content of phenolic compounds. For example, *Acetobacter pasteurianus* has been reported to increase the EGCG and theobromine content of black tea Kombucha (Shi et al., 2023). It is possible that the microorganisms in the starter culture used in this study *(K.rhaetius , D. prosopidis* and *Z. lentus*) may also play a role in the biodegradation of tea catechins, and this should be further investigated.

In contrast to the total phenolic content of Kombucha (Figure 6.4), the highest content of representative phenolic compounds determined by HPLC was observed in K8, however, the highest TPC determined by the Folin-Ciocalteu assay was found in K15. There are two possible reasons for these results. Firstly, the Folin-Ciocalteu assay is known to have some weaknesses in the determination of total phenolic content. It is possible that certain compounds such as peptides and amino acids in the black tea Kombucha also reacted with the reagents from the Folin-Ciocalteu assay, thus overestimating the total phenolic content of Kombucha (Malta & Liu, 2014; X. Wang et al., 2022). Secondly, only seven main bioactive phenolic compounds were measured in this study. Therefore, a variety of active phenolic compounds present in black tea Kombucha such as chlorogenic acid, rutin, cinnamic acid and quercetin were not quantified

by HPLC in the present study, resulting in the amount of total phenolic compounds being underestimated (Cardoso et al., 2020; de Noronha et al., 2022; Kumar & Joshi, 2016; La Torre et al., 2021).

Table 6.4 Phenolic compounds of Kombucha samples prepared under different fermentation conditions determined by HPLC.

Sample	Phenolic compounds (μg/mL)						
	Gallic acid	**Theobromine**	**Caffeine**	**EGC**	**EC**	**EGCG**	**ECG**
K1	1.99±0.02	0.96±0.25	20.33±0.32	ND	ND	ND	ND
K2	6.05±0.14	2.66±0.08	44.96±0.37	ND	ND	ND	ND
K3	5.46±0.22	1.94±0.17	37.91±1.12	ND	ND	ND	ND
K4	17.68±0.64	6.74±0.04	118.55±1.23	ND	0.01±0.00	ND	2.12±0.07
K5	13.49±0.05	6.24±0.18	101.17±0.21	ND	ND	ND	ND
K6	29.54±0.23	11.66±0.42	164.01±2.29	ND	0.10±0.02	ND	9.89±0.35
K7	14.50±0.10	6.35±0.23	109.79±0.03	ND	0.05±0.02	ND	4.47±0.10
K8	69.30±0.28	28.67±0.86	362.52±2.09	ND	0.21±0.03	0.01±0.00	11.11±0.67
K9	7.44±0.08	2.54±0.05	56.46±0.58	ND	ND	ND	ND
K10	8.95±0.15	4.37±0.13	75.37±0.70	ND	0.01±0.00	ND	ND
K11	19.11±0.08	6.21±0.76	81.16±0.00	ND	ND	ND	4.52±0.45
K12	13.16±0.23	5.49±0.43	95.33±0.68	ND	0.01±0.00	ND	5.64±0.05
K13	12.07±0.09	5.39±0.07	87.50±0.09	ND	ND	ND	5.02±0.25
K14	4.36±0.03	1.60±0.01	30.96±0.20	ND	ND	ND	ND
K15	16.93±0.28	6.64±0.01	107.73±0.70	ND	ND	ND	ND
K16	13.09±0.16	5.46±0.22	96.30±0.58	ND	0.03±0.00	ND	5.43±0.03
K17	22.45±0.01	9.50±0.16	146.22±3.17	ND	0.07±0.00	ND	11.49±0.48

The results were expressed as mean ± SD (n=2). ND: not detected. For the composition of the samples K1-K17, refer to Table 6.2.

Heatmap analysis was used to visualize the concentration of phenolic compounds (determined by HPLC) directly in black tea Kombucha prepared with seventeen different fermentation conditions (Figure 6.5A). As shown in Figure 6.5B, the 17 Kombucha samples were divided into five main clusters: A, B, C, D, and E based on the Euclidean distance, respectively. The six samples in cluster A accounted for 35% of total samples and had relatively low concentrations of most phenolic compounds, possibly due to low concentrations of black tea leaves. Cluster B (seven samples) accounted for 41% of total samples. The content of caffeine in Cluster C was relatively higher than Kombucha samples in Clusters A and B. Cluster D (K15) had the highest TPC and TFC of all five clusters. Cluster E (K8) had the most abundant EGCG, EC, caffeine, theobromine, and gallic acids among the 17 samples.

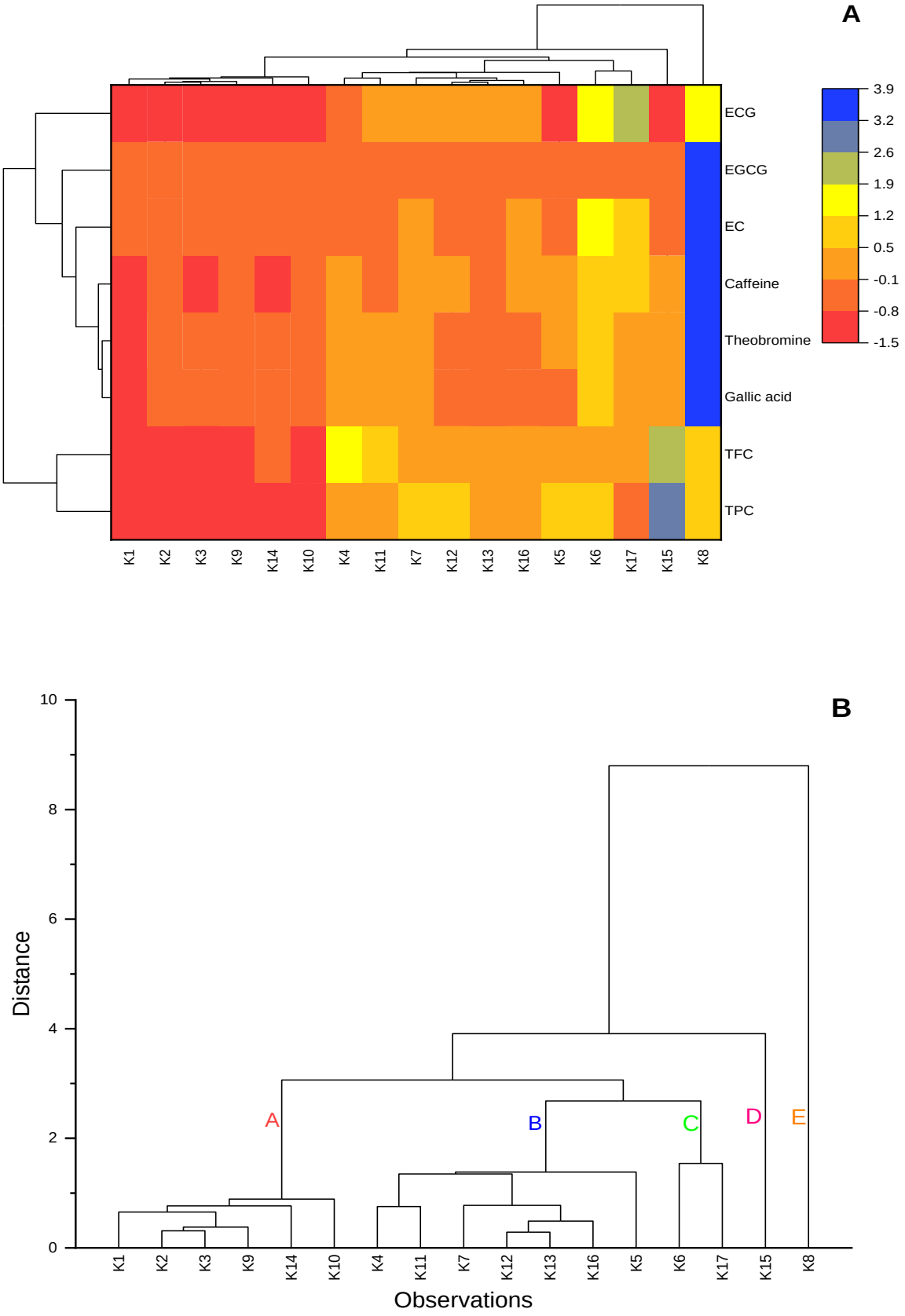

Figure 6.5 (A) Heatmap of quantified phenolic compounds in Kombucha prepared under different conditions. (B) Hierarchical cluster dendrogram.

6.4.6 Antioxidant activity of black tea Kombucha

Antioxidant activity is one of the most reported beneficial effects of Kombucha beverages (Ahmed et al., 2020; Chu & Chen, 2006; Kilic & Sengun, 2023; Zou et al., 2021). The *in vitro* antioxidant activity of black tea Kombucha prepared using 17 different fermentation conditions was assessed by two different assays, DPPH and ABTS+. As shown in Figure 6.6A, all 17 Kombucha samples exhibited ABTS+ free radical scavenging activities with levels, ranging from 22.63 to 551.1 µg TE/mL ($p<0.05$). The highest ABTS+ free radical scavenging activity was observed in K15 (highest tea concentration 12 g/L), followed by K8. Kombucha samples (K4, K5, K6, K7, K12, K13, K16 and K17) had similar ABTS+ radical scavenging capacities (~160 µg TE/mL). Low ABTS+ free radical scavenging activity was found in K1, K2, and K10 (lowest tea concentration 1.5g/L). The DPPH assay is a reliable method used for determination of the antioxidant capacity of bioactive compounds, food components and other biological substances (Kedare & Singh, 2011; Sun et al., 2015; Xiao et al., 2020). In this study, the DPPH free radical scavenging activity of black tea Kombucha was between 20.78±2.95% to 91.28±0.45% (Figure 6.6B). Similar to the ABTS+ assay, the highest DPPH percentage was observed for K15 followed by K8. Based on ANOVA analysis, the black tea leaf concentration was the most important factor affecting the antioxidant capacities of black tea Kombucha evaluated by both methods. Therefore, a higher black tea leaf concentration results in stronger antioxidant activity of Kombucha.

Several studies have indicated that the antioxidant activity of Kombucha is mainly due to the presence of polyphenols and catechins (Tanticharakunsiri et al., 2021; Ulusoy & Tamer, 2019; Zou et al., 2021). In the current study, the results from the two antioxidant activity methods (DDPH and ABTS+) showed a strong positive correlation with TPC and TFC ($0.83<r<0.88$, Figure 6.7B), which was consistent with previous studies (Ahmed et al., 2020; Amjadi et al., 2023; Kilic & Sengun, 2023; La Torre et al., 2021). Many studies have documented the high

antioxidant capacity of tea polyphenol monomers such as gallic acid, caffeine, chlorogenic acid, rutin, ferulic acid and catechins (Amjadi et al., 2023; Sun et al., 2015). In the current study, six individual phenolic compounds (gallic acid, theobromine, caffeine, EC, EGCG, and ECG) were correlated with antioxidant activity ($0.16<r<0.65$). ECG showed the strongest positive correlation with DPPH ($r=0.65$), while the highest correlation with ABTS+ was for caffeine ($r=0.62$) followed by gallic acid ($r=0.61$).

Gallic acid, caffeine, and tea catechins have been used as antioxidant agents to prevent lipid peroxidation (Ulusoy & Tamer, 2019; Vieira et al., 2020; Yen et al., 2002). Therefore, the high concentration of these dominant phenolic components (gallic acid, caffeine, and theobromine) in Kombucha samples (K8 and K6) makes them a rich source of these bioactive components. The presence of vitamin C and organic acids produced during fermentation may be also responsible for some of the antioxidant capacity of Kombucha (Ahmed et al., 2020; Bortolomedi et al., 2022; X. Wang et al., 2022). Certain organic acids, especially acetic acid, gluconic acid, and glucuronic produced during Kombucha fermentation may also have synergistic effects on the concentrations of phenolic compounds, thus enhancing the antioxidant activity of Kombucha (Aung & Eun, 2021; Ebrahimi Pure, 2016; Malbaša et al., 2011).

Reported antioxidant activities of Kombucha vary greatly between studies, from low DPPH radical scavenging activities (10.17%-18.03%) from snake fruit Kombucha (Zubaidah et al., 2018), to moderate antioxidant activities (39.0%-69.2% DPPH) for black tea Kombucha (Chu & Chen, 2006), to high antioxidant activities (83.61% to 98.41% DPPH) of Kombucha prepared from different substrates (oolong tea, echium amoenum, royal lotus pollen, and butterfly pea) (Amjadi et al., 2023; Tanticharakunsiri et al., 2021; Wongthai et al., 2021). Although our results indicated that the black tea leaf concentration was the most significant factor affecting the antioxidant activity of black tea Kombucha ($p<0.05$), previous studies

suggested that the type of tea base, carbon source, the yeast and AAB metabolites, fermentation time, and fermentation temperature could affect the overall antioxidant activity of the Kombucha product to different extents (Jayabalan et al., 2014; Kilic & Sengun, 2023; Muhialdin et al., 2019; Srihari & Satyanarayana, 2012; Ulusoy & Tamer, 2019).

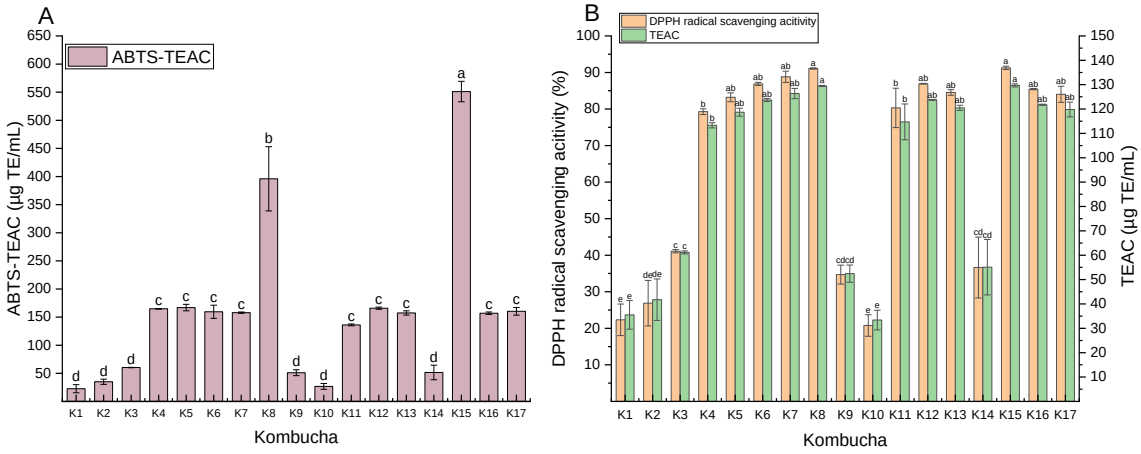

Figure 6.6 ABTS+ radical scavenging activity (A) and DPPH free radical scavenging activity (B) of Kombucha samples. The results were expressed as mean ± SD (n=3). Different lowercases (a-d) above the chart bars indicated the significant differences between samples ($p<0.05$). For the composition of the samples K1-K17, refer to Table 6.2.

6.4.7 Antimicrobial activity of black tea Kombucha

As expected, the utilisation of different fermentation conditions led to the production of Kombucha tea broth samples with different levels of antimicrobial activities against the tested pathogenic microorganisms (Table 6.5). The absence of transparent halos surrounding the agar wells indicated the resistance of *P. aeruginosa* to the antimicrobial effects of all 17 black tea Kombucha samples in the present study. The results showed that K11 and K15 exhibited antimicrobial activities against three pathogens (*S. aureus, E. coli*, and *B. cereus*), while K4, K5, K7, K12, K13, K16 and K17 showed antimicrobial effects against *E. coli* and *B. cereus*. Samples K3, K6 and K14 only exhibited antimicrobial activity against *E. coli*. Kombucha samples K1, K2, K8, K9 and K10 did not exert bactericidal activities against any of the four

pathogenic microorganisms tested. The agar well diffusion assay indicated that the strongest antimicrobial activity of black tea Kombucha was K11, with an inhibition diameter of 16.00 mm (*E. coli*), followed by K11 with 14.00 mm (*S. aureus*) and K15 with 14.00 mm (*E. coli*).

Table 6.5 Antimicrobial activity of Kombucha samples prepared under different fermentation conditions.

	Inhibition zone diameter (mm)			
	Tested microorganisms			
Sample	*S. aureus* MCTIC 4163	*E. coli* NCTIC 8196	*P. aeruginosa* MUA26	*B. cereus* MU-A44
K1	6.00±0.00^{c}	6.00±0.00^{g}	6.00±0.00	6.00±0.00
K2	6.00±0.00^{c}	6.00±0.00^{g}	6.00±0.00	6.00±0.00
K3	6.00±0.00^{c}	9.00±0.00^{f}	6.00±0.00	6.00±0.00
K4	6.00±0.00^{c}	13.33±1.15^{bc}	6.00±0.00	8.00±0.00
K5	6.00±0.00^{c}	12.67±0.58^{bcd}	6.00±0.00	8.67±0.58
K6	6.00±0.00^{c}	11.00±0.00^{de}	6.00±0.00	6.00±0.00
K7	6.00±0.00^{c}	12.00±0.00^{cd}	6.00±0.00	8.67±0.58
K8	6.00±0.00^{c}	6.00±0.00^{g}	6.00±0.00	6.00±0.00
K9	6.00±0.00^{c}	6.00±0.00^{g}	6.00±0.00	6.00±0.00
K10	6.00±0.00^{c}	6.00±0.00^{g}	6.00±0.00	6.00±0.00
K11	14.00±0.00^{a}	16.00±0.00^{cd}	6.00±0.00	12.00±0.00
K12	6.00±0.00^{c}	11.67±0.58^{cde}	6.00±0.00	8.67±0.58
K13	6.00±0.00^{c}	11.67±0.58^{cde}	6.00±0.00	8.67±0.58
K14	6.00±0.00^{c}	10.00±0.00^{ef}	6.00±0.00	6.00±0.00
K15	8.33±0.57^{b}	14.00±0.00^{b}	6.00±0.00	9.33±1.16
K16	6.00±0.00^{c}	12.00±0.00^{cd}	6.00±0.00	8.33±0.58
K17	6.00±0.00^{c}	12.67±2.08^{bcd}	6.00±0.00	9.00±0.00
Positive control	52.00	25.00	16.00	20.00

The results were expressed as mean ±SD (n=3). Different lowercase letter (a-h) in the same column indicated significant differences between samples ($p<0.05$); 5% trigene was used as positive control; For the composition of the samples K1-K17, refer to Table 6.2.

The antimicrobial activities of black tea Kombucha against a broad spectrum of pathogenic microorganisms responsible for several infective diseases has been widely reported (Ansari et al., 2019; Battikh et al., 2012; Battikh et al., 2013; Ivanisova et al., 2020; Sreeramulu et al., 2000). This antimicrobial activity is commonly attributed to Kombucha's low pH and the presence of organic acids, especially acetic acid produced during fermentation, which inhibit the growth of a broad range of Gram-positive and Gram-negative pathogenic bacteria (Ivanisova et al., 2020; Villarreal-Soto et al., 2018). This occurs as the undissociated parts of acetic acid and other organic acids in Kombucha such as glucuronic acid molecules can cause cytoplasmic acidification thus destroying the pathogenic bacterial cells (Cetojevic-Simin et al.,

2008; Velicanski et al., 2014). In this study, strong positive correlations (r=0.59-0.93, Figure 6.7B) were found between titratable acidity and antimicrobial activity against the tested microorganisms; which was consistent with previous studies (Cetojevic-Simin et al., 2008; Greenwalt et al., 1998). However, here, negative correlations were observed between pH and antimicrobial activities against *S. aureus, E. coli* and *B. cereus* (Figure 6.7B). The antimicrobial activity of Kombucha against the tested microorganisms increased at higher titratable acidity activities, as the strongest antimicrobial potential was found in sample K11 which had the highest TA.

Various studies have indicated that phenolic, condensed tannins and flavonoid compounds in Kombucha are potential antimicrobial agents (Bhattacharya et al., 2018; Nyiew et al., 2022; Shahbazi et al., 2018; Sreeramulu et al., 2000; Zubaidah et al., 2018). A high antimicrobial activity was observed in Kombucha sample K15 which had the highest total phenolic content and flavonoid concentrations (Figure 6.7B and Table 6.5). Consistent with previous observations, there were also positive correlations ($0.18<r<0.70$) between TPC and TFC and antimicrobial activity against tested microorganisms in the present study; (Battikh et al., 2013; Bhattacharya et al., 2018). ECG concentrations also had positive correlations with antimicrobial activity against *E. coli* and *B. cereus* (Figure 6.7B). Therefore, the phenolic content of black tea Kombucha may be responsible for some of the antimicrobial effects against certain pathogens (Mizuta et al., 2020). The results obtained in this study showed that the antimicrobial activity of black tea Kombucha may be influenced by the titratable acidity, total phenolic/flavonoid content, added tea substrates, starter culture and fermentation conditions. The Kombucha samples with the strong antimicrobial activities (K11 and K15 all prepared with 12 g/L black tea and fermented for 14 days) in this study could be useful as potential sources of antimicrobial agents in functional beverages.

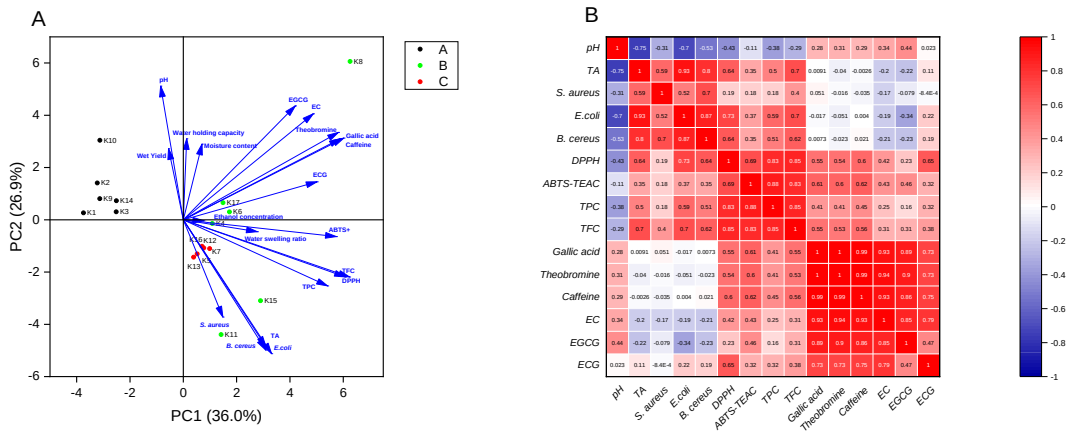

Figure 6.7 (A) Principal component analysis (PCA) biplot of Kombucha prepared under seventeen fermentation conditions. (B) Pearson's correlation coefficient of Kombucha samples.

6.4.8 Principal component analysis

The principal component analysis (PCA) is the most used method for reducing the dimensionality of multiple experimental parameters and variables (Kherif & Latypova, 2020). To visualise the relationship between the eight physicochemical characteristics (pH, TA, moisture content, water swelling ratio, water holding capacity, wet yield, and ethanol); six bioactive compounds (gallic acid, theobromine, caffeine, EC, EGCG and EGC); TPC and TFC; antioxidant activity (DDPH and ABTS+); and antimicrobial effects *(B. cereus*, *S. aureus*, and *E. coli*), PCA was performed on the results of the 17 Kombucha samples prepared under the different fermentation conditions. The results from the biplot (Figure 6.7A) showed that the first two principal components explained 62.9% of the total variance. The principal component (PC)1 displayed 36.0% and PC2 explained 26.9% of total variance, respectively. All the parameters contributed positively to the PC1 except pH and wet yield.

The 17 Kombucha samples were clearly separated into three clusters based primarily on black tea leaf concentrations (1.5 g/L, 6.75 g/L and 12 g/L), and plotted in three quadrants (I, II, IV). Kombucha samples prepared with low black tea concentrations (1.5 g/L) were located close to each other in quadrant II (top left), indicating low variances between these samples. As the

black tea leaf concentration increased, Kombucha samples tended to position on the positive side of PC1, and three samples (K11, K15 and K8) were distributed along the edge of PC2. These results indicated that bioactive components may be improved by increasing the black tea leaf concentrations. The biplot also indicated that Kombucha samples (K6, K17 and K8), positioned in quadrant I (top right), showed good correlation with the six bioactive components, moisture content and water holding capacity. Finally, Kombucha samples (K5, K7, K12, K13, and K16) prepared with 6.25 g/L black tea as well as three Kombucha samples prepared with the highest black tea levels (12g/L) were plotted in quadrant IV (lower right). K4 correlated with water swelling ratio and ABTS+, whereas K11 showed better correlations with antimicrobial activities against three pathogens than the other samples in quadrant IV. Furthermore, K15 also exhibited good correlations with higher antioxidant activity (DPPH), TPC, and TFC, as well as antimicrobial activities. Overall, K8 with high phenolic components, K11 with strong antimicrobial activities, and K15 with good functional activities (antioxidant and antimicrobial activities) were considered as desirable formulas and fermentation processes to produce high-quality black Kombucha beverages.

6.5 Conclusion

In the present study, the Plackett-Burman design was applied for the first time to determine the most significant fermentation factors affecting the physicochemical and functional characteristics of black tea Kombucha prepared with a New Zealand Kombucha starter culture composed of *K. rhaeticus*, *D. prosopidis*, and *Z. lentus*. The results indicated that SCOBY concentrations, black tea infusion time, sugar concentration and previous Kombucha inoculum volume were the key factors affecting WSR, wet yield and WHC ($p<0.05$). The antioxidant activity of black tea Kombucha is likely due to the presence of phenolic compounds particularly caffeine, gallic acid and theobromine in black tea as it is mainly influenced by the black tea leaf concentration. The titratable acidity and total phenolic/flavonoid content may contribute

to the antimicrobial activity against three pathogens. Kombucha produced using the K15, K11 and K8 fermentation processes and formulations can be considered potential functional fermented beverages due to their high levels of bioactive components and excellent antioxidant and antimicrobial activities. However, further studies should be conducted to optimize the levels of these desirable bioactive components and beneficial activities.

Chapter 7. General discussion, and recommendation for future study

7.1 General Discussion

Despite recent increases in consumer awareness of the health-promoting effects of Kombucha and the associated increase in global Kombucha sales, the industrial scale-up of Kombucha still faces challenges. These challenges are partially attributed to the limited scientific knowledge on the dynamics of the microbiological composition of the starter culture, the lack of information about the impact of fermentation process parameters on the bioactive metabolites and health-promoting characteristics in the final fermented products (Eyles et al., 2021; Mandow, 2021). Additionally, there are no standardised fermentation processes to ensure production of consistent high-quality Kombucha products which meet the stipulated standard alcohol content in New Zealand (<0.5 % ABV) (FSANZ, November 2014). This study aimed to determine the microbiological characteristics of a New Zealand Kombucha starter culture (Chapter 3), evaluate the probiotic potential of the microorganisms isolated from commercial Kombucha products (Chapters 4 and 5), and investigate the impact of different fermentation process parameters on the levels of the bioactive compounds and functional activities of black tea Kombucha (Chapter 6).

The microbial characteristics of a New Zealand Kombucha starter culture during fermentation were investigated in Chapter 3. It is well known that there are limited efficient microbiological media commercially available for the isolation of acetic acid bacteria (AAB) alone. Therefore, a selective medium composed of glucose, yeast extract, mannitol, absolute ethanol, and glacial acetic acid (GYMEA) was designed to effectively isolate AAB in this study (Chapter 3). The GYMEA developed and used here for AAB isolation effectively inhibited the growth of other undesirable microorganisms such as lactic acid bacteria (LAB) and yeast thus enabling the efficient isolation of AAB. In addition, the developed medium was easier to prepare and more cost-effective than the traditional media such as glucose yeast extract calcium carbonate agar

(GYC). The dominant AAB from the Kombucha starter culture was identified as *Komagataeibacter rhaeticus* DST GL02 which was responsible for the formation of the cellulosic pellicle. The dominant yeast belonged to *Zygosaccharomyces lentus* CBS 8574 with *Debaryomyces prosopidis* JCM 9913 considered a minor microorganism due to its presence at low levels (10.3%) (Chapter 3). To our knowledge, this is the first study to characterise and identify the dominant fermenting microorganisms in black tea Kombucha fermented with a New Zealand starter culture. The use of these findings will contribute to the stable production of high-quality Kombucha via the manipulation of microorganisms in the starter culture.

Many commercial Kombucha products are perceived to be probiotic or good sources of "live cultures" due to their inherent microorganisms. Thus, Kombucha has attracted more attention and grown rapidly in the probiotic beverage market as an alternative dairy-free probiotic source to confer health effects particularly to consumers with lactose intolerance. The scientific evidence about the probiotic characteristics of microorganisms in Kombucha is limited. Therefore, to help fill this knowledge gap, this study evaluated the probiotic potential of representative AAB (n=9) and, yeast (n=15) strains isolated from Kombucha in New Zealand (Chapter 4 and Chapter 5). The findings from Chapter 4 and Chapter 5 indicated that all three AAB strains of *Acetobacter musti* LOAAB1, and *Gluconobacter potus* (LOAAB2 and GBAAB3), and two yeast strains of *Pichia kudraivzevii* GBY1 and *Saccharomyces cerevisiae* GBY2 may be promising novel probiotic strains due to their excellent probiotic and safety characteristics. Among the five representative isolates, two yeast strains (GBY1, GBY2) showed better tolerance to high temperatures (39 and 42°C), low pH (2.00) and $NaCl_{aqueous}$ (2%, 4% and 6.5% w/v), much higher auto-aggregation (>95%, 24 h), co-aggregation with pathogens (>65%), and hydrophobicity rate (>60%), and stronger bile salt hydrolase activity than the three AAB strains (Table 7.1). These two yeast trains (GBY1 and GBY2) may be the most promising probiotic cultures for applications in fermented foods and beverages. To the

author's knowledge, this is the first study to evaluate the probiotic potential of fermenting microorganisms especially *A. musti* LOAAB1 and *G. potus* (LOAAB2, GBAAB3) isolated from a Kombucha starter culture and commercial Kombucha products in New Zealand, which also makes Kombucha a potential good source of probiotics (Chapter 5). Therefore, the findings in Chapter 4 and Chapter 5 provide strong scientific evidence for the *in vitro* screening and selection of novel probiotic strains isolated from Kombucha and provide more options for consumers with lactose intolerance and other allergens including dairy.

Table 7.1 Summary of probiotic characteristics of AAB and yeast strains isolated from New Zealand Kombucha.

Probiotic characteristics	**Yeast (Chapter 4)**		**AAB (Chapter 5)**		
	GBY1	GBY2	LOAAB1	LOAAB2	GBAAB3
Growth at different temperature					
Growth at 37℃	+	+	+	+	+
Growth at 39℃	+	+	N/A	N/A	N/A
Growth at 42℃	+	+	N/A	N/A	N/A
Tolerance to $NaCl_{aq}$ (w/v)					
1%	+	+	+	+	+
2%	+	+	-	-	-
4%	+	+	-	-	-
6.5%	+	+	-	-	-
Tolerance to low pH					
pH 2	+	+	-	-	-
pH 3	+	+	+	+	+
Tolerance to bile salts (w/v)					
0.5%	+	+	+	+	+
1.0%	+	+	+	+	+
1.5%	+	+	+	+	+
Cell surface characteristics					
Auto-aggregation (24 h, %)	98.95±0.25	98.53±0.39	86.55±10.95	82.33±1.48	90.40±1.97
Coaggregation with *S. aureus* (%)	78.41±1.74	86.76±1.76	30.72±1.02	43.00±3.57	53.47±0.77
Coaggregation with *B. cereus* (%)	75.86±2.48	80.33±1.40	13.27±2.47	24.76±4.25	21.13±0.93
Coaggregation with *P. aeruginosa* (%)	78.83±1.06	83.39±1.92	18.41±0.90	25.78±1.03	22.70±0.56
Coaggregation with *E. coli* (%)	66.73±1.39	73.01±1.68	30.18±1.54	21.19±1.03	14.35±0.57
Hydrophobicity with chloroform (%)	83.87±0.98	64.59±0.22	47.22±0.38	42.12±0.38	50.20±0.67
Functional activities					
Antioxidant activity (%)	89.72±0.17	92.37±0.07	94.40±0.55	91.01±0.72	93.39±0.64
Bile salt hydrolase activity (mm)	23.33±1.16	16.00±0.00	N/A	N/A	N/A

Note: "+" indicated positive results; "-" indicated the negative results; "N/A" indicated not available.

The effect of different fermentation parameters (fermentation temperature, fermentation time, sugar concentrations, black tea concentrations, tea infusion time, inoculum and SCOBY concentrations) on the physicochemical and functional activities of Kombucha were determined using the Plackett-Burman design in Chapter 6. The results showed that

concentrations of SCOBY, sugar, inoculum, and black tea infusion time were significant ($p<0.05$) factors influencing the physicochemical characteristics of the cellulosic pellicle produced during the Kombucha fermentation. The concentration of black tea was the most important factor affecting the total phenolic and flavonoid content, dominant individual phenolic compounds (caffeine, gallic acid and theobromine) and antioxidant activities of black tea Kombucha. Whereas the high titratable acidity, total phenolic and flavonoid content contribute to the strong antimicrobial activities of black tea Kombucha. To the best of our knowledge, this is the first study to investigate the effect of main fermentation parameters on the physicochemical characteristics and functional activities (antioxidant and antimicrobial activities) of black tea Kombucha prepared with a defined New Zealand starter culture. The results in Chapter 6 suggested that the formulation and process parameters used to prepare K15, K11 and K8 could be used as potential strategies for manufacturers to produce Kombucha with high bioactive compounds and stronger functional activities. Furthermore, the use of the fermentation process parameters used to prepare K15, K11 and K8 may also help the standardisation of the industrial scale-up production process.

The findings from this study will contribute to better understanding of the microbiological characteristics of the dominant fermenting microorganisms during Kombucha fermentation which is important for manufacturers to control the quality of Kombucha. The current research also provides scientific evidence of the probiotic potential of the microorganisms isolated from Kombucha. The most promising strains should be further investigated to support claims on Kombucha products as probiotic beverages. Such scientifically substantiated claims may attract more consumers to Kombucha and therefore contribute to gaining more market share in the functional beverage category. This study also identified the most important fermentation parameters affecting physicochemical characteristics of black tea Kombucha such as the acidity and ethanol concentrations which could contribute to a better control of the industrial scale-up

production process. This will both lower the risk of health concerns to consumers and should also reduce products recall due to excess ethanol and acidity levels in commercial Kombucha products. Additionally, the information gained from the current study especially the effect of fermentation parameters on functional activities and bioactive compounds may help producers develop customised functional beverages for consumers by precisely monitoring the fermentation parameters and formulations.

7.2 Recommendations for future research

- This study provides an understanding of the dominant microorganisms of black tea Kombucha during fermentation by traditional culture-based methods. However, the distribution and proportion of the microbial composition during fermentation are still not fully understood. Other culture-independent methods, such as 16S amplicon sequencing using next-generation sequencing, whole genome sequencing, and metagenomic sequencing are recommended to increase our understanding of the overall fermenting microbial communities involved in Kombucha fermentation.
- This thesis only evaluated the tolerance of probiotic strains to single-factor stresses such as tolerance to NaCl, bile salts, different temperatures, and low pH. *In vivo* trials need to be carried out to determine whether these potential probiotic microorganisms could actually survive in the human gastrointestinal tract environment and potentially confer health benefits to the host.
- Previous studies have shown that certain probiotic bacteria or yeast strains can confer antidiabetic, anti-inflammatory, anticancer, and cholesterol-lowering effects (Gebre et al., 2023; Srinivas et al., 2017; Won et al., 2021). However, whether the probiotic strains isolated from New Zealand Kombucha have similar health effects has not been determined. Hence, further *in vivo* research is needed to explore other beneficial effects of the potential probiotic strains from Kombucha.

- Only seven representative phenolic compounds from black tea Kombucha were identified and quantified in this study. Previous studies have found several phenolic compounds and other bioactive compounds such as organic acids and vitamins in Kombucha (Aung et al., 2022; Bortolomedi et al., 2022; Cardoso et al., 2020). The presence of these bioactive compounds has been associated with a variety of functional activities including antioxidant, antimicrobial and antiproliferative activities of Kombucha (de Miranda et al., 2022). Therefore, presence and quantity of additional phenolic compounds (caffeic acid, chlorogenic acid, ferulic acid), flavonoids (rutin, quercetin, catechin, theaflavin), organic acid (acetic acid, gluconic acid, glucuronic acid), and vitamins (B1, B6, B12 and C) in the black tea Kombucha solutions should also be determined in any future work.
- This study emphasises the physicochemical, microbial, and functional properties of traditional black tea Kombucha. The use of alternative raw materials such as different types of tea (jasmine, oolong, green, kawakawa), fruit infusions (Kiwifruit, cherry, goji berry, blackberry, grape, coconut water), herb and spices (yerba-mate, mint, lemongrass, sage), coffee, and milk used in Kombucha fermentation should be explored in future studies. Moreover, different types of sugars such as unrefined sugar, molasses and other fermentable carbohydrates could also be investigated as replacements for sucrose to produce Kombucha with a different taste or containing other nutrients such as amino acids, minerals, and vitamins (Muhialdin et al., 2019). Therefore, more research needs to be conducted to exploit the bioactive compounds, physicochemical, functional, and sensory characteristics of Kombucha prepared with alternative raw materials. The use of these alternative materials may result in Kombucha with different sensory characteristics, chemical composition, and functional properties (Freitas et al., 2022; Leonarski et al., 2022).

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Appendices

Appendix 1. DRC 16 forms

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Boying Wang
Name and title of main supervisor:	Dr Tony Mutukumira
In which chapter is the manuscript/published work?	Chapter 2, 3.
What percentage of the manuscript/published work was contributed by the student?	95%

Describe the contribution that the student has made to the manuscript/published work:

Planning and design of experiments, conducting the experiments, data acquisition and analysis, writing and review of the papers.

Please select one of the following three options:

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  Wang, B., Rutherfurd-Markwick, K., Zhang, X.-X., Mutukumira, A. N. (2022b). Kombucha: Production and Microbiological Research. Foods, 11(21), 3456. https://doi.org/10.3390/foods11213456
  Wang, B., Rutherfurd-Markwick, K., Naren, N., Zhang, X.-X., & Mutukumira, A. N. (2023). Microbiological and

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  Wang, B., Rutherfurd-Markwick, K., Liu, N., Zhang, X.-X., Mutukumira, A. N. (2024)
  Evaluation of the probiotic potential of yeast isolated from kombucha in New Zealand, Current Researchin in Food Science. https://doi.org/10.1016/j.crfs.2024.100711.

- ( ) It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Student's signature:	Boying Wang Digitally signed by Boying Wang DN: cn=Boying Wang , c=NZ, email=2372434479@qq.com Date: 2024.03.12 21:02:37 +13'00'	Main supervisor's signature:	Dr Tony Mutukumira Digitally signed by Dr Tony Mutukumira DN: cn=Dr Tony Mutukumira, c=NZ, o=Massey University, ou=SF&AT, email=a.n.mulukumira@massey.ac.nz Reason: I agree to the terms defined by the placement of my signature on this document Location: Auckland Date: 2024.03.12 16:12:24 +08'00'

This form should be placed at the beginning of each relevant thesis chapter.

Appendix 2. Supplementary materials of Chapter 2

Appendix 2.1 Commonly used media for the isolation, cultivation, and differentiation of acetic acid bacteria.

Medium	Components (g/L) or (mL/L)
Glucose yeast extract carbonate (GYC)	Glucose (100), yeast extract (10), calcium carbonate (20), bacteriological agar (15)
Glucose yeast extract (GY)	Glucose (20), yeast extract (10), bacteriological agar (20)
Yeast extract Peptone Mannitol (YPM)	Yeast extract (5), mannitol (25), peptone (3), bacteriological agar (12)
Reinforced Acetic acid ethanol (RAE)	Glucose (40), yeast extract (10), peptone (10), Na_2PO_4*$2H_2O$ (3.38), citric acid x H_2O, ethanol (20), acetic acid (50), bacteriological agar (20)
Malt yeast extract (MYA)	Malt extract (15), yeast extract (5), Bacteriological agar (15)
Basal medium ethanol (BME)	Yeast extract (0.5), vitamin-free casamino acids (3), ethanol (3), bacteriological agar (15)
Carr	Yeast extract (30), ethanol (20), bromocresol green (0.022), bacteriological agar (20)
Glucose yeast extract acetic acid ethanol (GYAE)	Glucose (50), yeast extract (10), acetic acid (10), ethanol (20), bacteriological agar (15)
Glucose yeast extract ethanol calcium carbonate (GYEC)	Glucose (10), yeast extract (10), calcium carbonate (20), ethanol (30), bacteriological agar (15)
Glucose yeast extract peptone (GYP)	Glucose (30), yeast extract (5), peptone (2), bacteriological agar (15)
Hestrin-Schramm (HS)	Glucose (20), yeast extract (5), peptone (5), Na_2HPO_4 (2.7).
Sorbitol yeast extract peptone (SYP)	Sorbitol (50), yeast extract (5), peptone (3), bacteriological agar (15)
Yeast extract glucose mannitol (YGM)	Glucose (20), mannitol (20), yeast extract (10), acetic acid (5), ethanol (20)

Appendix 2.2 Commonly used media for the isolation, cultivation and differentiation of yeast from food products.

Medium	Component (g/L) or (mL/L)
Sabouraud glucose agar (SGA)	Glucose (10), Peptone (10) bacteriological agar (15)
Potato dextrose agar (PDA)	Glucose (20), potato infusion (500), bacteriological agar (15)
Malt extract agar (MEA)	Malt extract (20), peptone (5), bacteriological agar (15)
Acidified potato dextrose agar (APDA)	Glucose (20), potato infusion (500), lactic acid solution (5), bacteriological agar (15)
Acidified Tryptone glucose yeast extract agar (ATGY)	Tryptone (5), glucose (100), yeast extract (5), glacial acetic acid (10), bacteriological agar (15)
Oxytetracycline glucose yeast extract agar (OGY)	Glucose (20), agar (12), yeast extract (5), oxytetracycline solution (10)
Yeast extract glucose chloramphenicol (YGC) agar	Glucose (20), agar (14.9), yeast extract (5), Chloramphenicol (0.1)
Rose Bengal chloramphenicol agar (RBC)	Glucose (10), Papaic digest of soybean meal (5), KH_2PO_4 (1), $MgSO_4$*$7H_2O$ (0.5), Rose Bengal (0.05), chloramphenicol solution (10)
Dichloran Rose Bengal chloramphenicol agar (DRBC)	Glucose (10), peptone (5), KH_2PO_4 (1), $MgSO_4$*$7H_2O$ (0.5), Rose Bengal (5% w/v, 0.5), chloramphenicol (0.1), dichloran (0.2% w/v in ethanol, 1), bacteriological agar (15)

Appendix 3. Supplementary materials of Chapter 3

Table A3.1 Sequences obtained from the representative AAB and yeast strains.

Sample code	Sequence
D5T1AAB1	TGCAGTCGCACGAACCTTTCGGGGTTAGTGGCGGACGGGTGAGTAACGCGTAGG GATCTGTCCATGGGTGGGGGATAACTTTGGGAAACTGAAGCTAATACCGCATGAC ACCTGAGGGTCAAAGGCGCAAGTCGCCTGTGGAGGAACCTGCGTTCGATTAGCT AGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCGATAGCTGGTCTGAGAGGA TGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA GTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGTGTG AAGAAGGTTTTCGGATTGTAAAGCACTTTCAGCGGGGACGATGATGACGGTACC CGCAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGG GGCAAGCGTTGCTCGGAATGACTGGGCGTAAAGGGCGCGTAGGCGGTTGTTACA GTCAGATGTGAAATTCCCGGGCTTAACCTGGGGGCTGCATTTGATACGTGATGAC TAGAGTGTGAGAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGAT ATTGGGAAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCATGACTGACGCTG AGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT GTAAACGATGTGTGCTGGATGTTGGGTGACTTTGTCATTCAGTGTCGTAGTTAAC GCGATAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAA TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG CAGAACCTTACCAGGGCTTGACATGCGGAGGCTGTGTCCAGAGATGGGCATTTC TCGCAAGAGACCTCCAGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTG AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCTTTAGTTGCCATCA CGTCTGGGTGGGCACTCTAAAGGAACTGCCGGTGACAAGCCGGAGGAAGGTGG GGATGACGTCAAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAAT GGCGGTGACAGTGGGAAGCCAGGTGGTGACACCGAGCCGATCTCAAAAAGCCG TCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCTAGTA ATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC GTCACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCGAACCGCAAGGAC GCAGCCGA
D7T2AAB10	GTCGCACGAACCTTTCGGGGTTAGTGGCGGACGGGTGAGTAACGCGTAGGGATC TGTCCATGGGTGGGGGATAACTTTGGGAAACTGAAGCTAATACCGCATGACACC TGAGGGTCAAAGGCGCAAGTCGCCTGTGGAGGAACCTGCGTTCGATTAGCTAGT TGGTGGGGTAAAGGCCTACCAAGGCGATGATCGATAGCTGGTCTGAGAGGATGA TCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTG GGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGTGTGAAG AAGGTTTTCGGATTGTAAAGCACTTTCAGCGGGGACGATGATGACGGTACCCGC AGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGC AAGCGTTGCTCGGAATGACTGGGCGTAAAGGGCGCGTAGGCGGTTGTTACAGTC AGATGTGAAATTCCCGGGCTTAACCTGGGGGCTGCATTTGATACGTGATGACTAG AGTGTGAGAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATT GGGAAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCATGACTGACGCTGAGG CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAA ACGATGTGTGCTGGATGTTGGGTGACTTTGTCATTCAGTGTCGTAGTTAACGCGA TAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGA CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAG AACCTTACCAGGGCTTGACATGCGGAGGCTGTGTCCAGAGATGGGCATTTCTCG CAAGAGACCTCCAGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGA TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCTTTAGTTGCCATCACGTC TGGGTGGGCACTCTAAAGGAACTGCCGGTGACAAGCCGGAGGAAGGTGGGGAT GACGTCAAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCG GTGACAGTGGGAAGCCAGGTGGTGACACCGAGCCGATCTCAAAAAGCCGTCTC AGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCTAGTAATC GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT CACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCGAACCGCAAGGACGC AGCCGA
D11T1AAB16	TGCAGTCGCACGAACCTTTCGGGGTTAGTGGCGGACGGGTGAGTAACGCGTAGG GATCTGTCCATGGGTGGGGGATAACTTTGGGAAACTGAAGCTAATACCGCATGAC

	ACCTGAGGGTCAAAGGCGCAAGTCGCCTGTGGAGGAACCTGCGTTCGATTAGCT AGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCGATAGCTGGTCTGAGAGGA TGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA GTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGTGTG AAGAAGGTTTTCGGATTGTAAAGCACTTTCAGCGGGGACGATGATGACGGTACC CGCAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGG GGCAAGCGTTGCTCGGAATGACTGGGCGTAAAGGGCGCGTAGGCGGTTGTTACA GTCAGATGTGAAATTCCCGGGCTTAACCTGGGGGCTGCATTTGATACGTGATGAC TAGAGTGTGAGAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGAT ATTGGGAAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCATGACTGACGCTG AGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT GTAAACGATGTGTGCTGGATGTTGGGTGACTTTGTCATTCAGTGTCGTAGTTAAC GCGATAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAA TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG CAGAACCTTACCAGGGCTTGACATGCGGAGGCTGTGTCCAGAGATGGGCATTTC TCGCAAGAGACCTCCAGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTG AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCTTTAGTTGCCATCA CGTCTGGGTGGGCACTCTAAAGGAACTGCCGGTGACAAGCCGGAGGAAGGTGG GGATGACGTCAAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAAT GGCGGTGACAGTGGGAAGCCAGGTGGTGACACCGAGCCGATCTCAAAAAGCCG TCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCTAGTA ATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC GTCACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCGAACCGCAAGGAC GCA
D11T2AAB17	TGCAGTCGCACGAACCTTTCGGGGTTAGTGGCGGACGGGTGAGTAACGCGTAGG GATCTGTCCATGGGTGGGGGATAACTTTGGGAAACTGAAGCTAATACCGCATGAC ACCTGAGGGTCAAAGGCGCAAGTCGCCTGTGGAGGAACCTGCGTTCGATTAGCT AGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCGATAGCTGGTCTGAGAGGA TGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA GTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGTGTG AAGAAGGTTTTCGGATTGTAAAGCACTTTCAGCGGGGACGATGATGACGGTACC CGCAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGG GGCAAGCGTTGCTCGGAATGACTGGGCGTAAAGGGCGCGTAGGCGGTTGTTACA GTCAGATGTGAAATTCCCGGGCTTAACCTGGGGGCTGCATTTGATACGTGATGAC TAGAGTGTGAGAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGAT ATTGGGAAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCATGACTGACGCTG AGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT GTAAACGATGTGTGCTGGATGTTGGGTGACTTTGTCATTCAGTGTCGTAGTTAAC GCGATAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAA TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG CAGAACCTTACCAGGGCTTGACATGCGGAGGCTGTGTCCAGAGATGGGCATTTC TCGCAAGAGACCTCCAGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTG AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCTTTAGTTGCCATCA CGTCTGGGTGGGCACTCTAAAGGAACTGCCGGTGACAAGCCGGAGGAAGGTGG GGATGACGTCAAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAAT GGCGGTGACAGTGGGAAGCCAGGTGGTGACACCGAGCCGATCTCAAAAAGCCG TCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCTAGTA ATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC GTCACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCGAACCGCAAGGAC GCA
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	CATGCCTGTTTGAGCGTCATTTCCTTCTCAAACATTCGTGTTTGGTGGTGAGTGA TACTCTGTGTAACAGCTTGAGTTAGCTTGAAATTGTAAGCCTTTTGGGTGCGTTG GCAAGATGTTTTGGGCGGAGACGCCCAGGCAGCCGATTTTCTTTACCAAATGTC GTATTAGGTTTTACCGACTCGGCAGACGGTGGAGCGGACTGTGTTCATTTTCCTT GTGCGCCTTGGCAAACAATGTTCTCAAAGCT
TFT1Y39	TAATCTGATTGAAGAAGTCTGAGGGAGCATACCAAAGGGACTGTTGTCTCTGCC GGGCCTGCGCTTAGTTGCGCGGTCTAGTGGGGGAGTGGTTCTGCTCTTTTCAGCT TTTACAATTAACACACAGTGGAGTTTCTACTTTTCTTCTTCTTTGGGATTGGGCTC CGGCCCTGTCCCAGAGGTAAACACAAACAATTTTTTTATTTTATTTTAAAAAGTC AAACAAAAAAACAAAAACAAAATATTCAAAACTTTCAACAACGGATCTCTTGGT TCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAAT TCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCAGGGGG CATGCCTGTTTGAGCGTCATTTCCTTCTCAAACATTCGTGTTTGGTGGTGAGTGA TACTCTGTGTAACAGCTTGAGTTAGCTTGAAATTGTAAGCCTTTTGGGTGCGTTG GCAAGATGTTTTGGGCGGAGACGCCCAGGCAGCCGATTTTCTTTACCAAATGTC GTATTAGGTTTTACCGACTCGGCAGACGGTGGAGCGGACTGTGTTCATTTTCCTT GTGCGCCTTGGCAAACAATGTTCTCAAAGCT

Appendix 4. Standard curve for DPPH (Chapter 4 and Chapter 5)

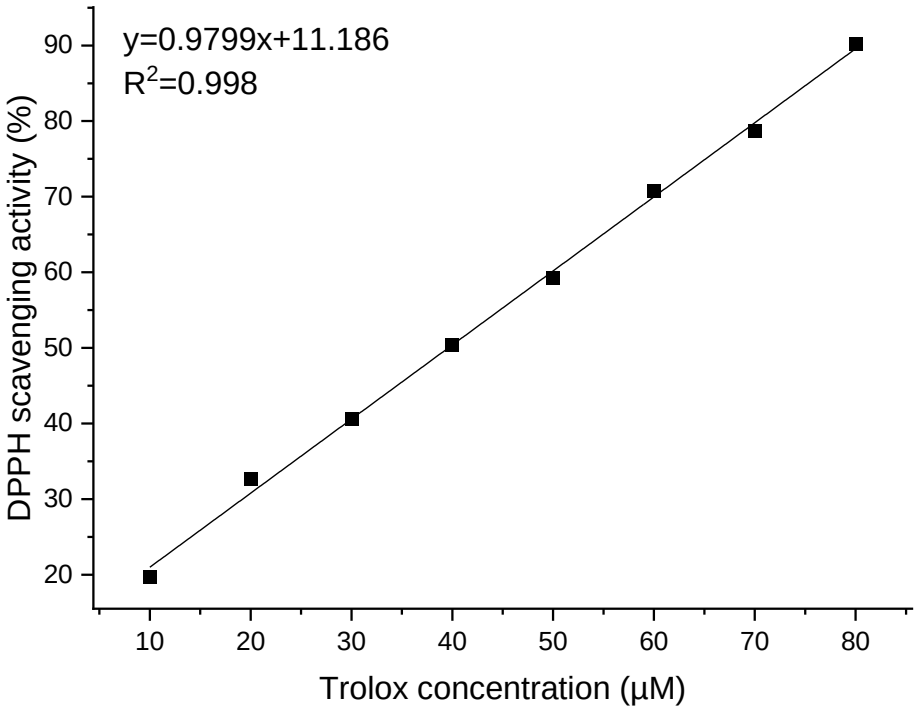

Figure A4.1 The standard curve for DPPH (517 nm).

Appendix 5. S Standard curve for total phenolic content, total flavonoid content, DPPH and ABTS (Chapter 6)

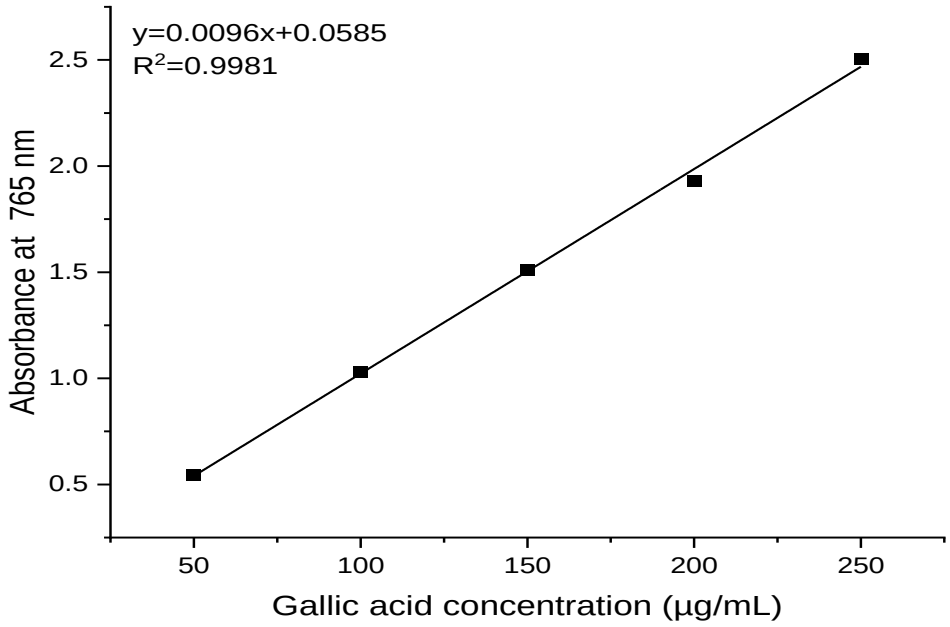

Figure A5.1 The standard curve for total phenolic content at 765 nm.

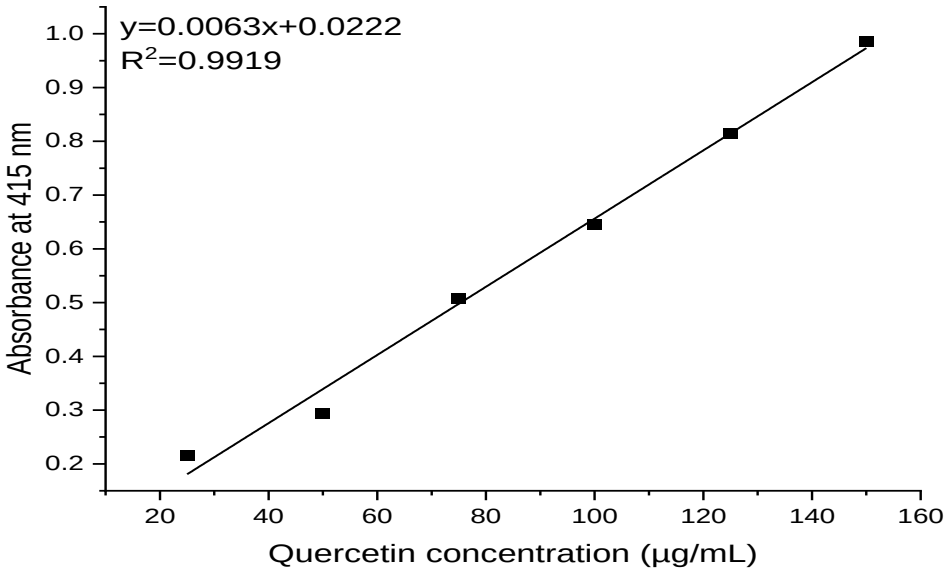

Figure A5.2 The standard curve for total flavonoid content at 415 nm.

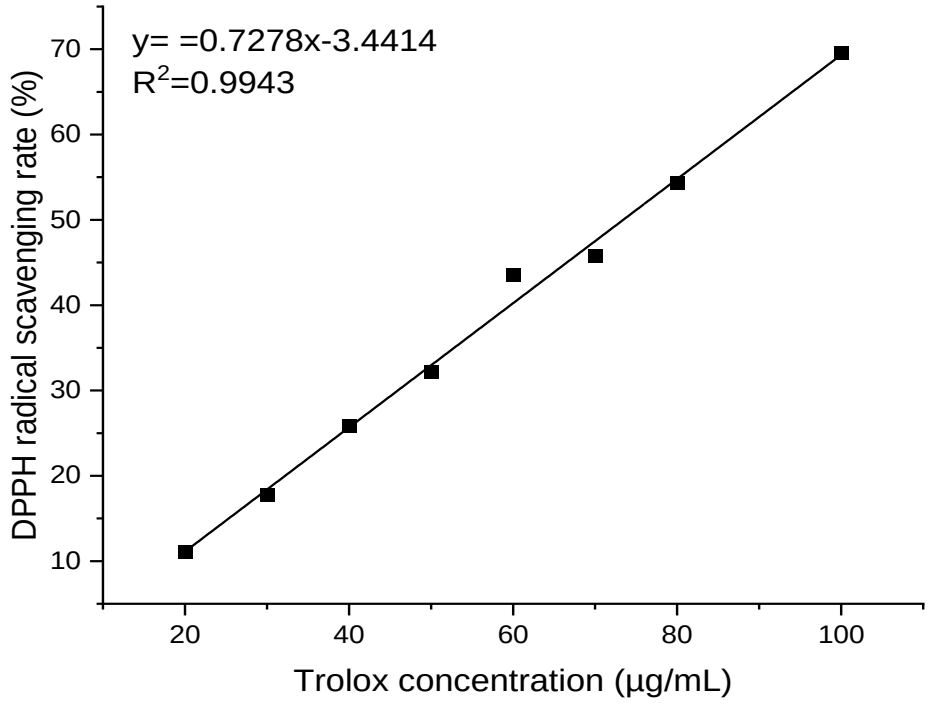

Figure A5.3 The standard curve for DPPH free radical scavenging activity.

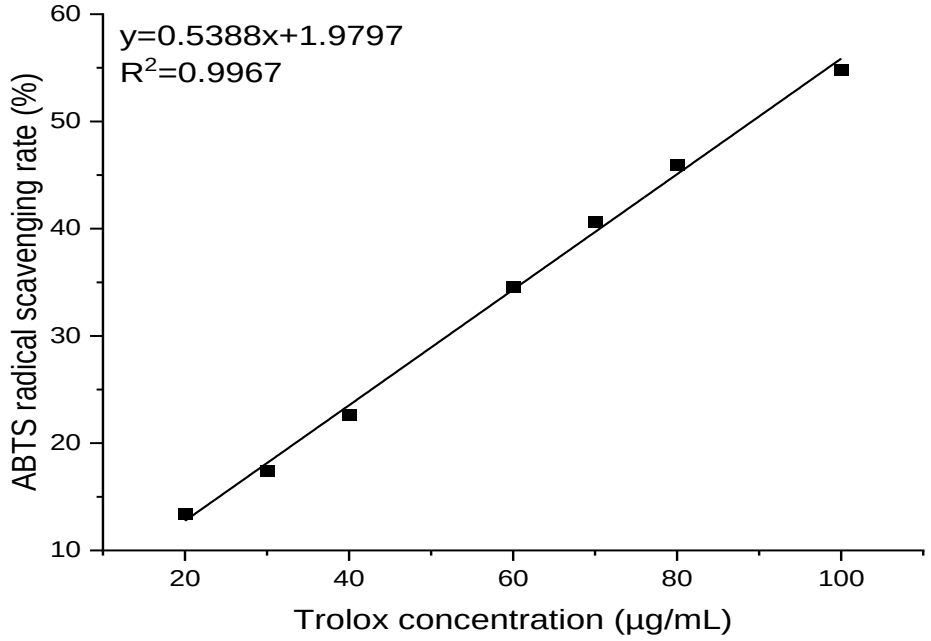

Figure A5.4 The standard curve for ABTS free radical scavenging activity.

Appendix 6. HPLC standard curve for individual phenolic compounds (Chapter 6)

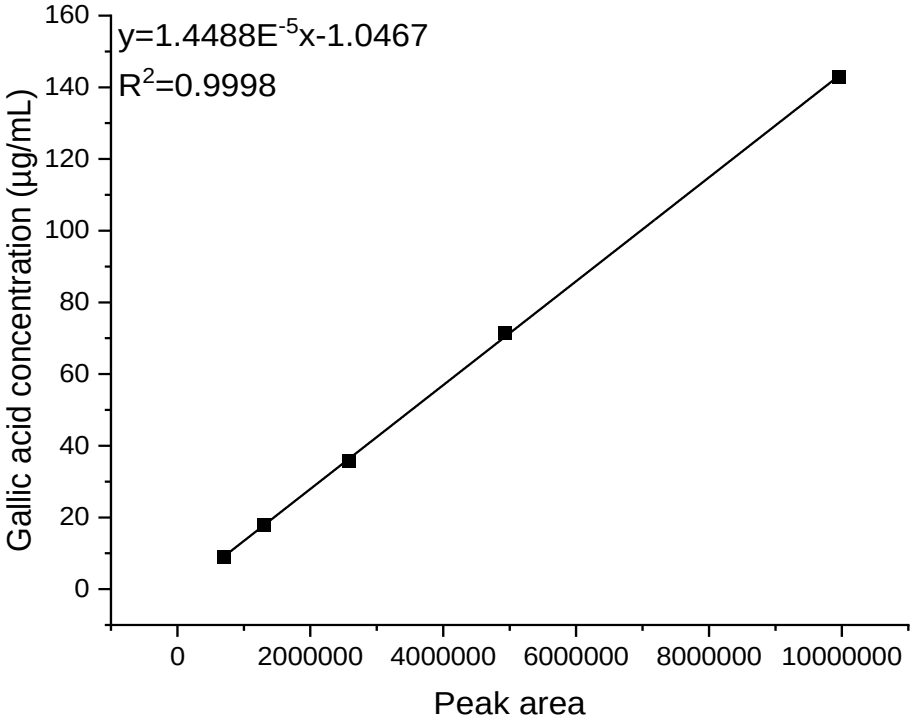

Figure A6.1 HPLC standard curve for gallic acid.

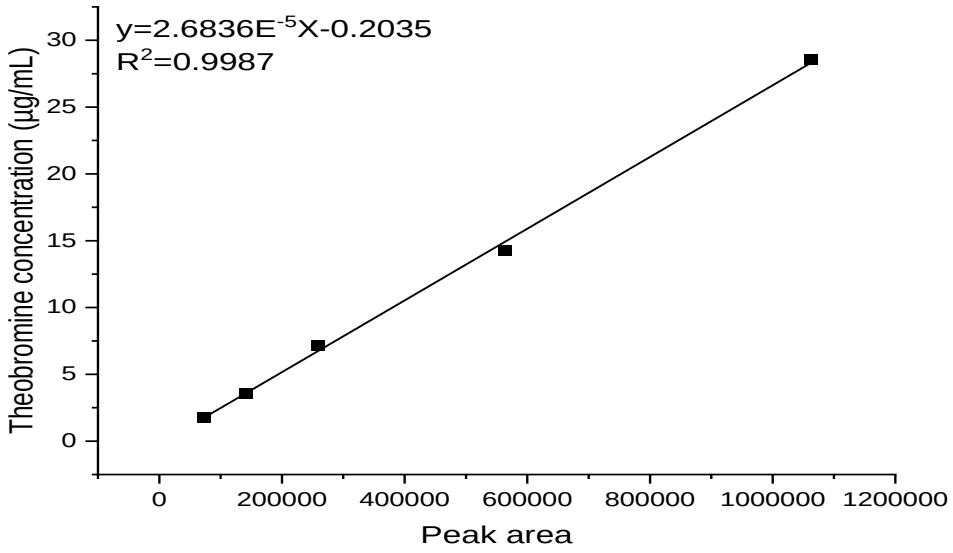

Figure A6.2 HPLC standard curve for theobromine.

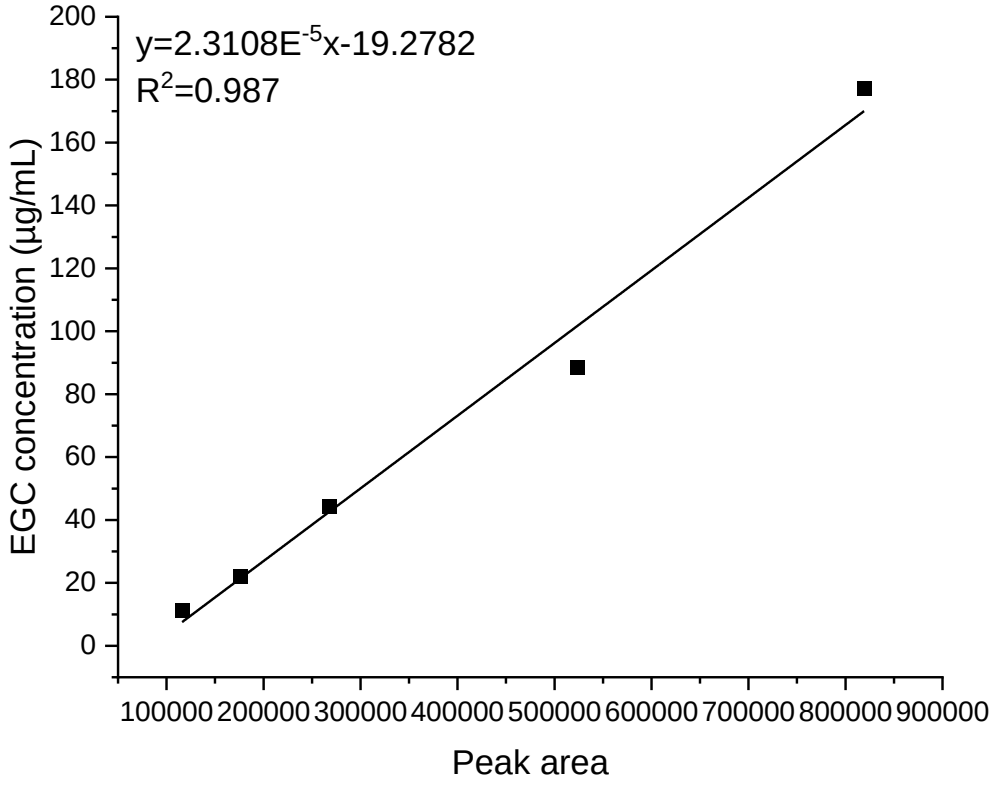

Figure A6.3 HPLC standard curve for EGC.

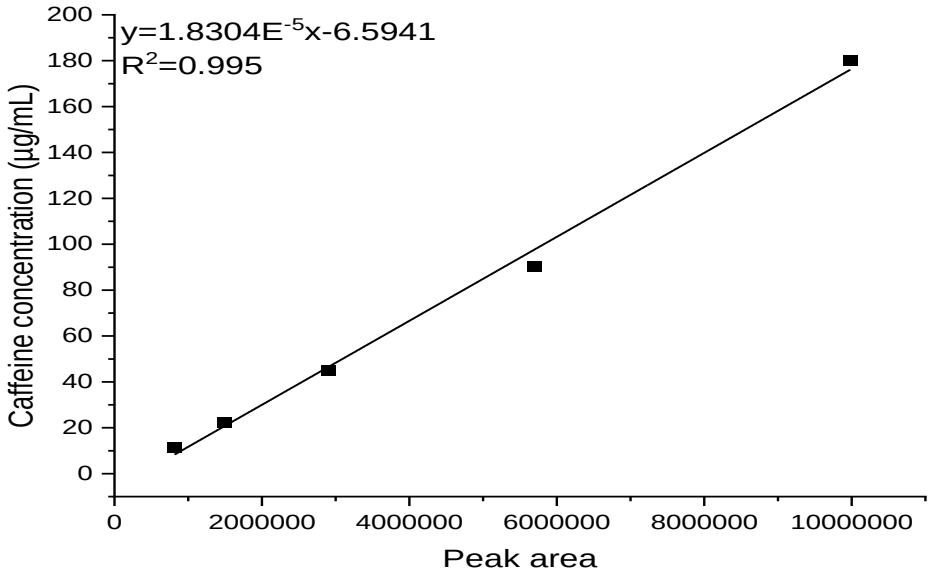

Figure A6.4 HPLC standard curve for caffeine.

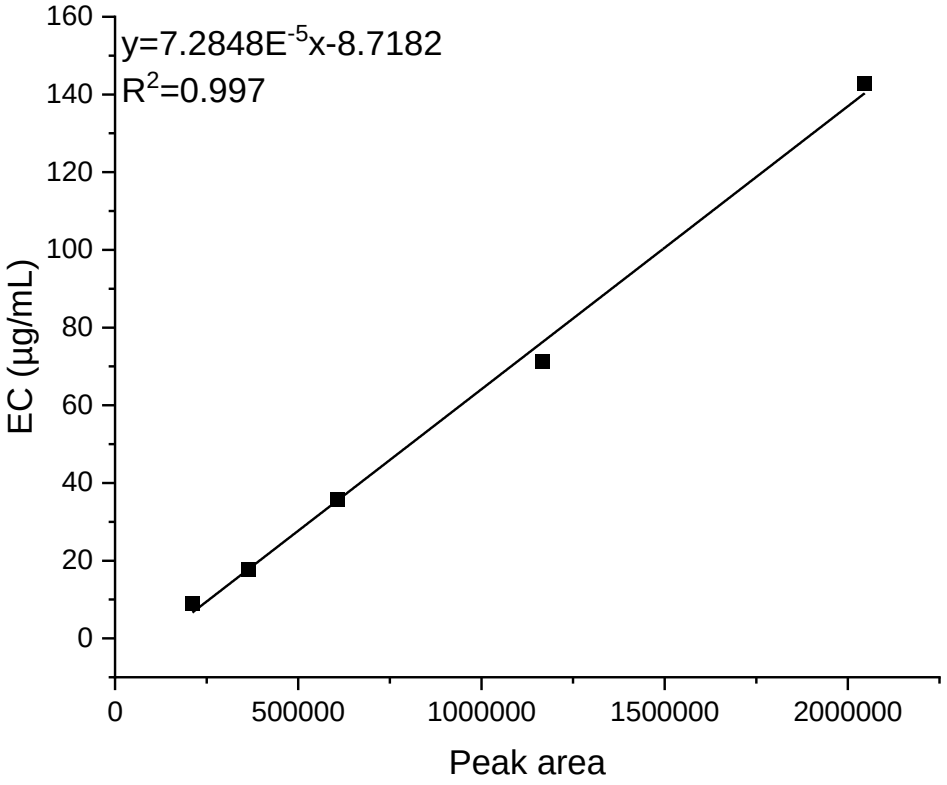

Figure A6.5 HPLC standard curve for EC.

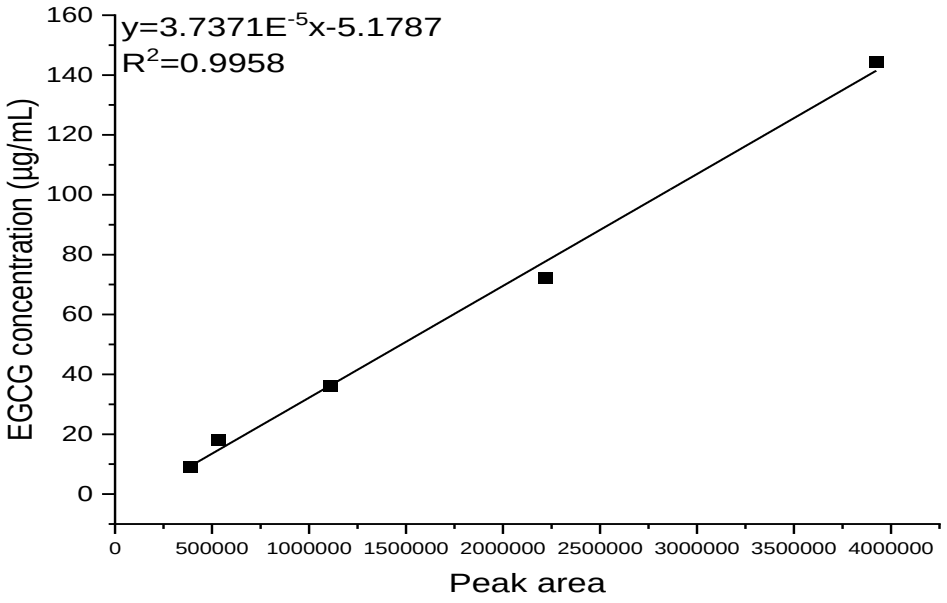

Figure A6.6 HPLC standard curve for EGCG.

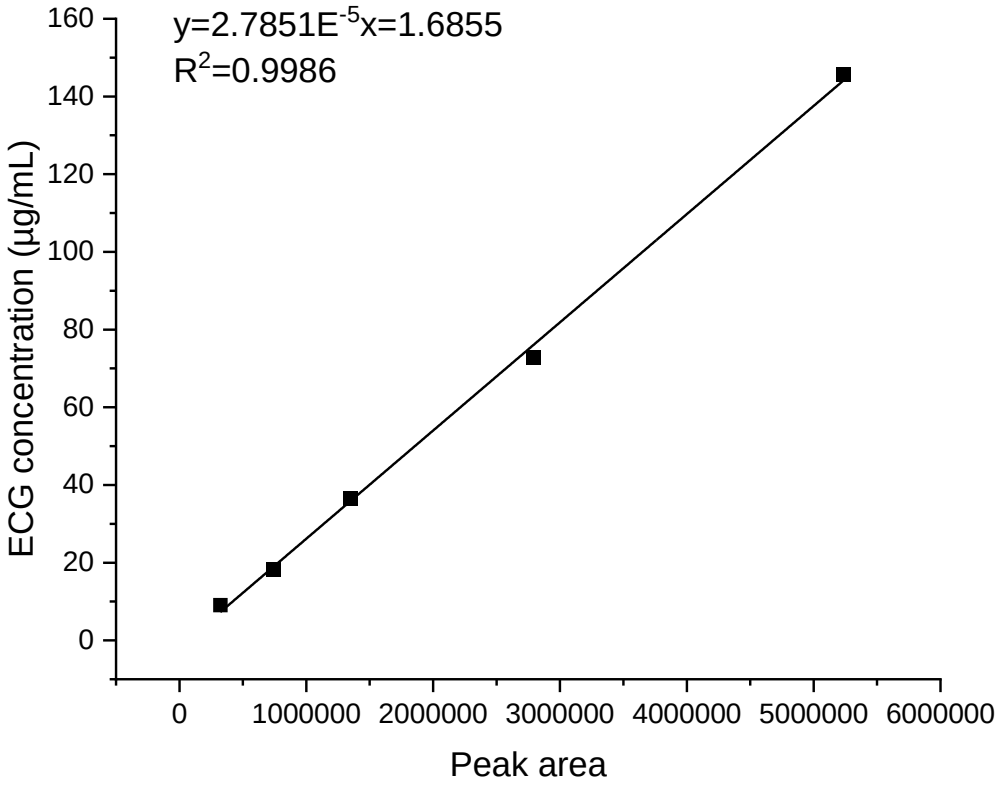

Figure 6.7 HPLC standard curve for ECG

Appendix 7. Retention time and concentration of standard phenolic compounds determined by HPLC (Chapter 6)

Table A7.1 Retention time and concentration of validated HPLC phenolic compounds standards.

Standards	Retention time min)	Concentration (µg/mL)				
		Stock	Dilution 1	Dilution 2	Dilution 3	Dilution 4
Gallic acid	9.286	142.85	71.43	35.71	17.85	8.92
Theobromine	16.685	28.57	14.29	7.14	3.57	1.79
EGC	21.010	177.14	88.57	44.29	22.14	11.07
Caffeine	24.475	180.29	90.14	45.07	22.54	11.27
EC	27.342	142.85	71.43	35.71	17.85	8.92
EGCG	28.888	144.29	72.14	36.07	18.03	9.01
ECG	42.703	145.71	72.86	36.43	18.21	9.11

Appendix 8. HPLC chromatograms

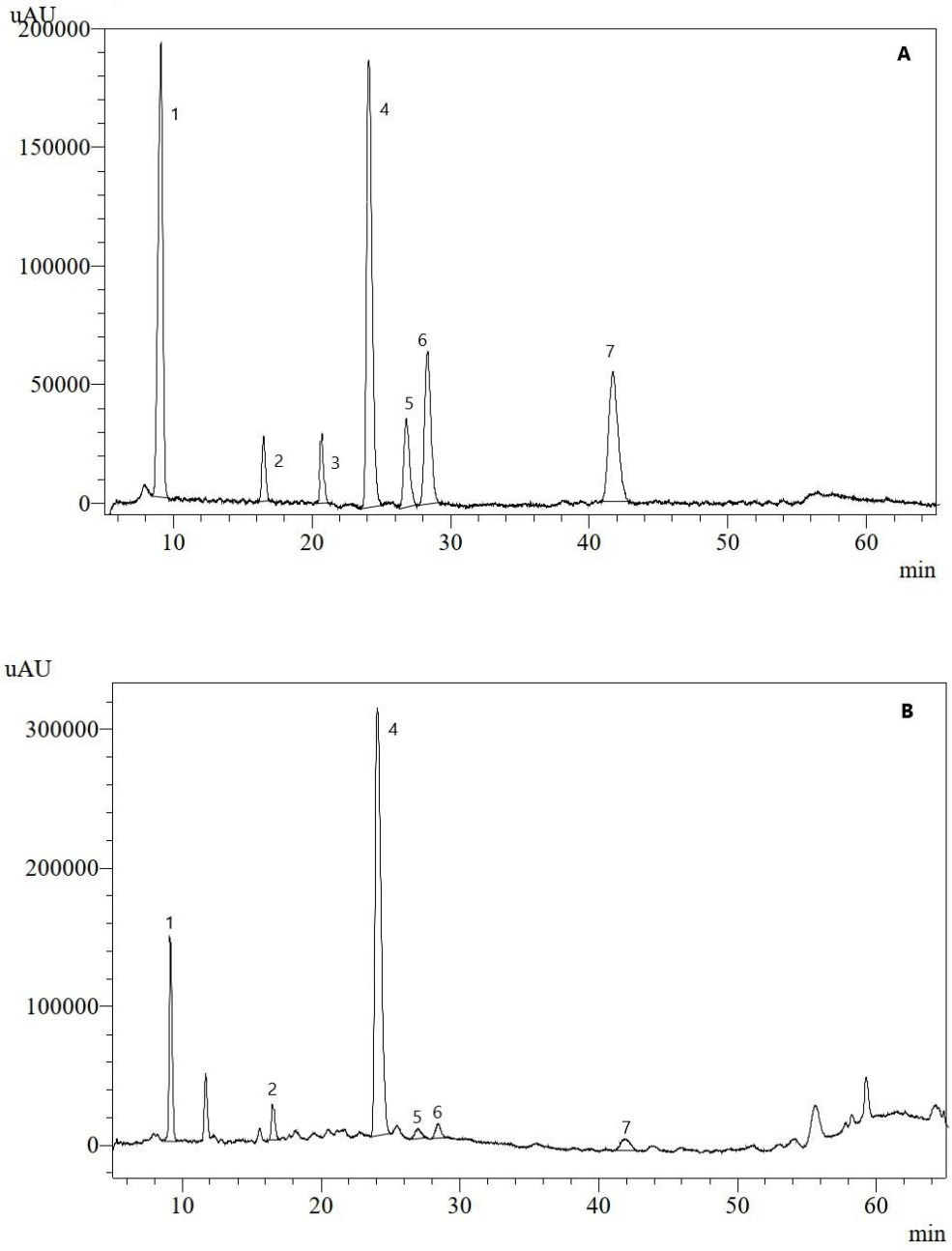

Figure A8.1 HPLC chromatograms of the standard phenolic compounds (A) and Kombucha sample K6 (B). Peak identification: 1. Gallic acid; 2. Theobromine; 3. EGC; 4. Caffeine; 5. EC; 6. EGCG; 7. EGC.

Appendix 9. ANOVA analysis of physicochemical and functional activities of black tea Kombucha (Chapter 6)

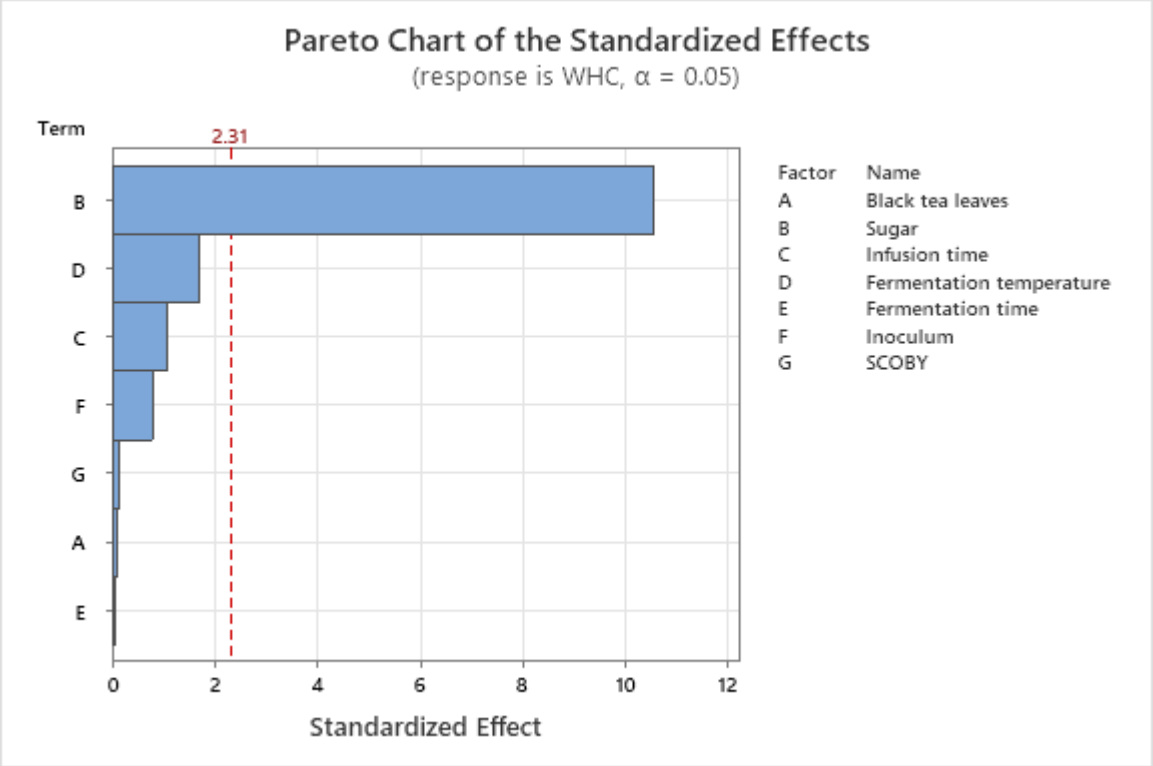

Figure A9.1 Pareto chart of the standardized effects of water holding capacity.

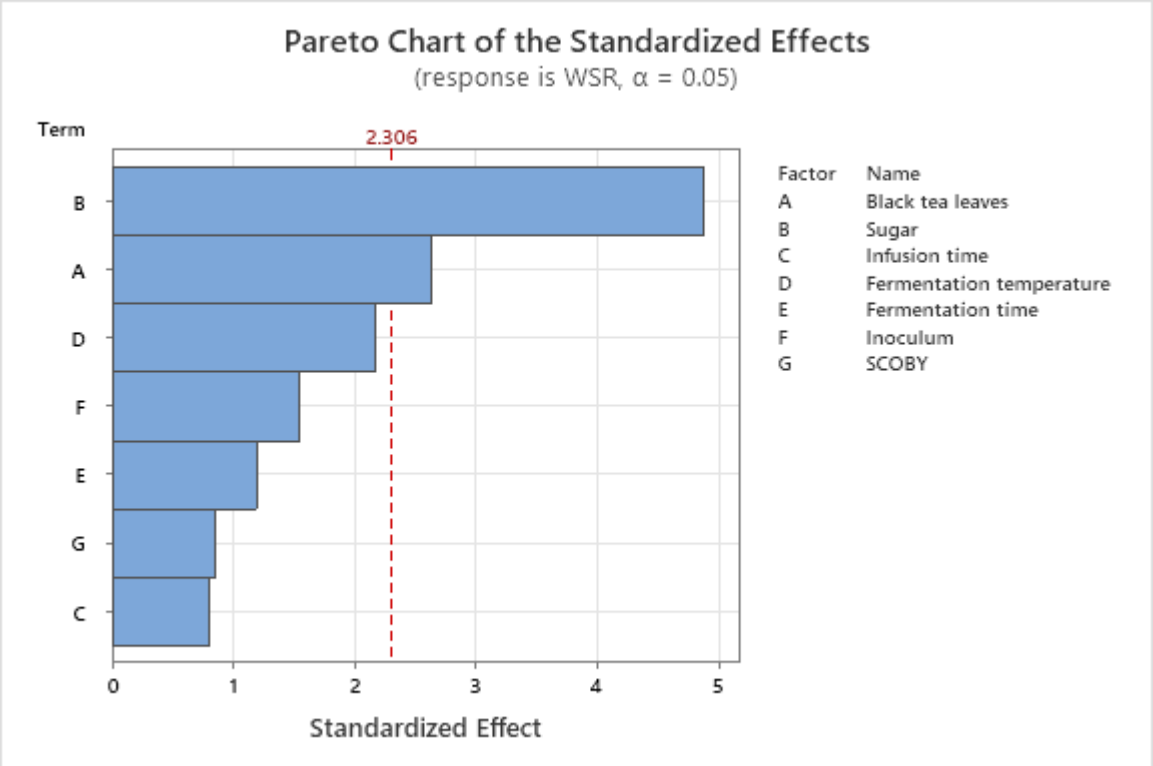

Figure A9.2 Pareto chart of the standardised effects of water swelling ratio.

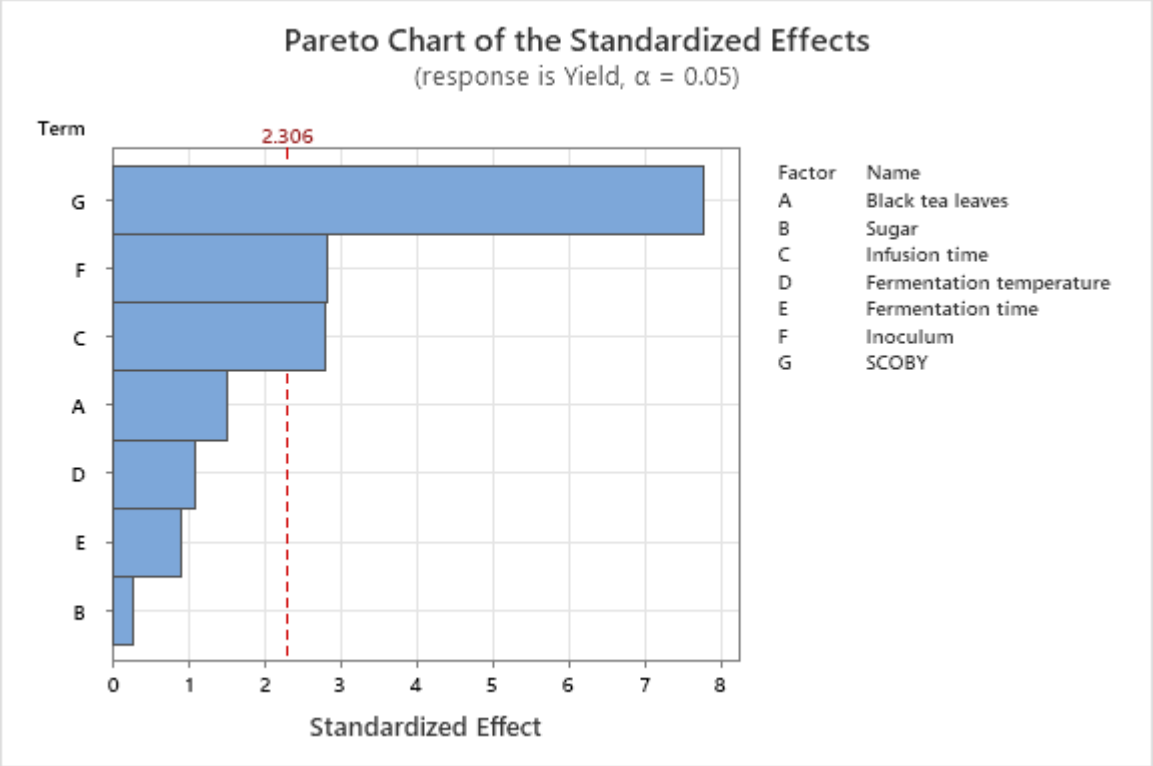

Figure A9.3 Pareto chart of the standardised effects of wet yield.

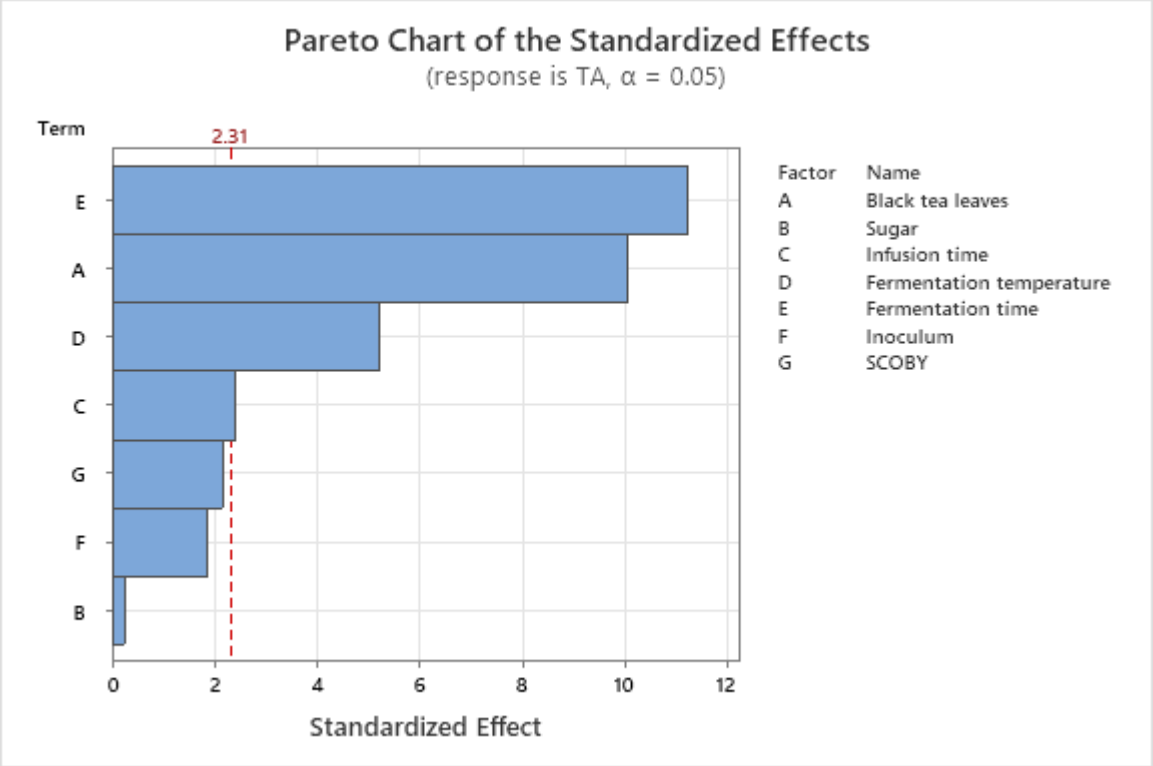

Figure A9.4 Pareto chart of the standardised effects of titratable acidity.

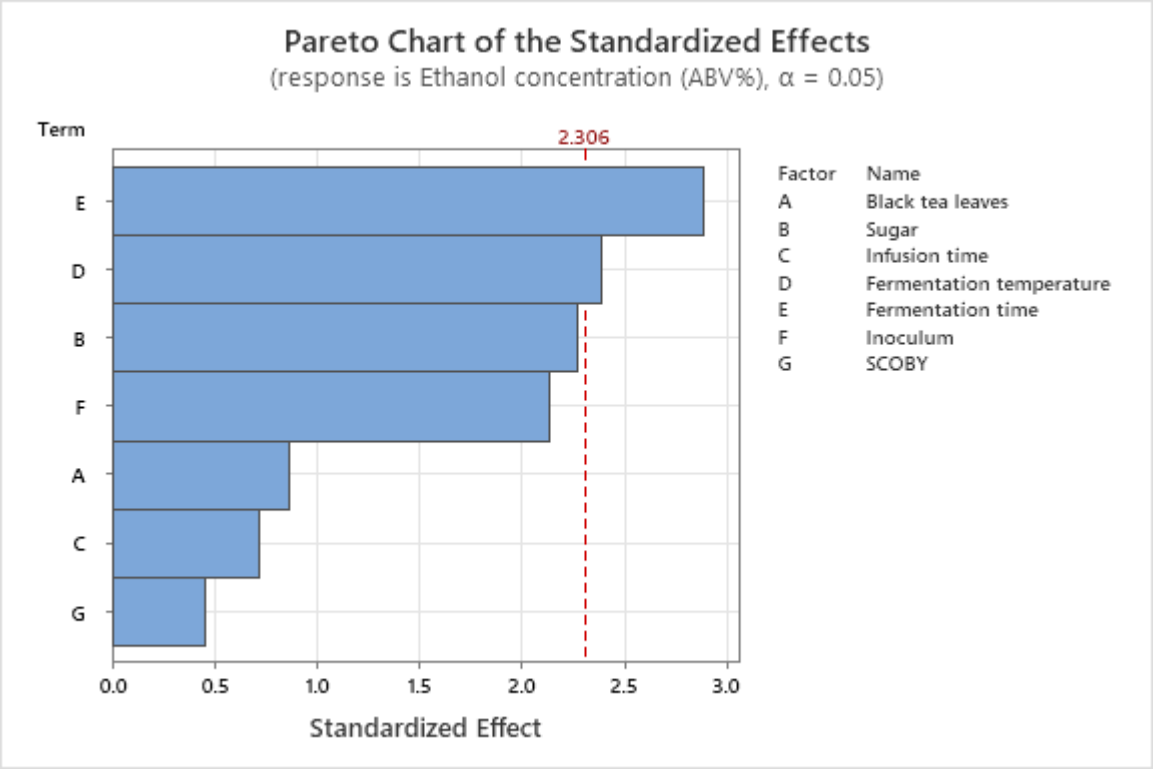

Figure A9.5 Pareto chart of the standardised effects of ethanol concentration.

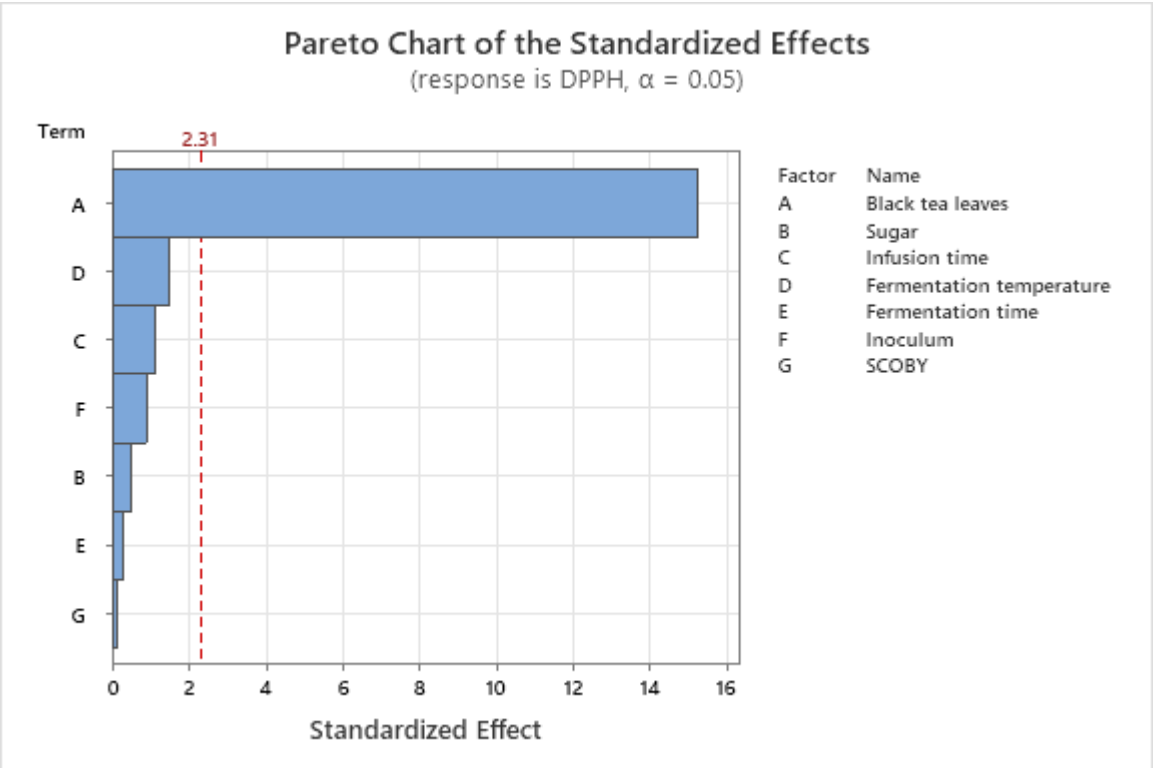

Figure A9.6 Pareto chart of the standardized effects of DPPH.

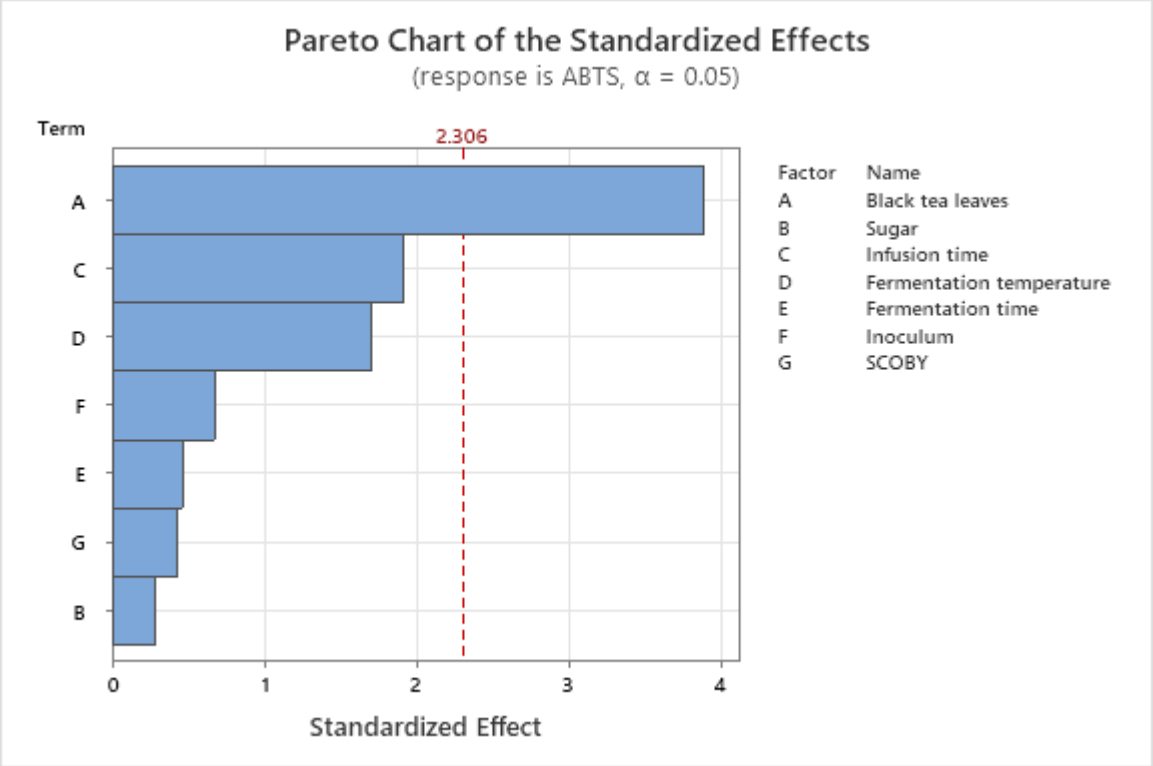

Figure A9.7 Pareto chart of the standardized effects of ABTS.

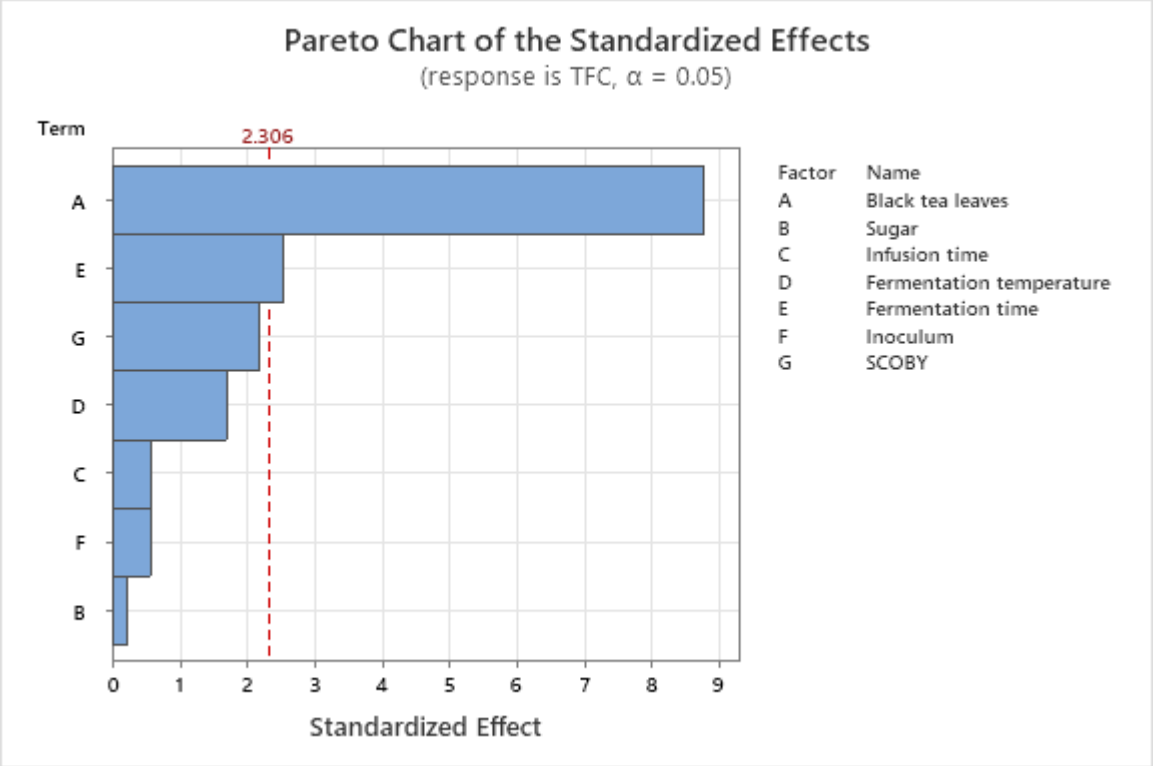

Figure A9.8 Pareto chart of the standardised effects of total flavonoid content.

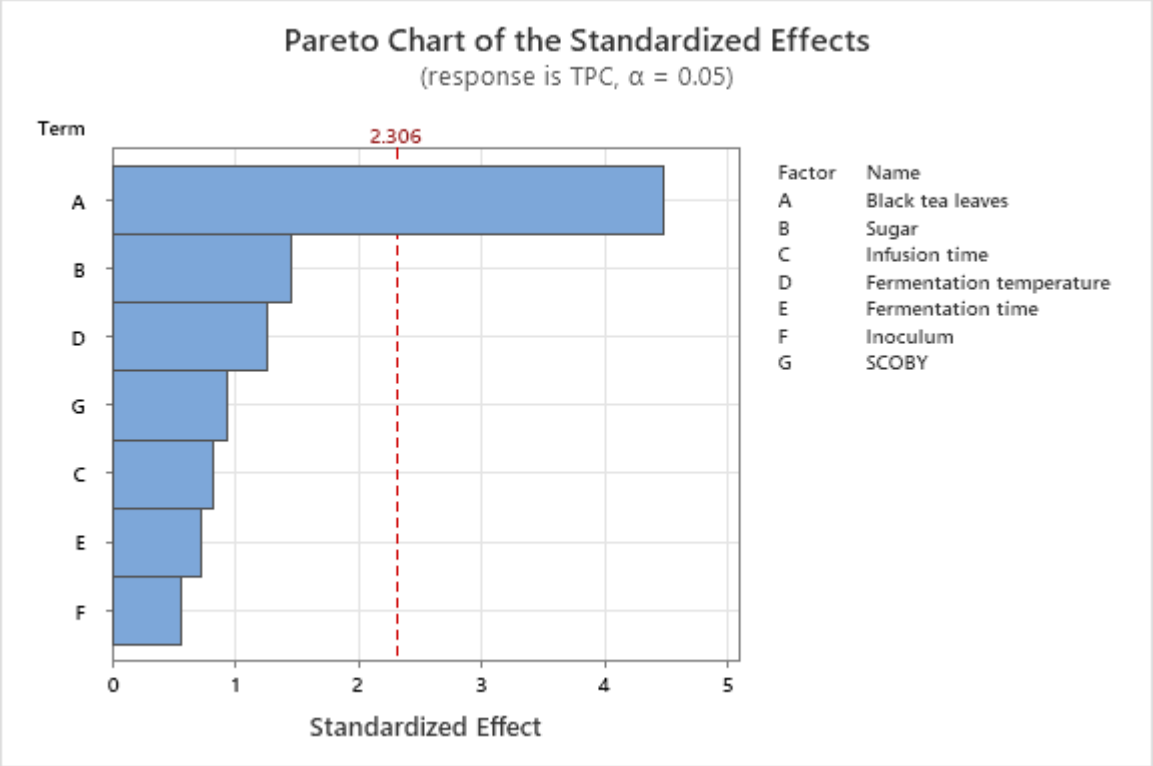

Figure A9.9 Pareto chart of the standardised effects of the total phenolic content.

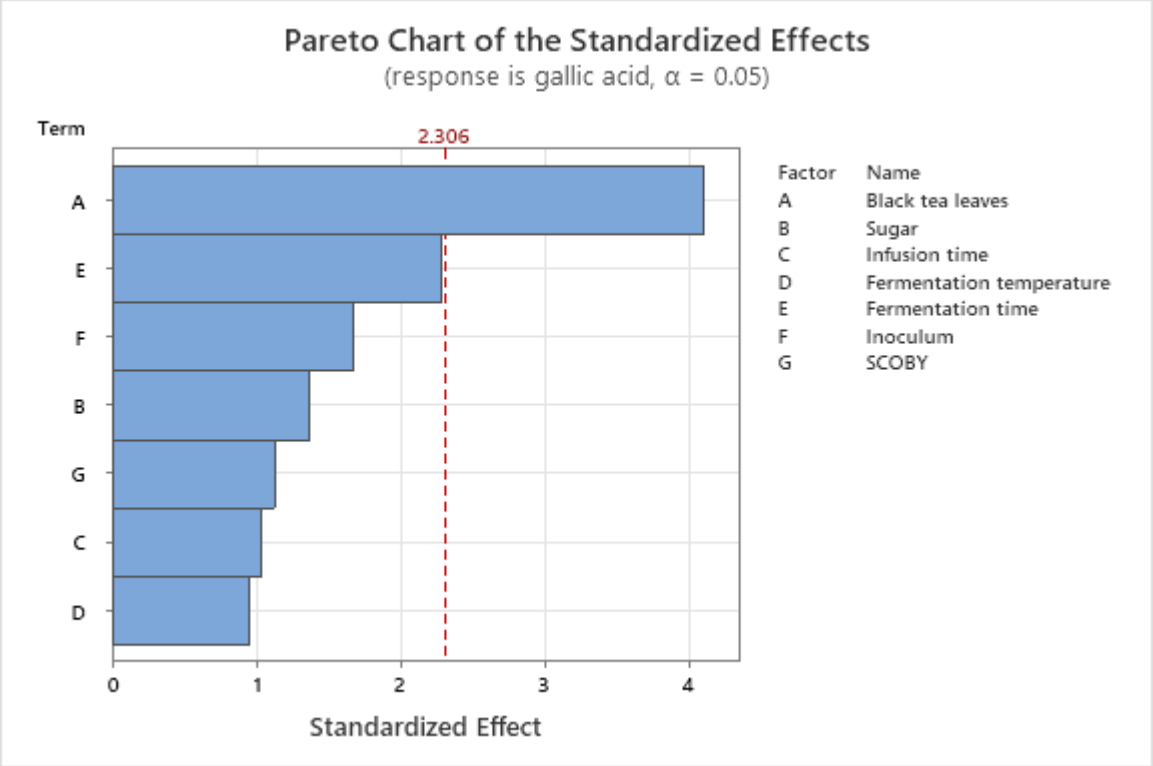

Figure A9.10 Pareto chart of the standardised effects of the gallic acid determined by HPLC.

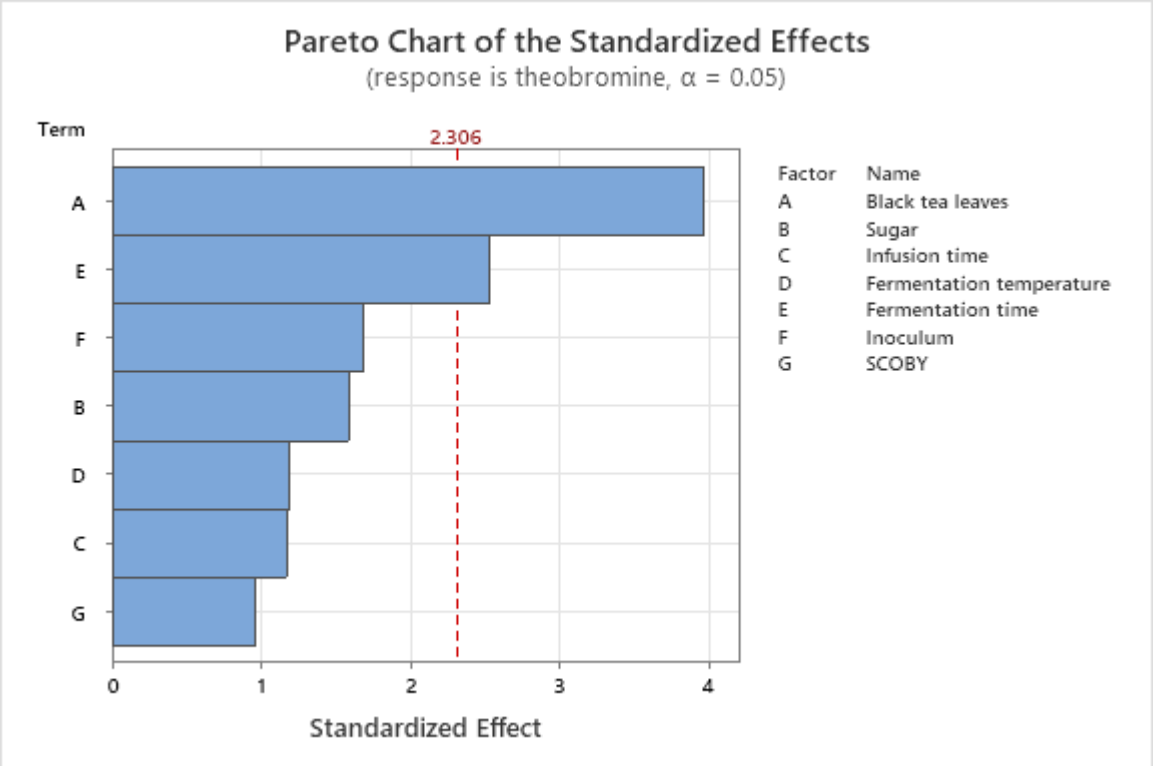

Figure A9.11 Pareto chart of the standardised effects of the theobromine determined by HPLC.

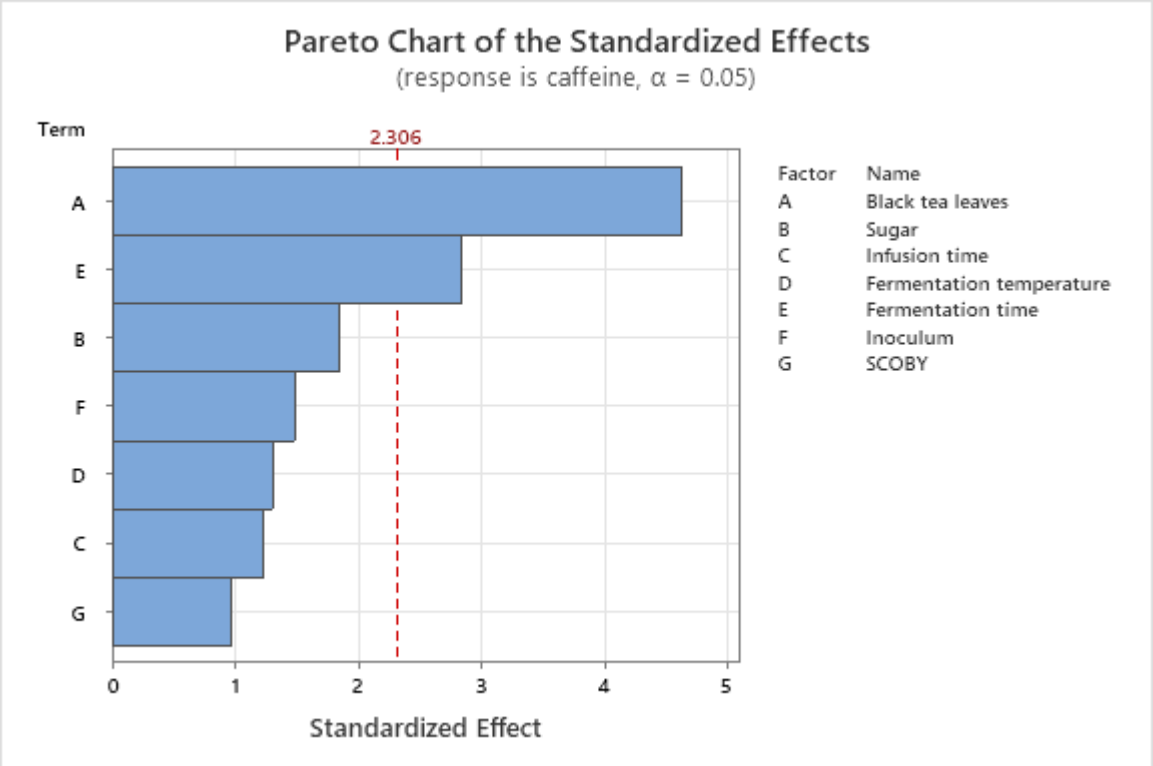

Figure A9.12 Pareto chart of the standardised effects of the caffeine determined by HPLC.