Characteristics of Dominant Acetic Acid Bacteria and Yeasts in Kombucha Sold in New Zealand

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ABSTRACT

Kombucha is a sparkling sugared tea beverage fermented with a symbiotic culture of acetic acid bacteria (AAB) and yeast. Despite the increase in the demand of the beverage due to its perceived health benefits and naturalness, its microbial composition, presumed to be probiotic is unknown at consumption. The microbial composition of the cultures are therefore important to processors and consumers. This study characterised the predominant AAB and yeasts present in Kombucha sold in New Zealand. AAB and yeasts were isolated from six Kombucha samples using the glucose yeast extract peptone mannitol (GYPM) and yeast extract glucose chloramphenicol (YGC) media, respectively. The phenotypic identification of AAB and yeast was achieved by morphological and biochemical characterisations. The biochemical analysis of AAB included the oxidation of ethanol, ketogenesis of glycerol, oxidation of lactate and acid produced from different carbohydrates. Yeasts were identified using the API 32C kit and molecular sequencing of 23S rRNA whereas the AAB were identified using the 16S rRNA sequencing. The pH of the Kombucha samples ranged between 3.21 and 3.90 and the titratable acidity (TA) varied from 0.38-0.43%. The total soluble solids (TSS) ranged from 1.87 to 7.00 ºBrix. The microorganisms were only found in domestic Kombucha samples and not in the imported samples. Mean AAB counts ranged from 4.97±0.06 to 5.63±0.02 log CFU/mL, while yeast counts ranged from 4.75±0.10 to 5.69±0.01 log CFU/mL. Dominant AAB species were identified as *Gluconobacter oxydans* and *Acetobacter musti*, and the yeasts were *Dekkera bruxellensis, Schizosaccharomyces pombes, Hanseniaspora valbyensis, Brettanomyces anamalus, Pichia kudriavzevii* and *Saccharomyces cerevisiae* in the analysed Kombucha samples sold in New Zealand. The yeast community was more complex and variable than AAB community in the analysed Kombucha samples.

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LIST OF ABBREVIATIONS AND SYMBOLS

AAB	Acetic acid bacteria
ADH	Alcohol dehydrogenase
AE	Acetic acid ethanol medium
ALDH	Aldehyde dehydrogenase
ALFP	Amplified length fragments polymorphism
ANOVA	Analysis of variance
APDA	Acidified potato dextrose agar
ATGY	Acidified tryptone glucose yeast extract agar
BME	Basal medium ethanol
BLAST	Basic local alignment search tool
CFU	Colony forming per unit
DBB	Diazonium blue B colour test
DGGE	Denaturing gradient gel electrophoresis
DHA	Dihydroxyacetone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPA	Dye-pour-plate auxanogram
DRBC	Dichloran rose Bengal chloramphenicol agar
DSL	D-saccharide acid-1,4 lactone
DSM	Dextrose sorbitol mannitol agar
ERIC-PCR	Enterobacterial repetitive intergenic consensus polymerase chain reaction
FY	Fructose yeast extract
FSANZ	Food tandards Australia New Zealand
g	grammes
g	Gravity
GY	Glucose yeast extract medium
GYAE	Glucose yeast extract acetic acid ethanol medium
GYC	Glucose yeast extract carbonate medium
GYE	Glycerol yeast extract medium
GYEC	Glucose yeast extract ethanol calcium carbonate medium
GYP	Glucose yeast extract peptone medium

GYPM	Glucose yeast extract peptone mannitol medium
HS	Hestrin-Sachramm medium
ITS	Internal transcribed spacer
KHP	Potassium hydrogen phthalate
L	Litre
LAB	Lactic acid bacteria
mL	millilitre
MEA	Malt extract agar
MYA	Malt yeast extract medium
NCBI	National centre for biotechnology information
OGY	Oxytetracycline glucose yeast extract agar
PCR	Polymerase chain reaction
PCR-EIA	Polymerase chain reaction enzyme immunoassay
PCR-RELP	Restriction fragment length polymorphism analysis of polymerase chain reaction-amplified fragments
PDA	Potato dextrose agar
PVP	Polyvinylpyrrolidone
RAE	Reinforced acetic acid ethanol medium
RAPD	Random amplification of polymorphic DNA
RBC	Rose Bengal chloramphenicol agar
REP-PCR	Repetitive extragenic palindromic polymerase chain reaction
r RNA	Ribosomal ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
SCOBY	Symbiotic culture of bacteria and yeast
SD	Standard deviation
SGA	Sabouraud glucose agar
SYP	Sorbitol yeast extract peptone medium
TA	Titratable acidity
TCA	Tricarboxylic acid
TSS	Total soluble solids
YGC	Yeast extract glucose chloramphenicol medium
YGM	Yeast extract glucose mannitol medium
YPD	Yeast extract peptone dextrose

YPE	Yeast extract peptone ethanol medium
YPM	Yeast extract peptone mannitol medium
UDPGc:	Uridine diphospho-glucose
µm	Microlitre
UYT	Uni-yeast-tek

CHAPTER 1 INTRODUCTION

1.1 Background of Kombucha

Kombucha is a popular sparkling tea beverage with a long history and the product is consumed worldwide. The beverage originated from China, then it spread to Japan, Russia, Germany and other middle east countries (Dufresne & Farnworth, 2000). The taste of Kombucha is mildly acidic and alcoholic, which is similar to apple cider (Marsh et al., 2014). The taste of Kombucha can vary from slightly fruity, sour and fizzy to vinegar-like depending on the fermentation conditions (Goh et al., 2012).

The microbial community in Kombucha is dominated by a symbiotic culture of acetic acid bacteria (AAB) and yeast, commonly called SCOBY or Tea Fungus. The composition of SCOBY is influenced by geographic location, weather, and local yeasts and bacteria species (Jayabalan et al., 2014). Some small concentration of lactic acid bacteria (LAB) species have also been isolated from Kombucha (Bogdan et al., 2018). The presence of LAB has been reported to enhance the synthesis of glucuronic acid and improve the antimicrobial activity of Kombucha (Nguyen et al., 2015). Regular consumption of Kombucha is perceived to confer health benefits to the host (Dufresne & Farnworth, 2000). Nowadays, Kombucha has been sold as a commercial beverage on scale up manufacturing. The Kombucha global market was valued more than one billion in 2016 and expected to reach two billion before 2020 according to Lumina Intelligence (2018).

1.2 Significance of Kombucha starter culture during fermentation

Kombucha is commonly fermented by a complex SCOBY starter cultures (Tea fungus) in a base of sugared tea infusion at ambient temperature for 7-10 days (Jayabalan et al., 2014). Brewed black tea is the most common ‘tea substrate’ that has been used through the history of Kombucha. Other substrates can be also used, including green tea, oolong tea, and medicinal herbs such as lemon balm or peppermint (Velicanski et al., 2013). To achieve the desired fermentation, other materials, like table sugar is also added.

Sucrose is the most popular sugar substrate that provides basic nutrients for the growth of cultures during fermentation. The concentration of sucrose added varies from 5% to 15% (w/v) (Greenwalt et al., 2000).

During fermentation, sucrose is firstly hydrolysed by yeasts into fructose and glucose, and further oxidised into carbon dioxide which is responsible for the sparkling characteristic of Kombucha (Villarreal-Soto et al., 2018). The AAB convert glucose to gluconic acid then to glucuronic acid which is associated with the hepatoprotective effect of Kombucha (Jayabalan et al., 2014). The presence of AAB also contributes to the production of acetic acid which provides the sourness of Kombucha. The production of acetic acid also stimulates the yeasts to produce more ethanol (Kumar & Joshi, 2016). The produced ethanol and acetic acid can work together to exhibit the antimicrobial effect against potential pathogenic microorganisms during fermentation. There are two components that develop during Kombucha fermentation which are the floating pellicle that rests on the top and the liquid tea broth (Chakravorty et al., 2016). The tea fungus can be recovered and used for the next fermentation. The fermented tea broth is the final product that is consumed.

After fermentation, Kombucha consists of complex components including organic acids such as acetic, gluconic, glucuronic acid, vitamin C and vitamin B group, trace elements such as Cu, Fe, and polyphenols like catechins. The composition of the beverage varies between products which is affected by several factors including the composition of starter culture, fermentation conditions and substrates added (Martinez Leal et al., 2018). The compounds are also responsible for the antioxidant, anticancer and probiotic activities of Kombucha (Watawana et al., 2016).

It is important to understand the composition of starter cultures involved in Kombucha fermentation and their metabolic activities for better control of the fermentation process. The interaction between different species may stimulate or interfere with the growth rate of other species and their characteristics could influence metabolic activities of Kombucha (Villareal-Soto et al., 2018). However, there are limited studies on the composition of the starter culture of Kombucha and their characteristics. The main challenges in understanding the SCOBY of Kombucha are the diversity and complexity

of the microbial communities (Chakravorty et al., 2016). The variation of SCOBY could be attributed to climatic and geographic conditions, localised species of AAB and yeasts or contamination between microorganisms (Mayser et al., 1995). The identified cultures and their characteristics may allow manufacturers to control the fermentation process to produce safe, high quality products. Also, there are more opportunities for the starter cultures in different areas of consumer products. In the food industry, the cellulose pellicle produced during Kombucha fermentation has been used as food thickener, dietary fibre and binder (Goh et al., 2012). Additionally, bacterial cellulose has been also considered as a potential ingredient to produce artificial skin to stimulate the healing process (Czaja et al., 2006). Dried tea fungus has been used for removing metal pollutants from wastewater because of its strong adsorbent ability (Jayabalan et al., 2014).

1.3 Aim and objectives

Aim: The overall aim of the project was to characterise the dominant AAB and yeasts present in selected Kombucha beverages sold in New Zealand.

Objectives

1. To determine the acidity of Kombucha samples by measuring pH and conducting acid-base titrations;
2. To measure the total soluble solids of Kombucha samples using the refractometer;
3. To enumerate and isolate AAB and yeasts from Kombucha samples;
4. To characterise AAB isolates from Kombucha using microbiological and biochemical tests;
5. To characterise the isolated yeasts using API 32C kit; and,
6. To conduct DNA sequencing of representative isolates of yeasts and AAB using polymerase chain reaction (PCR).

CHAPTER 2 LITERATURE REVIEW

2.1 History of Kombucha

Kombucha is a traditional fermented tea beverage that has been consumed since 220 B.C. in China during the Tsin Dynasty, for its detoxifying and energizing properties (Dufresne & Farnworth, 2000). It is a sparkling beverage with slightly sweet and acidic flavour. It tastes like apple cider and can be easily made at home or in a factory (Jayabalan et al., 2014; Steinkraus, 1995). After doctor Kombu took the tea fungus from Korea to Japan, the fermented tea was used to cure digestive diseases of the Emperor Inkyo (Dufresne & Farnworth, 2000; Jayabalan et al., 2014). Therefore, the name, 'Kombucha' origins from the founder's name "Kombu" and in Japanese, tea is called, "cha". Kombucha was exported to Russia as "Tea Kvass" and then spread to Eastern Europe around 20th century. Kombucha became very popular in Russia because of its 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 1960s, Switzerland scientists indicated that the benefits of drinking Kombucha were similar to yoghurt (Jayabalan et al., 2014). Nowadays, Kombucha is being mass produced and sold as a commercial beverage with different flavours globally.

2.2 Production of Kombucha

Kombucha is commonly fermented from sugared tea and SCOBY. To prevent contamination from pathogenic microorganisms, the production process is generally prepared under high sanitary conditions (Greenwalt et al., 2000). There are multiple choices for Kombucha substrates, which include green tea, oolong tea and black tea, as well as some medicinal herbs including lemon balm, peppermint, thymes and sage or their combination have been also used (Velicanski et al., 2013).

For Kombucha fermentation, tea leaves or tea bags are added to boiled water and infused for around 5-10 min, followed by filtration to remove tea leaves (Dufresne & Farnworth, 2000). Then approximately 50 to 150 g/L (5% to 15%, w/v) sucrose or

brown sugar is dissolved in the tea and the solution is cooled to room temperature (Hesseltine, 1965). The cooled tea is transferred to a sterile wide-mouth container, and the “mushroom” (tea fungus) is laid on the surface of solution. The vessel is covered with a sterile cloth or paper towel to prevent insects such as fruit flies and other undesirable cross-contamination affecting the fermentation. The fermentation generally continues for 7 to 10 days at room temperature (20ºC to 30ºC) (Figure 2.1). The fermentation temperature for the starter ranges from 18ºC to 26ºC (Jayabalan et al., 2014). During the fermentation, a newly formed jelly-like daughter tea fungus membrane is formed which floats on the surface of broth. The cellulosic tea fungus is removed and kept together with a small amount of tea broth for next fermentation. The daughter tea fungus can grow up to 2 cm thick and cover the surface of container (Steinkraus, 1995; Dufresne & Farnworth, 2000; Jayabalan et al., 2014; Kumar & Joshi, 2016). After fermentation, the liquid broth is filtered with a clean cloth and stored in a sealed container at 4ºC for further processing such as packaging.

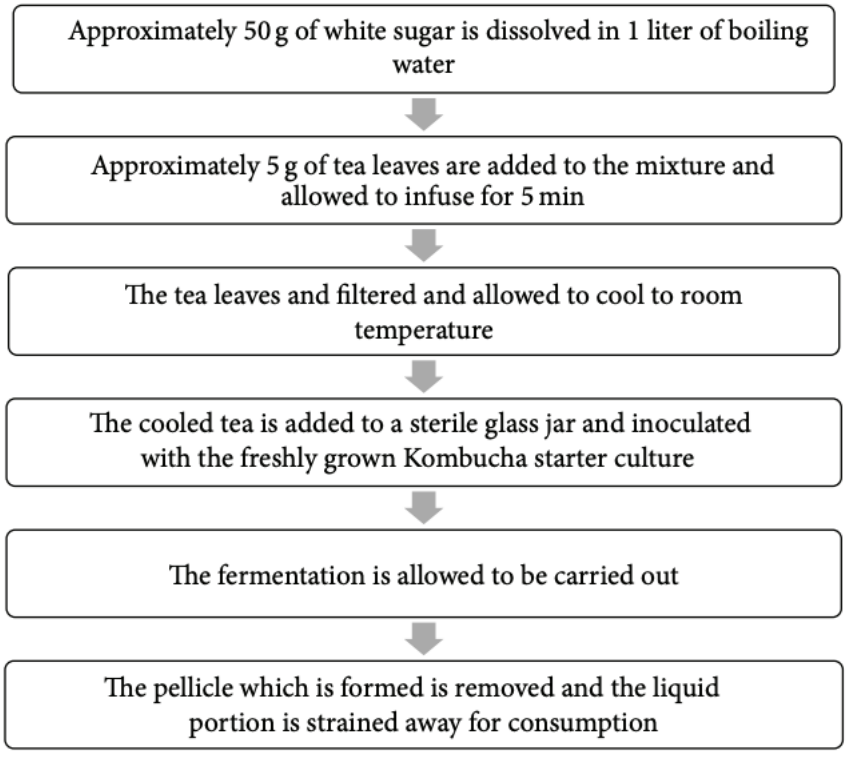


Figure 2. 1 General processing of Kombucha (Watawana et al., 2016).

2.3 Microbiological characteristics of Kombucha

The Kombucha microbial community can be classified into two parts, the floating cellulosic biofilm and the liquid broth (Chakravorty et al., 2016). The symbiotic culture of bacteria and yeast in Kombucha is called SCOBY and may vary between fermentations (Dufresne & Farnworth, 2000; Villarreal-Soto et al., 2018). The main metabolic activity of Kombucha fermentation processes are described as Figure 2.2. Sucrose is hydrolysed by yeast cells into fructose and glucose which are metabolised by yeasts to produce ethanol and carbon dioxide. Fructose is preferably utilised as substrate rather than glucose (Sievers et al., 1995). The ethanol produced is then further metabolised by AAB to produce acetic acid, thereby reducing the ethanol content in Kombucha. Glucose is transformed by AAB into gluconic acid and glucuronic acid. Yeasts provide vitamins and other nutrients to support the bacterial growth by autolysis (Villarreal-Soto et al., 2018). Purine derivatives from tea in medium such as caffeine and theophylline provide the essential nitrogen source for the metabolism of tea fungus culture. Green tea contains higher caffeine (5%) than black tea (2%) which provide more nitrogen for the tea fungus culture (Velicanski et al., 2013).

Figure 2. 2 Metabolic activity of Kombucha (Villarreal-Soto et al., 2018).

2.3.1 AAB starter culture in Kombucha

2.3.1.1 AAB in the production of fermented foods

AAB play an important role in the fermented food and beverage industry. The bacteria are widespread in the environment and can be found on sugary or acidic substances such as fruits and flowers (Montet & Ray, 2016). AAB are widely used in the production of several 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 bacteria are also used to oxidise sugars, alcohol and sugar alcohols into organic acids, aldehydes and ketones by the oxidative fermentation process (Lynch et al., 2019).

2.3.1.2 Dominant AAB present in Kombucha

AAB and gluconic acid-producing bacteria are the dominant prokaryotes in the Kombucha culture. The microbial community in Kombucha varies between fermentations and impacts on the biochemical properties of the beverage (Karabiyikli & Sengun, 2017). The bacterium in the cellulosic biofilm (tea fungus) is reported to be *Acetobacter (A.) xylinum* (Dufresne & Farnworth, 2000; Jarrell et al., 2000; Jayabalan et al., 2014; Villarreal-Soto et al., 2018). The production of biofilm in Kombucha involves the synthesis of the cellulose precursor-uridine diphospho-glucose (UDPGc) (Figure 2.3), which can be synthesised from different carbon sources such as ethanol, sucrose, and glycerol (Villarreal-Soto et al., 2018). The floating cellulose network produced by *A. xylinum* enhances the association between moulds and other bacteria on non-agitated medium. Caffeine, theophylline and theobromine in Kombucha facilitate the production of the *A. xylinum* cellulose network (Jayabalan et al., 2016; Fontana et al., 1991). The other main AAB in Kombucha are *Bacterium gluconicum* (Jayabalan et al., 2010; Reiss, 1994), *Acetobacter aceti*, *Acetobacter pasteurianus,* (Mukadam et al., 2016) and *Glucobacter oxygendans* (Liu et al., 1996). Recently, *Komagataeibacter* and *Gluconoacetobacter* species have been reported in Kombucha including *Komagaeibacter kombuchae* (Dutta & Gachhui, 2007), *Komagataeibacter saccharivorans* (Mukadam et al., 2016), and *Gluconacetobacter sacchari* (Trovatti et al., 2011). *Gluconacetobacter* sp. A4 (G.sp. A4) was the main functional bacteria isolated from Kombucha which synthesised D-saccharide acid-1,4 lactone (DSL)

(Yang et al., 2010). Nitrogen-fixing *Acetobacter nitrogenifigens* spp. and the nitrogen fixing and cell-producing *Gluconactobacter kombucahe* sp.*nov* were also isolated from Kombucha (Dutta & Gachhui, 2006).

Figure 2. 3 Cellulose synthesized by *A. xylinum* (Villarreal-Soto et al., 2006).

2.3.2 Yeasts starter cultures in Kombucha

2.3.2.1 Characteristics of yeasts

Yeasts are defined as a group of eukaryotic unicellular fungi which reproduce by budding or fission. Some yeasts can be reproduced by both budding and fission (Deak, 2007). Yeasts have been used for food and beverages fermentation for a long history because of their ability to hydrolyse different substrates to produce valuable fermented final products such as beer (Buzzini et al., 2017). Yeasts are currently classified into Ascomycetous and Basidiomycetous yeasts, according to their molecular phylogenies (Deak, 2007). The fungi are facultative anaerobes which can grow without the presence of oxygen. The presence of oxygen allows the yeasts to convert sugars to carbon dioxide and energy. Under anaerobic conditions, sugars are converted to ethanol, glycerol and carbon dioxide by the fungi (Bekatorou et al., 2006). The *Saccharomyces*

(*S.*) *sensu stricto* complex was identified as the first human-used yeasts. Meanwhile, *S. cerevisiae* is the most common food grade yeast, also known as the baker's yeasts which is widely used in bakery products, beer-brewing, and winemaking. Molasses from sugar by-products are usually used for producing the baker's yeast. Additionally, food-grade yeasts are utilised as food additives, flavouring agents and substrates for microbiological agar, as well as feedstocks (Bekatorou et al., 2006). Nowadays, some non-*Saccharomyces* species yeasts such as the genera *Candida, Debaryomuces, Kluyveromyces, Yarrowia and Zygosaccharomyces* are attracting more attention from industry as starter cultures for food and non-food products (Buzzini et al., 2017). The key function of yeasts in Kombucha is to facilitate the fermentation of the sugar to ethanol (Teoh et al., 2004).

2.3.2.2 Dominant yeasts present in Kombucha

Yeast species may vary between Kombucha products and out-number the bacteria (Matei et al., 2018). Watawana et al. (2016) reported that the genus *Zygosaccharomyces* comprise of up to 84% of yeast genera in the pellicle layer, while Martinez leal et al. (2018) found more than 95% of *Zygosaccharomyces*. Some other yeast genera have been found in Kombucha including *Candida, Hnaseniaspora, Pichia, Brettaanomyces, Sachhaaromyces, Kloeckera, Torulospora, Mycotorula, Mycoderma, Lanchancea* (Jayabalan et al., 2016). The common yeasts in kombucha and their characteristics are shown Table 2.1 and Table 2.2.

Table 2. 1 Common yeast species isolated from Kombucha and their 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 centre	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., 2016; 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. 2 Other yeasts isolated from Kombucha in different regions

Species	Country/Region	Reference
Brettanomyces (B.) lambicus	Germany	(Mayser, et al., 1995)
B. custerisii	Germany	(Mayser, et al., 1995)
B. intermedius	dn	(Jayabalan et al., 2016)
B. claussenii	dn	(Jayabalan et al., 2016)
Candida (C.) albican	Japan	Jayabalan et al., 2016
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 Arabia	(Kozaki et al., 1972)
C. obtuse	Taiwan	(Teoh et al., 2004)
C. stellata	Australia	(Teoh et al., 2004)
Kloeckera apiculata	dn	(Jayabalan et al., 2016)
Kluyceromyces africanus	dn	(Jayabalan et al., 2016)
Pichia (P.) fermentans	dn	(Kumar & Joshi, 2016)
P. membranefaciens	dn	(Jayabalan et al., 2010)
Torulaspora delbrueckii	Australia	(Teoh et al., 2004)
Torulopsis famata	Japan	(Kozaki et al., 1972)
Zygosaccharomyces Kombucahensis sp.	Russia	(Kurtzman et al., 2001)

Note: dn - Data not available

2.4 Isolation of AAB and yeasts in Kombucha

2.4.1 Isolation, enumeration and preservation of AAB

AAB can be isolated from some fermented beverages, vinegar, cider and beers (Komagata et al., 2014). The isolation and enrichment media are designed based on the metabolism and nutritional requirements of the AAB. The carbon sources are obtained mainly from D-mannitol and D-glucose. In addition, ethanol and acetic acid with different concentrations are added to the medium. The nitrogen sources are obtained from peptone and yeast extract. Some mineral salts including KH_2PO_4, Na_2PO_4 and $MgSO_4$ are added to the medium to recover the acetic acid bacteria cells (Gomes et al., 2018). Composition of culture media shown in published reports are commonly used for the isolation and cultivation of AAB (Table 2.3). The cultivation techniques include streaking, spread-plate technique, pour plate method and drop plate technique (Da Silva et al., 2018). Meanwhile, pour plating and spread plating methods are commonly used for the enumeration of microorganisms. The spread plate method is preferred as it protects the microorganisms from high temperature of molten medium and allow the exteriorization of the morphology and characteristics of colonies. However, the traditional pour plating method may be not applicable to determine the AAB population

due to the non-culturable cells (Vegas et al., 2010). The population of non-culturable AAB can be measured using real-time polymerase chain reaction (RT-PCR). 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 culture is commonly achieved by sub-culturing, storage under mineral oils, freeze-drying and cryopreservation. The sub-culturing methods involve transferring the existing colonies to a fresh medium successively and then store them at low temperature (0-4ºC) to maintain the viability of AAB cells for several weeks to few months. The low storage temperature retards the metabolic activities of the cells. However, the preservation method requires frequent refreshing as the cells may die under low temperatures and due to the production of wastes during metabolic activities. The method is also not convenient to store a large amount of cultures as it is time-consuming as well as the possibility for cross-contamination and genetic changes (De Vero et al., 2017).

Isolated cultures can be also preserved under sterile mineral oil to inhibit dehydration and reduce metabolic activities at controlled temperatures (15ºC to 18ºC). This method allows the cells to be preserved for several months to years. The reactivation of the culture is performed by streaking a portion of the colony onto appropriate agar plates.

Freeze-drying is one of the best methods for long-term storage of AAB cultures. The metabolic activities of the cells are reduced to maintain viability. The culture is readily frozen, and the moisture is removed by sublimation (Greaves, 1964). The moisture level should be controlled from 1 to 3% (w/v) for long-term preservation. The freeze-dried cells are suspended in the medium with cryoprotective agents such as polyvinylpyrrolidone (PVP), dimethyl sulfoxide (DMSO), glycerol, dextran, skimmed milk, mannitol, sucrose, inositol, trehalose and malt extract.

Cryopreservation is one of the most effective methods to preserve bacterial cultures for a long period. The frozen culture can be stored in the freezer at temperatures between -70ºC and -150ºC. The cell damage caused by ultra-low temperature can be prevented

by adding cryoprotectant such as glycerol (10-25%) and DMSO (5%). However, glycerol is not suitable for the AAB that form cellulose structure such as *Komagataeibacter xylinu*s. In these instances, DMSO is preferable as it can maintain stability and high viability without affecting the cellulose structure (Wiegand & Klemm, 2006).

Table 2. 3 Dominant isolation, cultivation and differentiation media for acetic acid bacteria

Medium	Composition	Reference
Glucose yeast extract carbonate (GYC) medium	Glucose (100g/L), yeast extract (10g/L), calcium carbonate (20g/L), bacteriological agar (15g/L)	(Sievers & Swing,2005)
Glucose yeast extract (GY) medium	Glucose (20g/L), yeast extract (10g/L), bacteriological agar (20g/L)	(Yamada & Yukphan, 2008)
Acetic acid ethanol medium (AE)	Glucose (5g/L), yeast extract (3g/L) peptone (4g/L), acetic acid (30g/L), ethanol (30 ml/L), bacteriological agar (9g/L)	(Gullo et al., 2006)
Reinforced Acetic acid ethanol (RAE) medium	Glucose (40g/L), yeast extract (10g/L), peptone (10g/L), Na_2PO_4*$2H_2O$ (3.38g/L), citric acid x H_2O, ethanol (20ml/L), acetic acid (50ml/L), bacteriological agar (20g/L)	(Mamlouk & Gullo, 2013)
Yeast extract Peptone Mannitol (YPM) medium	Yeast extract (5g/L), mannitol (25g/L), peptone (3g/L), bacteriological agar (12g/L)	(Gullo et al., 2006)
Malt yeast extract (MYA)	Malt extract (15g/L), yeast extract (5g/L), Bacteriological agar (15g/L)	(Gullo et al., 2006)
Basal medium ethanol (BME)	Yeast extract (0.5g/L), vitamin-free casamino acids (3g/L), ethanol (3ml/L), bacteriological agar (15g/L)	(Cleenwerk et al., 2007)
Carr medium	Yeast extract (30g/L), ethanol (20 ml/L), bromocresol green (0.022g/L), bacteriological agar (20g/L)	(Carr, 1968)
Glucose yeast extract acetic acid ethanol medium (GYAE)	Glucose (50g/L), yeast extract (10g/L), acetic acid (10g/L), ethanol (20 ml/L), bacteriological agar (15g/L)	(Cleenwerck et al., 2009)
Glucose yeast extract ethanol calcium carbonate (GYEC) medium	Glucose (10g/L), yeast extract (10g/L), calcium carbonate (20g/L), ethanol (30ml/L), bacteriological agar (15g/L)	(Wu et al., 2012)
Glucose yeast extract peptone (GYP) medium	Glucose (30g/L), yeast extract (5g/L), peptone (2g/L), bacteriological agar (15g/L)	(Entani, et al., 1985)
Hestrin-Schramm (HS) medium	Glucose (20g/L), yeast extract (5g/L), peptone (5g/L), Na_2HPO_4 (2.7g/L).	(Hestrin & Schramm, 1954)
Sorbitol yeast extract peptone (SYP) medium	Sorbitol (50g/L), yeast extract (5g/L), peptone (3g/L), bacteriological agar (15g/L)	Sievers & Swing, 2005)
Yeast extract glucose mannitol (YGM) medium	Glucose (20g/L), mannitol (20g/L), yeast extract (10g/L), acetic acid (5ml/L), ethanol (20ml/L)	(Ohmori et al.,1980)
Yeast extract peptone ethanol (YPE) medium	Yeast extract (10g/L), peptone (5g/L), ethanol (20ml/L), bacteriological agar (15g/L)	(Fungelsang & Edwards, 2007)

2.4.2 Isolation and enumeration of yeasts

The conventional enumeration of yeasts is carried out by spread plating as it allows microorganisms to be exposed to oxygen and avoid stress caused by hot or warm culture medium (Da Silva et al., 2018). In general, peptone water (0.1%) is widely used for dilution although the use of 40% or 50% glucose diluent with MY50G agar is recommended to determine the occurrence of xero-tolerant yeasts from high sugar intermediate water activity foods. There are also numerous commercial media available for the isolation and cultivation of yeasts from food (Table 2.4). These media provide the basic nutrients to support the growth of yeasts and inhibit the presence of bacteria and moulds. Traditional acidified media (pH 3.5-5) such as malt extract agar (MEA) and potato dextrose agar (PDA) are widely used for general isolation purposes. The pH should be adjusted by adding appropriate amounts of hydrochloride, phosphoric or tartaric acids after sterilization as the agar may be hydrolysed by acid during autoclaving (Deak, 2007; Kurtzman et al., 2011). Acidified media are recommended for yeast isolated from high-acid food such as pickles and fruit purees. Acidified media supress the growth of undesirable bacteria. Some common antibiotics such as oxytetracycline, penicillin G, chlortetracycline, chloramphenicol, gentamicin and streptomycin sulphate may be also added to the media to achieve the same goal. It is important to control the concentration of some antifungal antibiotics to suppress the growth of yeasts rather than filamentous fungi. For example, gentamicin (50 mg/L) inhibits the growth of yeasts (Bank & Board, 1987). Preservative-resistant yeasts can be detected on acidified malt extract agar with pour plating (Pit & Hocking, 1985).

Xerotolerant yeasts can cause spoilage in foods (a_w 0.65-0.85). These yeasts are commonly detected on modified basal media by increasing sugar content to lower the water activity such as malt extract yeast extract agar with 30% glucose (MY30G) (Andrew et al., 1997) and dichloran 18% glycerol agar (DG18). The yeasts cultures are commonly incubated at 25ºC for 5 days before enumeration. Higher temperature incubation (40-45ºC) is only for thermophiles and lower temperature incubation (4-15ºC) for psychrotrophic fungi (Beuchat, 1993). Longer incubation is recommended for xero-tolerant yeast.

Table 2. 4 Dominant isolation, cultivation and differentiation media for yeasts from food

Medium	Composition	Reference
Sabouraud glucose agar (SGA)	Glucose (10g/L), Peptone (10g/L)	(Deak, 2007)
Potato dextrose agar (PDA)	Glucose (20g/L), potato infusion (500ml/L), bacteriological agar (15g/L)	(Atlals, 2004)
Malt extract agar (MEA)	Malt extract (20/L), peptone (5g/L), bacteriological agar (15g/L)	(Deak, 2007)
Acidified potato dextrose agar (APDA)	Glucose (20g/L), potato infusion (500 ml/L), lactic acid solution (5ml/L), bacteriological agar (15g/L)	(Deak, 2007
Acidified Tryptone glucose yeast extract agar (ATGY)	Tryptone (5g/L)， glucose (100g/L), yeast extract (5g/L), glacial acetic acid (10ml/L), bacteriological agar (15g/L)	(Deak, 2007)
Oxytetracycline glucose yeast extract agar (OGY)	Glucose (20g/L)， agar (12g/L), yeast extract (5g/L), oxytetracyline solution (10ml/L)	(Deak, 2007)
Yeast extract glucose chloramphenicol (YGC) agar	Glucose (20g/L), agar (14.9g/L), yeast extract (5g/L), Chloramphenicol (0.1g/L)	(Deak, 2007)
Rose Bengal chloramphenicol agar (RBC)	Glucose (10g/L), Papaic digest of soybean meal (5.0g/L), KH_2PO_4 (1g/L), $MgSO_4{*}7H_2O$ (0.5g/L), Rose Bengal (0.05g/L), chloramphenicol solution (10ml/L)	(Deak, 2007)
Dichloran Rose Bengal chloramphenicol agar (DRBC)	Glucose (10g/L), peptone (5g/L), KH_2PO_4 (1g/L), $MgSO_4{*}7H_2O$ (0.5g/L), Rose Bengal (5% w/v, 0.5ml/L), chloramphenicol (0.1g/L), dichloran (0.2% w/v in ethanol, 1ml/L), bacteriological agar (15g/L)	Deak (2007)

2.5 Phenotypic identification of acetic acid and yeasts in Kombucha

2.5.1 Phenotypic identification of AAB

Traditional classification of AAB species are differentiated by cellular morphology, flagellation, physiological and biochemical properties. Morphological examination includes both direct observation as a colony grown on specific solid medium and microscopy. The colony examination involves size, form, elevation, and colour as shown in Table 2.5. The cellular morphology of bacteria includes the cell shape, size and response to Gram reaction, motility, spore-forming and cellular arrangement.

Table 2. 5 Morphology and classification of bacteria

Characteristic	Direct observation (colony)	Microscopic observation (cell)
Size	Colony size (mm)	Cell size (μm)
Shape	● Circular. ● Irregular ● lenticular or filamentous.	● Cocci: spherical or oval ● Bacilli: rod shaped cell ● Spirilla: rigid spiral forms ● Vibrio: comma shaped curved rod ● Actinomycetes: branched filamentous bacteria
Texture	● Mucoid ● Fluid or viscous ● Butyrous ● Friable	N/A
Margin	The edge of colony may be varied from: entire, undulated and filamentous	N/A
Cell arrangement	N/A	● In pairs: diplococci ● In chains: streptococci ● In groups: tetrad or sarcina ● Grape like clusters: staphylococci
Evaluation	The depth and cross-sectional appearance of a colony	N/A
Staining	N/A	Gram-positive: purple Gram-negative: pink red
Optical	● Transparent ● Opaque	N/A
Colour	● Some colonies produce typical pigment such as yellow, red and orange ● A few pigments are fluorescent under UV light	N/A

Source :(Schleifer et al., 2009; Kurtzman et al., 2011)
Note: N/A = not applicable

AAB belong to the family *Acetobacteraceae* and are classified into acetous or acidophilic groups. Currently, AAB are divided into 17 genera, however, only *Acetobacter*, *Gluconobacter*, *Gluconacetobacter* and *Komagataeibacter* are mostly used in food industry (Montet & Ray, 2016; Lynch et al., 2019). In terms of morphology and physiology, AAB are commonly Gram-negative, aerobic rods or ellipsoidal and non-spore forming cells. Their cells can be single, in pairs, or clusters. Cell sizes range from 0.4 to 1.0 μm by width and 0.8 to 4.5 μm by length (Tanasupawat et al., 2017). They are mostly catalase positive and oxidase negative. The optimum growth temperature varies from 25ºC to 30ºC, the higher temperature (>34ºC) environment can lower the growth of AAB significantly. Some thermotolerant strains can grow at temperatures up to 42ºC (Lynch et al., 2019). AAB can still grow under acidic conditions ranging from pH 3.0-4.0 and pH 5.0-6.5. Two types of membrane-bound

enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) play a role in the conversion of ethanol to acetic acid (Sievers & Swing, 2005).

The classification of AAB comprise five chemical properties which are presence of catalase, oxidation of ethanol to acetic acid, overoxidation of ethanol to water and CO_2, oxidation of lactate to CO_2 and water, ketogenesis from glycerol and hydrolyse D-glucose to different acids (Frateuer, 1950). Initially, the AAB were classified into *Acetobacter* and *Gluconobacter*. The genus of *Acetobacter* has peritrichous flagella and can oxidise acetate and lactate. In contrast, the genus of *Gluconobacter* lacks the ability to oxidise acetate and lactate, however, they can all oxidise D-glucose to 2-ketogluconate and 5-ketogluconate. The main difference between genera *Acetobacter* and *Gluconobacter* is determined by the presence of ubiquinone. Ubiquinone 9 is commonly found in the genus *Acetobacter*, while Ubiquinone-10 is present in the genus *Gluconobacter* (Yamada et al., 1968). In 1997, A new genus, *Gluconacetobacter*, was reported with partial 16 ribosomal sequencing techniques and the species containing Q10 were classified as *Gluconacetobacter* (Cleenwerck & DeVos, 2008). Another new genus, *Komagataebacter*, was introduced based on 16s rRNA gene sequencing, phenotypic properties and different morphology from *Gluconacetobacter* (Yamada et al., 2012). The 11 species from *Gluconacetobacter* were classified as *Komagataeibacter*. Compared with *Gluconacetobacter*, the genus *Komagataeibacter* was non-motile, unable to produce water-soluble brown pigment on the glucose peptone yeast extract and calcium carbonate medium. The genus *Gluconacetobacter* could produce 2,5-diketo-D-glouconate but the *Komagataeibacter* could not. All AAB can oxidise different sugars such as glucose, fructose, galactose, mannose, ribose and xylose through the cytoplasmic hexose monophosphate pathway (Mamlouk & Gullo, 2013). Furthermore, AAB are able to oxidize sugar alcohols such as glycerol and convert it to dihydroxyacetone (DHA). Additionally, the ability to produce the cellulose structure is mainly found in the genera *Gluconacetobacter* and *Komagataebacter*; these differential properties may help distinguish AAB to genus or species levels. The main biochemical characteristics of the genera AAB applied in the food industry are shown in Table 2.6.

Table 2. 6 Differential characteristics of the genera *Acetobacter, Gluconacetobacter, Gluconobacter* and *Komagataeibacter* commonly associated with food

Characteristic	*Acetobacter*	*Gluconacetobacter*	*Gluconobacter*	*Komagataeibacter*
Cell shape	Ellipsoidal to rods	Ellipsoidal to rods	Ellipsoidal to Rods	Coccoid to rods
Cell size (μm)	0.4-1.0x1.0-3.0	0.5-0.9x1.0-2.0	0.6-1.0x1.0-3.0	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
H_2S formation	+	+	+	ND
Growth on 0.35% acetic acid containing medium	+	+	+	+
Growth in the presence of 1% KNO_3	-	-	-	ND
Growth on methanol as carbon source	+/-	-	-	ND

Characteristics	*Acetobacter*	*Gluconacetobacter*	*Gluconobacter*	*komagataeibacter*
Growth on the presence of 30% D-glucose	-	+/-	+/-	ND
Growth on 1% of glucose	+	+	+	+
Ketogenesis (dihydroxyacetone) from glycerol	+	+	+	+
Acid production from				
1) Glycerol	+/-	+	+	ND
2) D-Mannitol	-	-	+	-
3) Raffinose	-	-	-	ND
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; Komagata et al., 2014)

Note: "+" 90% or more strains shown positive results； "-" 90% or more strains shown negative results; ND. : not determined; "+/-": some strains are positive or negative.

2.5.2 Phenotypic identification of yeasts

The phenotypic identification of yeasts is mainly achieved by morphological, physiological tests and rapid commercial yeast identification kit (ID 32 C) (Lin & Fung, 1987). Traditionally, it is time-consuming to conduct the physiological identification tests to species level. The morphological characteristics of yeasts are important in their identification as they may be produced by different modes shown in Table 2.7. Cellular morphological tests are usually obtained by the wet mount method through suspending the culture in saline and mixing with dyes such as India ink, lactophenol cotton blue, calcofluor white or methylene blue staining (Sangeetha & Thangadurai, 2013). Germ tube test is a rapid confirmation test for *Candida* (*C.*) *albicans*. The identical yeast colonies are inoculated in 0.5 to 1.0 ml of sterile serum (human, bovine or rabbit) and incubated between 35-37ºC for 2-3 h then examined for the presence of germ tube using the wet mount method microscopically at 40X. A positive germ tube result shows filamentous extension from a yeast cell without constriction at the original point (Lin & Fung, 1987). However, the results of the germ tube are affected by temperature and some strains of *Candida stellatolae* may show positive results which interfere with the identification of *C. albicans*.

Table 2. 7 Morphology of yeasts

Different reproduction mode of yeasts	Morphology characteristics of yeasts
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 produce a terminal conidium
Sexual reproduction in ascomycetous yeasts	Parent cell-bud conjugation Gametangial conjugation Heterothallism conjugation Conjugation between hyphae
Sexual reproduction in basdiomycetous yeasts	Budding haplophase Dikaryotic hyphal phase or self-spore forming diplophase

Source: (Boekhout & Robert, 2003)

The physiological and biochemical tests are carried out after morphology observation. Physiological and biochemical tests shown in Table 2.8 are conducted with

representative purified colonies. Additionally, commercial identification kits such as API 32C and API 20C are also used for rapid yeast identification (Deak, 2007).

Table 2. 8 Physiological and biochemical characteristics of yeasts

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)

2.5.2.1 Assimilation of carbohydrates and nitrogen

Assimilation tests of carbohydrate are the major biochemical tests used to determine the ability of yeast to utilise a specific carbohydrate as sole carbon source to identify the yeast culture to species level (Pincus et al., 2007). The auxanographic method, Wikerham broth technique and the assimilation agar slant techniques are most common three techniques applied in carbohydrate assimilation tests. Beijerinck (1889) introduced the auxanographic technique in which the yeast colonies are suspended in molten basal agar medium and the mixture is poured in a petri dish, or the yeast culture could be also inoculated by streaking on the surface of solidified agar. Then different dry carbohydrates are placed on the same agar and examined the growth of yeasts (Kurtzman et al., 2011). The solid agar method allows many sugar assimilations tests to be conducted on one plate and results are obtained in a short time (few days). A modified approach called dye-pour-plate auxanogram (DPPA) involves the inoculation of the yeast culture in a tube slant with agar containing a single carbon source and pH indicator (bromocresol purple) (Land et al., 1975). The method can be used to test different carbon substrates on one plate rather than using several tubes which reduces the incubation time from several weeks to 24-48 h. The colour change from purple to yellow after one-two days incubation is considered positive. The method is quicker, reproducible, and less interpretable than the turbidity measurement-based methods and suitable for clinical laboratories (Lin & Fung, 1987).

Yeasts can utilise different types of nitrogen sources including nitrate, nitrite, glucosamine, creatine and creatinine (Kurtzman et al., 2011). The method for nitrogen

assimilation tests is similar to the carbohydrate assimilation tests except nitrogen base is used instead of carbon. Nitrate assimilation tests are usually carried out in the Wickerham yeast carbon liquid medium containing 0.78g/L KNO_3 and 1% peptone is used as positive control (Lin & Fung, 1987). Some of the growth may interfere with the results as the growth of cells may use the soluble nitrogenous compounds from the cells and ammonium sulphate in the medium. Hence, doubtful growth should be confirmed by the nitrite test (Kurtzman et al., 2011). Sulfanilic acid and dimethyl alpha-naphthylamine can be added to the yeast culture. The red pink colour indicates a positive result of the presence of nitrite. The absence of colour changes due to growth yeast colonies may be a negative result and should be confirmed with the addition of zinc powder. The absence of colour change after zinc addition indicates that the yeasts assimilated both nitrite and nitrate completely. A modified auxanographic method was introduced to use basal medium and bromothymol blue colour indicator. The yeast culture is streaked on the plates, then 1% KNO_3 and 4x yeast carbon base were placed onto a paper disk on the agar surface. The samples are incubated at 25ºC for 1-2 days. A positive nitrate assimilation result is shown by colour change from light green to dark blue around the disk. This modified method is quicker and has higher accuracy (Ling & Fung, 1987).

2.5.2.2 Fermentation of carbohydrates

Carbohydrate fermentation test is used to determine the ability of yeast to ferment a sole carbon source and it is generally determined by observing the formation of CO_2 (Kurtzman et al., 2003). However, this method is not very reliable for slow fermenting yeast species which may not produce enough CO_2 during incubation. In this case, further confirmation tests should be conducted. Durham tubes are used for observing the production of CO_2. D-glucose, D-galactose, sucrose, maltose, lactose, trehalose and raffinose are the most common sugar substrates used to characterise yeast species by sugar fermentation. The basal medium consists of 2% sugar substrate solution (w/v), appropriate concentration of yeast extract and peptone. The presence of gas (CO_2) in Durham tubes indicates positive results (Lin & Fung, 1987).

2.5.2.3 Growth at different temperatures

Most yeasts grow optimally between 20-28ºC; however, some yeast species can grow at higher or lower temperatures (Sinclair & Stokes, 1965). For example, yeasts detected from cold regions grow better between 4-15ºC whereas *Cyniclomyces gultulatus* requires 35-37ºC to grow optimally (Leeming & Notman, 1987). Therefore, growth at different temperatures is an effective method to differentiate some yeasts. The yeasts can be inoculated onto appropriate media such the malt extract agar for several days at the test temperature (Ling & Fung, 1987).

2.5.2.4 Growth in vitamin-free medium

Wickerham (1951) introduced a test for yeasts to grow in medium containing minerals without the presence of all vitamins or individual vitamins as a diagnostic property of the cultures. The test is carried out by inoculating yeast cells in a vitamin-free medium tube at suitable conditions. To conduct the test, a loopful of cell culture from the first tube is transferred to a second vitamin-free medium tube as the growth in the first tube may be caused by the vitamin from the inoculum (Ling & Fung, 1987).

2.5.2.5 Growth in high osmotic pressure environments

Yeasts can grow under different glucose and salt concentrations which can be used for identification. The ability to grow in high sugar concentration is achieved by inoculating the yeast culture on either 50% or 60% (w/w) glucose agar media (Kurtzman et al., 2011). Wickerham (1951) also developed a sodium chloride (10%, w/w) medium containing glucose (5%) for identification purposes.

2.5.2.6 Urease test and Diazonium Blue B colour test (DBB)

Urease and DBB tests are important for differentiating basidiomycetous and ascomycetous yeasts (Deak, 2007). Urease found in yeast cells hydrolyses urea and turns it alkaline. This activity is often strongly detected in basidiomycetous yeast such as genera *Crytococcus* and *Rhodotorula*. The presence of a deep red colour after 4-20 h incubation in urease broth indicates urease activity (Kurtzman et al., 2011). However, urease activity is not often found in ascogenous species (Ling & Fung,1987). Similarly,

only the basidimycetous yeasts turn a dark red colour after reacting with DBB reagent, whereas no colour change is observed from the ascogenous species (Deak, 2007).

2.5.2.7 Starch hydrolysis test

Some yeasts produce starch-like extracellular polysaccharides and form a blue to greenish blue colour with iodine solution (Kurtzman et al., 2011). The starch hydrolysis test should be conducted under acidic (pH<5) conditions for yeast culture. However, some species from the genus *Leucosporidium* can still produce extracellular amyloids compounds and give a positive result above pH 5. The test is conducted by inoculating the yeast culture on agar or in broth containing D-glucose for about one to two weeks. Following incubation, iodine-potassium iodide mixed solution is added to the medium. The formation of a dark blue colour on medium indicates the presence of starch-like compounds (Kurtzman et al., 2011).

2.5.8 Commercial identification systems

Some commercial identification systems based on the conventional carbon fermentation or nitrogen assimilation reactions have been developed for rapid and accurate yeast identification in combination with the morphological characterisation as well as other additional required tests (Deak, 2007). The ability of yeast to grow on different carbon source and nitrogen source can be determined by turbid formation or colour change in the presence of pH indicator (Ling & Fung, 1987). Description of common commercial systems such as API 20 C are summarised in Table 2.9.

Table 2. 9 Characterization of common commercial identification system

Commercial system	Description	Incubation condition	Correctly identified	Reference
API 20 C	19 carbon assimilation test and 1 control test in 20 strips	30ºC for 72 h	98.9%	(Germain & Beauchesne,1991)
API Candida	5 carbohydrate and 7 enzyme colorimetric test in 10 strips	35ºC for 18-24 h	97.4%	(Fricker-Hidalgo, 1996)
API 32 C	● 29 assimilation tests (carbohydrate, organic acids, and amino acids); 1negative control,1 susceptibility test (cycloheximide) and 1 colorimetric tests (esculin) in 32 wells. ● Includes 63 different species in database	30ºC for 48 h	92.0%	(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	96.0%	(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., 1991)
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)

2.6 Genomic Identification of AAB and yeasts

2.6.1 Genomic identification of AAB

It is difficult to identify AAB to species levels with phenotypic characteristics only (Gomes et al., 2018). Compared with conventional biochemical and physiological identification, genomic techniques are more reliable and rapid (EI-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 (Andre-Barrao et al., 2017). Restriction fragment length polymorphism analysis of PCR-amplified fragments (PCR-RFLP) of the 16S rRNA gene, 16-23S rRNA and space region between 16S-23S rRNA genes, DNA amplification and direct sequencing of 16S rRNA gene and 16S-23S ITS, denaturing gradient gel electrophoresis (DGGE) of partial 16S rRNA gene, real-time PCR (RT-PCR) are the main methods used to determine AAB to species level (Andre-Barrao et al., 2017). For identification to strain level, random amplification of polymorphic DNA (RAPD), amplified length fragments polymorphism (ALFP), enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), repetitive extragenic palindromic PCR (REP-PCR) are the most used methods (Andre-Barrao et al., 2017). The differences and characteristics of these DNA-based techniques are shown in Table 2.10.

Table 2. 10 Common molecular techniques applied in AAB identification

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
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.

Source: (Andre-Barrao et al., 2017; Sorkhen et al., 2007)

2.6.2 Genomic identification of yeasts

Different rapid commercial kits such as API 20 C and 32 C are more convenient in yeast identification, however, there would be minor differences in biochemical profiles due to variabilities in test conditions. The commercial kits also have to correlate to morphological observations to identify the yeasts to species level. For instance, the species from *Dekkera* are the anamorphs of *Brettanomyces* and they are deficient in sexual characteristics, hence, the biochemical profiles of these yeasts with limited morphological features are not stable and therefore difficult to differentiate (Kurtzman,1998). The PCR and sequencing of genes are more accurate, reliable, and reproducible than phenotypic tests (Latouche et al., 1997). The ribosomal deoxyribonucleic acid (rDNA) is the most common target in yeast identification. The rDNA regions include 18S, 5.8S, 26S subunits separated by internal transcribed spacers (ITS) called ITSI and ITS2. Several universal primers are available for these ribosomal rDNA regions. The large subunit of rDNA D1 or D2 regions are also chosen for genomic sequencing of clinical yeasts (Pincus et al., 2007). PCR-enzyme immunoassay (PCR-EIA) method uses amplified rDNA by PCR to detect the yeasts with labelled probes. Real-time PCR methods are widely used in detecting the fungal pathogens such as the genus *Candida* as the test takes less than one hour and is more sensitive than traditional PCR techniques (Mackay, 2004). Other PCR-based techniques such as AFLP, randomly amplified polymorphic DNA (RAPD), and repetitive-sequence-based PCR (rep-PCR) are also effective in the rapid identification of yeasts (Pincus et al., 2007).

2.7 Conclusion

Kombucha is a refreshing ‘live’ fermented beverage and its popularity is partially derived from this characteristic. This review has shown that there is insufficient published information on the dominant yeasts and AAB in the commercial Kombucha beverage at point of sale as well as during fermentation. Therefore, there is need to analyse the composition of the dominant symbiotic starter culture of the two main types of microorganisms in the fermented beverage. Also, this review has shown that currently, there is hardly any information on the availability of commercial media for

the isolation of the AAB. The phenotypic identification of AAB and yeast can be achieved by biochemical tests and commercial kit like the API 32C, respectively. However, previous studies have shown that genomic techniques such as PCR provide more accurate, rapid and reliable identification of the microbiological composition of the AAB and yeasts.

CHAPTER 3 MATERIALS AND METHODS

3.1 Sampling

The main objective of this study was to identify the dominant AAB and yeasts in selected commercial Kombucha sold in New Zealand. The acidity and total soluble solids were also measured in the commercial products. Post-fermentation, residual sugar may be retained in Kombucha which may be fermented during storage if the conditions are favourable. Therefore, the survival of the dominant AAB and yeast are affected by sugar concentration and storage conditions. Six Kombucha branded beverages (one case of four bottles of each brand) were randomly purchased from the local retail supermarkets in Auckland, New Zealand and then coded as shown in Table 3.1. The samples had been stored under chilled conditions (≈7° C) in the retail shop before purchasing. Following purchasing, the samples were transported (≈4 °C) to the Food Microbiology Laboratory at Massey University, Auckland and kept chilled (≈4° C) until required for further analysis. Based on the food labelling information on the bottled products, three branded samples were produced in New Zealand and the remaining three were manufactured in Australia and the USA. The flavours of the Kombucha products were randomly selected for each brand.

Table 3. 1 Description of the commercial Kombucha samples

*Sample codes	Flavour	Country of Origin
DO	Chai spices and ginger	New Zealand
GB	Lemon and ginger	New Zealand
LO	Feijoa	New Zealand
AM	Peach and mango	Australia
RE	Original	Australia
WO	Original	USA

*Note: Designated for the study.

3.2 Description of the experiments

The study was conducted in three phases. The physico-chemical characteristics of Kombucha samples were determined in phase I. The AAB and yeast cultures present in commercial Kombucha samples were isolated/enumerated and purified in Phase II. There are abundant growth media available for the isolation and cultivation of AAB

isolates, however, there is limited commercial media available now (Gomes et al., 2018). All the media used in this study were prepared or modified based on the previous studies. All the experiments in phase I and phase II were done in triplicate except the enumeration of AAB and yeast which were duplicated. In phase III, the isolates were characterised using biochemical tests and API 32C kits. Further tests were conducted using PCR and genomic sequencing to identify the isolates. Chemicals and reagents used in this study were of reagent grade or higher. In the characterisation experiments, freshly grown young cultures were used.

Phase I: Physico-chemical characteristics of Kombucha samples

During fermentation of Kombucha, organic acids and other organic compounds are produced through the metabolism of sugars thereby reducing the concentration of total soluble solids (Zubaidah et al., 2019). The produced organic acids are responsible for the increase in acidity. Therefore, acidity and total soluble solids of Kombucha samples were determined in this phase.

3.3 Determination of acidity and total soluble solids

3.3.1 pH

pH was determined using the method of Nielsen (2017). The Sartorius glass electrode pH meter (Model PH-11, Germany) was used to measure the pH of the Kombucha samples at ambient temperature (~23°C). The pH meter was calibrated using pH 4.0 and pH 7.0 standard buffer solutions before measurement. To measure pH, about 50 mL of each sample were transferred to a clean beaker and the glass electrode was immersed in the solution. The pH reading was recorded after it had been stabilised.

3.3.2 Titratable acidity of Kombucha

Titratable acidity was determined using the acid-base titration (Nielsen, 2017). The titration commenced with the standardisation of sodium hydroxide (NaOH) solution using potassium hydrogen phthalate (KHP) (Sigma-Aldrich, New Zealand).

3.3.2.1 Standardisation of aqueous sodium hydroxide

The standardisation of sodium hydroxide solution was achieved using the method described by Nielsen (2017). About 0.1M NaOH was standardised by titration against potassium hydrogen phthalate (KHP). The KHP was prepared by drying at 120ºC for about 2 h and then kept in a desiccator until required for use. One (1.0000) gramme (g) dried KHP was dissolved in 50 mL deionized water. About 1-3 drops of phenolphthalein solution indicator were added into the KHP solution and mixed completely. Then, aqueous sodium hydroxide solution (0.1M NaOH) was titrated against the KHP to the first persistent pink colour. The volume of NaOH was recorded and the titrations were repeated until the readings were consistent. The concentration (molarity) of standardised NaOH solution was calculated using Equation 3.1.

$$M_{NaOH} = \frac{w_{KHP}}{MW_{KHP} \times V_{NaOH}} \quad \text{.....Equation 3.1}$$

M_{NaOH} = normality of NaOH (M)
W_{KHP} = weight of KHP (g)
MW_{KHP} = molecular weight of KHP (204.228 g/mol)
V_{NaOH} = volume of NaOH (mL)

3.3.2.2 Analysis of titratable acidity

The determination of the TA of the Kombucha samples was slightly modified from Waisundara (2018). Thirty (30) mL of each Kombucha sample were measured into a 50-mL glass measuring cylinder and transferred into a clean beaker. Carbon dioxide in air was allowed to escape from the Kombucha solution for 3-5 h under chilled condition (4ºC) (Essawet et al., 2015). Aliquots of Kombucha samples (30 mL) were withdrawn for titration against the standardised sodium hydroxide to pH 8.2 measured by the Sartorius glass electrode (Waisundara, 2018; Nieslen, 2017). The TA was expressed as % of acetic acid g per g of sample as the acetic acid is the major organic acid in Kombucha (Equation 3.2) (Essawet et al., 2015; Greenwalt et al., 1998).

% Titratable acidity of acetic acid = $\frac{V_{NaOH} * M_{NaOH} * 6.0053}{V_{sample}}$ …….. Equation 3.2

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

3.3.3 Determination of total soluble solids

The TSS of Kombucha samples were determined according to Amarasinghe et al. (2018). A refractometer (Atago, pr-32 alpha, UK) was calibrated using distilled water following the supplier's instructions. The Kombucha sample was mixed by swirling in the original bottle, then 1-2 drops of sample were transferred onto the prism of the instrument. The prism should be completely covered by the specimen before recording the reading. The ºBrix reading () was allowed to stabilise before recording. The prism was rinsed with distilled water and dried with lens tissue between each measurement. The TSS were expressed as grammes of sugar in 100 g of the aqueous test sample (ºBrix).

Phase II: Enumeration, isolation and purification of AAB and yeasts in Kombucha

Kombucha is fermented by a complex symbiotic culture of AAB and yeast commonly called SCOBY (Kozyrovska et al., 2012). Despite the popularity of the beverage worldwide, very little is unknown about the constituent fermenting microorganisms in the SCOBY, which are key to the production of consistent, high quality products (Jayabalan et al., 2014). Kombucha cultures are perceived to be probiotic and knowledge of their presence in the beverage at the time of consumption is important to health-conscientious consumers (Kozyrovska et al., 2012). The information is also important to the producers for better control of their processes (Sinir et al., 2019). Therefore, the isolation of AAB and yeasts from kombucha was carried out by plating serial-diluted samples on appropriate selective media. The cell morphology of representative colonies from AAB and yeasts were stained and then examined under the oil-immersion of the microscope (Model HBO 50/AC, Germany). The isolates were divided into different groups based on their morphology and then purified on appropriate agar for further characterisation tests (Figure 3.1) (Mukadam et al., 2016).

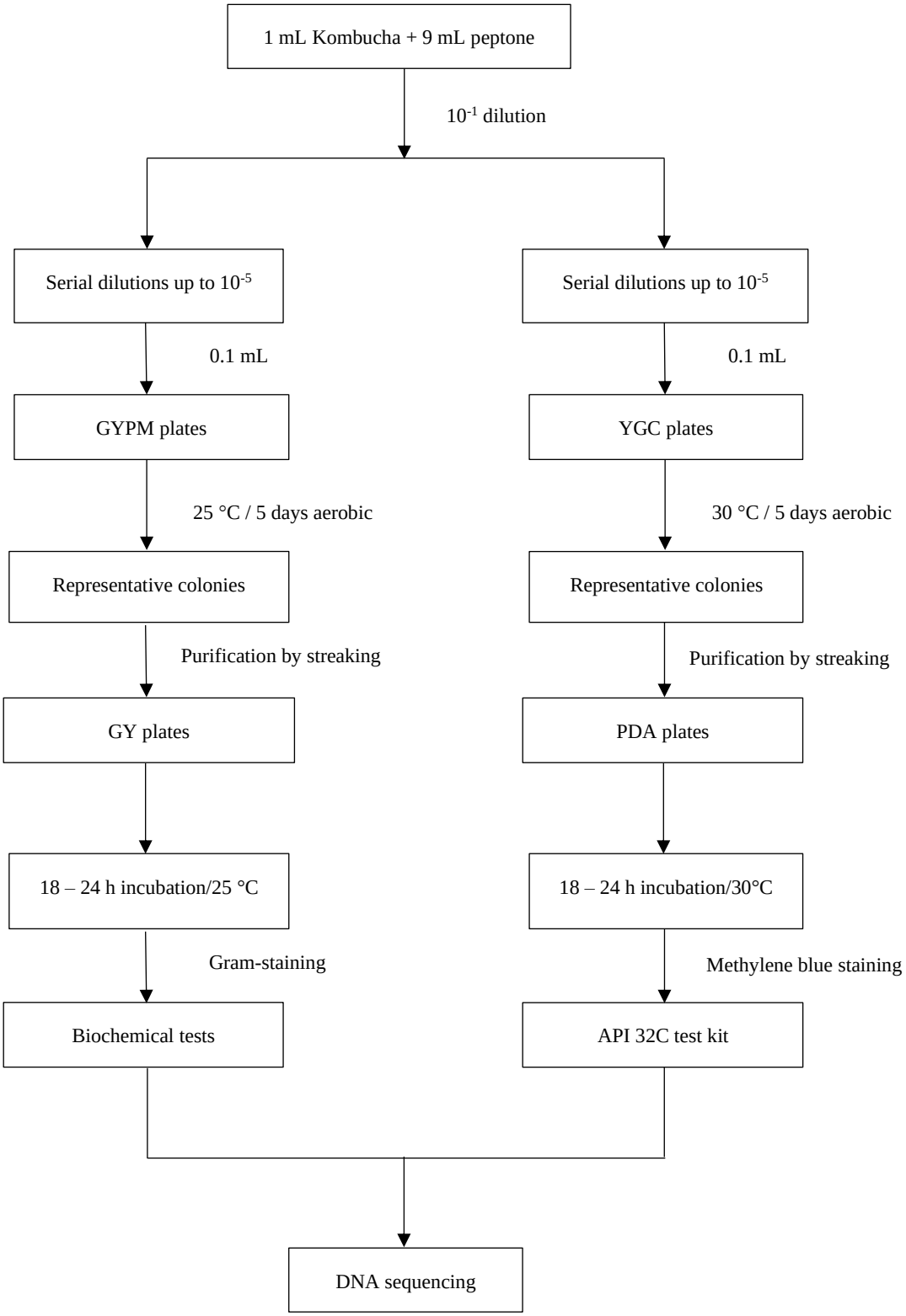


Figure 3. 1 Microbiological analysis of AAB and yeasts in Kombucha

3.4 Isolation of AAB and yeasts in Kombucha

Isolation of AAB in Kombucha samples was performed on modified Glucose Yeast Extract Peptone Mannitol (GYPM) agar containing 20 g/L D-glucose (ThermoFisher, New Zealand), 25g/L D-mannitol (ThermoFisher, New Zealand), 5g/L yeast extract (Sigma-Aldrich, New Zealand), 3g/L peptone (Sigma-Aldrich, New Zealand), 12 g/L bacteriological agar (ThermoFisher, New Zealand) according to the method by Gomes et al. (2018). The GYPM agar was prepared in the laboratory by mixing the ingredients according to the formulation and then dissolved in 1 L distilled water. The agar suspension was autoclaved at 121ºC for 15 min. Once the sterile medium was cooled to about 50ºC, 0.1g/L cycloheximide (Sigma-Aldrich, New Zealand) and 1mL/L pimaricin (Sigma-Aldrich, New Zealand) were filtered using 0.22 μm filter (type, Pall Corporation, UK) and added to the medium to inhibit the growth of yeast and lactic acid bacteria, respectively. Isolation of yeast was performed on Yeast extract Glucose Chloramphenicol (YGC) agar (ThermoFisher, New Zealand). The YGC agar was prepared following the manufacturer's instruction. The molten agar was poured into sterile Petri dishes and swirled to cool for solidification. The plates were stored at 4ºC for preservation. Before streaking, the plates were placed in an incubator at 37 ºC for 10-15 min to remove moisture.

Kombucha samples for plating were prepared as shown in Figure 3.1. The samples were mixed thoroughly with a vortex mixer (VM-10, WiseMix®, Germany) for 10 sec. After incubation, five well-developed representative isolated colonies from each sample on GYPM and YGC plates were streaked on Glucose yeast extract agar (GY) which contained 50 g/L D-glucose, 10 g/L yeast extract and 15g/L bacteriological agar and potato dextrose agar (PDA), respectively. The GY agar plates were prepared following the same procedure as described for the GYPM. The PDA was prepared following the manufacturer's instruction (ThermoFisher, New Zealand). The morphology of the colonies such as pigment production and colony size were recorded.

3.5 Selection and purification of AAB and yeasts

3.5.1 Morphology of AAB

The microscopic examination of AAB was conducted by Gram-staining and examination under oil-immersion of the microscope (Claus, 1992; El-Salam, 2012). Five representative pure colonies of each sample isolated on GYPM agar were Gram-stained. To Gram stain, one drop of distilled water was added to a clean slide and a young bacterial colony (18-24 h) was transferred by a sterile loop to the water droplet. The colony and the water droplet were mixed to produce a smear on the slide. Then the bacterial smear was dried in an incubator at 35 °C and then heat-fixed for 3-5 sec. The smear was flushed with crystal violet for 1 min, washed under running potable water and the excess water was removed using a soft tissue. The smear was flooded with Gram's iodine for 1 min and rinsed with running water. About 3-4 drops of ethanol were added to the slide and rinsed with running water. Safranin was added to the slide and left for 30 sec, then gently rinsed off with running water followed by air-drying. The Gram-stained cell isolates were examined under oil immersion (x1000) with a Carl Zeiss Transmission light microscope (Model HBO 50/AC, Germany). The cell sizes were measured with the AxioVision microscope software version 4.8.1 and cell shapes were also recorded. The representative Gram-negative cells with distinct cell morphology were presumed to be AAB (Yamada, 2008; Sievers & Swing, 2005). Cells of AAB are commonly Gram-negative, ellipsoidal to rod-shaped when examined under the microscope.

3.5.2 Morphology of yeasts

The cell morphology of yeast cells was examined using the methylene blue staining method (Painting & Kirsop, 1990; Matthews, 1914). Five representative pure colonies of each sample isolated from YGC agar were stained with methylene blue. The methylene blue stain was prepared by dissolving 0.01 g methylene blue powder (Sigma-Aldrich, New Zealand) in 10 mL distilled water and 2 g sodium citrate dihydrate (Sigma-Aldrich, New Zealand) were added. The solution was mixed well, and distilled water was added to the solution to make to 100-mL in a glass measuring cylinder. A drop of methylene blue was transferred onto a clean microscope slide and a young yeast colony (18-24 h) was transferred by a sterile loop to the methylene solution. The

coverslip was placed gently on the slide to avoid trapping air bubbles. Then the stained culture was examined x1000 under oil immersion with a Carl Zeiss transmission light microscope (Model HBO 50/AC, Germany). The cell sizes were measured using the AxioVision microscope software version 4.8.1 and cell shape was also recorded. The representative cells with distinct cell morphology (such as the formation of budding and fission) were chosen for further purification (Kurtzman et al., 2011).

3.6 Purification of AAB and yeast isolates

After microscopic observation, distinct AAB and yeast isolates were purified by successive streaking on GY agar and PDA agar, respectively. The AAB and yeast cultures were stored in 67% glycerol (w/w) at -80ºC for longer preservation and, also on GY agar or PDA agar at 4ºC with monthly sub-culturing until required for further studies (Du Toit & Lambrecht, 2002).

PHASE III: Phenotypic and molecular characterisation of AAB and yeasts

The purified AAB isolates from GY plates were characterised using biochemical tests described in section 3.7 and genotype analysis with 16S rRNA sequencing. Purified yeast isolates from PDA plates were subjected to metabolic tests using the API 32C kit (bioMerieux, France) described in section 3.8 and genotype analysis using 26S rRNA sequencing.

3.7 Phenotypic characteristics of AAB

The characterisation of AAB was achieved by conducting the following tests (i) catalase test, (ii) oxidation of lactate and acetate, (iii) ketogenesis of glycerol to dihydroxyacetone (DHA), (iv) oxidation of ethanol to acetic acid and, over-oxidation to carbon dioxide and water, (v) formation of water-soluble brownish pigment, (vii) acid production from different sugars, sugar alcohols and alcohol substrates (Gomes et al, 2018; Yamada, 2014). AAB are commonly Gram-negative, catalase-positive and oxidase negative. Their cells are ellipsoidal to rod-shaped and arranged singly, in pairs or chains. The *Acetobacter* prefer to grow at mesophilic temperature with optimum

growth of 25-30ºC (Gullo et al., 2018; Mamlouk & Gullo, 2013; Sievers & Swings, 2005). Based on these biochemical characteristics, the following tests were carried out to classify the AAB to genus or species level (Gomes et al., 2018). All the tests in section 3.7 were conducted with a loopful of purified AAB young cultures (18-24 h) and were done in triplicate.

3.7.1 Catalase test

Catalase catalyses the hydrolysis of H_2O_2 into water and oxygen which can be detected by rapid formation of air bubbles (Equation 3.3). Catalase test was carried out according to the slide-drop method of Reiner (2010). The mechanism of the test differentiates between microorganisms that can produce the catalase enzyme to neutralize the oxidative damage of hydrogen peroxide.

$$2H_2O_2 + \text{Catalase} \rightarrow 2H_2O + O_2 \quad \text{..... Equation 3.3}$$

A few drops of 6% H_2O_2 solution (v/v) (Sigma-Aldrich, New Zealand) were added onto a sterile microscope slide. The young purified colonies from each sample were transferred by a sterile inoculating loop to the 6% H_2O_2 drops on the slide. The formation of air bubble was recorded as positive for the catalase test. The results were examined over a dark background.

3.7.2 Oxidase test

Oxidase test was carried out according to the slide-drop method described by Shields and Cathcart (2010). The analysis determines the bacterial respiration involves the electron transport chain and may utilise the cytochrome oxidase to catalyse the oxidation of cytochrome c and to produce water. Tetra-methyl-p-phenylenediamine dihydrochloride is used as an artificial electron donor in the oxidase test. The reagent changes from colourless to dark blue when it is oxidised by cytochrome c. The young purified colonies were transferred by sterile loops onto oxidase strips (Oxoid, UK) and

observed for colour change. The appearance of a blue/purple colour within 30 sec indicated a positive reaction.

3.7.3 Growth of presumptive AAB isolates at different temperatures

AAB are commonly mesophilic and the optimum growth temperature ranges from 25 to 30ºC with some species able to grow at 37ºC (Komagata et al., 2014). The young purified colonies were streaked on solidified GY agar plates and incubated aerobically at selected temperatures (25ºC, 30ºC, 37ºC) for 3-7 days. The growth of colonies indicated a positive result.

3.7.4 Growth of presumptive AAB on different media

3.7.4.1 Growth of presumptive AAB on Glucose Yeast extract calcium Carbonate (GYC) agar

The GYC medium is one of the most common media used to isolate the AAB (Mukadam et al., 2016; Mamlouk & Gullo, 2013). Growth on GYC (50 g/L D-glucose, 10 g/L yeast extract, 20 g/L calcium carbonate,15 g/L bacteriological agar was conducted with slight modifications of the method of Yamada et al. (1976). All the ingredients were dissolved in 1000 mL distilled water. The agar was sterilised at 110ºC for 10 min. The molten agar was poured onto Petri dishes and mixed well as calcium carbonate does not dissolve easily. The purpose of adding calcium carbonate in the medium was to neutralise the acids produced by the AAB. The appearance of a transparent region surrounded by colonies was considered positive (De Vero et al., 2017). The young purified colonies were streaked on the GYC agar plates and incubated at 30ºC for 5-7 days under aerobic conditions.

3.7.4.2 Growth of presumptive AAB on 0.35% (w/v) acetic acid medium (pH 3.5)

The ability to grow on 0.35% (w/v) acetic acid containing medium has been used as a criterion to distinguish AAB since not all the genera from AAB can grow on the medium such as the genus *Asai* (Lynch et al., 2019). The 0.35% (w/v) acetic acid medium (pH 3.5) contained 3.5 ml/L glacial acetic acid (Sigma-Aldrich, New Zealand), 10 g/L yeast extract and 10 g/L D-glucose. To prepare the medium, all the solid ingredients were dissolved in 976.5 mL distilled water. Then 3.5 mL glacial acetic acid

was added dropwise into the solution under the fume hood and mixed completely. Ten (10) mL of the medium were dispensed into test tubes using a dispenser. The medium was autoclaved at 121ºC for 15 min, after which it was adjusted to pH 3.5 with 1 M hydrochloric acid. The young purified colonies were inoculated in the 0.35% (w/v) acetic acid containing medium. The cultured broth tubes were incubated at 30ºC for 2-5 days. The growth of bacteria was confirmed by the appearance of turbidity.

3.7.4.3 Growth of presumptive AAB on 30% (w/v) D-glucose medium

The D-glucose agar was used to determine the ability of the isolates to grow under high osmotic pressure environment (Lynch et al., 2019). The medium contained 300 g D-glucose, 10 g yeast extract and 15 g bacteriological agar in 1 L distilled water. The ingredients were dissolved in 1 L distilled water and then sterilised at 110ºC for 10 min. The young purified colonies were streaked on the D-glucose agar plates and incubated at 30ºC for 5-7 days. The growth of colonies on the D-glucose medium indicated positive results.

3.7.4.4 Growth of presumptive AAB on methanol medium

The growth of presumptive AAB on methanol medium of AAB was conducted described by Hanmoungjai et al. (2007) with slight modifications. The methanol agar consisted of 8 mL methanol (Sigma-Aldrich, New Zealand), 5 g yeast extract, and 15 g bacteriological agar. All the ingredients were mixed in 1L distilled water and sterilised at 121ºC for 15 min. The young purified colonies were streaked on methanol-containing agar plates and incubated at 30ºC for 5-7 days. The growth of colonies indicated positive results.

3.7.4.5 Growth of presumptive AAB on dextrose sorbitol mannitol (DSM) agar

DSM agar was used as a primary selective agar to isolate and differentiate the genera *Gluconobacter* and *Acetobacter* based on the method of Cirigliano (1982). The agar consisted of the following ingredients: 10 g peptone (ThermoFisher, New Zealand), 3 g yeast extract, 15 g calcium lactate (Sigma-Aldrich, New Zealand), 1 g D-sorbitol (ThermoFisher, New Zealand), 2 g D-mannitol, 1 g monopotassium phosphate (Sigma-Aldrich), 0.02 g manganese sulphate monohydrate (Sigma-Aldrich, New Zealand) and

15 g bacteriological agar in 1 L distilled water. Bromocresol purple (0.03 g) and 0.0295 g brilliant green (Sigma-Aldrich, New Zealand) was added to the medium as colour indicator. Calcium lactate was dissolved with mild heating on magnetic hot plate stirrer (Torrey Pines Scientific, USA) before sterilisation to avoid cloud formation of the medium. Then the medium was autoclaved at 121ºC for 15 min. Cycloheximide (0.004 g) was added to inhibit the growth of yeast. The cycloheximide was filtered with 0.22 μm filters before addition to the medium. The pH of the medium was adjusted to 4.2 - 4.4 with 1 M hydrochloric acid. The young purified colonies were streaked on DSM agar plates and incubated at 30ºC for 5-7 days. The growth of colonies and colour change on DSM agar were examined.

The growth of *Acetobacter* colonies changes the colour of the medium from green to purple, with the production of white precipitate in the early incubation periods. After prolonged incubation, a transparent zone around colonies develops. Meanwhile, the growth of *Gluconobacter* maintains the green colour of the medium during incubation (Cirigliano, 1982).

3.7.4.6 Growth of presumptive AAB on glutamate agar

The growth of AAB colonies on glutamate medium was conducted with the method of Asai et al. (1964). The glutamate agar contained 10 g glucose, 5 g sodium glutamate (Sigma-Aldrich, New Zealand), 1g KH_2PO_4 (Sigma-Aldrich, New Zealand), 0.2 g $MgSO_4 \cdot 7H_2O$ (Sigma-Aldrich, New Zealand), 0.1 g KCl (Sigma-Aldrich, New Zealand), and 20 g bacteriological agar prepared in 1 L mL distilled water. All the ingredients were mixed well and autoclaved at 121ºC for 15 min. The young purified colonies were streaked on glutamate agar plates and incubated at 30ºC for 7-10 days. The growth of colonies on glutamate agar indicated a positive result.

3.7.5 Oxidation of ethanol and acetic acid

The oxidation of ethanol to acetic acid and, over-oxidation of alcohol to CO_2 and water by AAB were determined using Carr medium (Gomes et al., 2018). To prepare Carr's medium, 30 g yeast extract, 0.022 g bromocresol purple (Sigma-Aldrich, New Zealand) and 20 g bacteriological agar were dissolved in 980 mL distilled water and sterilised at

121ºC for 15 min then allowed to cool. Absolute ethanol (Sigma-Aldrich, New Zealand) was added to the medium and mixed well to avoid loss of ethanol by evaporation.

The young purified colonies were streaked on Carr medium and incubated at 30ºC for 5-7 days. The growth of colonies and any colour change of medium were recorded as positive. The oxidation of ethanol to acetic acid by AAB reaction is shown in Equation 3.4. Some AAB genera can oxidize ethanol to acetic acid, which increases the acidity (reduces pH) of the medium, thereby changing the colour of the medium from purple to yellow (Gomes et al., 2018). The bacterial isolates that could further oxidise the ethanol to CO_2 and H_2O would change the colour of the medium from yellow to purple during the extended incubation period (Song et al., 2002). In contrast, isolates that cannot oxidise acetic acid to CO_2 and water would maintain the yellow colour after extended incubation.

$$CH_3CH_2OH \rightarrow CH_3CHO + 2H \rightarrow CH_3COOH + 2H \quad \text{……Equation 3.4}$$

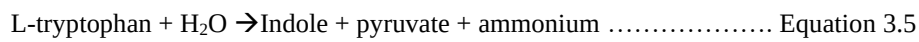
3.7.6 Alcoholic tolerance test

The AAB can grow at different ethanol concentrations which are used to differentiate AAB isolates (Gullo et al., 2005). The alcoholic tolerance test was slightly modified from Klawpiyapamornkun et al. (2015) and Gullo et al. (2005). This test was carried out to determine the ability of the bacterial isolates to grow at different ethanol concentrations. The basal medium was prepared by mixing 5 g yeast extract and 20 g bacteriological agar in 1 L distilled water. The medium was autoclaved at 121ºC for 15 min and then cooled before the additions of different levels of ethanol. Absolute Ethanol (ThermoFisher, New Zealand) concentrations of 2%, 4%, 6%, 8% and 10% (v/v) were added to the basal medium after sterilisation to avoid evaporation of the alcohol. The young purified colonies were streaked on different ethanol agar plates and incubated at 30ºC for 5-7 days. The growth of colonies on plates indicated the tolerance of the isolates to ethanol at the respective concentration.

3.7.7 Production of H_2S, indole and motility tests

AAB can be motile or non-motile; if motile, the flagella are peritrichous or polar depending on the genus. Most species are not able to produce H_2S and indole (Sievers & Swings, 2005). The SIM (sulphide indole motility) medium was used to test the production of H_2S, indole and cell motility, which was slightly modified from Visser et al. (1985). Briefly, 20 g tryptone (Sigma-Aldrich, New Zealand), 6.1 g meat extract (ThermoFisher, New Zealand), 0.2 g ferrous ammonium sulphate (Sigma-Aldrich, New Zealand), 0.2 g sodium thiosulfate (Sigma-Aldrich, New Zealand) and 3.5 g bacteriological agar were mixed in 1 L distilled water and heated to boiling to dissolve. About 10 mL of the medium were dispensed into each test tube and autoclaved at 121ºC for 15 min.

The ferrous ammonium sulphate and sodium thiosulfate in the medium were used as indicators for the formation of H_2S. Sodium thiosulfate functions as a substrate for the enzymatic reduction and ferrous sulphate reacts with H_2S to form an insoluble black precipitate (ferrous sulphide in the medium). Tryptone is added in the medium to determine the production of indole as tryptone contains a high amount of tryptophan. Tryptophan can be metabolised by some microorganisms to produce tryptophanase resulting in the production of three main final products comprising indole, ammonia and pyruvic acid shown in Equation 3.5.



The formation of indole indicates the degradation of tryptophan and can be confirmed by adding Kovac's Reagent. The indole reacts with the active ingredient, p-dimethylaminobenzaldehyde in Kovac's Reagent and turns the solution to pinkish red. Motile microorganisms can diffuse from the centre of the stab line.

The young purified colonies were inoculated by stabbing the middle of the medium in the tube, about two-thirds of the depth of medium with a sterile needle. The tubes were incubated at 30ºC for 3-5 days aerobically. The indole test was confirmed by adding a

few drops of Kovac's Reagent on the surface of the medium and observing the colour change within a minute.

Interpretation of results

The blackening (colour) of medium developed along the stab line indicates a positive result for H_2S (Visser et al., 1985). The presence of red colour after the addition of Kovac's Reagent shows the production of indole and the yellow colour indicates a negative result. A positive motility result is shown by the formation of a diffuse zone from the stabbed line of incubation, while growth along the line of inoculation shows a negative result.

3.7.8 Gelatine hydrolysis test

The absence of gelatine liquefaction has been detected from some AAB genera such as *Acetobacter* and *Gluconobacter* (Sievers & Swing, 2005). The gelatine hydrolysis test was carried out according to dela Cruz and Torres (2012). The test determines the ability of a microorganism to produce gelatinase. Gelatine is commonly produced when collagen is boiled. The gelatinases are proteases that are produced to help some bacteria to digest gelatine. Gelatine hydrolysis involves two reactions. Firstly, gelatine is degraded to polypeptides by gelatinase, and polypeptides are then degraded to amino acids. Gelatine hydrolysis test uses nutrient gelatine medium or with the nutrient gelatine stab method. Gelatine in the medium serves as the solidifying agent and substrate for gelatine hydrolysis. The digestion of gelatine would disable the solidification of the medium after cold treatment. To prepare the medium, 5 g peptone, 3.0 g meat extract and 120 g gelatine (ThermoFisher, New Zealand) were mixed in 1000 mL distilled water and mildly heated to dissolve completely. Then about 5 mL of medium was dispensed to culture tubes and sterilised by holding at 121ºC for 15 min.

The procedure for conducting the gelatine test is shown in Figure 3.2. A young culture was stabbed by a sterile needle to about two-thirds depth of the gelatine medium tube. The tubes were incubated at 30ºC for a week and the liquefaction of gelatine was observed every day. The liquefaction of gelatine was confirmed by immersing the medium tubes in an ice bath for 30 min. The presence of liquefaction was recorded as positive results.

Figure 3. 2 Gelatine hydrolysis using the stabbing method (dela Cruz & Torres, 2012)

3.7.9 Oxidation of acetate and lactate

The oxidation of acetate and lactate by the AAB is used to distinguish the different genera of the bacteria (Gomes et al., 2018). The oxidation of acetate was conducted using the method modified from Asai (1964). The acetate agar contained 15 g sodium acetate, 30 g yeast extract and 20 g bacteriological agar in 1 L distilled water. Bromothymol blue was added as pH colour indicator. In our study, calcium lactate (15 g) was added instead of sodium lactate (Sigma-Aldrich, New Zealand) for the oxidation of lactate (Franke et al, 1999). All the ingredients were mixed completely in 1000 mL distilled water. The calcium lactate was dissolved in a small amount of water with mild heating on a hot plate (Torrey Pines Scientific, USA) before sterilisation to avoid the formation of cloudiness in the medium. The pH of the medium was adjusted to pH 6.4 by adding 1M HCl and then sterilised at 121ºC for 15 min. The molten medium was dispensed into Petri dishes to solidify and then stored in the refrigerator until required for use.

The young purified colonies were streaked on the solidified acetate and lactate agar plates, respectively. The plates were incubated aerobically at 30ºC for 5-7 days. The plates were examined for growth and colour changes of the developed colonies. The medium changes from yellow to blue indicated positive results as the oxidation of the substrate turns the medium to alkaline.

3.7.10 Production of cellulose

Bacteria from the family *Acetobacteraceae* are widely used for bacterial cellulose production and mainly from the genus *Komagataeibacter* (Semjonovs et al., 2017). The production of cellulose was determined with the modified method of Lavasani et al. (2017) and Semjonovs et al. (2017). The Hestrin-Schramm (HS) broth medium was prepared by mixing 20 g D-glucose, 5 g peptone, 2.7 g Na_2HPO_4 (Sigma-Aldrich, New Zealand), and 1.15 g citric acid (Sigma-Aldrich, New Zealand) in 1000 mL distilled water (Hestrin & Schramm, 1954). About 10 mL broth medium was dispensed into glass bottles and sterilised at 121ºC for 15 min.

The young purified colonies were inoculated into the HS medium and incubated aerobically at 30ºC for 3-5 days. One mL of well-mixed broth culture was transferred into a 2-mL centrifuge tube and mixed with 1 mL of 0.1N NaOH solution. The solution was heated at 90ºC on a hot plate (Benchmark Scientific, USA) at medium heat level for 30 min because the cellulose structure does not breakdown under alkaline conditions at high temperature. The insoluble pellicle after heat treatment in the alkaline solution indicates the formation of cellulose (positive results).

3.7.11 Ketogenesis of glycerol to dihydroxyacetone (DHA)

The ketogenesis activity of glycerol was determined with a modified method from Swing et al. (1992). The glycerol yeast extract medium (GYE) consisted of 30 g glycerol,10 g yeast extract, and 20 g bacteriological agar in 1000 mL distilled water. All the ingredients were mixed, dissolved and then sterilised at 121ºC for 15 min. Benedict's solution was prepared by dissolving 17.3 g copper sulphate pentahydrate (Sigma-Aldrich, New Zealand) in distilled water in a 100-mL volumetric flask (A). Anhydrous sodium carbonate (100 g) (Sigma-Aldrich, New Zealand) and 173 g sodium citrate (Sigma-Aldrich, New Zealand) were dissolved in 500 mL distilled water (B). The copper sulphate solution was added to solution B and made up to 1 L solution with distilled water. Benedict's Reagent is used to test the presence of monosaccharides and mainly disaccharides. The copper (II) ions in the reagent react (oxidise) with the reducing sugar group C=O. In this reaction, Cu^{2+} are reduced to Cu^{+} to produce a red precipitate (Cu_2O), shown in Equation 3.6. Benedict's Reagent changes to different

colours ranging from blue to green, yellow, orange, or red depending on the amount of reducing sugars present (Damondarran & Parkin, 2017).

$$2Cu(OH)_2 + R\text{-}COH \rightarrow R\text{-}COOH + Cu_2O + H_2O \quad \text{……Equation 3.6}$$

The young purified colonies were streaked on GYE medium and incubated at 30ºC for 3-5 days. Benedict's Reagent was flooded on the surface of the agar and incubated for 3 h at 30ºC. The appearance of an orange-red colour indicated the ketogenesis of glycerol.

3.7.12 Formation of γ-pyrone from D-glucose and D-fructose

Production of γ-pyrone from D-glucose and D-fructose is used to differentiate the AAB genera (Mamlouk & Gullo, 2013). The formation of γ-pyrone from D-glucose and D-fructose was conducted with a modified method of Swing (1992). The GY and fructose yeast extract broth (FY) contained 10 g yeast extract and 50 g/L glucose/fructose with 1000 mL distilled water. About 10 mL of the medium was dispensed in test tubes and sterilised at 121ºC for 15 min. The 5% $FeCl_3$ solution (w/v) was prepared by dissolving 5 g $FeCl_3$ (Sigma-Aldrich, New Zealand) in 95 mL distilled water in a volumetric flask.

The young purified colonies were inoculated into the appropriate broth at 30ºC for 5-7 days in a shaking incubator (150 rpm) (KBLee 1001, DAIKI SCIENCES, Korea) aerobically. A few drops of $FeCl_3$ (5%, w/v) were added into the broth and then observed for colour change. The appearance of a dark brown colour indicated the formation of γ-pyrone.

3.7.13 Carbohydrate fermentation test

The AAB demonstrate different carbohydrate fermentation profiles, which can be used to differentiate the genera of the bacteria (Komagata et al., 2014). The carbohydrate fermentation test was carried out according to the modified method of Arifuzzanman et al. (2014). The basal medium consisted of 10 g carbohydrate source, 5 g yeast extract, and 10 g peptone with 0.02 g bromocresol purple as pH indicator. Mannitol, D-glucose and glycerol were chosen as sole carbon sources to determine the carbohydrate

fermentation pattern of the AAB isolates. All the ingredients were mixed thoroughly and dispended into test tubes. The medium was sterilised at 121ºC for 15 min.

The young purified colonies were inoculated to the appropriate medium respectively and incubated at 30ºC for 7-10 days, aerobically. The colour and changes in turbidity of the medium were examined. For a positive result, the medium changes from purple to yellow due to acid formation which reduces the pH.

3.7.14 Nitrate reduction test

Some Gram-negative bacteria can utilise nitrate as a final electron acceptor during anaerobic metabolism by producing nitrate reductase (Buxton, 2011). Nitrite may be reduced to different nitrogenous products depending on the different enzymes such as a nitric oxide (NO), nitrous oxide (N_2O) and nitrogen (N_2) shown in Figure 3.4. Nitrites form a red compound when they react with sulfanilic acid (Reagent A) to form diazotized sulfanilic acid. Also, nitrites can react with the α-naphthylamine solution (Reagent B) to develop a red azo dye shown in Figure 3.4. The acetic acid in the Reagents A and B acidify the NO_2^- to HNO_2. Zinc powder is a strong reducing agent and it catalyses the reduction of nitrate to nitrite.

The nitrate reduction test of AAB was carried out with the modified method of Franke et al. (1999). The test determines the ability of microorganisms to reduce nitrate to nitrite and to differentiate them among the species (Buxton, 2011). The nitrate peptone medium was used which consisted of 10 g peptone and 2 g KNO_3 in 1000 mL distilled water. The medium was dispensed into test tubes and sterilised at 121ºC for 15 min. The sulfanilic acid solution (Reagent A) was prepared by dissolving 8 mL sulfanilic acid (Sigma-Aldrich, New Zealand) in 1 L 5 N acetic acid. The Reagent B was prepared by dissolving 6 mL N, N-dimethyl-α-naphthylamine (Sigma-Aldrich, New Zealand) in 1 L 5 N acetic acid. The 5 N acetic acid was prepared by mixing 287 mL glacial acetic acid and 713 mL distilled water.

The young purified colonies were inoculated into nitrate peptone medium and incubated at 30ºC for 3-5 days, aerobically. A few drops of Reagent A and Reagent B were added to the tubes. The colour change was examined after a few minutes. In the

absence of colour change after adding Reagents A and B, a small amount of Zn powder is added to the medium and mixed completely. The colour change is observed after 10-15 min at room temperature. The development of cherry red colour after adding Reagents A and B or the presence of colourless after the addition of Zn powder indicates a positive result.

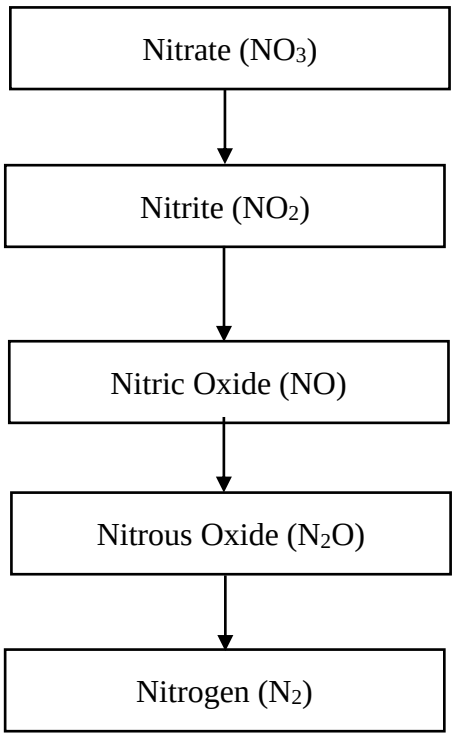


Figure 3. 3 Nitrate reduction pathway (Buxton, 2011)

Figure 3. 4 Reaction of nitrate reduction test (Buxton, 2011)

3.8 Tests for yeasts using API ID 32 kit

The API ID 32 C kit system was used for further characterisation of the yeasts based on different miniaturised assimilation of carbohydrate tests following the manufacturer's instructions (BIOMERIEUX, France). PDA plates were used for the cultivation of yeast colonies. Each purified colony from different samples was streaked on PDA plates and incubated at 30ºC for 24 to 48 h. One or several young pure colonies from the PDA plates were transferred to the API suspension medium (2 mL) to mediate a turbidity equivalent to 2 McFarland standards. The McFarland standard is used as a turbidity reference to estimate the approximate number of bacteria in a liquid suspension (Ramani et al., 1998). McFarland standard is a chemical suspension prepared by mixing 1% barium chloride and 1% of sulfuric acid. The 2 McFarland standard solution was prepared as shown in Equation 3.7 barium chloride and sulfuric acid react to form barium sulphate precipitate and induce turbidity in the solution.

2 McFarland standards = 0.2 mL % barium chloride + 9.8 mL 1% of sulfuric acid ……Equation 3.7

The bacterial cell density of 2 McFarland standard solution is approximately 6 $\times 10^8$ CFU/mL which is equivalent to an absorbance of 0.451 at 600 nm (Parthasaradhi & Kumari, 2018).

About 250 µL of the suspension with turbidity equivalent to 2 McFarland were transferred to the API C medium ampule and homogenized. Of the cell suspension, 135 µL were carefully dispensed into each ampule of API 32 C strips with sterile pipettes respectively and then incubated at 30ºC for 24-48 h. The development of turbidity of each sample was compared with the control (blank) sample. The appearance of heavy turbidity after incubation of the sample indicates a positive reaction. The general procedure of the API 32 C system is shown in Figure 3.5 (Biomerieux, France). The identification of the yeast cells was obtained from the APIWEB^{TM} software database V (https://apiweb.biomerieux.com/strip/12).

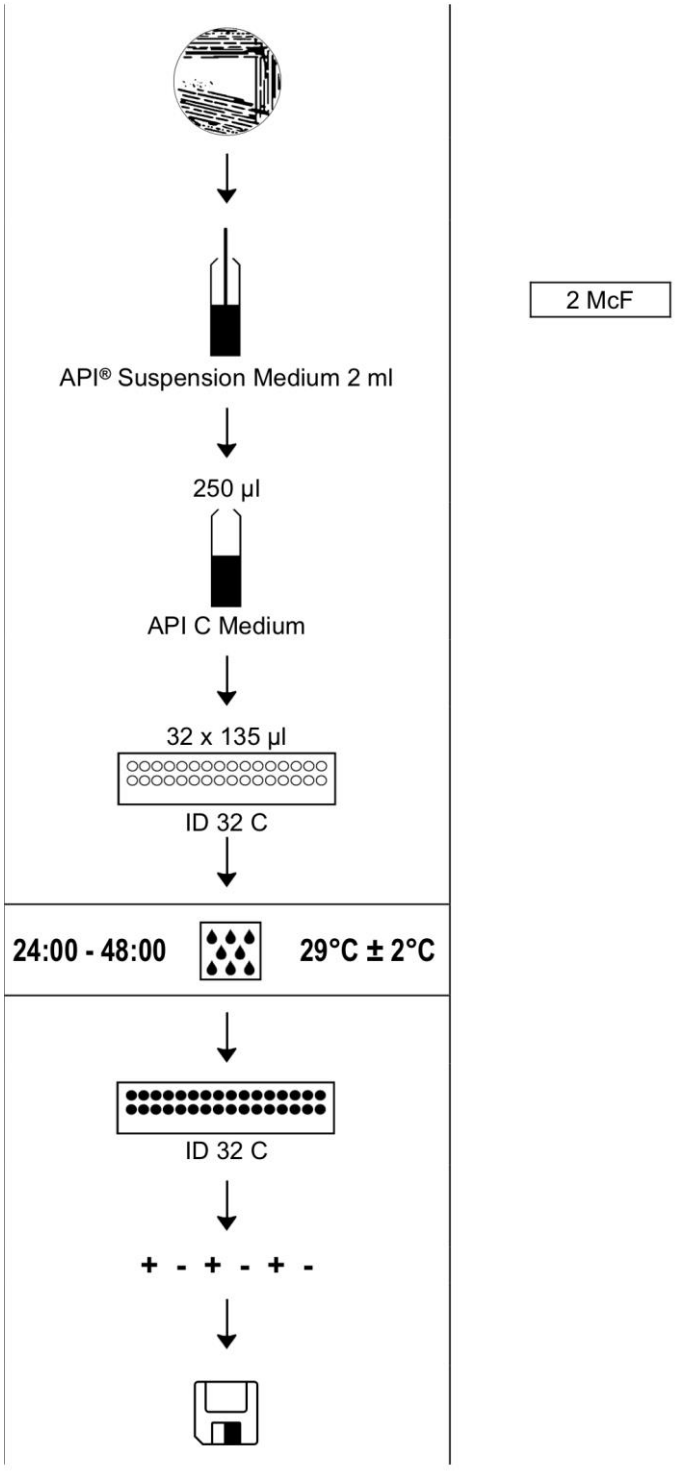


Figure 3. 5 General procedure of API 32 C system (BIOMERIEUX, 2011)

3.9 Molecular characterisation of AAB and yeasts

3.9.1 DNA extraction from AAB isolates and preparation stock cells

3.9.1.1 Purification of AAB isolates for sequencing

The young purified colonies were inoculated in GY broth medium and incubated at 30ºC for 5-7 days. The cell density was adjusted to an absorbance reading between 0.2-0.3 at OD_{600} using a visible spectrophotometer (Amersham Biosciences, UK). The culture medium was then transferred to 50 mL sterile conical centrifuge tubes and centrifuged at 6000 *g* (Heraeus^{TM}Pico^{TM}17 Microcentrifuge, Germany) for 5 min. The supernatant was discarded, and a small amount of distilled water was added and centrifuged at 6000 *g* for 1 min. The flow-through (filtrate) was discarded and the rinse step was repeated for 2-3 times. About 0.5 mL sterile distilled water were added to the pellet and mixed thoroughly by vortexing for one min. The suspension was transferred into 2-mL sterile microcentrifuge tubes consisting of 1.5 mL sterilised glycerol and thoroughly mixed by vortexing. The recovered cells were designated as the 'stock of cells' which was sealed with adhesive sealing film (Sigma-Aldrich, New Zealand) and stored at 4ºC for further analysis.

3.9.1.2 DNA extraction from AAB isolates

The DNA extraction of AAB colonies was achieved by using DNeasy®Blood &Tissue kit (Qiagen, Germany) following the manufacturer's instructions (Figure 3.6). One (1) mL freshly incubated GY broth suspension was prepared as described in section 3.8.1.1 at an absorbance reading between 0.05-0.3 at OD_{600} (10^8-10^9 CFU/mL) and.1.5 mL was pipetted into a sterile microcentrifuge tube. A suspension with an absorbance above 0.3 should be diluted with sterile GY broth until the suspension cell density falls into the range of 0.05-0.3 at $OD_{600.}$ The maximum cell density should not exceed 10^9 CFU/mL. One (1) mL pure AAB culture with the appropriate cell density was centrifuged at 5000 *g* for 10 min. The supernatant was discarded, and the bacterial pellet was resuspended in 180 µL buffer ATL (Qiagen, Germany). Four (4) µL RNase A (1.00 mg/mL) was added to the suspension and mixed by vortexing for 15 sec and incubated at 37ºC for 1 h. After incubation, 20 µL of proteinase K (Qiagen, Germany) were added to the suspension and mixed thoroughly by vortexing for 15 sec. Buffer AL (Qiagen, Germany) (200 µl) was added to the suspension and vortexed for 15 sec. The mixture

was incubated at 56ºC on a hot plate (Benchmark Scientific, USA) for 1.5 h. After incubation, 200 µL ethanol (96%-100%) were added to the sample and mixed completely by vortexing. The mixture was transferred by pipetting into the DNeasy Mini spin column placed in a 2-mL sterile microcentrifuge tube and centrifuged at 6000 *g* for 1 min. The filtrate and the microcentrifuge tube were discarded, and a fresh 2-mL sterile microcentrifuge tube was used. Buffers AW1 and Buffer AW2 were prepared by adding appropriate volumes of 96%-100% ethanol according to the manufacturer's instruction for first-time use. Buffer AW1 (500 µL) was added to the Dneasy spin column and centrifuged at 6000 *g* for 1 min, the flow-through and 2 mL sterile microcentrifuge tubes were discarded. The DNeasy Mini spin column was placed in fresh sterile microcentrifuge tubes. This was followed by adding 500 µL Buffer AW2 to the column, centrifuging at 17000 *g* for 5 min and drying the membrane. To prevent the carry-over of ethanol, the DNease Min spin column was placed in a fresh 2-mL sterile microcentrifuge tube and centrifuged at 17000 *g* for another one min. The flow-through and collection tube was discarded, then the DNeasy spin column was placed in a sterile 2-mL sterile microcentrifuge tube. One hundred (100) µL Buffer AE were pipetted into the DNeasy membrane and incubated at room temperature for one min. The addition of Buffer AE should not exceed 200 µL into the 2-mL microcentrifuge tube as the eluant would be not able to come into contact with the DNeasy Mini spin column. The suspension was centrifuged at 6000 *g* for one min to give the DNA elution. The DNA yield can be increased by repeating the elution step.

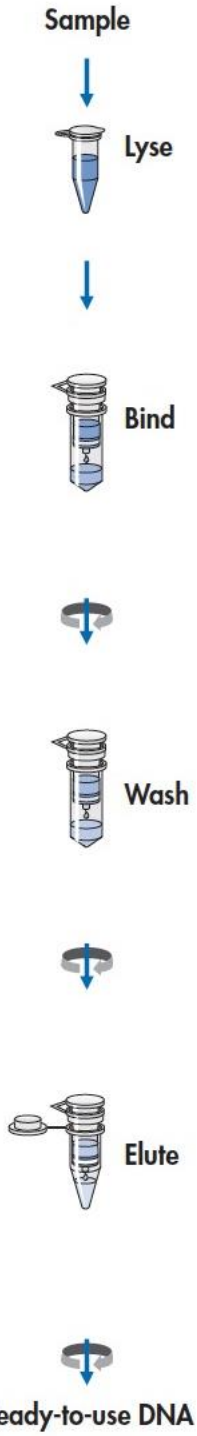


Figure 3. 6 DNA extraction (QIAGEN, 2006)

3.9.1.3 Analysis of purity of nucleic acid

The nucleic acid concentrations and purity were determined by measuring the absorbance of the DNA isolates at 260 nm and 280 nm in a spectrophotometer (GENOVA NANO, UK). The light absorbed at 260 nm is proportional to nucleic acid concentration according to the Beer-Lambert Law (Koetsier & Eric, 2019). The ratio

of absorbance at 260 nm and 280 nm (A_{260}/A_{280}) is an indicator of nucleic acid purity. A ratio of A_{260}/A_{280} between 1.85-1.88 indicates the presence of pure double stranded DNA. A higher A_{260}/A_{280} ratio (>2.0) indicates the presence of pure RNA. A low A_{260}/A_{280} ratio may be caused by a low concentration of nucleic acid or residual reagent such as phenol associated with extraction (Koetsier & Eric, 2019). The NANODROP spectrophotometer was calibrated by the AE buffer. About 1-2 µL sample were pipetted on the lower measurement pedestal to measure the absorbance ratio.

3.9.2 Purification of yeast isolates for sequencing

The yeast extract peptone dextrose (YPD) broth was prepared according to the manufacturer's instruction. A loopful of purified yeast colonies was inoculated into the YPD broth medium and incubated at 25ºC for 3-5 days. The cell density was measured with a spectrophotometer and adjusted to an absorbance reading between 0.2-0.3 at OD_{600}. The culture medium was then transferred to 50-mL sterile conical centrifuge tubes and centrifuged at 6000 *g* for 5 min. The supernatant was discarded, and a small amount of distilled water was added and centrifuged at 6000 *g* for 1 min. The flow-through (eluant) was discarded and the step was repeated for 2-3 times. About 0.5 mL of sterile distilled water were added to the pellet and mixed thoroughly by vortexing. The suspension was transferred to 2-mL sterile microcentrifuge tubes consisting of 1.5 mL of sterilised glycerol and mixed thoroughly by vortexing. The cell stock was stored at 4ºC for further analysis.

The PDA plates were prepared as previously described in section 3.6 and sealed with adhesive sealing film to prevent contamination during handling.

3.9.3 DNA sequencing of AAB and yeasts

3.9.3.1 PCR reactions of AAB

The cell stock of three representative AAB colonies after characterisation described in section 3.9.2 were prepared in 2-mL microcentrifuge tubes containing 67% glycerol at ambient temperature (23°C) and transported to Macrogen Inc. (Seoul, Korea) for DNA sequencing. Universal primers 785F (5' GGATTAGATACCCTGGTA 3') and 907R

(5’CCGTCAATTCMTTTRAGTTT 3’) were used to amplify 16S rRNA genes. The specimen was transported by FedEx Courier, an international airfreight service.

3.9.3.2 PCR reactions of yeasts

The DNA extraction and purification, PCR amplification and purification, and sequencing were carried out by Macrogen Inc. (Seoul, Korea). Six representative samples with more than 94% identification and seven unknown samples after the API 32C tests were transported by FedEx air freight service prepared in 67% glycerol in 2-mL microcentrifuge tubes at ambient temperature (23ºC). The universal primers LR0R (5’ ACCCGCTGAACTTAAGC 3’) and LR7 (5’TACTACCACCAAGATCT 3’) were used for amplifying the 26S rRNA genes.

3.10 Data analysis

Data on pH, titratable acidity and total soluble solids were analysed by Microsoft Excel 2016 (Microsoft, USA) using descriptive statistics. Analysis of variance-one way (ANOVA-ONEWAY) of the data on pH, TA and TSS were done using the IBM SPSS version 26 (IBM, USA) to determine the significant difference of the means ($p<0.05$). The 16 sRNA sequencing of the AAB isolates and 26S rRNA sequencing of yeast isolates were determined by using the Basic Local Alignment Search Tool (BLAST) database of the National Centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov/, NCBI).

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Phase I: Physico-chemical characteristics of Kombucha samples

4.1.1 Acidity of Kombucha

The acidity (pH and TA) of six samples of Kombucha is shown in Table 4.1. pH and TA are probably the two most common concepts used to express the acidity in food analysis (Sadler & Murphy, 2010). Titratable acidity indicates the total acid concentration in the food sample, which expresses better acid impact on food flavour than pH. However, the TA is not able to reflect the free hydronium ions (H^+) concentration as pH. Therefore, pH and TA are frequently used together to express the acidity of food products. Acidity is an important factor to monitor the fermentation process of Kombucha. The formation of acidity is attributed to the metabolic activity of the AAB and yeasts that produce organic acids such as acetic acid and gluconic acid (Chen & Liu, 2000). The variation of the hydrogen ion concentration can stimulate or inhibit the growth of the fermenting microorganism in Kombucha (Neffe-Skocinska et al., 2017). Most AAB species can grow at pH ranging from 3.5 to 8.5 and some species have been reported to grow below 3 (Komagata et al., 2014). For yeasts, the optimum growth pH is between pH 4.5 and 6.0 (Neffe-Skocinska et al., 2017). The mean pH of six samples of Kombucha sold in New Zealand was between 3.21 and 3.90, while the TA ranged from 0.38-0.43%, respectively. The highest pH was detected from sample DO with spices and ginger flavour. The LO with Feijoa flavor had the lowest pH. However, the lowest TA was 0.38% from sample DO and sample GB with lemon and ginger flavours. Sample WO with traditional flavour had the highest TA (0.43%). The variations between pH and TA could be attributed to the buffer characteristics of Kombucha broth. Carbon dioxide produced during fermentation dissociates in the aqueous solution to produce amphiprotic hydrocarbonate anion (Essawet et al., 2015). The anion reacts with hydrogen ions from other organic acids in the fermentation system which inhibits change in pH. The differences in TA between the Kombucha samples provided better expression of acidity than pH due to the buffer characteristics of Kombucha as discussed earlier. Therefore, TA should be used as a parameter to determine the fermentation endpoint instead of pH.

Kombucha containing herbal tea plant powder such as *Acaicia arabica* was reported to have pH ranging from 4.0-6.0, with TA of 2.5-5.0 g/L after fermentation for seven days (Waisundara, 2018). Another study on Kombucha containing medicinal herbs reported pH 2.95 with TA 4.95g/L after fermentation for nine days (Velićanski et al., 2018). After 14 days of fermentation, Kombucha fermented from different cultivars of snake fruit recorded pH 3.12 to 3.38 and TA 1.52-1.72% after fermentation for 14 days (Zubaidah et al., 2019). Imported Kombucha samples have variable ($p<0.05$) acidity compared with the New Zealand products. The differences in acidity may be influenced by different microbial starter cultures, substrate content (tea and sugar), and the fermentation conditions (Neffe-Skocinska et al., 2017). For instance, longer fermentation time may result in higher acid concentration (Chen & Liu, 2000). Currently, there is no standardised pH range for fermented Kombucha beverages which results in the variation of the products. Kombucha with less than pH 2.5 has been reported to contain good sensory characteristics (Neffe-Skocinska et al., 2017; Nummer, 2013). To achieve a pleasant sour flavour, the TA of Kombucha should be 4-4.5g/L (Waisundara, 2018).

Table 4. 1 Mean acidity and total soluble solids of six Kombucha samples

Sample	Origin	Flavour	pH*	TA (%) *	TSS (ºBrix)
LO	New Zealand	Feijoa	3.21±0.01	0.42±0.04	3.70±0.00
DO	New Zealand	Chai species and gingers	3.90±0.01	0.38±0.02	7.00±0.00
GB	New Zealand	Lemon and Ginger	3.43±0.04	0.38±0.02	4.00±0.00
AM	Australia	Peach & Mango	3.54±0.01	0.40±0.02	2.47±0.06
RE	Australia	Original	3.40±0.01	0.40±0.02	1.87±0.06
WO	US	Traditional	3.45±0.02	0.43±0.01	6.50±0.00

*Data are expressed as mean±SD; n=3; TA = Titratable acidity; TSS = Total soluble solids.

4.1.2 Total soluble solids of Kombucha

The TSS of the six samples of Kombucha are shown in Table 4.1. Total soluble solids of Kombucha indicate the sugar content dissolved in the beverage and it is expressed as ºBrix (Zubaidah et al., 2019). The TSS of the six samples varied from 1.87 to 7.00 ºBrix ($p<0.05$). The highest TSS was observed from sample DO and the lowest was

from sample RE. The variation of the TSS from the six samples suggested the different sugar levels in the Kombucha samples. A previous study on Indonesian Kombucha fermented from various snake fruit contained TSS ranging from 12.43 to 14.08 ºBrix, which was much higher than the levels obtained in our study (Zubaidah et al., 2019).

4.2 Phase II: Enumeration, isolation and purification of AAB and yeasts in Kombucha

4.2.1 Morphology of AAB grown on GYPM

The morphology of grown AAB colonies from three Kombucha samples (LO, GB and DO) produced in New Zealand were similar to those reported by previous authors (Sievers & Swing, 2005). No colonies that resembled AAB were observed on the agar plates of imported samples (AM, RE and WO). Typical morphology of AAB colonies have been described as circular, entire and the colour varies from beige to brownish (Komagata et al., 2014). The absence of AAB in the imported products may be explained by the heat treatment subjected to the products after fermentation during manufacturing. It has been reported that some Kombucha products may be pasteurised around 70ºC for 10 minutes after the end of fermentation. The products may also have been subjected to heat-treatment, filtration, centrifugation or other treatments to partially remove microbial cells to control post-fermentation that may increase acidity (Screeramulu et al., 2000; Liamkaew et al., 2016).

The morphology of the colonies present on GYPM plates were placed into three groups shown in Table 4.2. Colonies in group I and group II were isolated from sample LO and colonies in group III were isolated from sample GB. The colonies from group I were opaque with smooth surfaces and light brownish colour after incubation for three days at 30ºC. Then the colour of the colonies turned brownish-red with prolonged incubation. Colonies from group II appeared to be pale yellow colour, convex, shiny, and smooth surfaces. The diameter of colonies ranged from 1.0 to 1.2 mm. For group III, most of the colonies appeared to have smooth surfaces and light brownish colour. The colonial appearance of *Acetobacter* are commonly circular, cream to beige colour on glucose yeast extract peptone agar. The genus *Glucoacetobacter* colonies are light

brown to brownish, and most species produce water-soluble brownish pigment. Colonies from the genus *Gluconobacter* are entire, smooth and glisten while some strains form pink colonies (Yamada, 2016). The genus *Komagataeibacter* are circular, smooth or rough, raised or umbonate (Sievers & Swing, 2005; Komagata et al., 2014). The morphology of group I and group III colonies are similar to the genus *Glucoacetobacter* and *Gluconobacter*. The appearance of colonies from group II were similar to the genus *Acetobacter* reported by Komagata et al. (2014).

Table 4. 2 Appearance of colonies grown on GYPM agar plates

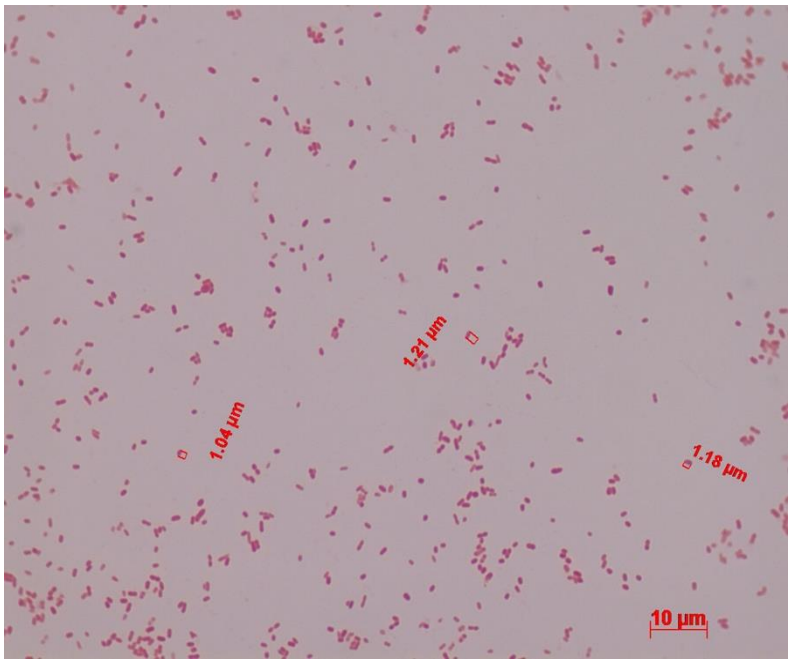
Colony Codes	Appearance
Group I （LO-AAB1, LO-AAB3, LO-AAB5）	Brownish red, circular, entire, smooth surface
Group II (LO-AAB2, LO-AAB-4)	Pale yellow, convex, with shiny, smooth surfaces
Group III (GB-AAB1, GB-AAB2)	Light brownish and shiny smooth surface

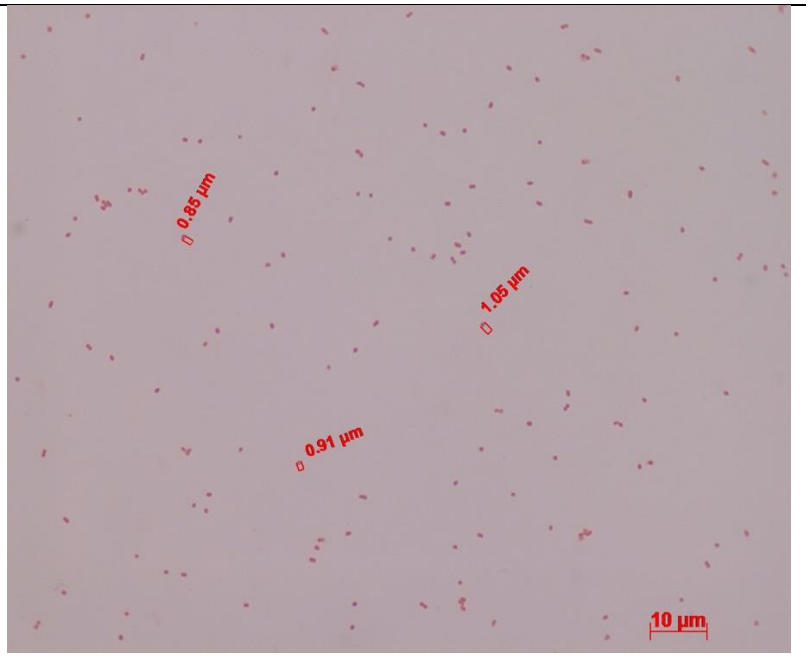
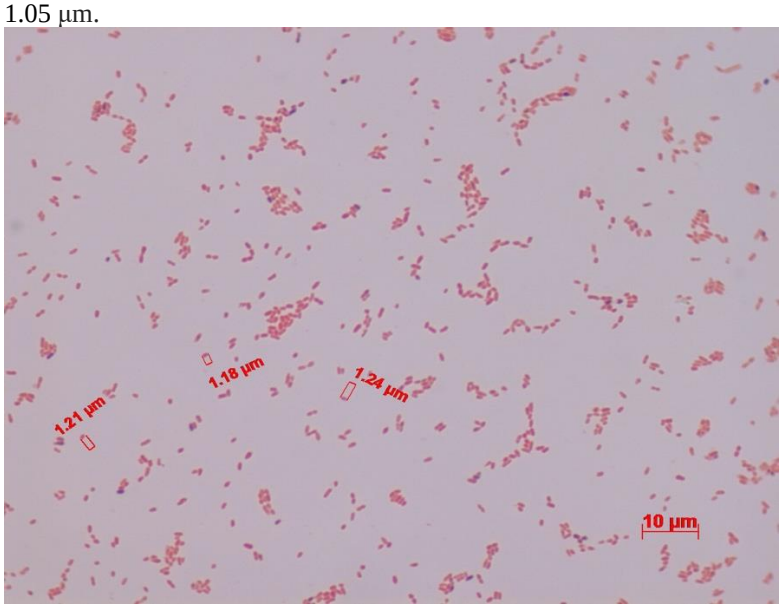
All the five colonies isolated from sample LO and two colonies from sample GB were Gram-negative and were presumed to be AAB based on their morphology and Gram stain reactions (Table 4.3) (Gomes et al., 2018). All the cells from the three groups were rod-shaped and had similar cell length of around 1μm, but their cell arrangements were slightly different. Cells of colonies in the three groups (I, II, III) appeared singly or paired, but the cell colonies of group III also appeared in chains. The majority of AAB cells are ellipsoidal or rod shaped (Komagata et al., 2014). The cells the genus *Acetobacter* occur in single, short chains or longer chains. The genus *Gluconacetobacter* cells occur in single or in pairs. Cells from the genus *Gluconobacter* commonly occur singly or paired and rarely in chains. Some irregular cells have been also observed from this genus (Sievers & Swing, 2005). Therefore, group III colonies may belong to the genus *Acetobacter* or *Gluconobacter* as the cells occurred in chains.

It is most likely that the three colonies from sample GB and the five colonies from DO were not bacteria, as the mean cell length was longer than 5 μm (Kurtzman et al., 2011). Cells of some colonies resembled the characteristics of yeasts with visible budding and fission structures which agreed with previous studies (Kurtzman et al., 2011; Boekhout & Robert, 2003). Thus, these cells were more likely to be yeasts instead of AAB. Although the GYPM is not selective for AAB, it was formulated to promote the propagation of the bacteria. Therefore, it is not uncommon for other microorganisms including yeasts to grow on this medium. According to our knowledge, there is no

commercial selective media for the isolation of AAB, probably due to the limited studies on the identification of AAB in food products including Kombucha. In the absence of a selective medium, the colonies grown on GYPM were Gram-stained to examine their cell morphology. The morphology of the Gram-negative cells grown on GYPM resembled that of the AAB (Gomes et al., 2018).

Table 4. 3 Cell morphology of colonies developed on GYPM agar plates

Group of isolates and codes	Gram-stained cells and characteristics of cells
Group I (LO-AAB1 LO-AAB3 LO-AAB5)	 Rod shaped; occurred in singly or paired. Cell length ranged from 1.00 to 1.25 µm.

Group II (LO-AAB2, LO-AAB4).		Rod shaped, occurred in singly or paired. Cell length ranged from 0.85-1.05 µm.
Group III (GB-AAB1 GB-AAB2)		Rod shaped, occurred in singly paired or chained. Cell length ranged from 1.00-1.25 µm.

Note: Group I = isolates from sample LO; Group II = isolates from sample LO; Group III = isolates from sample GB.

Based on the available information, it seems that there is no published information on AAB in fermented Kombucha at point of sale in New Zealand. Suspect AAB were only found in samples LO and GB manufactured in New Zealand. The AAB present in GB (5.63±0.02 log CFU mL) were higher than the bacteria found in LO (4.97±0.06 log CFU mL). The higher cell population density of suspect AAB found in GB may correspond to a favourable higher fermentation rate during production (Fernadez-Perez et al., 2010). However, continuous fermentation of Kombucha during storage or at retail

level is undesirable as this can increase the alcohol content under anaerobic environment which may be undesirable to consumers and against local non-alcoholic beverage ethanol content regulations (Gomes et al., 2018). In this study, the enumeration was conducted by the standard plate count method, however, the traditional method may be unsuitable for the enumeration of AAB as some species can form continuous biofilm structures on the surface of solid medium such as GY agar (Fernadez-Perez et al., 2010). Some exopolysaccharides such as levans, cellulose and dextrans produced by AAB stimulate the formation of biofilm and interfere with the colony counting on agar plates (Gullo & Giudici, 2008). Additionally, conventional plate count method is not valid for quantifying the non-culturable AAB as it can under-estimate the viable cells. Therefore, the suspect AAB counts from two brands could only be considered as a rough estimate for the dominant culturable AAB cell numbers isolated from Kombucha samples. It is therefore recommended to use methods that are more accurate such as epifluorescent direct count techniques applied in water treatment for enumeration of AAB (Mesa et al., 2003).

4.2.2 Morphology of yeast colonies grown on YGC agar

The isolation and enumeration of yeasts from Kombucha samples were conducted on the selective YGC agar. Suspect yeast colonies were recorded from three sample products (LO, GB and DO) produced in New Zealand. The absence of developed colonies on YGC plates from imported products may be caused by heat treatment after fermentation as previously discussed.

The yeast cell counts present in the three samples ranged from 10^4 to 10^5 CFU/mL (Table 4.4). The highest yeast cell concentrations were found in samples DO and GB, whereas sample LO had the lowest cells. Yeast concentrations of Romania kombucha were reported to range from 10^5-10^6 CFU/mL after 10 days fermentation (Matei et al., 2018). However, the yeast population of Australia Kombucha samples were up to 10^8 CFU/g (Teoh et al, 2003). The huge difference of yeast count may be caused by the product was chosen at different time periods. Our Kombucha samples were collected at the point of sale but previous studies analysed the beverages immediately after fermentation.

Table 4. 4 Yeast counts of three domestic Kombucha samples

Samples	Log CFU/mL
LO	4.75±0.10
GB	5.69±0.01
DO	5.57±0.07

Note: n=2; ± = SD of the mean of duplicate analyses.

There were potentially seven groups of colonies with yeast-like morphology isolated from three New Zealand-produced samples (Table 4.5). Most of the colonies were white to cream in colour, with a circular shape and entire margins. A summary of the appearance of the appearance of the colonies is shown in Table 4.5. The morphology of yeast colonies described in group I and group VII were in agreement with previous studies, which were isolated from Taiwan Kombucha samples and identified as *Brettanomyces bruxellensis* (Liu et al., 1996).

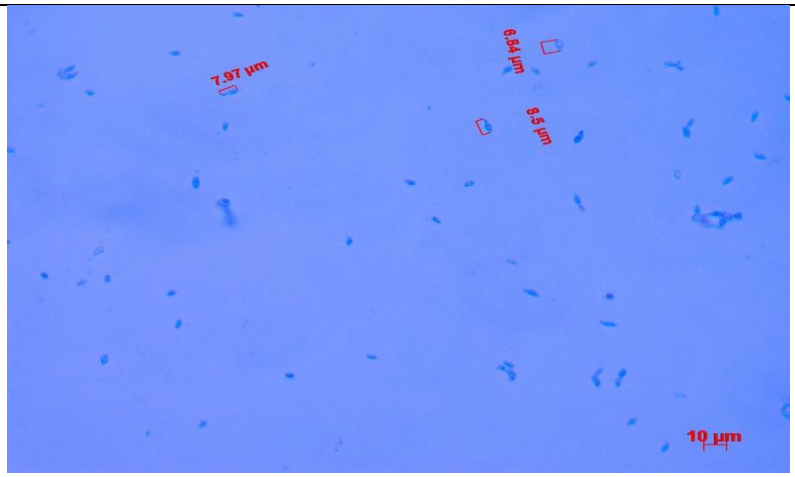
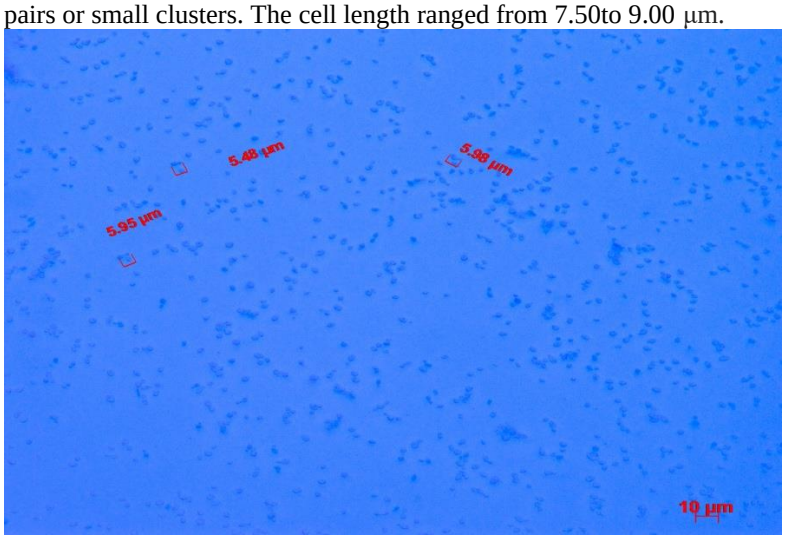
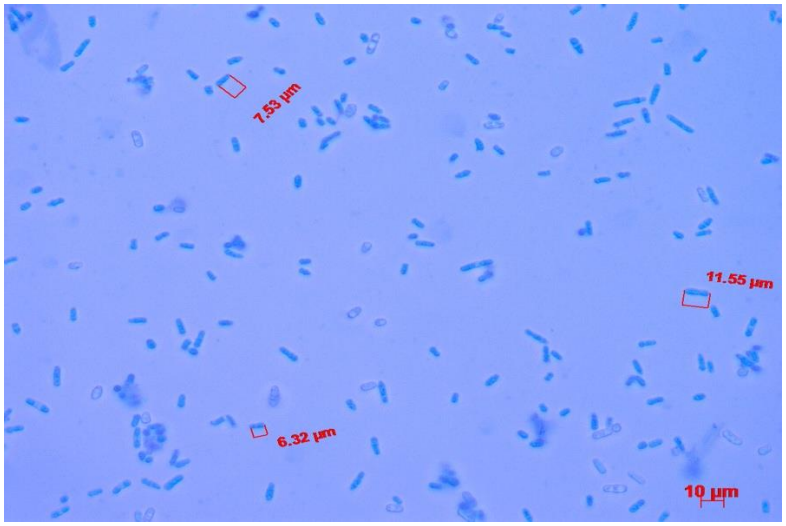
Table 4. 5 Appearance of colonies grown on PDA plates

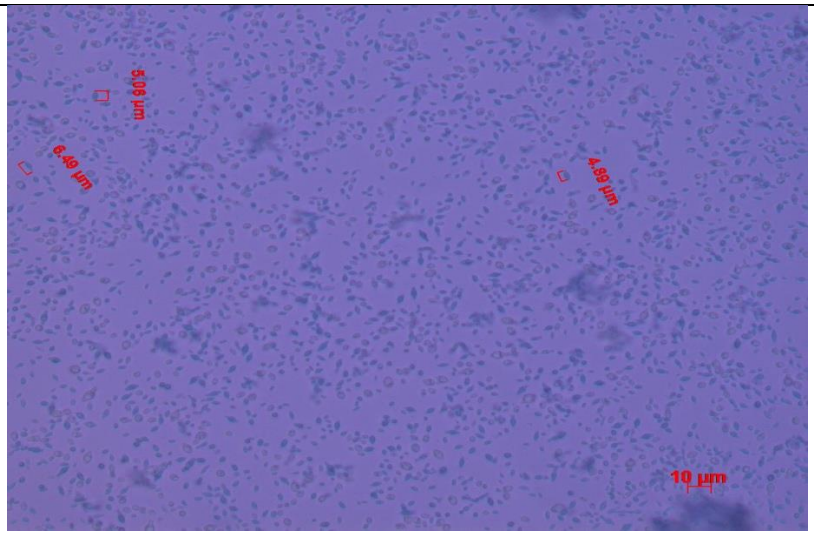
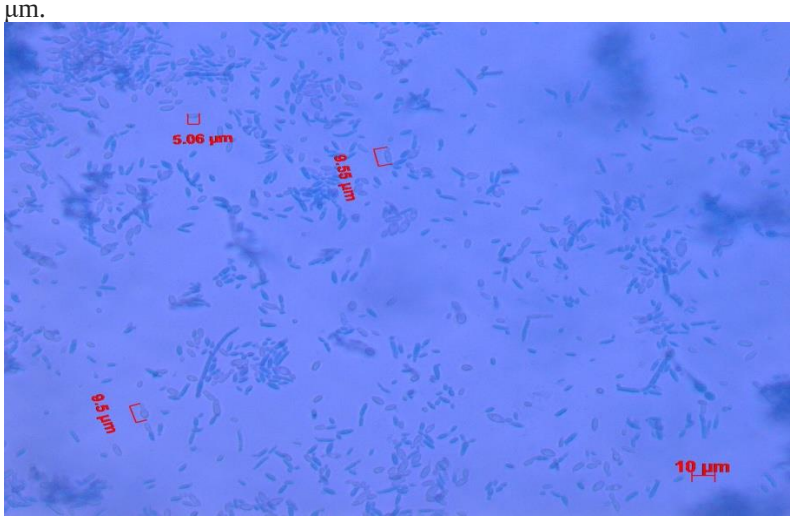
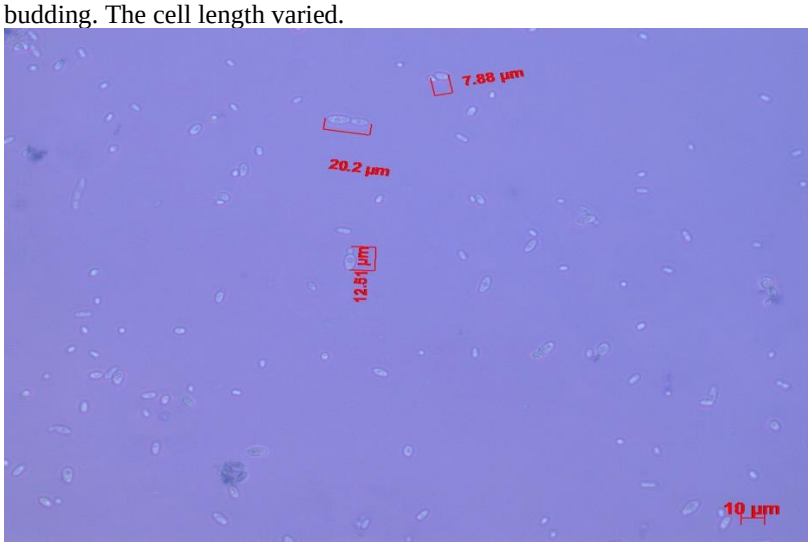
Colony code	Appearance
Group I (LO-yeast 1, DO-yeast 5)	Raised, white creamy colour, dull and smooth surface with entire margin.
Group II (LO-yeast 2, LO-yeast 3, LO yeast 4)	Circular and white cream colour, shiny, flat and smooth surface.
Group III (LO-yeast 5)	Circular, glistening surface, raised elevation, and entire margin
Group IV (DO-yeast 1, DO- yeast 2, DO-yeast 3, DO- yeast 4, DO-yeast 6)	White to creamy colour, smooth and glossy surface, umbonate elevation in the centre and entire margin.
Group V (DO-yeast 7, DO-yeast 8, DO-yeast 9, DO-yeast 10, GB-yeast 6, GB-yeast 7, GB-yeast 8)	Circular and slightly umbonate in the centre; creamy colour, shiny and smooth surface entire margin.
Group VI (GB-yeast 1, GB-yeast 5)	Circular, off-white colour, slightly umbonate in the centre, flat and smooth surface, entire or lobate margin.
Group VII (GB-yeast 2, GB-yeast 3, GB-yeast 4)	Circular, cream colour, flat and smooth surface.

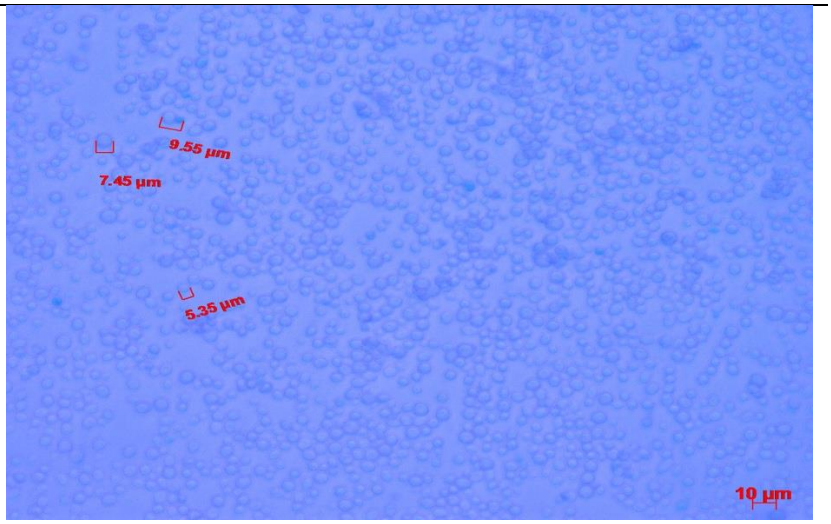
The cell morphology of the 23 representative colonies were placed into seven main groups after microscopic observation with methylene blue staining (Table 4.6). Methylene blue is one of the most rapid and objective stain to observe viable yeast cells. The dye penetrates into yeast cells, turning the viable yeast cells colourless due to the enzymatic reaction. In contract, the dead cells remain blue (Kwolek-Mirek & Zadrag-Tecza, 2014). The cells could be therefore differentiated by their shape, cell size, occurrence and the formation of budding or pseudomycelium under microscopic

observation. All the cells from seven groups were singly, paired or in small clusters as described in Table 4.6. The cell size varied between groups. The formation of budding was found in groups I, IV, V and VI. In group I, group II and group VI, the cells were ovoidal, or ellipsoidal shaped. Group III yeast cells were rod-shaped with the presence of septum, which divided the cells into two parts. Hence, it was predicted to be fission yeast. This fission reproduction feature can be observed in the yeast of *Schizosaccharomyces pombe*, which has been isolated from Australia Kombucha samples (Teoh, 2003). Cells from group IV were apiculate, spindle-like shaped. Ogival or cylindrical to elongate shaped cells were detected from group V. The pseudomycelium was produced and consisted of branched and filamentous cells. The morphological characteristics were reported from *Brettanomyces anomalus*, isolated from a spoiled carbonated beverage (Smith & Van Grinsven, 1984). The cells in group VII were spherical, ovoidal or elongated, which were consistent with *Saccharomyces cerevisiae*, isolated from Taiwan Kombucha called Haipao (Liu et al., 1996).

Table 4. 6 Cell morphology of colonies developed on YGC plates

Groups of isolates and codes	Methylene-stained and characteristics of cells
Group I (LO-yeast 1, D.O-yeast 5)	 Ovoidal, ellipsoidal, ogival to elongate shape, budding, occurred in single, pairs or small clusters. The cell length ranged from 7.50to 9.00 µm.
Group II (LO-yeast 2, LO-yeast 3, LO yeast 4)	 Ovoidal or ellipsoidal shape, occurred in single, pairs or small clusters. The cell length ranged from 5.40 to 6.00 µm.
Group III (LO-yeast 5)	 Cylindrical rod-shaped, occurred in single, pairs or small clusters, reproduced by fission. The cell length varied from 6.30 to 11.60 µm.

Group IV (DO-yeast 1, DO- yeast 2, DO-yeast 3, DO- yeast 4, DO-yeast 6)	 Apiculate, spindle-like, spheroidal, and ovoidal shape, budding and round ascospore; occurred in single or in pairs. The cell size varied from 4.50-6.50 µm.
Group V (DO-yeast 7, DO-yeast 8, DO-yeast 9, DO-yeast 10, GB-yeast 6, GB-yeast 8)	 Ellipsoidal, ogival or cylindrical to elongate; occurred in single, pairs short chains or cluster; branched filamentous cells formed; Pseudomycelium; budding. The cell length varied.
Group VI (GB-yeast 1, GB-yeast 5)	 Ovoidal，ellipsoidal and elongated shape; occurred in single, pairs or small clusters. The cell length varied.

Group VII (GB-yeast 2, GB-yeast 3, GB-yeast 4).	 Spherical, ovoidal or elongated shape. They were arranged in single or in small clusters. The cell length varied.

4.2.3 Purification of AAB and yeast isolates

Following the morphological examination of the AAB and yeast cells from representative colonies, there were seven suspect Gram-negative AAB and 23 suspect yeasts colonies from three NZ-produced sample products. The seven AAB colonies were placed into three groups according their morphology. Groups I and II were isolated from sample LO and group III isolates were obtained from sample GB. For suspect yeast cells, there were seven groups isolated from samples LO, GB and DO.

4.3 Phase III: Phenotypic and genotypic identification of AAB and yeasts

4.3.1 Phenotypic characteristics of AAB colonies

Seven rod-shaped Gram-negative isolates were divided into three groups according to their morphological characteristics. The Gram-negative reactions of the isolated agreed with previous studies (Komagata et al., 2014). The AAB belong to family *Acetobacteraceae*, which are divided into acetous and the acidophilic groups (Gomes et al., 2018). The AAB are included in the acetous group and there are eighteen genera in this group (Sievers & Swing, 2015). The genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter* and *Komagataeibacter* are the most important AAB associated with food (Lynch et al., 2019). The phenotypic and differential characteristics of the isolates are shown in Table 4.7. Based on their biochemical profiles, group I and III isolates

were presumed to belong to the genus *Gluconobacter*, and group II isolates to the genus *Acetobacter*.

Table 4. 7 Growth and biochemical characteristics of AAB isolates from Kombucha samples

Parameter	Group I	Group II	Group III
Catalase reaction	+	+	+
Oxidase reaction	-	-	-
Growth at different temperature			
Growth at 25ºC	+	+	+
Growth at 30ºC	+	+	+
Growth at 37ºC	+	+	+
Growth on different media			
GYC plates	+	+	+
0.35% (w/v) acetic acid medium (pH=3.5)	+	+	+
30% (w/v) D-glucose medium	-	-	-
DSM medium	-	-	-
Methanol medium	w	+	w
Glutamate medium	+	w	+
Oxidation of ethanol to acetic acid	+	+	+
Oxidation of acetic acid to water	-	+	-
Alcoholic tolerance test (v/v):			
2%	+	+	+
4%	+	+	+
6%	+	+	+
8%	+	+	+
10%	-	-	-
Formation of H_2S, indole and motility tests:			
H_2S	-	-	-
Indole	-	-	-
Motility	-	-	-
Gelatine hydrolysis test	-	-	-
Oxidation of acetate and lactate:			
Acetate	-	+	-
Lactate	-	+	-
Production of cellulose	-	-	-
Ketogenesis from glycerol to DHA	+	+	+
Formation of γ -pyrone from:			
D-glucose	+	-	w
D-fructose	+	-	w
Acid produced from			
D-glucose	+	+/-	+
Mannitol	+	-	+
Glycerol	+	-	+
Nitrate reduction	-	-	-
*Probable genus	*Gluconobacter*	*Acetobacter*	*Gluconobacter*

Note: (+) Indicated positive reaction; (-) Indicate negative reaction; (+/-) some strains were positive or negative; (w): weakly positive; *The probable genera were also reported based on previous studies (Komagata et al., 2014; Sievers & Swing, 2015; Yamada, 2016); experiments were replicated 3 times.

4.3.1.1 Oxidase and catalase reaction

Seven isolates from the three groups (I, II, and III) showed no colour changes on oxidase strips and no visible gas productions in 6% H_2O_2 (Table 4.7). These reactions indicated that the isolates were oxidase negative and catalase positive, which agreed with the majority of AAB species (Klawpiyapamornkun et al., 2015; Gomes et al., 2018).

4.3.1.2 Growth of isolates at different temperatures

AAB are commonly mesophilic and their optimum growth temperature is around 30 ºC (Komagata et al., 2014). Most AAB species belonging to the genera *Acetobacter* can grow at 37ºC, and most species of the genus *Gluconacetobacter* grow between 15ºC and 30ºC but not at 37ºC. Additionally, some species of the genus *Gluconobacter* can also grow at 35ºC. The optimum growth temperature for this genus lies between 25 ºC and 30 ºC (Yamada, 2016). All the seven isolates of three groups (I, II, III) obtained in this study were able to grow at 25ºC, 30ºC and 37ºC after 5-7 days incubation on GY agar plates (Table 4.7).

4.3.1.3 Growth of isolates on different media

Growth on glucose yeast extract calcium carbonate (GYC) agar plates

Bacterial cells from the three groups (I, II, III) were able to grow aerobically at 30 ºC on GYC agar plates after incubation for 5-7 days (Table 4.7). Growth of cells was confirmed by the formation of a transparent clear zone surrounding the colonies (De Vero et al., 2016). The formation of brownish colour colonies was observed in the isolates from group I. The production of water-soluble brown pigment was reported in a few species from the genera *Gluconobacter* and *Gluconacetobacter* (Gomes et al., 2018). Therefore, the group I isolates may belong to one of these two genera.

Growth on medium containing 0.35% (v/v) acetic acid (pH 3.5)

Growth of isolates on medium containing 0.35% acetic acid (pH 3.5) has been used as a criterion to differentiate the genera of food related AAB as some genera can grow in

the presence of 0.35% acetic acid but others cannot (Komagata et al., 2014). The acetic acid medium inoculated with isolates of the three groups (I, II, III) of became turbid after 2-5 days incubation at 30ºC and a brown red colour was observed from the group I tubes (Table 4.7). Results showed that all the isolates could grow in the presence of 0.35% (v/v) acetic acid medium.

Growth on medium containing 30% (w/v) D-glucose

All the isolates from the three groups were not able to grow on 30% (w/v) D-glucose in agar, suggesting that the cells were not tolerant to high osmotic pressure environment (Table 4.7). Most species from the genus *Acetobacter* are not able to grow in the presence of 30% (w/v) D-glucose whereas only some strains from the genus *Gluconobacter* are able to grow in high sugar environment (Lynch e al., 2019).

Growth on medium containing methanol

All seven isolates of three groups (I, II, III) could grow in medium containing methanol (Table 4.3.1). However, isolates from group II showed better growth than group I and III. These two group isolates could only develop few colonies around the streak lines on the agar that were difficult to observe, which suggested that these isolates utilised methanol weakly.

Growth on Dextrose Sorbitol Mannitol (DSM) agar plates

DSM agar was used as a primary selective isolation medium of AAB to differentiate between the genera *Gluconobacter* and *Acetobacter* according to different colour changes (Cirigliano, 1982). All the isolates from the three groups were not able to grow on this medium after 7 days incubation at 30°C (Table 4.7). This may be caused by the presence of some bacterial inhibitors in the medium including sodium deoxycholate and crystal violet. Sodium deoxycholate is commonly used to achieve cell lysis and inhibit the growth of majority species from *Acetobacter*. Crystal violet can completely inhibit the growth of *A. aceti* subspecies (Cirigliano, 1982). Therefore, this medium was considered unsuitable and ineffective to isolate AAB from the Kombucha samples.

It is therefore recommended to modify the composition of the medium to achieve the desired results.

Growth on glutamate agar plates

The ability to grow on glutamate agar plates is an important characteristic to differentiate the AAB. The genera *Acetobacter* and *Gluconobacter* do not grow on glutamate agar, whereas the *Gluconacetobacter* spp. and *Komagataeibacter* spp. can grow on this medium (Malimas et al., 2017). Group I and group III isolates showed weak growth around the streaked lines, and well-developed colonies were observed from group II isolates. The weak growth of isolates from the group (I, II, III) can affect the identification of the bacteria to genus level and therefore, the results should be examined with other phenotypic tests for confirmation.

4.3.1.4 Oxidation of ethanol and acetic acid

The AAB are well-known for their high efficiency of oxidizing ethanol to acetic acid in the presence of oxygen (Gomes et al., 2018). The oxidation of ethanol by AAB is tested using the Carr medium (Carr, 1979). The plated Carr medium agar turned from purple to yellow after incubation for 2 days at 30°C due to the production of acid (Gomes et al., 2018). The results indicated that the isolates were tolerant to alcohol due to their ability to utilise ethanol as carbon source. Agar plates inoculated with group I and group III isolates remained yellow with extended incubation of 3-5 days. In contrast, group II plates turned from yellow to purple due to pH increase, which indicated the acetic acid was further oxidised to water and CO_2. The ethanol oxidation is catalysed by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) which are bound to the outer surface of the cytoplasmic membrane (Mamlouk & Gullo, 2013). The ADH of the *Acetobacter* species is more stable than the *Gluconobacter* species in presence of acetic acid which may explain their higher acid yield than the *Gluconobacter* species. Moreover, *Acetobacter* species can further oxidise acetic acid to water and CO_2 in the tricarboxylic acid (TCA) cycle when the ethanol is depleted. In contrast, the *Gluconobacter* species are not able to further oxidize the acetic acid due to the deficiency of the succinate dehydrogenase and α-ketoglutarate dehydrogenase (Raspor & Goranovic, 2008). The ability to oxidise acetic acid to CO_2 and water is

called acetate oxidation or over-oxidation which differentiates the genus *Gluconobacter* from other genera as it does not have this ability. Therefore, it is possible that group I and group II belonged to the genus *Gluconobacter* (Gomes et al., 2018).

4.3.1.5 Alcoholic tolerance test

The ethanol tolerance of AAB varies between species or strains (Gullo & Giudici, 2008). Seven AAB isolates (Table 4.7) were tested for their ability to grow on different ethanolic (2% to 10%) agar for 5-7 days at 30 ºC. All three groups of isolates were able to grow on agar plates containing 2%-8% ethanol (v/v). Group II isolates showed better growth on the ethanolic agar media than groups I and III at the same concentrations as more colonies were observed around the streaked lines. However, no colonies developed on the 10% (v/v) ethanolic agar, which suggested that the maximum tolerance level for the isolates may be between 8% and 10% (v/v) ethanol. According to Kumar & Joshi (2016), Kombucha is commonly considered as a non-alcoholic beverage as the ethanol is expected to be less than 0.5%. However, regulated ethanol is different across the regions. For example, Food Standards Australia New Zealand (FSANZ) stipulate that the alcoholic beverages must provide a statement of alcohol content if the beverage contained more than 1.15% alcohol by volume (FSANZ, 2014).

4.3.1.6 Oxidation of acetate and lactate

Some genera such as *Acetobacter* and *Gluconacetobacter* can oxidise acetate and lactate which may be used to differentiate them from the genera that cannot oxidise acetate and lactate such as the *Gluconobacter* (Gomes et al., 2018). Group II colonies were blue after 5-7 days incubation at 30ºC on the sodium acetate and calcium lactate containing agar media. The appearance of the blue colour indicated the oxidation of acetate and lactate to CO_2 and water, thereby turning the media alkaline (Asai et al., 1964). Group I and group III colonies remained yellow under the same experimental conditions, indicating the absence of oxidation of acetate and lactate. Hence, the group I and group III isolates were presumed to belong to the genus *Gluconobacter* as these two biochemical tests are effective in differentiating isolates to genus level (Komagata et al., 2014).

4.3.1.7 Formation of H_2S and indole, motility and gelatine hydrolysis tests

Formation of H_2S, indole and motility tests were determined using the Sulphide Indole Motility (SIM) medium and gelatine hydrolysis test was carried out using the gelatine medium. For all the isolates in the three groups, there was a weak diffusion zone from the stabbed centre. However, there was no black formation along the stab line, and no red colour formed after adding Kovac's reagent in the SIM medium for all the isolates, which indicated the absence of H_2S, indole production and motility (Visser et al., 1985). The medium in the tube remained solid after cooling in the ice bath, which indicated negative results (Cruz & Torres, 2012). The AAB genera such as *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* cannot produce H_2S, indole and hydrolyse gelatine to liquid. The species from these three genera can be nonmotile or motile (Sievers & Swing, 2005). Hence, the results of the isolates of the three groups were consistent with the AAB properties due to the absence of H_2S, indole, motility and hydrolysis of gelatine (Sievers & Swing, 2005).

4.3.1.8 Production of cellulose

Bacterial cellulose has been widely applied in different areas such as wound-dressing and prolonged medicine dosage materials due to its excellent water absorption properties and ability to form strong, and flexible structures (Layasani et al., 2017). It is commonly produced by the AAB especially the genus *Komagataebacter* which can be isolated from Kombucha (Semjonovs et al., 2017). The Hestrin-Schramm (HS) medium was used to determine the production of cellulose in AAB (Layasani et al., 2017; Semjonoys et al., 2017). After incubation for 5 days, there were no pellicles formed on the surface of the medium and the transparent medium turned turbid. After heating 1.5-2 mL of the medium in 0.1N NaOH solution at 90ºC/30 min, there were no undissolved components were detected for all three groups of isolates; thus, indicating that there were no cellulose components produced.

4.3.1.9 Ketogenesis of glycerol to DHA

The production of DHA was confirmed with the presence of orange red colour on the plated glycerol yeast extract medium after the addition of Benedict's Reagent (Swing

et al., 1992). All three groups of isolates were able to grow in the glycerol yeast extract (GYE) medium after incubation at 30ºC for 5-7 days. Therefore, the isolates produced DHA by oxidising glycerol. DHA has been associated with the production of cosmetics, wine and medicine and using AAB species with glycerol is cost-effective. The AAB genera *Acetobacter*, *Gluconacetobacter*, *Gluconobacter* and *Komagataeibacter* can convert glycerol to DHA (Vu et al., 2016). DHA produces a sweet pleasant aroma and refreshing taste. In winemaking, AAB can utilise glycerol as a carbon source to produce DHA, which can react with several amino acids including proline to produce a "crust-like" taste (Drysdale & Fleet, 1988). Glucose and fructose are converted to glycerol by yeasts during Kombucha fermentation (Murugesan et al., 2009). Therefore, the production of DHA from glycerol by the three groups of isolates may affect the sensory properties of Kombucha.

4.3.1.10 Formation of γ-pyrone from D-glucose and D-fructose

Formation of γ-pyrone from D-glucose and D-fructose was confirmed with the appearance of a dark brown colour after adding 5% (w/v) $FeCl_3$ on glucose yeast extract and fructose yeast extract media respectively, following incubation for 5-7 days with shaking. The red colour developed immediately for the group I and group III isolates after adding a few drops of 5% (w/v) $FeCl_3$ solution on both D-glucose- and D-fructose-containing media, whereas group I formed a darker red colour than group III. In contrast, no obvious colour change was observed for the group II isolates. Therefore, the results suggest that group I and III isolates produced γ-pyrone components from both D-glucose and D-fructose. The γ-pyrone formation has been associated with the formation of water-soluble brownish pigment according to the synthesis pathway of γ-pyrone components (Kadere et al., 2008). Group I colonies were brown and group III colonies were light brown which is consistent with the presence of γ-pyrone components.

4.3.1.11 Carbohydrate fermentation test

Generally, *Acetobacter* species can ferment a limited range of carbohydrates and show variable reactions with D-glucose (Komagata et al., 2014). The species cannot produce acid from D-mannitol and glycerol (Mamlouk & Gullo, 2013). Some *Gluconobacter* species can produce acid from D-mannitol, glycerol, D-xylose and ethanol (Komagata

et al., 2014). The carbohydrate fermentation profiles of the isolates were determined according to their reactions in different media containing glucose, mannitol and glycerol, respectively. Group I and group III isolates produced acid from glucose, mannitol and glycerol after incubation for 7 days/30°C as the medium turned purple to yellow. In contrast, group II isolates did not produce acid from mannitol, glucose and glycerol which agreed with the previous study about *Acetobacter* conducted by Komagata et al. (2014). The ability to produce acid from different carbohydrate substrates can differentiate the isolates between the genera when used in conjunction with other biochemical reactions.

4.3.1.12 Nitrate reduction

Some *Acetobacter* species such as *Acetobacter pasteurianus* have demonstrated positive reaction with nitrate reduction, while other genera like *Gluconobacter* have not (Sievers & Swing, 2015). All three groups of isolates showed negative results of nitrate reduction by the development of cherry red colour after adding a small amount of Zn powder.

4.3.2 API 32C Tests of yeast isolates

Carbohydrate characterisation of 23 purified isolates in seven groups was carried out with API 32 tests following the manufacturer's instructions (https://apiweb.biomerieux.com/strip/12) and previous studies (Ramani et al., 1998). The formation of turbidity was considered positive. The metabolic profile of each group of isolates with different substrates is shown in Table 4.8. The metabolic patterns of the yeast isolates were different among the seven groups of microorganisms. According to the results, the only similarity was that all seven groups of yeasts metabolised glucose. Results showed that one yeast isolate (LO-yeast 1) in group I metabolised acitidione and one yeast (DO yeast 5) in group I metabolised D-trehalose. Results showed that all the yeasts from group II had positive results with D-saccharose and D-raffinose.

Table 4. 8 Carbohydrate metabolism of yeast isolates obtained from Kombucha samples sold in New Zealand using the API 32C test kit

Capsule	**Substrate**	**Group I** (LO-yeast 1)	**Group I** (DO-yeast 5)	**Group II** (LO-yeast 2, LO-yeast 3, LO-yeast 4)	**Group III** (LO-yeast 5)	**Group IV** (DO-yeast 1,2,3,4 and 6)	**Group V** (DO-yeast 7, DO-yeast 8, DO-yeast 9, GB-yeast 6, GB-yeast 7 GB-yeast 8)	**Group V** (DO-yeast 10)	**Group VI** (GB-yeast 1, GB-yeast 5)	**Group VII** (GB-yeast 2, GB-yeast 3, GB-yeast 4)
1.0	D-Galactose (GAL)	-	-	-	-	-	+	+	-	-
1.1	Cycloheximide （Actidione）(ACT)	+	-	-	-	+	-	+	-	+
1.2	D-sacharose (sucrose) (SAC)	-	-	+	+	-	-	+	-	+
1.3	N-Acetyl-Glucosamine (NAG)	-	-	-	-	-	-	-	+	+
1.4	Lactic acid (LAT)	-	-	-	-	-	-	-	+	+
1.5	L-Arabinose (ARA)	-	-	-	-	-	-	-	-	+
1.6	D-Cellobiose (CEL)	-	-	-	-	+	-	+	-	+
1.7	D-Raffinose (RAF)	-	-	+	-	-	-	+	-	+
1.8	D-Maltose (MAL)	-	-	-	-	-	-	-	-	+
1.9	D-Trehalose (TRE)	-	+	-	-	+	-	-	-	+
1.A	Potassium 2-Ketogluconate (2-KG)	-	-	-	-	-	-	+	-	+
1.B	Methyl-alpha D-Glucopyranoside (MDG)	-	-	-	-	-	-	-	-	+
1.C	D-Mannitol (MAN)	-	-	-	-	-	-	-	-	+
1.D	D-Lactose (bovine origin) (LAC)	-	-	-	-	-	-	-	-	+
1.E	Inositol (INO)	-	-	-	-	-	-	-	-	+
1.F	No substrate (0)	-	-	-	-	-	-	-	-	+
0.0	D-Sorbitol (SOR)	-	-	-	+	-	-	+	-	+
0.1	D-Xylose (XYL)	-	-	-	-	-	-	-	-	+

0.2	D-Ribose (RIB)	-	-	-	-	-	-	-	-	+
0.3	Glycerol (GLY)	-	-	-	-	-	-	-	+	+
0.4	L-Rhamnose (RHA)	-	-	-	-	-	-	-	-	+
0.5	Palatinose (PLE)	-	-	-	-	-	-	-	-	+
0.6	Erythritol (ERY)	-	-	-	-	-	-	-	-	+
0.7	D-melibiose (MEL)	-	-	-	-	-	-	-	-	+
0.8	Sodium Glucuronate (GRT)	-	-	-	-	-	-	-	-	+
0.9	D-Melezitose(MLZ)	-	-	-	-	-	-	-	-	+
0.A	Potassium Glucuronate (GNT)	-	-	-	-	-	-	-	-	+
0.B	Levulinic acid (Lavullinate) (LVT)	-	-	-	-	-	-	-	-	+
0.C	D-Glucose (GLU)	+	+	+	+	+	+	+	+	+
0.D	L-sorbose (SBE)	-	-	-	-	-	-	-	+	+
0.E	Glucosamine (GLN)	-	-	-	-	-	-	-	-	-
0.F	ESCulin ferric citrate (ESC)	-	-	-	-	-	-	-	-	+

Note: (+) indicated positive results; (-) indicated negative results

Results of the metabolic profiles of the seven groups were then analysed using the apiweb^{TM} identification system software (https://apiweb.biomerieux.com/strip/12) to determine the most probable yeast species. Five different yeast species were identified which comprised *Kloeckera japonica*，*Candida (C.) glabrata*，*Candida (C.) colliculosa*，*Candida(C.) krusei* and *Cryptococcus humicola* in groups I, IV, V, VI, and VII with high percentages of identity shown in Table 4.9. The isolate LO yeast 1 from the group I and all group IV yeast isolates had high identification scores to species of *Kloeckera japonica* although they exhibited different fermentation profiles. These two groups of yeast were probably different strains of the species *Kloechera japonica*. The API identifications showed low discrimination or doubtful results of isolates in groups II, II and V except for the DO-yeast 10. The standard API 32 C database has 63 yeast species (Deak & Beuchat, 1993). Therefore, some of the isolates from the three groups in Table 4.9 could not be identified. Additionally, geographic location may also affect the identification of isolates using the API 32C system as the ID 32C database was based on U.S isolates (Ranami et al.,1998). Therefore, it is necessary to use molecular methods for further identification.

Table 4. 9 Reactions of yeast isolates using the API 32C kit

Sample Group (isolated identification code)	Probable identified species	% Probable Identification[§]	*Description of identification
Group I (LO-yeast 1)	*Kloeckera japonica*	94.0	Good identification
Group I (DO-yeast 5)	*Candida glabrata*	99.0	Very good identification
Group II (LO-yeast 2, LO-yeast 3, LO-yeast 4)	ND	N/A	N/A
Group III (LO-yeast 5)	ND	N/A	N/A
Group IV (DO- yeast 1,2,3,4 and 6)	*Kloeckera japonica*	99.0	Excellent identification
Group V (DO-yeast 7, DO-yeast 8, DO-yeast 9, GB-yeast 6, GB-yeast 7 GB-yeast 8)	ND	N/A	N/A
Group V (DO-yeast 10)	*Candida colliculosa*	98.1	Good identification
Group VI (GB-yeast 1, GB-yeast 5)	*Candida krusei*	99.7	Very good identification
Group VII (GB-yeast 2, GB-yeast 3, GB-yeast 4)	*Cryptococcus humicola*	98.9	Good identification

Note: ND = no data; N/A =not applicable; *description of the identification obtained from the software output at： https://apiweb.biomerieux.com/strip/12

A broad range of yeasts has been reported in Kombucha products including those found in our study shown in Table 4.9 (Ramdani & Abulreesh, 2010). The genus *Kloeckera* which shows typical apiculate yeasts were reported in Kombucha (Mayser et al., 1995). There is scanty information on the presence of *Kloeckera japonica* in Kombucha and only one species, *Kloeckera apiculate* was isolated from Formosa Kombucha beverage called Haipao (Liu et al, 1996). The genus *Candida* was widely isolated from Kombucha produced in different regions with a few reports on *Candida glabrata* in Kombucha (Dufresne & Farnworth, 2000). The presence of *Candida glabrata* is reported to be pathogenic with potential to causing high frequent mucosal and systemic infection (Fidel et al., 1999). The presence of the pathogenic yeast in Kombucha may be caused by cross-contamination during processing. However, some black tea fermented Kombucha beverage exhibited antifungal activity against *C. glabrata* after 21 days incubation (Battikh et al., 2012). *Candida colliculosa* is the anamorph of *Torulaspra delbruekii* which has been isolated from Australian Kombucha samples (Teoh et al., 2004). This ubiquitous yeast has been used for winemaking manufacturing for a long time due to its positive effect on the flavour of alcoholic beverages. Previous studies reported that this yeast species has higher fermentation purity and produce low levels of volatile acidity and undesirable components such as acetaldehyde (van Breda et al., 2013; Zhang et al., 2018). Some cellulosic yeasts such as *Candida krusei* was also isolated from German Kombucha products (Mayser et al., 1995). There is limited research on the presence of *Cryptococcus humicola* in Kombucha sample (Table 4.9). Therefore, the identification of the yeast species present in Kombucha was not complete, hence, more investigation should be carried out to determine the diversity of yeast species and their characteristics during the fermentation.

4.3.3 Molecular identification of AAB isolates using 16S RNA sequencing

Three representative AAB from each group were subjected to molecular identification using the 16S RNA sequencing. DNA of three bacterial clones was amplified with universal primers 785F and 907R. The BLAST was used to match the 16S rRNA sequences to specific bacterial taxa. The results of the sequencing analysis of representative pure AAB isolates are shown in Table 4.10.

Table 4. 10 Sequenced representative AAB isolates

Group Sample /isolate code	Primer used	Most possible identification of isolate from NCBI[3]	[1]Accession number	Probable Identification (%)
Group I (LO-AAB1)	785F/907R	*Gluconobacter oxydans*	NR_026118.1	99
Group II (LO-AAB2)	785F/907R	*Acetobacter musti*	HM162854.1	100
Group III (GB-AAB1)	785F/907R	*Gluconobacter oxydans*	NR_026118.1	99

Note: [1]Accession number obtained from Macrogen (https://dna.macrogen.com/); [2]NCBI - National Centre for Biotechnology Information.

Isolates of group I and group III were identified as *Gluconobacter (G.) oxydans.* However, the morphology of the colonies of the two groups was different; colonies of group I were brownish red and those of group III were light brown. In general, the brown pigment is not produced by the isolates of *G. oxydans* (Malimas et al., 2009). However, a red brownish pigment was observed during the growth of *G. oxydans* DSM3504 and the formation of pigment may be associated with the accumulation of 2,5-diketogluconic acid (Elfari et al., 2005). Therefore, differences in the morphology of the two groups (I, III) in our study suggested that the AAB isolates were different strains of the *G. oxydans.* Strains of *G. oxydans* are commonly used for the microbial synthesis of DHA that is the main component of cosmetic tanning agent in the pharmaceutical industry (Mamlouk & Gullo, 2013). Other applications of *G. oxydans* are associated with its enzymes. A novel enzyme arylesterase was isolated from *G.. oxydans* in wine, which may catalyse the formation of wine aromas (Montet & Ray, 2016). Group II isolates were identified as *Acetobacter musti.* The type strain Bo7^T was first introduced and isolated from grape must (Ferrer et al., 2016). The application of this species is limited, and more research should be carried out.

Although some phenotypic characteristics of the isolates were consistent with the molecular identification to genus level, the conventional tests are not reliable and time-consuming (Ei-Salam, 2012). Also, it is difficult to correctly identify AAB to species level using only biochemical and physiological characteristics. Low-resolution DNA-based molecular identification techniques using 16S RNA loci are now commonly used to identify organisms to the genus or species (Gomes et al., 2018; Ei-Salam, 2012).

4.3.4 Molecular identification of yeast isolates using 26S RNA sequencing

Thirteen representative yeast isolates from seven groups were subjected to molecular identification with 26S RNA sequencing, amplified with universal primers LR0R and LR7 (Table 4.11). The BLAST was used to identify matching yeast taxa . The 26S RNA sequencing is a very effective technique used to identify an unknown yeast sample to species level if the percentage of its sequence matched to the database is over or similar at 99% (Kurtzman & Robnnet, 1998).

Table 4. 11 Sequenced representative yeast isolates

Group Sample/isolate code	Primer	Most possible Microorganisms from [3]NCBI	[1]Accession number	Identity (%)
Group I (LO-yeast 1, DO-yeast 5)	LR0R/LR7	*Dekkera bruxellensis*	AM8500551.1	99
Group II (LO-yeast 3)	LR0R/LR7	*Predicted :Lucillia*	XR_002762854	94
Group III (LO-yeast 5)	LR0R/LR7	*Schizosaccharomyces pombe*	NG_0426491.1	99
Group IV (DO-yeast 1)	LR0R/LR7	*Hanseniaspora valbyensis*	NG_042630.1	99
Group V (DO-yeast 7, DO-yeast 8, DO-yeast 9, DO-yeast 10, GB-yeast 6, GB-yeast 8)	LR0R/LR7	*Brettanomyces anomalus*	AM850055.1	99
Group VI (GB-yeast 1)	LR0R/LR7	*Pichia kudriavzevii*	KX538801.1	100
Group VII (GB-yeast 2)	LR0R/LR7	*Saccharomyces cerevisiae*	CP033481.1	100

Note: [1]Accession number obtained from Macrogen (https://dna.macrogen.com/); [2]NCBI-National Centre for Biotechnology Information.

Representative isolates of group I (Table 4.11) were identified as *Dekkera bruxellensis* and group V isolates were similar to the *Brettanomyces anomalus.* The genus *Dekkera* is the ascospores (spore-forming) form of the genus *Brettanomyces*. *Dekkera bruxellensis* is known as the teleomorph of *Brettanomyces of anomalus* (Schifferdecker et al., 2014), which was isolated from the sourdough, feta cheese and other spontaneous fermented alcoholic beers (Fadda et al., 2001; Meroth et al., 2003). The acid-producing genus is well-adapted to Kombucha fermentation and contributes to the flavour due to the formation of acetic acid and the acetic acid ester (Teoh et al., 2003). *D. bruxellensis* also contributes to the aroma characteristic in some French wines (Schifferdecker et al., 2014). Low amounts of this genus contribute to a positive effect on metabolites in bread,

Lambic beer and Kombucha (Schifferdecker et al., 2014). It is advantageous to use *Brettanomyces* spp. since it produces an exotic flavour such as pineapple in craft beers. The genus *Dekkera* exhibits high resistance to cycloheximide, which blocks the protein synthesis in eukaryotic organisms (Steensels et al., 2015). This may explain why some yeast isolates could grow on the GYPM plates despite the addition of cycloheximide.

Isolates from group III (Table 4.11) matched with the species *Schizosaccharomyces (S.) pombe.* The occurrence of this fission yeast in Kombucha, grape juice and wine have been reported (Teoh et al., 2003). This species is commonly found in Kombucha fermentation due to its high fermentative ability and tolerance to high sugar and low pH environment (Teoh et al., 2003; Loira et al., 2018). Moreover, *S. pombe* can release high quantities of polysaccharides during the alcoholic fermentation. The high concentration of polysaccharides in a fermented beverage such as sparkling wine can provide better taste and longer aromatic persistence (Loira et al., 2018). Group IV isolates (Table 4.11) belonged to *Hanseniaspora valbyensis* which is the teleomorph of *Kloeckera japonica.* The genus *Hanseniaspora* are apiculate yeasts and often isolated from fruits and berries. They can release high amounts of volatile components which form a pleasant aroma similar to cider (Mayser et al., 1995). Group VI yeast isolates (Table 4.11) was identified as *Pichia kudriavzevii,* which is the teleomorph of *Candida krusei,* and is known to produce a pellicle structure in Kombucha (Mayser et al., 1995). Group VII yeast isolates were identified as *Saccharomyces* (*S.*) *cerevisiae* (Table 4.11). *S. cerevisiae* is the most common yeast culture applied in modern alcoholic beverage due to its high fermentation efficiency and tolerance to ethanol (Walker & Stewart, 2016). It was isolated from beer, wine, whisky and Kombucha (Villarreal-Soto et al., 2018; Walker & Stewart, 2016). The complex microbial interactions between different yeast species could lower the risks of a stuck fermentation, contribute to the formation of aroma and flavours, and modify the undesired components (Villarreal-Soto et al., 2018). Although *S. cerevisiae* was included in the API 32 C database, group VII isolates had different biochemical characteristics to the reference profiles. The isolates may be the different strains of *S. cerevisiae.* Lower identity (94%) results were obtained from group II (*Predict: Lucilla*) which has been not isolated from previous Kombucha SCOBY. There was limited information about this unclear species. More investigation

should be conducted to determine the characteristics of the undefined species and the microbial interaction between different yeast cultures in Kombucha SCOBY.

Table 4. 12 Sequenced yeast isolates using API 32 C

Group Sample/isolate code	API 32 C identified species	Species identified by 26S rRNA sequencing
Group I (LO-yeast 1)	*Kloeckera japonica*	*Dekkera bruxellensis*
Group I (DO-yeast 5)	*Candida glabrata*	*Dekkera bruxellensis*
Group II (Group II LO-yeast 2, LO-yeast 3, LO-yeast 4)	n.d.	*Predicted :Lucillia*
Group III (LO-yeast 5)	n.d.	*Schizosaccharomyces pombe*
Group IV (DO- yeast 1,2,3,4 and 6)	*Kloeckera japonica*	*Hanseniaspora valbyensis*
Group V (DO-yeast 7, DO-yeast 8, DO-yeast 9, GB-yeast 6, GB-yeast 7 GB-yeast 8)	n.d.	*Brettanomyces anomalus*
Group V (DO-yeast 10)	*Candida colliculosa*	*Brettanomyces anomalus*
Group VI (GB-yeast 1, GB-yeast 5)	*Candia Krusei*	*Pichia kudriavzevii*
Group VII (GB-yeast 2, GB-yeast 3, GB-yeast 4)	*Cryptococcus humicola*	*Saccharomyces cerevisiae*

Note: n.d. = no data

There were six different species from 13 yeast isolates obtained at 99-100% identification and one unclear (doubtful) species with 94% identification according to the 26S rRNA sequencing analysis (Table 4.11). However, most results were different from API 32 C identifications shown in Table 4.12. The sequencing results agreed with the morphological groups rather than the API 32C tests. Only group IV and group VII sequenced results matched with API 32 tests, which indicated that the API 32 C kit may be not suitable for identifying the domestic Kombucha yeast isolates. The conventional biochemical tests can vary as the test conditions change (Latouche et al., 1997). Hence, phenotypic profiles are not stable and limited. The molecular techniques such as PCR are potentially more accurate, fast, easy to carry out and reliable than the phenotypic characterization in the identification of yeasts to species level (Latouche et al., 1997).

CHAPTER 5 CONCLUSIONS

Six types commercial Kombucha beverages sold in New Zealand were characterised for acidity, TSS, dominant AAB and yeasts. All the six Kombucha samples belonged to the high acid products with pH ≤4.6. The sample products were characterised by high variation of TSS ($p<0.05$). The analyses Kombucha samples produced in New Zealand contained yeasts and AAB while none of these microorganisms were detected in the imported samples. The AAB counts of the three locally produced Kombucha samples ranged from 4.97 and 5.63 log CFU/mL whereas the yeasts counts ranged from 4.75 to 5.69 log CFU/mL at the point of purchase. Two dominant Gram negative AAB isolates found in the Kombucha samples were identified as *Gluconobacter oxydan*s NR_026118.1 and *Acetobacter musti* HM162854.1. Seven dominant yeast isolates were identified as *Dekkera bruxellensis* AM8500551.1, *Schizosaccharomyces pombe* NG_0426491.1, *Hanseniaspora valbyensis* NG_042630.1, *Brettanomyces anomalus* AM850055.1, *Pichia kudriavzevii* KX538801.1, *Saccharomyces cerevisiae* CP033481.1 with one unclear isolate *predicted as Lucillia* XR_002762854. The AAB and yeasts identified in this study are commonly found in fermented Kombucha. It would be interesting to study their fermentation profiles in Kombucha during fermentation with the aim of producing a defined starter culture for the beverage which presently does not exist globally.

CHAPTER 6 RECOMMENDATIONS

1. In this study, *Gluconobacter oxydan*s NR_026118.1 and *Acetobacter musti* HM162854.1 were identified from two New Zealand Kombucha samples. Two isolates of *Gluconobacter oxydans* had different colonial appearance which suggested that they could be different strains. The molecular techniques such as real time PCR which can identify isolates to strain level are recommended for more accurate results (Andre-Barrao et al., 2017).

2. Currently, there is no commercialised selective medium developed for the isolation of AAB. Therefore, it is desirable to develop a rapid and reliable selective medium for isolation and cultivation of AAB from food samples.

3. According to our knowledge, there is no defined cultures for Kombucha fermentation. Therefore, the next natural step following this study would be to develop a defined starter culture for Kombucha with predictable performance. Such an endeavour would produce products with predictable consumer sensory characteristics and other attributes. This would assist producers to control the fermentation processes and for the regulatory authorities to develop standards for the products.

4. The presence of LAB in Kombucha fermentation has received little attention despite their probiotic properties and potential health benefits. Further, the role of LAB in Kombucha is poorly understood. It is therefore recommended to identify the LAB in Kombucha and study their association between the yeasts and AAB.

5. Some yeast strains are also responsible for the aromatic properties of Kombucha while the AAB provide the sourness and sparkling characteristics. Thus, further research on the fermentation of the isolates obtained in this study is recommended.

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CHAPTER 8 APPENDICES

A. Raw data

A.1 Acidity of six brand Kombucha samples

Table A.1a pH of Kombucha sample (n=3)

Sample	pH run 1	pH run 2	pH run 3	Mean±SD
LO	3.21	3.20	3.22	3.21±0.01
DO	3.90	3.91	3.89	3.90±0.01
GB	3.39	3.44	3.46	3.43±0.04
AM	3.53	3.54	3.55	3.54±0.01
RE	3.39	3.39	3.41	3.40±0.01
WO	3.46	3.46	3.43	3.45±0.02

Table A.1b Titratable acidity of Kombucha sample (n=3)

Sample	0.1N NaOH volume used to pH 7.0 (ml)	Titratable acidity (% of acetic acid)	Mean±SD
LO	18.50	0.37	0.42±0.04
	21.20	0.42	
	22.90	0.46	
DO	17.90	0.36	0.38±0.02
	19.10	0.38	
	19.60	0.39	
GB	18.70	0.37	0.38±0.03
	17.30	0.35	
	20.50	0.41	
AM	18.80	0.38	0.40±0.02
	20.20	0.40	
	21.00	0.42	
RE	17.90	0.36	0.40±0.04
	20.90	0.42	
	21.10	0.42	
WO	22.00	0.44	0.43±0.01
	20.70	0.41	
	21.70	0.43	

Calculation sample:

% Titratable acidity of acetic acid=$\frac{V_{NaOH} x N_{NaOH} x 6.0053}{V_{sample}}$

LO run 1= [18.5x0.1x6.0053]/18.5=0.37% of acetic acid

A.2 Total soluble solids of six kombucha samples

Table A.2 TSS of Kombucha (n=3)

Sample	TSS run 1 (ºBrix)	TSS run 2 (ºBrix)	TSS run 3 (ºBrix)	Mean±SD
LO	3.70	3.70	3.70	3.70±0.00
DO	7.00	7.00	7.00	7.00±0.00
GB	4.00	4.00	4.00	4.00±0.00
AM	2.50	2.50	2.40	2.47±0.06
RE	1.80	1.90	1.90	1.87±0.06
WO	6.50	6.50	6.50	6.50±0.00

A.3 Enumeration of AAB and yeast

Table A.3a AAB count of Kombucha sample (n=2)

Sample	Run 1 (log CFU/ml)	Run 2 (log CFU/ml)	Mean±SD
LO	4.93	5.01	4.97±0.06
GB	5.65	5.62	5.64±0.02

Table A.3b yeast count of Kombucha sample (n=2)

Sample	Run 1 (log CFUml)	Run 2 (log CFU/ml)	Mean±SD
LO	4.85	4.69	4.76±0.10
DO	5.61	5.52	5.57±0.07
GB	5.70	5.68	5.69±0.01

A.4 API 32 C Results

A.4a API 32 C profiles for purified yeasts

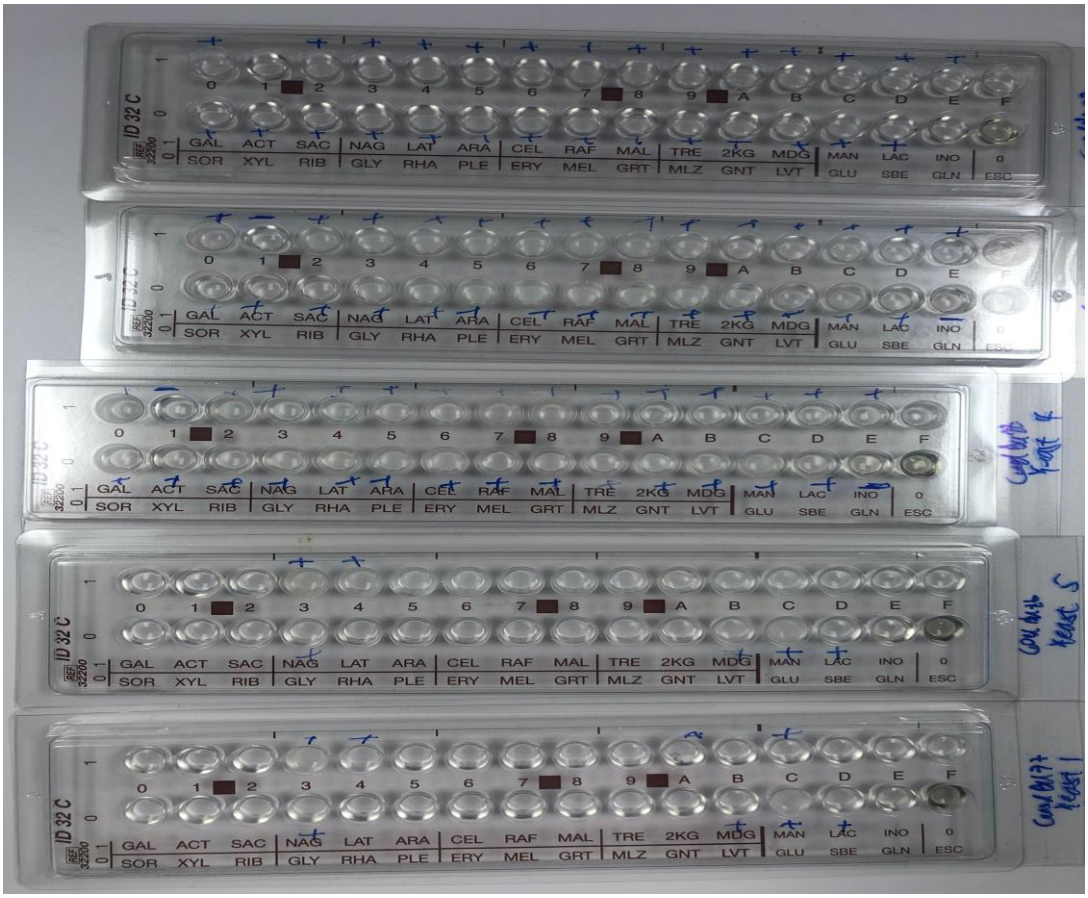


Figure A.4a Carbohydrate assimilation tests of different purified yeast cultures using API 32 C test kit

ID 32 C V4.0 Printout Export New test Modify

REFERENCE

DATE 5/19/20

COMMENT

GOOD IDENTIFICATION

Strip	ID 32 C V4.0
Profile	2 0 0 0 0 0 0 0 0 1
Note	

Significant taxa	**% ID**	**T**	**Tests against**			
Kloeckera japonica	**94.0**	**0.85**	**CEL 75%**			

Next taxon	**% ID**	**T**	**Tests against**			
Saprochaete capitata	**4.8**	**0.67**	**GLY 100%**			

Figure A.4b Results of API 32 C system (Group I-LO-yeast 1)

ID 32 C V4.0 Printout Export New test Modify

REFERENCE DATE 5/19/20

COMMENT

VERY GOOD IDENTIFICATION

Strip	ID 32 C V4.0
Profile	0 0 0 1 0 0 0 0 0 1
Note	

Significant taxa	% ID	T	Tests against			
Candida glabrata	99.0	1.0				

Next taxon	% ID	T	Tests against			
Kloeckera japonica	0.3	0.59	ACT 100%	CEL 75%		

Figure A.4c Results of API 32 C system (Group I -DO-yeast 5)

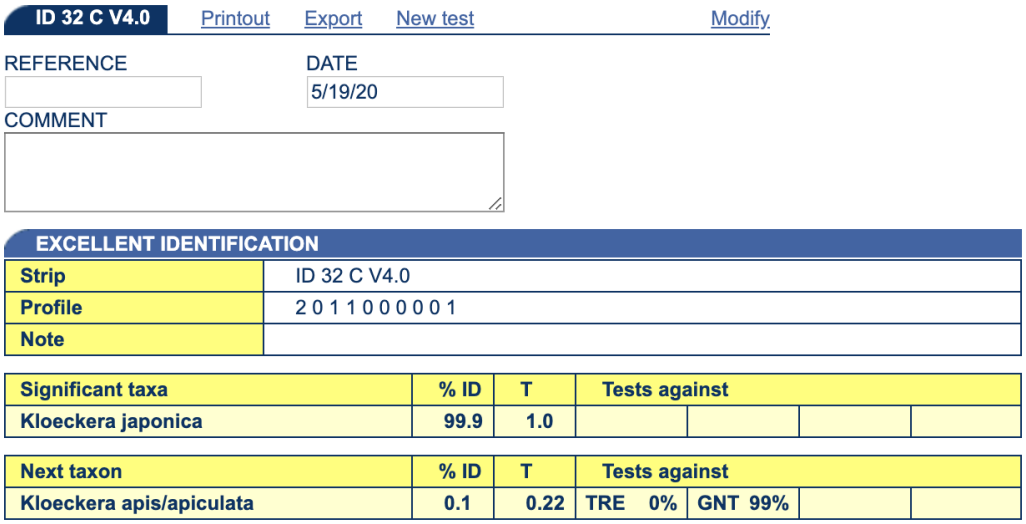


Figure A.4d Results of API 32 C system (Group IV)

ID 32 C V4.0 Printout Export New test Modify

REFERENCE DATE 5/19/20

COMMENT

GOOD IDENTIFICATION

Strip	ID 32 C V4.0
Profile	7 0 3 2 1 0 0 0 0 1
Note	ID. NOT VALID BEFORE 48 HOURS

Significant taxa	**% ID**	**T**	**Tests against**			
Candida colliculosa	**98.1**	**0.4**	**ACT 19%**	**LAT 82%**	**CEL 15%**	**MAN 78%**

Next taxon	**% ID**	**T**	**Tests against**			
Candida kefyr	**1.6**	**0.0**	**LAT 99%**	**CEL 12%**	**2KG 5%**	**XYL 83%**
			MAN 77%	**LAC 96%**		

Figure A.4e Results of API 32 C system (Group V-DO-yeast 10)

ID 32 C V4.0 Printout Export New test Modify

REFERENCE DATE 5/19/20

COMMENT

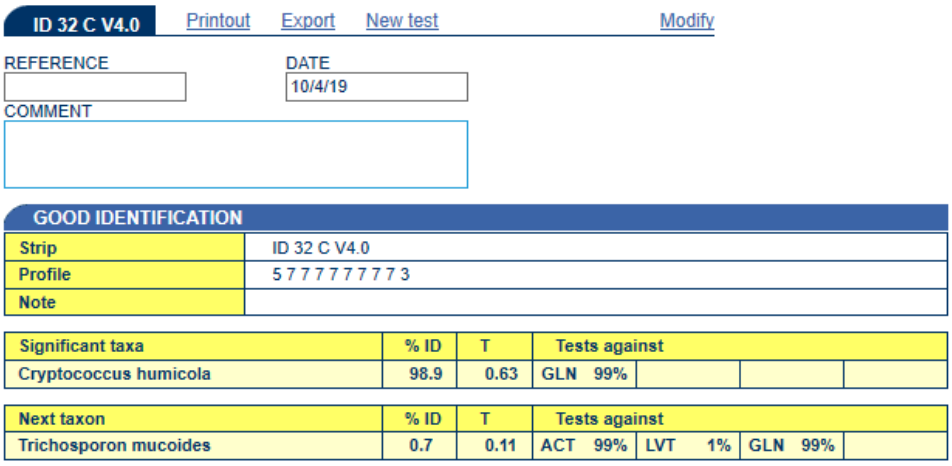
VERY GOOD IDENTIFICATION

Strip	ID 32 C V4.0
Profile	0 3 0 0 0 1 0 0 0 3
Note	ID. NOT VALID BEFORE 48 HOURS

Significant taxa	**% ID**	**T**	**Tests against**			
Candida krusei	**99.7**	**0.67**	**SBE 1%**			

Next taxon	**% ID**	**T**	**Tests against**			
Candida valida	**0.1**	**0.24**	**LAT 14%**	**SBE 0%**	**GLN 86%**	

Figure A.4f Results of API 32C system (Group VI)



ID 32 C V4.0 Printout Export New test Modify

REFERENCE

DATE 10/4/19

COMMENT

GOOD IDENTIFICATION

Strip	ID 32 C V4.0
Profile	5 7 7 7 7 7 7 7 7 3
Note	

Significant taxa	% ID	T	Tests against			
Cryptococcus humicola	98.9	0.63	GLN 99%			

Next taxon	% ID	T	Tests against					
Trichosporon mucoides	0.7	0.11	ACT 99%	LVT 1%	GLN 99%	

Figure A.5g Results of API 32C system (Group VII)

A.5 Sequenced representative purified AAB and yeast cultures

Table A.5a Sequenced representative purified AAB cultures

Sample	Primer 785F	Primer 970R
LO-AAB1	CGCCATGTGGTGCTGGATGTTGGGAACTTAGTTTTTCAGTGTCGAAGCT	TGGGCGTCTCCCCAGGCGGTGTGCTTAGCGCGTTAGCTTC
	AACGCGCTAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACT	GACACTGAAAAACTAAGTTTCCCAACATCCAGCACACATC
	CAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAA	GTTTACAGCGTGGACTACCAGGGTATCTAATCCTGTTTGCT
	TTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGCATGGGGAGGACCG	CCCCACGCTTTCGCGCCTCAGCGTCAGTATCGAGCCAGGT
	GTTCAGAGATGGACCTTTCTTCGGACCTCCCCGCACAGGTGCTGCATGG	TGCCGCCTTCGCCACCGGTGTTCTTCCCAATATCTACGAAT
	CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGC	TTCACCTCTACACTGGGAATTCCACAACCCTCTCTCGAACT
	GCAACCCTTGTCTTTAGTTGCCAGCACTTTCAGGTGGGCACTCTAGAGA	CTAGTCGTCACGTATCAAATGCAGTTCCCAGGTTAAGCCC
	GACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTCAAGTCCCT	GGGGATTTCACATCTGACTGTAACAACCGCCTACGCGCCC
	CATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGGTGACAG	TTTACGCCCAGTCATTCCGAGCAACGCTAGCCCCCTTCGT
	TGGGAAGCTATGTGGTGACACAATGCTGATCTCTAAAAGCCGTCTCAGT	ATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTT
	TCGGATTGTACTCTGCAACTCGAGTACATGAAGGTGGAATCGCTAGTAA	CTACGGGTACCGTCATCATCGTCCCCGTCGAAAGTGCTTT
	TCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACAC	ACAATCCGAAGACCTTCTTCACACACGCGGCATTGCTGGA
	CGCCCGTCACACCATGGGGAGTTGGTTCGACCTTAAGCCGGTGAGCGAA	TCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCT
	CCGCAAGGACGCAGCCGACCACGGACGGGTCAGCGACTGGGGTGAAGT	CCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCT
	CGACAAGGGGGGGCCCCAACAAGGGCACCCCCCGTTTTGCCCCCCCCCC	GATCATCCTCTCAAACCAGCTATCGATCATCGCCTTGGTA
	CCTCCGCGGCCCCGCCCGCCGTGGCGGGCCCCCGCGCCGTCGGCGCGGC	GGCCTTTACCCCACCAACTAGCTAATCGAACGCAGGTTCC

	CCCCCATCCCGCCGGGTTCCCCCCCCCCCGGGGGGGGACCGCCCCCCCC GTCTCGGCGGACCTCCGGTGCGCCGCGGGGGGCCCCCGCCGGCGCCCC GGGGACATCCCCGCGGGTTTCCCCCCCTCCCCGGGGGAGCCCCGCCCCC GCGTCCCTTTTCGGCCCGCCCCGTTTCCTTTCGATGGGGTCTATTTCCCC CTTCCCCTTTCCCCCCCCGGGTGGCTGGGCCCGGGGGGTTCGGCCGGGG GCCTTCCTTCTTTCGGTGGTGGGGTTTTTTTTTCCCCCCCCCCCCCCCGCG GGGGGCCTTTTTTTTTTTTTCTTTTTCCCCCCTCCTTTTCCCTCCTCCCCG GGCGGTTTTTGGGGGGTTGTCGGGGTGTGGGGGCCCCCTCTTGCCCCCG GTTTTTCCCCCCCCTTGGCGCGTCCTCCCCCCCCCCCTGGGGCGGGGGG GTGGGGGGGGTTTGCTTCCCCCCCCCGTG	TCCACAGGCGACTTGCGCCTTTGACCCTCAGGTATCATGC GGTATTAGCTCCAGTTTCCCGAAGTTGTCCCCCACCCGTG GATAGATCCCTACGCGTTACTCACCCGTCCGCCACTAAGG CCGAAACCTTCGTGCGACTTGCATGTGTTAAGCATGCCGC CAGCGTTCGCTCTACAAGAAAAAAACCTTAAAAAAAAAA AACCCTCTTTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTATTTTTTTATTTTATTTTTTTTTTTTTTTT AATATTTTTTATAAAAAAAAAAAAATTATTTTTTTTTTAAA ATTTAAATAAAAAAAATTTTTATAAATAAATATTTATAAA ATTTTTTTTTTTATTATTAAATTATTATTATTATTTATATAA AAAAAATAAAATATTTTATTTTTTTTTTTTATAAAAAATAA TTTTT
LO-AAB2	CCAATGTGTGCTGGATGTTGGGTGACTTAGTCATTCAGTGTCGTAGCTA ACGCGATAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTC AAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT TCGAAGCAACGCGCAGAACCTTACCAGGGCTTGTATGGAGAGGCTGTG TCCAGAGATGGGCATTTCCCGCAAGGGACCTTTCGCACAGGTGCTGCAT GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA GCGCAACCCTTATCTTTAGTTGCCAGCACGTCTGGGTGGGCACTCTAAA	CTGTGCGGGCGTCTCCAGGCGGTGTGCTTATCGCGTTAGC TACGACACTGAATGACTAAGTCACCCAACATCCAGCAATC GTTTACAGCGTGGACTACCAGGGTATCTAATCCTGTTTGCT CCCCACGCTTTCGCGCCTCAGCGTCAGTAATGAGCCGGTT GCCGCCTTCGCCACCGGTGTTCTTCCCAATATCTACGAATT TCACCTCTACACTGAGAATTCCACAACCCTCTCTCCACTCT AGTCTGCACGTATCAAATGCAGCTCCCAGGTTAAGCCCGG

	GAGACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTCAAGTCC TCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGGTGACA GTGGGAAGCCAGATGGCGACATCGTGCTGATCTCTAAAAGCCGTCTCAG TTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCTAGTA ATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACA CCGCCCGTCACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCGAA CCGCAAGGACGCAGCCGACCACGGTCGGGTCAGCGACTGGGGTGAATC AAAAGGGGGGGGCCCCAAAATAAAACCCTCAAGGTGTCCCTCCCCAAA TTAGGCTCCGGATTCCCGGGAGGTTATCCCCCTCCCCGGGGGCTGCATA CCTTAGTCGGTAACTGAGCCGGTGGGGGCGGTGAGGGGGAAGGCGTTC GGCGGGATTGGCAATGGGGGTACGCCATGGTGGGCGGGGGATCGGGCT GGCGGGGGGGAGGGCCGGGCCGGAGGGGAGGGAGGGCGGGGGGGGGAG GGTGGGGGGGGGGGGTGGGGGGGGGGGGGGGGTGGGGGGGGGGGGGGA GGGGGGGGGGGGGGGGGGGGGGGGAGGGGGGCGGGGGGGGGGGGTGT GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGTGGGG GGGTGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGAAG GGGGGGGGGGGCCCCGGGGGGGGGGGGGGGGGGGGGGGGG	GGATTTCACATCTGACTGTACAAACCGCCTACACGCCCTT TACGCCCAGTCATTCCGAGCAACGCTAGCCCCCTTCGTAT TACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCT GCGGGTACCGTCATCATCGTCCCCGCCGAAAGTGCTTTAC AATCCGAAAACCTTCTTCACACACGCGGCATTGCTGGATC AGGGTTGCCCCCATTGTCCAATATTCCCCACTGCTGCCTCC CGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGA TCATCCTCTCAGACCAGCTATCGATCATCGCCTTGGTAGG CCTTTACCCCACCAACAAGCTAATCGAACGCAGGCTCCTC CACAGGCGACTTGCGCCTTTGACCCTCAGGTGTCATGCGG TATTAGCTCCAGTTTCCCGGAGTTATCCCCCACCCATGGAC AGATACCTACGCGTTACTCACCCGTCCGCCACTAAGGCCG AAACCTTCGTGCGACTTGCATGTGTTAAGCATGCCGCCAG CGTTCGCTCTAGCGAAAAAGAAACCTTAAAAAAAAAAAT TAGCTTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT

		TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
GB-AAB1	CCGATGTGGTGCCTGGATGTTGGGAACTTAGTTTTTCAGTGTCGAAGCT AACGCGCTAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACT CAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAA TTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGCATGGGGAGGACCG GTTCAGAGATGGACCTTTCTTCGGACCTCCCGCACAGGTGCTGCATGGC TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG CAACCCTTGTCTTTAGTTGCCAGCACTTTCAGGTGGGCACTCTAGAGAG ACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTCAAGTCCTCA TGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGGTGACAGTG GGAAGCTACATGGTGACATGGTGCTGATCTCTAAAAGCCGTCTCAGTTC GGATTGTACTCTGCAACTCGAGTACATGAAGGTGGAATCGCTAGTAATC GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCG CCCGTCACACCATGGGAGTTGGTTCGACCTTAAGCCGGTGAGCGAACCG CAAGGACGCAGCCGACCACGGACGGGTCAGCGACTGGGGTGAGTCACA AGGGGGGGCCCCACAAAAAATTATCCCCCCCTTTGCCCCCCCCCCTCCC CCATACCTCACCCGGACGCCCGCCCCCCGGCCCGTGGGTGCGCCTCCGC AAGCGCCCGGATGCCACCCCCCCGGGGGGGGCTCCCGCCCCCTCGTCCG	CCAAGTCGCGTCTCCCAGGCGGTGTGCTTAGCGCGTTAGC TTCGACACTGAAAAACTAAGTTTCCCAACATCCAGCACAC ATCGTTTACAGCGTGGACTACCAGGGTATCTAATCCTGTTT GCTCCCCACGCTTTCGCGCCTCAGCGTCAGTATCGAGCCA GGTTGCCGCCTTCGCCACCGGTGTTCTTCCCAATATCTACG AATTTCACCTCTACACTGGGAATTCCACAACCCTCTCTCGA ACTCTAGTCGTCACGTATCAAATGCAGTTCCCAGGTTAAG CCCGGGGATTTCACATCTGACTGTAACAACCGCCTACGCG CCCTTTACGCCCAGTCATTCCGAGCAACGCTAGCCCCCTTC GTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTC TTCTACGGGTACCGTCATCATCGTCCCCGTCGAAAGTGCTT TACAATCCGAAGACCTTCTTCACACACGCGGCATTGCTGG ATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCC TCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGC TGATCATCCTCTCAAACCAGCTATCGATCATCGCCTTGGTA GGCCTTTACCCCACCAACTAGCTAATCGAACGCAGGTTCC TCCACAGGCGACTTGCGCCTTTGACCCTCAGGTATCATGC

	GACGCCCGGGCGGGGCCGGTGGCCCCGCCGGTCCTCCGGGGCGGGGCG GGGGGATTGCACCTCGGCCCCGGGGAGACCCCCCCACGCGGTGCCTTG GCGGGCGGGACGGTTCCCAAAGAAAGAACCCGCCCCCCCGCACATGAC CGCCCGAGGGCTGGGCAGGAAGATTACCCCACCCCTTCTTCTTAAAAAG AACGGGCATTCTTCCTCCCCCCTCCAAAAGAAGCCTTTGTTATTCCCAA AGAACCTTCCTTTTCCTCACGGCGGCCCTTGTGGGAAATAAAAACCTGG GCCCCCCCGCGTCAAAAAAAACCCCAATGGTTTCTCCCCCCCCCAAAAG AGAGGGTCCGGGGTGTTCTTTTCCCCACCGGGGGGGGAAATATTGTCTC CTCCCAAACCGGCAAAAACAAAAATATTGTGGCCTTTGGGGGGGGGGT TTTTTCCCCCCCCCCCCCCCCCCCCACTTCAAAGA	GGTATTAGCTCCAGTTTCCCGAAGTTGTCCCCCACCCGTG GATAGATCCCTACGCGTTACTCACCCGTCCGCCACTAAGG CCGAAACCTTCGTGCGACTTGCATGTGTTAAGCATGCCGC CAGCGTTCGCTCTAGAGGGGGAAAAATTAAAAAAAAAAA AAAGGCCCCGCCCCCCCCCCCCCCCCCCCCTCTCTTTTCTC TCTCCTCTCCCTCCCTCTCCCCCTCCCCCCTTCTTCCCCTCT CCCTTTCCTTCCCCTTTTTTTCCCTCTTTTCTTCCTCCCCCCC CCTTTTTTTTTTCTTTTTTTTTCCTCCCCTCTTCTTTCTCTTT TTTTTCTTTCCTTTTTTCCCCCCCCCCCTCTCTCTTTTTTTTT TTCTTTTTTCTCTCCCTTTCCTCTCCTCCCTTTTTTTCTTTCT CTTTTTTCTTCCCTTCTTTCTCCTCCTTTCCCCCCTTTTTTCC CCCCCCCCTCCTCTCTCCCTCTCTTTTCTTTCTCTCTCCCCC CCCCCCCCTTCCTTTCTTTTCTTCTCCCCTCTCTCTCTCTTCT CTTTTTCTCCTTTTCTTTTCCTTTTTTTTTTTCTCTCCCCCCCT CCCTCTCCTTCCTCCCCTTCCTCTTTTTCCTCCCTCTCCCCC TCCCTCCTCCTCCCCCCTTTTTTCTCTTTCCTCTTT

Table A.5b Sequenced representative purified yeast cultures

Sample	Primer LR0R	Primer LR7
LO-yeast 1	AATCGGGAGTAGAACAACAGGGATTGCCCCAGTAATGGCGAATGAAGCGG	CAACCTACCATGCTCACGCCAGAGGCTTCGTCACTGACCC
	CAAGAGCCCAAATTTGAAATCGGGCAACCGAGTTGTAATTTGGAGACGGG	CCACGCCTGCCTACTCGTCACAGAATTTATATTCCATCCGT
	ACACTAGAGAGGAGGAAGGCGATTAAGTGCCTTGGAACAGGCTGCCGTAG	GACGGCGAAGTATAGGTAACGCGCTTGAGCGCCATCCATT
	AGGGTGAGAGCCCCGTGAATCGCTGGAGACCGATCAATTAGTGCCCGCCG	TTCAGGGCTGGTTCATTCGGCCGGTGAGTTGTTACACAGT
	AAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTATATTCCAT	CCTTAGCGGTTTCCGACTTCCATGGCCACCGTCCGGCTGTC
	CTAAGGCTAAATATTAGCGAGAGACCGATAGCAAACAAGTACAGTGATGG	TAGATGGACCAACACCTTTTCTGGTGTCTGATGAGCGCGC
	AAAGATGAAAAGAACTTTGGAAAGAGAGTGAAATAGTACGTGAAATTGTT	ATTCCGGCACCTTAACTTCACGTTCGGTTCATCCCGCATCG
	GAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGGCCCGTTCC	CCAGTTCTGCTTACCAAAAATGGCCCACTAAAAGCTCTGC
	TCGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGTTCTGGGAG	ATTCAACTGTCCACGTTCAACTAAGCAACAAGGACTTCTT
	CCATATACGGGGTTCGTGAATGTGGCCCTTCGATTCTGTCGGAGGGTGTT	ACATATTTAAAGTTTGAGAATAGGCTGCAGCCTTTTCGGC
	ATAGCGCGGACATCTTGTGGCTAGCCGGGACCGGGGACTGCGGTGACTTG	CCCAGATCCTTTAATCATTCGCTTTACCTCATAAAACTGCT
	TCACCAAGGATGCTGGCAGAACGAGCAAATACCACCCGTCTTGAAACATG	ACGAGCTTCTGCTATCCTGAGGGAAACTTCGGCAGGAACC
	GACCAAGGAGACTAACGTCTATGCGAGTGTTTGGGTGGATAAAACCCATT	AGCTACTAGATGGTTCGATTAGTCTTTCGCCCCTATACCCA
	AACGCGGAATGAAAGTGAACGTAGGTCGGAGCCCCCTTGGGGGCGCACGA	AATTCGACGATCGATTTGCACGTCAGAACCGCTGCGAGCC
	TCGACCGATCCCGATGTTTTACCGGAGGGATTTGAGTAAGAGCATAGCTG	TCCACCAGAGTTTCCTCTGGCTTCACCCTATTCAGGCATAG
	TTGGGACCCGAAAGATGGTGAACTATGCCTGAATAGGGTGAAGCCAGAGG	TTCACCATCTTTCGGGTCCCAACAGCTATGCTCTTACTCAA
	AAACTCTGGTGGAGGCTCGCAGCGGTTCTGACGTGCAAATCGATCGTCGA	ATCCCTCCGGTAAAACATCGGGATCGGTCGATCGTGCGCC

	ATTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCTGGTTCC TGCCGAAGTTTCCCTCAGGATAGCAGAAGCTCGTAGCAGTTTTATGAGGT AAAGCGAATGATTAAAGGATCTGGGGCCGAAAAGGCTGCAGCCTATTCTC AAACTTTAAATATGTAAGAAGTCCTTGTTGCTTAGTTGAACGTGGACAGT TGAATGCAGAGCTTTTAGTGGGCCATTTTTGGTAAGCAGAACTGGCGATG CGGGATGAACCCAACGTGAAGTTTAAGGTGCCGGAATGGGCGCTCCTCCA CACCCCAAAAAGGGGTTGGTCCTCTTAAAACCCGGAGGGGGGCCTGGAAG TCGGAAACCCTTAAGACTGGGTATAACTCCCCGGCCGAAGAAACACCCCC	CCCAAGGGGGCTCCGACCTACGTTCACTTTCATTCCGCGTT AATGGGTTTTATCCACCCAAACACTCGCATAGACGTTAGT CTCCTTGGTCCATGTTTCAAGACGGGTGGTATTTGCTCGTT CTGCCAGCATCCTTGGTGACAAGTCACCGCAGTCCCCGGT CCCGGCTAGCCACAAGATGTCCGCGCTATAACACCCTCCG ACAGAATCGAAGGGCCACATTCACGAACCCCGTATATGGC TCCCAGAACCGATGCTGGCCCAGTGTAAACCAGGTGCACC CATCCACGAGGAACGGGCCGCTGCTAAACACCATGTCGG ATCAAATACCCTTCCCTTTCAACAATTTCACGTACTATTTC ACTCTCTTTCCAAAGTTCTTTTCATCTTTCCTCACTGTACTT GTTTGCTATCGGTCTCTCGCTAAAATTTAGCCTTAGAGGG AGATACACCCACTTAAAGTGCATTCCAACAAATTGAATCT TCGGCGGGGCTAATTGATCGGTTCCA
LO-yeast 3	AATCGGTAGTAGACAACAGGGATTGCCTTAGTAACGGCGAGTGAACAGGT AAGAGCTCAGATTTGAAAGGCACTTGTGCCGTTGTATTCTGAAGTTAGGA TTCTCGAAAACGACACCTAAGTTTTCTGGAAAGGAATGCCACAGAGGGTG ATAGCCCCGTACGGTGTTGACTCGATACAGAGTCCTAACATGGAGTCGAG TTGTTTGGGAATGCAGCTCAAATGGGTGGTATGCTCCATCTAAGGCTAAA TATTTGCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGAAAA	GAACGTGGATAGCTCCGCCCTAAGCTTCTACACAACCTCC ACGCCTGCCTACTCCACAACCGATAGTCACGGCTGTGGGC GAAGTATAGGTGGTACGCTTGAGCGCCATCCATTTTCAGG GCTAGTTCATTCGGCCGGTGAGTTGTTACACATTCCTTAGC GGATTCCGACTTCCATGGCCACCGTCCGGCTGTCTAGATG AACTAACACCTTTTGTGGTGTCTGATGAGCGTACACTCCG

	GAACTTTGAAAAGAGAGTGAAATAGTACGTGAAATTGTTGAAATGGAAGG	GCACCTTAACTTCGCGTTCGGTTCATCCCGCATCGCCAGTT
	GTAGGCCGCTAACCATGTAGGACCGTGTTTGGGGGGAAGATAAATGCTGT	CTGCTTACCAAAAATGGCCCACTAAAAGCTCTTCATTCAA
	AGAATGTGGCTCCTCGGAGTGTTATAGCTACAGTTCATGTTCCCATCCGA	GAGTCCAGGTTCACTTAAGTAACAAGGACGTTTTACATAT
	GCGCGAGGATCTCAGGTTCTACTAAATGGTGGTCTACCACCCGTCTTGAA	TCAAAGTTTGAGAATAGGTCAAGATCGTTTCAATCCCAAT
	ACACGGACCAAGGAGTCTATTGCCTGCGCGAGTATTTGGGTGTCAAACCC	ACCTCTAATCATTCGCTTTACCCCATAAAACTGATACGAG
	ATATGCGAAGTGAAAGCGAAAGATTCTAACTTTTGTTCAGAATCGGCCGA	CTTCTGCTATCCTGAGGGAAACTTCGGCAGGAACCAGCTA
	CCGTTTATAGAAACGGATTGAGTAGAGCGTAGGTGATGGGACCCGAAAGA	CTAGATGGTTCGATTAGTCTTTCGCCCCTATACCCAAATTT
	TGGTGAACTATACCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGGAGG	GACGATCGATTTGCACGTCAGAACCGCTGCGAGCCTCCAC
	CTCGCAGCGGTTCTGACGTGCAAATCGATCGTCAAATTTGGGTATAGGGG	CAGAGTTTCCTCTGGCTTCACCCTATTCAGGTATAGTTCAC
	CGAAAGACTAATCGAACCATCTAGTAGCTGGTTCCTGCCGAAGTTTCCCT	CATCTTTCGGGTCCCATCACCTACGCTCTACTCAATCCGTT
	CAGGATAGCAGAAGCTCGTATCAGTTTTATGGGGTAAAGCGAATGATTAG	TCTATAAACGGTCGGCCGATTCTGAACAAAAGTTAGAATC
	AGGTATTGGGATTGAAACGATCTTGACCTATTCTCAAACTTTGATATGTA	TTTCGCTTTCACTTCGCATATGGGTTTGACACCCAAATACT
	AAACGTCCTTGGTACTTAAGTGAACCTGGACTCTTGAATGAGAAGCTTTT	CGCGCAGGCAATAGACTCCTTGGGTCCGTGTTTCAAGACG
	AGGGGGCCATTTTTGGTAAGCAGAACTGGCGATGCGGGATGAACCGAACG	GGTGGTAGACCACCATTTAGTAGAACCTGAGATCCTCGCG
	CGAAGTTAAGGGGCCGGAGTGGACGCTCATCAAACCCCCAAAAAGGGGGT	CTCGGATGGGAACATGAACTGTAGCTATAACACTCCGAGG
	TAGTCTTTTAAAAACCCGGACGGGGGCCTGGGAAATCGGAACCCGCTAGG	AGCCACATTCTACAGCATTTATCTTCCCCCCAAACACGGT
	AAAGGGGGAAAAACCTCCCGGCCGGATGAATAACCCCCGAAGGGGGGGGG	CCTACATGGTTAGCGGCCTACCCTTCCATTTCAACAATTTC
	GTCCAGGGGCCCCCTATTTCCCCACCGGGGATTTTGTGTGGGGAGGGGGG	ACGTACTATTTCACTCTCTTTTTCAAAGTTCTTTTCATCTTT
		CCTTCACAGTACTTGTTTCGCTATCGGGCTCTCGCAAATAT

		TTAGCCTTAGATGGAGCATACCACCCATTTGAGCTGCATT CCCAACAACTCGACTCCCTGGTTAGGACTCTGGATCGAGT CAACACCGGACGGGGGTATTACCCTCCGGGGGGATTCCTT TCCAGAAAAACAAAAAGGGGGGGTTTTCGAGAAAACCT AAATTCCCAAAAAAAAAAGGGGCACAAGGGCTTTTCAAT TCGGAGTCTTACTGGGTCCCCCCGGTATAAGGAAAACCCC GG
LO- yeast 5	CATCCCGGTAAGACAAAGACCATGGTTCACTCAGTAACGGCGAGTGAAGC GGGAAAAGCTCAAATTTGAAATCTGTCGCATTTCTTTTGTTGTCCGAGTT GGAATTTCAAGAAGCTGCTTTGACTGTAGACCATCGGTCTAAGTTCCTTG GAACAAGACGTCAGACAGGGTGACAGGCCCTTCTTTGGGCGATTGGAAAT GCCATATAAACCCCCTTCGAATACTGAGTCGCTGGGCTGCCCCTCCCAAT GCCTGAGACATTGCATCGATAGTCAATATTGGTGAACCCCCGACTACGTA AAAGGCCAACGGACCAAATATGAAAAGAAATTTGAAAGACAATTAAACCC TCCGTGAAATTTCCGAAAGGGAAGCCTTTGAAATCAGTCTTACCCGTCCG AAATCCTTATTTTCTTCCCGAGACTATGAGCTCTGAACCTGTGGGACGTC AGCATCCCTTTTCGGGGGCGGAAAAAAAATAAGGGAAGGTGGCTTTCCCG GGTCCTGCTGGGGAGTGTTTATTCCCCTTGGTGTAATACGCTCACTGGGG AATGAGGACTGCCGCATCATGCCCAGGACGCTGACATATGGTTTTCACTG	ATCACTCTCGACAGCTCACGCCCAAGGCTTCGTCACAAAC CTCCACGCCTGCCTACTCGTCTGAGCTTCTCAAAGCTAACC CAGACGGTGAGGTATGGGTAGTACGCTTAAGCGCCATCCA TTTTCAGGGCTAGTTCATTCGGCAGGTGAGTTGTTACACA CTCCTTAGCGGATTCCGACTTCCATGGCCACCGTCCTGCTG TCTAGATGAACTAACACCTTTTCTGGTGTCTGATGAGCGT ACATTCCGGCACCTTAACCTCACGTTCGGTTCATCCCGCAT CGCCAGTTCTGCTTACCAAAAATGGCCCACTAGAAACTCT CATTCGATGGCCCACGTCCAATTAAGCGACAAGGGCGTCT TACATATTTAAAGTTTGAGAATAGGTTGAGGAAATTCCTT CCCCAAGACCTCTAATCATTCGCTTTACCTCATAAAACTG ATCTGAGTTTCTGCTATCCTGAGGGAAACTTCGGCAGGAA

	GCCCGTCTTGAAACACCGACCAAGGAGTCTAGCATCTATGCCAATGTTTG GGTGAAGAAAACCCATCCGCCAAATGAAAATGAATGCATGTGGGAACCCC CCTGTGGCGTGCACCATCTACCGACCCGGAAGTTTGTCCATGGAAGGGTT TGAGTAAGAACATATCTGTTGGGACCCCGCAAGATGGTGGAACTATGCCC TGAAATAGGGAGAAACCCGAGGAAACTCTGGTGGAGGCCTCGTAAAGATT CTGACGTGCAAATCGATCTTCAAATTTGGGTATAAGGGGCCGAAAGACTA AATCGAAACCATCTAGTAAGCTGGTTCCTTGCCGAAGTTTCCCTCCAGGA TAGCAGAAACTCCAGATCAGTTTTTATGGAGGTAAAGCCGAATGATTAGA GGTCTTGGGGAAAGGAATTTCCTCAACCTATTCCTCAAAACTTTAAATAT TGTAAAACCGCCCTTGTCGCCTAAATTGGAAAGTGGGCCCATCCGAATGG	CCAGCTACTAGATGGTTCGATTAGTCTTTCGCCCCTATACC CAAATTTGAAGATCGATTTGCACGTCAGAATCTCTACGAG CCTCCACCAGAGTTTCCTCTGGCTTCACCCTATTCAGGCAT AGTTCACCATCTTTCGGGTCCCAACAGCTATGCTCTTACTC AAACCCTTCCATTGACAAACTTCCGGGTCGGTCGATGGTG CACGCCACAAGGGCGTTCCCACCTGCATTCACTTTCATTTC GCGGATGGGTTTTCATCACCCAGACACTCGCATAGATGCT AGACTCCTTGGTCCGTGTTTCAAGACGGGCCATTGAAAAC CATTATGTCAGCATCCTTGGCACGAAGCCGCAGTCCTCAG TCCCCAGTGGACGTATTACAACAAGGGCTATAAACACTCC CCAGGCAGAACCCGGAAAGCCACCTTCCCTTATTCTTTTTC CGCCCCCGAAAACTGATGCTGACCTACCACAGGTTCAGAG TGCATAGTCTCGCGAAGAGACTACTGATCTCACCCAGGTA AGACTGATTTCCAATGCTTCCCTTTCAGCAATTTCACGTAC TATTTAACTCTCTTTTCAAAGTTCTTTTCATCTTTCGATCAC TCTACTTGTTCGCTATCGGTCTCTCGCCAATATTTAGCTTT AGATGAAATTTACCACCCATTTAGAGCTGCATTCCCAAAC AACTCGACTCTTCAAAAGGGCTTTTTTTGGCAATTCCAATC

		GACCAGAAAAGGGGGTTCGCCCCTCCCTGAAGTCCGGTTT CAAGGAA
DO- yeast 1	AATCGGGGAGAAGAACCACTGGGATTACCTTAGTAACGGCGAGTGAAGCG GTAAAAGCTCAAATTTGAAATCTGGTACTTTCAGTGCCCGAGTTGTAATT TGTAGAATTTGTTTTTGATTTGGTCCTTGTCTATGTTCCTTGGAACAGGA CGTCATAGAGGGTGAGAATCCCGTTTGGCGAGGATACCATTTCTTTGTAA AACTTTTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAAGTGGGTGG TAAATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGT ACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACG TGAAATTGTTGAAAGGGAAGGGCATTTGATCAGACATGGTGTTTTTTGCA TGCACTCGCCTCTCGTGGGCTTGGGCCTCTCAAAAATTTCACTGGGCCAA CATCAGTTTTGGCGGTAGGATAAATCATTAGGAACGTAGCTTCTTCGGGA GTATTATAGCCTAGTGGAATACTATCAGCCGGGACTGAGGACTGCGCTTT ATGCAAGGATGTTGGCATAATGGTTAAATGCCGCCCGTCTTGAAACACGG ACCAAGGAGTCTAACGTCTATGCGAGTGTTTGGGTGTAAAACCCGTACGC GTAATGAAAGTGAACGTAGGTTGGGGCCTTCGGGTGCACAATCGACCGAC CTTGATGTTTTCGGATAGGTTTGAGTAAGAGCATAGCTGTTGGGACCCGA AAGATGGTGAACTATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTG GAGGTTCGTAGCGGTTCTGACGTGCAAATCGATCGTCGAATTTGGGTATA	CGAGCGTACAAGCATCACTGCCTAGGCTTCGTCACTGACC TCCACGCCTGCCTACTCGTCAGGGCATCAAATTAACCCTG ACGGTGGAGTATAGGTAATACGCTTGAGCGCCATCCATTT TCAGGGCTAGTTCATTCGGCCGGTGAGTTGTTACACACTC CTTAGCGGATTCCGACTTCCATGGCCACCGTCCGGCTGTCT AGATGAACTAACACCTTTTGTGGTGTCTGATGAGCGTACA TTCCGGCACCTTAACTCCACGTTCGGTTCATCCCGCATCGC CAGTTCTGCTTACCAAAAATGGCCCACTAGAAACTCTTCA TTCATATGTCCACGTTCAATTAAGTAACAAGGACTTCTTAC ATATTTAAAGTTTGAGAATCGGTCAAGGACATTTCATCCC CGGTACCGCTAATCATTCGCTTTACCTCATAAAACTGATA CGAGTTTCTGCTATCCTGAGGGAAACTTCGGCAGGAACCA GCTACTAGATGGTTCGATTAGTCTTTCGCCCCTATACCCAA ATTCGACGATCGATTTGCACGTCAGAACCGCTACGAGCCT CCACCAGAGTTTCCTCTGGCTTCACCCTATTCAGGCATAGT TCACCATCTTTCGGGTCCCAACAGCTATGCTCTTACTCAAA CCTATCCGAAAACATCAAGGTCGGTCGATTGTGCACCCGA

	GGGGCGAAAGACTAATCGAACCATCTAGTAGCTGGTCCCTGCCGAAGTTT CCCTCAGGATAGCAGAAACTCGTATCAGTTTTATGAGGTAAAGCGAATGA TTAGCGGTACCGGGGATGAAATGTCCTTGACCGATTCTCAAACTTTAAAT ATGTAAGAAGTCCTTGTTACTTAATTGAACGTGGACATATGAAATGAAGA GTTTCTAGTGGGCCATTTTTGGTAAGCAGAACTGGCGATGCGGGATGAAC CGAACGTGGAGTTAAAGGGGCCGGAATGGACGCTCATCAGAACCCCCAA	AGGCCCCAACCTACGTTCACTTTCATTACGCGTACGGGTTT TACACCCAAACACTCGCATAGACGTTAGACTCCTTGGTCC GTGTTTCAAGACGGGCGGCATTTAACCATTATGCCAACAT CCTTGCATAAAGCGCAGTCCTCAGTCCCGGCTGATAGTAT TCCACTAGGCTATAATACTCCCGAAGAAGCTACGTTCCTA ATGATTTATCCTACCGCCAAAACTGATGTTGGCCCAGTGA AATTTTTGAGAGGCCCAAGCCCACGAAAAGGCGAGTGCA TGCAAAAAACACCATGTCTGATCAAATGCCCTTCCCTTTC AACAATTTCACGTACTTTTTCCTCTCTTTTTCAAAGTTCTTT TCATCTTTCATCACTGGACTTGGTTCGCTATCGGGCTCTCG CCACAATTTAGCTTTTAAAGGGAATTTACCACCCCACTTTG AGCTGCATTCCCAACAACTCGACTCTTCCAAAAAAGTTTA TAAAAGAAAAGGGATCCTCTCCCCACCGGA
DO- yeast 5	AACCCGTGGGCAAGACCAACAGGGATTGCCCCAGTAATGGCGAATGAAGC GGCAAGAGCCCAAATTTGAAATCGGGCAACCGAGTTGTAATTTGGAGACG GGACACTAGAGAGGAGGAAGGCGATTAAGTGCCTTGGAACAGGCTGCCGT AGAGGGTGAGAGCCCCGTGAATCGCTGGAGACCGATCAATTAGTGCCCGC CGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTATATTCC ATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAACAAGTACAGTGAT	CATTGTAACAGGCTCCGCCAGAGACTTCGTCACTGACCCC CACGCCTGCCTACTCGTCACAGAATTTATATTCCATCCGTG ACGGCGAAGTATAGGTAACGCGCTTGAGCGCCATCCATTT TCAGGGCTGGTTCATTCGGCCGGTGAGTTGTTACACAGTC CTTAGCGGTTTCCGACTTCCATGGCCACCGTCCGGCTGTCT AGATGGACCAACACCTTTTCTGGTGTCTGATGAGCGCGCA

	GGAAAGATGAAAAGAACTTTGGAAAGAGAGTGAAATAGTACGTGAAATTG	TTCCGGCACCTTAACTTCACGTTCGGTTCATCCCGCATCGC
	TTGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGGCCCGTT	CAGTTCTGCTTACCAAAAATGGCCCACTAAAAGCTCTGCA
	CCTCGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGTTCTGGG	TTCAACTGTCCACGTTCAACTAAGCAACAAGGACTTCTTA
	AGCCATATACGGGGTTCGTGAATGTGGCCCTTCGATTCTGTCGGAGGGTG	CATATTTAAAGTTTGAGAATAGGCTGCAGCCTTTTCGGCC
	TTATAGCGCGGACATCTTGTGGCTAGCCGGGACCGGGGACTGCGGTGACT	CCAGATCCTTTAATCATTCGCTTTACCTCATAAAACTGCTA
	TGTCACCAAGGATGCTGGCAGAACGAGCAAATACCACCCGTCTTGAAACA	CGAGCTTCTGCTATCCTGAGGGAAACTTCGGCAGGAACCA
	TGGACCAAGGAGACTAACGTCTATGCGAGTGTTTGGGTGGATAAAACCCA	GCTACTAGATGGTTCGATTAGTCTTTCGCCCCTATACCCAA
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	GATCGACCGATCCCGATGTTTTACCGGAGGGATTTGAGTAAGAGCATAGC	CCACCAGAGTTTCCTCTGGCTTCACCCTATTCAGGCATAGT
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	GGAAACTCTGGTGGAGGCTCGCAGCGGTTCTGACGTGCAAATCGATCGTC	TCCCTCCGGTAAAACATCGGGATCGGTCGATCGTGCGCCC
	GAATTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCTGGTT	CCAAGGGGGCTCCGACCTACGTTCACTTTCATTCCGCGTT
	CCTGCCGAAGTTTCCCTCAGGATAGCAGAAGCTCGTAGCAGTTTTATGAG	AATGGGTTTTATCCACCCAAACACTCGCATAGACGTTAGT
	GTAAAGCGAATGATTAAAGGATCTGGGGCCGAAAAGGCTGCAGCCTATTC	CTCCTTGGTCCATGTTTCAAGACGGGTGGTATTTGCTCGTT
	TCAAACTTTAAATATGTAAGAAGTCCTTGTTGCTTAGTTGAACGTGGACA	CTGCCAGCATCCTTGGTGACAAGTCACCGCAGTCCCCGGT
	GTTGAATGCAGAGCTTTTAGTGGGCCATTTTTGGTAAGCAGAACTGGCGA	CCCGGCTAGCCACAAGATGTCCGCGCTATAACACCCTCCG
	TGCGGGATGAACCGAACGTGAAATTAAGGTGCCGGAATGGCCGCTCTTCA	ACAGAATCGAAGGGCCACATTCACGAACCCCGTATATGGC
	GAACCCAGAAAAGGGGTGGGCCCTTTAGAAACCCGGAAGGGGGGGCTGGG	TCCCAGAACCGATGCTGGCCCAGTGTAAACCAGGTGCACC
		CATCCACGAGGAACGGGCCGCTGCTAAACACCATGTCGG

	AAAAACGGAAACCCTTGGGGGGGGAAAAAACCCCCGGGCCAAAAAACCAC CCCCCCAAAAAGGGGGGGGCCCCCCGCCCC	ATCAAATACCCTTCCCTTTCAACAATTTCACGTACTATTTC ACTCTCTTTTCCAAAAGTCTTTTCATCTTTCCTCACCGGGA CTTGTTTGCTATCGGGCTCTCGCTAAAATTTAACCCTAGAA GGGAGAAAACCACCCACTTTAAAGTGGTTTTCCAAAAAAA TTGAATTTTTCGCGGGGGACAAATTGATTGGGTTCCCCCG AATTACGGGGGTTTCCCCCCTACGGGGGCCGGGTCAAGGA CTAAAGCTTCCCCCCCCCTAGGGGCCGGTCCAAAAATAAA AAAGGGGGGG
DO- yeast 7	CACCGGGGAGCAGAACAACAGGGATTGCCCCAGTAATGGCGAATGAAGCG GCAAGAGCCCAAATTTGAAATCAGGCCCTCGTGGCTTGAGTTGTAATTTG GAGACGGGATACTAGAGAGAGGGAGGGCGACTAAGTGCCTTGGAACAGGCT GCCGTAGAGGGTGAGAGCCCCGTGAGTCGCGTGAACTCGATCAATTAGTGCC CGCCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTATATTC CATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAACAAGTAC AGTGATGGAAAGATGAAAAGAACTTTGGAAAGAGAGTGAAATAGTACGTG AAATTGTTGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGG CCCGTTCCTCGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGT TCTGGGAGCCATATACGGGGCAGGCGAATGTGGCCCTTCGATTCTGTCGG AGGGTGTTATAGCGCCAGCCAGACGTGGCTATCCGGGACCGGGGACTGCG	CAATCTCCAGGCTCACGCCACAGGCTTCGTCACTGACCCC CACGCCTGCCTACTCGTCACAGAGTTTGTATTCCATCCGTG ACGGCGAAGTATAGGTAACGCGCTTGAGCGCCATCCATTT TCAGGGCTGGTTCATTCGGCCGGTGAGTTGTTACACAATC CTTAGCGGTTTCCGACTTCCATGGCCACCGTCCGGCTGTCT AGATGGACCAACACCTTTTCTGGTGTCTGATGAGCGCGCA TTCCGGCACCTTAACTTCACGTTCGGTTCATCCCGCATCGC CAGTTCTGCTTACCAAAAATGGCCCACTAAAAGCTCTGCA TTCAACTGTCCACGTTCAACTAAGCAACAAGGACTTCTTA CATATTTAAAGTTTGAGAATAGGCTGAAGCCTTTTCGGCC TCAGATCCTTTAATCATTCGCTTTACCTCATAAAACTGCTA

	GCGATTCTATCGCCAAGGATGCTGGCAGAACGAGCAAATACCGCCCGTCT TGAAACATGGACCAAGGAGACTAACGTCTATGCGAGTGTTTGGGTGGATA AAACCCATTAACGCGGAATGAAAGTGAACGTAGGTCGGAGCCCTCTCGGG GGCGCACGATCGACCGATCCCGATGTTTTAGCGGAAGGATTTGAGTAAGA GCATAGCTGTTGGGACCCGAAAGATGGTGAACTATGCCTGAATAGGGTGA AGCCAGAGGAAACTCTGGTGGAGGCTCGCAGCGGTTCTGACGTGCAAATC GATCGTCGAATTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTA GCTGGTTCCTGCCGAAGTTTCCCTCAGGATAGCAGAAGCTCGTAGCAGTT TTATGAGGTAAAGCGAATGATTAAAGGACTGAGGCCGAAAAGGCTTCAGC CTATTCTCCAACTTTAAATATGTAAGAAGTCCTTGTTGCTTAATTGAACGTGG ACAGTTGAATGCAAACTTTTATGGGGCCTTTTTTGGTAAACAAAACTGGGGA TGCGGAATAAACCAACACGGGAAAATAAGGGGGCCGGAATGGCCCTCTCTA AACCCCAAAAAAGGGGGGGCCCTTTAAACCCGGGGGGGGCTGGG	CGAGCTTCTGCTATCCTGAGGGAAACTTCGGCAGGAACCA GCTACTAGATGGTTCGATTAGTCTTTCGCCCCTATACCCAA ATTCGACGATCGATTTGCACGTCAGAACCGCTGCGAGCCT CCACCAGAGTTTCCTCTGGCTTCACCCTATTCAGGCATAGT TCACCATCTTTCGGGTCCCAACAGCTATGCTCTTACTCAAA TCCTTCCGCTAAAACATCGGGATCGGTCGATCGTGCGCCC CCGAGAGGGCTCCGACCTACGTTCACTTTCATTCCGCGTT AATGGGTTTTATCCACCCAAACACTCGCATAGACGTTAGT CTCCTTGGTCCATGTTTCAAGACGGGCGGTATTTGCTCGTT CTGCCAGCATCCTTGGCGATAGAATCGCCGCAGTCCCCGG TCCCGGATAGCCACGTCTGGCTGGCGCTATAACACCCTCC GACAGAATCGAAGGGCCACATTCGCCTGCCCCGTATATGG CTCCCAGAACCGATGCTGGCCCAGTGTAAACCAGGTGCAC CCATCCACGAGGAACGGGCCGCTGCTAAACACCATGTCGG ATCAAATACCCTTCCCTTTCACCAATTTCACGTACTATTTC CTCTCTTTTCAAAGTCTTTTCATCTTTCCTCACTGGACTGGT TGGTATCGGGCTCTCGCTAAAATTTAGCCTTAGAGGAAGA ACCACCCACTTTAAGTGGTTTCCCAACAAATCGAATTTTTG TGGGGGTTATTGTTCAAGTTCCCCGACT

DO-yeast 8	AACGGTATGGAAGACCAACAGGGATTGCCCCAGTAATGGCGAATGAAGCG	AAAACCTGTAACCAGGGCTCCGCCACAGGCTTCGTCACTG
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	GAGACGGGATACTAGAGAGAGGGAGGGCGACTAAGTGCCTTGGAACAGGCT	CCGTGACGGCGAAGTATAGGTAACGCGCTTGAGCGCCATC
	GCCGTAGAGGGTGAGAGCCCCGTGAGTCGCGTGAACTCGATCAATTAGTGCC	CATTTTCAGGGCTGGTTCATTCGGCCGGTGAGTTGTTACAC
	CGCCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTATATTC	AATCCTTAGCGGTTTCCGACTTCCATGGCCACCGTCCGGCT
	CATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAACAAGTAC	GTCTAGATGGACCAACACCTTTTCTGGTGTCTGATGAGCG
	AGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAATAGTACGTG	CGCATTCCGGCACCTTAACTTCACGTTCGGTTCATCCCGCA
	AAATTGTTGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGG	TCGCCAGTTCTGCTTACCAAAAATGGCCCACTAAAAGCTC
	CCCGTTCCTCGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGT	TGCATTCAACTGTCCACGTTCAACTAAGCAACAAGGACTT
	TCTGGGAGCCATATACGGGGCAGGCGAATGTGGCCCTTCGATTCTGTCGG	CTTACATATTTAAAGTTTGAGAATAGGCTGAAGCCTTTTC
	AGGGTGTTATAGCGCCAGCCAGACGTGGCTATCCGGGACCGGGGACTGCG	GGCCTCAGATCCTTTAATCATTCGCTTTACCTCATAAAACT
	GCGATTCTATCGCCAAGGATGCTGGCAGAACGAGCAAATACCGCCCGTCT	GCTACGAGCTTCTGCTATCCTGAGGGAAACTTCGGCAGGA
	TGAAACATGGACCAAGGAGACTAACGTCTATGCGAGTGTTTGGGTGGATA	ACCAGCTACTAGATGGTTCGATTAGTCTTTCGCCCCTATAC
	AAACCCATTAACGCGGAATGAAAGTGAACGTAGGTCGGAGCCCTCTCGGG	CCAAATTCGACGATCGATTTGCACGTCAGAACCGCTGCGA
	GGCGCACGATCGACCGATCCCGATGTTTTAGCGGAAGGATTTGAGTAAGA	GCCTCCACCAGAGTTTCCTCTGGCTTCACCCTATTCAGGCA
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	GATCGTCGAATTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTA	GCCCCCGAGAGGGCTCCGACCTACGTTCACTTTCATTCCG
	GCTGGTTCCTGCCGAAGTTTCCCTCAGGATAGCAGAAGCTCGTAGCAGTT	CGTTAATGGGTTTTATCCACCCAAACACTCGCATAGACGT

	TTATGAGGTAAAGCGAATGATTAAAGGATCTGAGGCCGAAAAGGTTTAGC CTATTCTCAACTTTAATATGTAGAAAGTCCTTGTTGCTTAGTTGAACGTGGAC AGTTGAATGCAGAGCTTTTATGGGGCCATTTTTGGTAAGCAGAACTGGCGAT GCGGATGAACCGAACGGAAATTAAGGGGCCGGAAGGGGGGTCTTCAAACCC AAAAAAGGGGTGGGCCCTTTAAAACCCGAAGGGGGCCGGAAGGGAAACGCT AGGGTTTGTTAAACTCCCGCCCAAAGACCCCCCCCAAAAAGGGGGGCTCACC GGTCCTTTTTTTCCCCCCCGGGGGAAAAACATTTGG	TAGTCTCCTTGGTCCATGTGTCAAGACGGGCGGTATTTGCT CGTTCTGCCAGCATCCTTGGCGATAGAATCGCCGCAGTCC CCGGTCCCGGATAGCCACGTCTGGCTGGCGCTATAACACC CTCCGACAGAATCGAAGGGCCACATTCGCCTGCCCCGTAT ATGGCTCCCAGAACCGATGCTGGCCCAGTGTAAACCAGGT GCACCCATCCACGAGGAACGGGCCGCTGCTAAACACCAT GTCGGATCAAATACCCTTCCCTTTCACCAATTTCACGTACT ATTTCACTCTCTTTTCAAAGTTCTTTTCATCTTTCCATCACT GGACTTGTTTGCTATCGGGCTCTCGCTAATATTTAACCCTT AGATGGAAGATACCACCCACTTAGAACTGCATTCCCAAAA AATCGAATTTTTCGGCGGGGACTAATTGATTCGGGTTTCC CCGAATCCCGGGGGTTTTCCCCCCTCTGGAGACCCTGT
DO-yeast 9	AATCGTGAGCAAGAGCAACAGGGATTGCCCCAGTAATGGCGAATGAAGCG GCAAGAGCCCAAATTTGAAATCAGGCCCTCGTGGCTTGAGTTGTAATTTG GAGACGGGATACTAGAGAGAGGGAGGGCGACTAAGTGCCTTGGAACAGGCT GCCGTAGAGGGTGAGAGCCCCGTGAGTCGCGTGAACTCGATCAATTAGTGCC CGCCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTATATTC CATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAACAAGTACAGTGAT GGAAAGATGAAAAGAACTTTGGAAAGAGAGTGAAATAGTACGTG	CGAAACGTACCCAGGCTCCGCCACAGGCTTCGTCACTGAC CCCCACGCCTGCCTACTCGTCACAGAGTTTGTATTCCATCC GTGACGGCGAAGTATAGGTAACGCGCTTGAGCGCCATCCA TTTTCAGGGCTGGTTCATTCGGCCGGTGAGTTGTTACACA ATCCTTAGCGGTTTCCGACTTCCATGGCCACCGTCCGGCTG TCTAGATGGACCAACACCTTTTCTGGTGTCTGATGAGCGC GCATTCCGGCACCTTAACTTCACGTTCGGTTCATCCCGCAT

	AAATTGTTGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGG CCCGTTCCTCGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGT TCTGGGAGCCATATACGGGGCAGGCGAATGTGGCCCTTCGATTCTGTCGG AGGGTGTTATAGCGCCAGCCAGACGTGGCTATCCGGGACCGGGGACTGCG GCGATTCTATCGCCAAGGATGCTGGCAGAACGAGCAAATACCGCCCGTCT TGAAACATGGACCAAGGAGACTAACGTCTATGCGAGTGTTTGGGTGGATA AAACCCATTAACGCGGAATGAAAGTGAACGTAGGTCGGAGCCCTCTCGGG GGCGCACGATCGACCGATCCCGATGTTTTAGCGGAAGGATTTGAGTAAGA GCATAGCTGTTGGGACCCGAAAGATGGTGAACTATGCCTGAATAGGGTGA AGCCAGAGGAAACTCTGGTGGAGGCTCGCAGCGGTTCTGACGTGCAAATC GATCGTCGAATTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTA GCTGGTTCCTGCCGAAGTTTCCCTCAGGATAGCAGAAGCTCGTAGCAGTT TTATGAGGTAAAGCGAATGATTAAAGGATCTGAGGCCGAAAAGGCTTCAG CCTATTCTCAAACTTTAAATATGTAAGAAGTCCTTGTTGCTTAATTGAACGTG GACAGTTGAATGCAGAGCTTTTTATGGGGCCTTTTTTGGTAAGCAGAACTGG CGATGCGGGATGAACCCAACGGGAAAATTAAGGTGCCGGAATGGGCGCTCT TCAAAACCACAAAAAAGGGGTGGGGCCCTCTTAAAAACCCGACGGGGGGCC TTGGAAATCGGAAACCC	CGCCAGTTCTGCTTACCAAAAATGGCCCACTAAAAGCTCT GCATTCAACTGTCCACGTTCAACTAAGCAACAAGGACTTC TTACATATTTAAAGTTTGAGAATAGGCTGAAGCCTTTTCG GCCTCAGATCCTTTAATCATTCGCTTTACCTCATAAAACTG CTACGAGCTTCTGCTATCCTGAGGGAAACTTCGGCAGGAA CCAGCTACTAGATGGTTCGATTAGTCTTTCGCCCCTATACC CAAATTCGACGATCGATTTGCACGTCAGAACCGCTGCGAG CCTCCACCAGAGTTTCCTCTGGCTTCACCCTATTCAGGCAT AGTTCACCATCTTTCGGGTCCCAACAGCTATGCTCTTACTC AAATCCTTCCGCTAAAACATCGGGATCGGTCGATCGTGCG CCCCCGAGAGGGCTCCGACCTACGTTCACTTTCATTCCGC GTTAATGGGTTTTATCCACCCAAACACTCGCATAGACGTT AGTCTCCTTGGTCCATGTTTCAAGACGGGCGGTATTTGCTC GTTCTGCCAGCATCCTTGGCGATAGAATCGCCGCAGTCCC CGGTCCCGGATAGCCACGTCTGGCTGGCGCTATAACACCC TCCGACAGAATCGAAGGGCCACATTCGCCTGCCCCGTATA TGGCTCCCAGAACCGATGCTGGCCCAGTGTAAACCAGGTG CACCCATCCACGAGGAACGGGCCGCTGCTAAACACCATGT CGGATCAAATACCCTTCCCTTTCAACAATTTCACGTACTAT

		TTCACTCTCTTTCCAAAGTTCTTTTCATCTTTCCATCACTGG ACTTGGTTGCTATCGGGCTCTCGCTAATATTTAGCCTTAGA AGGAAATATCAC
DO- yeast 10	ACGGGAGTAAGAAAACAACAGGGATTGCCCCAGTAATGGCGAATGAAGCG GCAAGAGCCCAAATTTGAAATCAGGCCCTCGTGGCTTGAGTTGTAATTTG GAGACGGGATACTAGAGAGAGGGAGGGCGACTAAGTGCCTTGGAACAGGCT GCCGTAGAGGGTGAGAGCCCCGTGAGTCGCGTGAACTCGATCAATTAGTGCC CGCCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTATATTC CATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAACAAGTACAGTGAT GGAAAGATGAAAAGAACTTTGGAAAGAGAGTGAAATAGTACGTGAAATTGT TGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGGCCCGTTCCT CGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGTTCTGGGAGCCA TATACGGGGCAGGCGAATGTGGCCCTTCGATTCTGTCGGAGGGTGTTATAGC GCCAGCCAGACGTGGCTATCCGGGACCGGGGACTGCGGCGATTCTATCGCCA AGGATGCTGGCAGAACGAGCAAATACCGCCCGTCTTGAAACATGGACCAAG GAGACTAACGTCTATGCGAGTGTTTGGGTGGATAAAACCCATTAACGCGGAA TGAAAGTGAACGTAGGTCGGAGCCCTCTCGGGGGCGCACGATCGACCGATC CCGATGTTTTAGCGGAAGGATTTGAGTAAGAGCATAGCTGTTGGGACCCGAA AGATGGTGAACTATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGGA	CAAACCGTACCATGCTCACGCCAACAGGCTTCGTCACTGA CCCCCACGCCTGCCTACTCGTCACAGAGTTTGTATTCCATC CGTGACGGCGAAGTATAGGTAACGCGCTTGAGCGCCATCC ATTTTCAGGGCTGGTTCATTCGGCCGGTGAGTTGTTACAC AATCCTTAGCGGTTTCCGACTTCCATGGCCACCGTCCGGCT GTCTAGATGGACCAACACCTTTTCTGGTGTCTGATGAGCG CGCATTCCGGCACCTTAACTTCACGTTCGGTTCATCCCGCA TCGCCAGTTCTGCTTACCAAAAATGGCCCACTAAAAGCTC TGCATTCAACTGTCCACGTTCAACTAAGCAACAAGGACTT CTTACATATTTAAAGTTTGAGAATAGGCTGAAGCCTTTTC GGCCTCAGATCCTTTAATCATTCGCTTTACCTCATAAAACT GCTACGAGCTTCTGCTATCCTGAGGGAAACTTCGGCAGGA ACCAGCTACTAGATGGTTCGATTAGTCTTTCGCCCCTATAC CCAAATTCGACGATCGATTTGCACGTCAGAACCGCTGCGA GCCTCCACCAGAGTTTCCTCTGGCTTCACCCTATTCAGGCA TAGTTCACCATCTTTCGGGTCCCAACAGCTATGCTCTTACT

	GGCTCGCAGCGGTTCTGACGTGCAAATCGATCGTCGAATTTGGGTATAGGGG CGAAAGACTAATCGAACCATCTAGTAGCTGGTTCCTGCCGAAGTTTCCCTCA GGATAGCAGAAGCTCGTAGCAGTTTTATGAGGTAAAGCGAATGATTAAAGG ATCTGAGGCCGAAAAGGCTTCAGCCTATTCTCAAACTTTAAATATGTAAGAA GTCCTTGTTGCTTAGTTGAACGTGGACAGTTGAATGCAGAGCTTTTATGGGG CCTTTTTGGTAAGCAGAACTGGCGATGCGGGATGAACCCAAACGGAAATTA AGGGGCCGGAATGGCCGC	CAAATCCTTCCGCTAAAACATCGGGATCGGTCGATCGTGC GCCCCCGAGAGGGCTCCGACCTACGTTCACTTTCATTCCG CGTTAATGGGTTTTATCCACCCAAACACTCGCATAGACGT TAGTCTCCTTGGTCCATGTTTCAAGACGGGCGGTATTTGCT CGTTCTGCCAGCATCCTTGGCGATAGAATCGCCGCAGTCC CCGGTCCCGGATAGCCACGTCTGGCTGGCGCTATAACACC CTCCGACAGAATCGAAGGGCCACATTCGCCTGCCCCGTAT ATGGCTCCCAGAACCGATGCTGGCCCAGTGTAAACCAGGT GCACCCATCCACGAGGAACGGGCCGCTGCTAAACACCAT GTCGGATCAAATACCCTTCCCTTTCAACAATTTCACGTACT ATTTCACTCTCTTTTCAAAGTTCTTTTCATCTTTCCATCACC GGGACTTGGTTGGCTATCGGGCTCCTCGCTAAAATTTAAC CCTTAGAAGGGAATATACCACCCACTTTAAACTGGATCCC CAAAAAAATCGAATCTTTTGGGGGGGCCTAATTGGTTCAA GTTCACCCGAATCCCGGGGGTTTTCCCCCTTTTAGGGAGC
GB-yeast 1	ATCCCGGAAGGAAAGACAACAGGGATTGCCTCAGTAGCGGCGAGTGAAGC GGCAAGAGCTCAGATTTGAAATCGTGCTTTGCGGCACGAGTTGTAGATTG CAGGTTGGAGTCTGTGTGGAAGGCGGTGTCCAAGTCCCTTGGAACAGGGC GCCCAGGAGGGTGAGAGCCCCGTGGGATGCCGGCGGAAGCAGTGAGGCCCT	AAATGCGTCGACAGCTCCGCCCAGGCTTCGTCACAAACCC CCACGCCTGCCTACTCGCCAAGGCCTTGCGCCATGGCGGC GAAGTATAGGTAACACGCTTGAGCGCCATCCATTTTCAGG GCTAGTTCATTCGGCCGGTGAGTTGTTACACACTCCTTAGC

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	GAAGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAACAGCACGTGAAA	GCACCTTAACTTCGCGTTCGGTTCATCCCGCATCGCCAGTT
	TTGTTGAAAGGGAAGGGTATTGCGCCCGACATGGGGATTGCGCACCGCTG	CTGCTTACCAAAAATGGCCCACTAAAAGCTCTGCATTCAA
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	GGCCAGATGCTGCGTGCGGGGACCGAGGACTGCGGCCGTGTAGGTCACGGA	ACGTCTAATCATTCGCTTTACCTCATAAAACTGATACGAG
	TGCTGGCAGAACGGCGCAACACCGCCCGTCTTGAAACATGGACCAAGGAGT	CTTCTGCTATCCTGAGGGAAACTTCGGCAGGAACCAGCTA
	CTAACGTCTATGCGAGTGTTTGGGTGTGAAACCCGTACGCGTAATGAA	CTAGATGGTTCGATTAGTCTTTCGCCCCTATACCCAAATTC
	AGTGAACGTAGGTCGGACCCCCTGCCCTCGGGGAGGGGAGCACGATCGAC	GACGATCGATTTGCACGTCAGAACCGCTACGAGCCTCCAC
	CGATCCCGATGTTTATCGGAAGGATTTGAGTAGGAGCATAGCTGTTGGGA	CAGAGTTTCCTCTGGCTTCACCCTATTCAGGCATAGTTCAC
	CCCGAAAGATGGTGAACTATGCCTGAATAGGGTGAAGCCAGAGGAAACTC	CATCTTTCGGGTCCCAACAGCTATGCTCCTACTCAAATCCT
	TGGTGGAGGCTCGTAGCGGTTCTGACGTGCAAATCGATCGTCGAATTTGG	TCCGATAAACATCGGGATCGGTCGATCGTGCTCCCCTCCC
	GTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCTGGTTCCTGCCGA	CGAGGGCAGGGGGTCCGACCTACGTTCACTTTCATTACGC
	AGTTTCCCTCAGGATAGCAGAAGCTCGTATCAGTTTTATGAGGTAAAGCG	GTACGGGTTTCACACCCAAACACTCGCATAGACGTTAGAC
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	TAATATGTAAGAAGTCCCTGTTGCTTATTGAACGCGGACGTTTGAATGCA	TGCCAGCATCCGTGACCTACACGGCCGCAGTCCTCGGTCC
	GAGCTTTTATGGGGCCATTTTTGGTAAGCAGAACTGGCGATGCGGGATGA	CCGCACGCAGCATCTGGCCCTGGCTATAACACTCCGAAGA
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GB-yeast 2	CAACGGAAGAAGGACAAACCGGGATTGCCTTAGTAACGGCGAGTGAAGCG GCAAAAGCTCAAATTTGAAATCTGGTACCTTCGGTGCCCGAGTTGTAATT TGGAGAGGGCAACTTTGGGGCCGTTCCTTGTCTATGTTCCTTGGAACAGG ACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTA AAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTG GTAAATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAG TACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTAC GTGAAATTGTTGAAAGGGAAGGGCATTTGATCAGACATGGTGTTTTGTGC CCTCTGCTCCTTGTGGGTAGGGGAATCTCGCATTTCACTGGGCCAGCATC AGTTTTGGTGGCAGGATAAATCCATAGGAATGTAGCTTGCCTCGGTAAGT	CGAAAGTCGACCGACCTTACGGTCTAGGCTTCGTCACTGA CCTCCACGCCTGCCTACTCGTCAGGGCATCATATCAACCC TGACGGTAGAGTATAGGTAACACGCTTGAGCGCCATCCAT TTTCAGGGCTAGTTCATTCGGCCGGTGAGTTGTTACACACT CCTTAGCGGATTCCGACTTCCATGGCCACCGTCCGGCTGT CTAGATGAACTAACACCTTTTGTGGTGTCTGATGAGCGTG TATTCCGGCACCTTAACTCTACGTTCGGTTCATCCCGCATC GCCAGTTCTGCTTACCAAAAATGGCCCACTAAAAGCTCTT CATTCAAATGTCCACGTTCAATTAAGTAACAAGGACTTCT TACATATTTAAAGTTTGAGAATAGGTCAAGGTCATTTCGA

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GB- yeast 6	AACAGGTAGAAAGACCAACAGGGATTGCCCCAGTAATGGCGAATGAAGCG GCAAGAGCCCAAATTTGAAATCAGGCCCTCGTGGCTTGAGTTGTAATTTG GAGACGGGATACTAGAGAGAGGGAGGGCGACTAAGTGCCTTGGAACAGGCT GCCGTAGAGGGTGAGAGCCCCGTGAGTCGCGTGAACTCGATCAATTAGTGCC CGCCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTATATTC CATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAACAAGTACAGTGAT GGAAAGATGAAAAGAACTTTGGAAAGAGAGTGAAATAGTACGTGAAATTGT TGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGGCCCGTTCCT CGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGTTCTGGGAGCCA TATACGGGGCAGGCGAATGTGGCCCTTCGATTCTGTCGGAGGGTGTTATAGC GCCAGCCAGACGTGGCTATCCGGGACCGGGGACTGCGGCGATTCTATCGCCA AGGATGCTGGCAGAACGAGCAAATACCGCCCGTCTTGAAACATGGACCAAG GAGACTAACGTCTATGCGAGTGTTTGGGTGGATAAAACCCATTAACGCGGAA TGAAAGTGAACGTAGGTCGGAGCCCTCTCGGGGGCGCACGATCGACCGATC CCGATGTTTTAGCGGAAGGATTTGAGTAAGAGCATAGCTGTTGGGACCCGAA	AAATTGATACCAGGCTCACGCCACAGGCTTCGTCACTGAC CCCCACGCCTGCCTACTCGTCACAGAGTTTGTATTCCATCC GTGACGGCGAAGTATAGGTAACGCGCTTGAGCGCCATCCA TTTTCAGGGCTGGTTCATTCGGCCGGTGAGTTGTTACACA ATCCTTAGCGGTTTCCGACTTCCATGGCCACCGTCCGGCTG TCTAGATGGACCAACACCTTTTCTGGTGTCTGATGAGCGC GCATTCCGGCACCTTAACTTCACGTTCGGTTCATCCCGCAT CGCCAGTTCTGCTTACCAAAAATGGCCCACTAAAAGCTCT GCATTCAACTGTCCACGTTCAACTAAGCAACAAGGACTTC TTACATATTTAAAGTTTGAGAATAGGCTGAAGCCTTTTCG GCCTCAGATCCTTTAATCATTCGCTTTACCTCATAAAACTG CTACGAGCTTCTGCTATCCTGAGGGAAACTTCGGCAGGAA CCAGCTACTAGATGGTTCGATTAGTCTTTCGCCCCTATACC CAAATTCGACGATCGATTTGCACGTCAGAACCGCTGCGAG CCTCCACCAGAGTTTCCTCTGGCTTCACCCTATTCAGGCAT

	AGATGGTGAACTATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGGA GGCTCGCAGCGGTTCTGACGTGCAAATCGATCGTCGAATTTGGGTATAGGGG CGAAAGACTAATCGAACCATCTAGTAGCTGGTTCCTGCCGAAGTTTCCCTCA GGATAGCAGAAGCTCGTAGCAGTTTTATGAGGTAAAGCGAATGATTAAAGG ATCTGAGGCCGAAAAGGCTTCAGCCTATTCTCAACTTTAATATGTAAGAAAT CCTTGTTGCTTATTTGAACGGGGACAGTTGAATGCGAAGTTTTATGGGGCCTT TTTGGGAAGCAAAATGGCGATGCGGATGAACCCAACGTGAAAATTAAGGGG CGGAAGGTGGTCTTCAAACCAAAAAAAGGGTGGGGCCTTTTAAAACCCGAA CGGGGGCCTGGGAAAGGGAAACGCCCCGGGGTGGGGGAAAACTCCCCGCCC AG	AGTTCACCATCTTTCGGGTCCCAACAGCTATGCTCTTACTC AAATCCTTCCGCTAAAACATCGGGATCGGTCGATCGTGCG CCCCCGAGAGGGCTCCGACCTACGTTCACTTTCATTCCGC GTTAATGGGTTTTATCCACCCAAACACTCGCATAGACGTT AGTCTCCTTGGTCCATGTTTCAAGACGGGCGGTATTTGCTC GTTCTGCCAGCATCCTTGGCGATAGAATCGCCGCAGTCCC CGGTCCCGGATAGCCACGTCTGGCTGGCGCTATAACACCC TCCGACAGAATCGAAGGGCCACATTCGCCTGCCCCGTATA TGGCTCCCAGAACCGATGCTGGGCCCAGTGTAAACCAGGT GCACCCATCCACGAGGAACGGGCCGCTGCTAAACACCAT GTCGGATCAAATACCCTTCCCTTTCACCAATTTCACGGACT ATTTTCCTCTCTTTTCAAAAGTCTTTTCATCTTTCCACACCG GGACTTGTTTGCTATCGGGCTCCCGCCAAAAATTTAACCC TTAGAAGGGAAAATACCACCCCACTTTAAAGTGGTTTTCC CAACAAAATCGAAATTTTG
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	CTGGGAGCCATATACGGGGCAGGCGAATGTGGCCCTTCGATTCTGTCGGA	CTTACATATTTAAAGTTTGAGAATAGGCTGAAGCCTTTTC
	GGGTGTTATAGCGCCAGCCAGACGTGGCTATCCGGGACCGGGGACTGCGGC	GGCCTCAGATCCTTTAATCATTCGCTTTACCTCATAAAACT
	GATTCTATCGCCAAGGATGCTGGCAGAACGAGCAAATACCGCCCGTCTT	GCTACGAGCTTCTGCTATCCTGAGGGAAACTTCGGCAGGA
	GAAACATGGACCAAGGAGACTAACGTCTATGCGAGTGTTTGGGTGGATAA	ACCAGCTACTAGATGGTTCGATTAGTCTTTCGCCCCTATAC
	AACCCATTAACGCGGAATGAAAGTGAACGTAGGTCGGAGCCCTCTCGGGG	CCAAATTCGACGATCGATTTGCACGTCAGAACCGCTGCGA
	GCGCACGATCGACCGATCCCGATGTTTTAGCGGAAGGATTTGAGTAAGAG	GCCTCCACCAGAGTTTCCTCTGGCTTCACCCTATTCAGGCA
	CATAGCTGTTGGGACCCGAAAGATGGTGAACTATGCCTGAATAGGGTGAA	TAGTTCACCATCTTTCGGGTCCCAACAGCTATGCTCTTACT
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	CTGGTTCCTGCCGAAGTTTCCCTCAGGATAGCAGAAGCTCGTAGCAGTTT	CGTTAATGGGTTTTATCCACCCAAACACTCGCATAGACGT
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	AGTTGAATGCAAGCTTTTATGGGGCCTTTTTGGTAACAAAACTGGGGATCCG	CCGGTCCCGGATAGCCACGTCTGGCTGGCGCTATAACACC
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B. Statistical outputs

B.1 One-way ANOVA analysis of pH results

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	5	0.788644	0.157729	465.43	0.000
Error	12	0.004067	0.000339		
Total	17	0.792711			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0184089	99.49%	99.27%	98.85%

Means

Sample	N	Mean	StDev	95% CI
AM	3	3.54000	0.01000	(3.51684, 3.56316)
DO	3	3.90000	0.01000	(3.87684, 3.92316)
GB	3	3.4300	0.0361	(3.4068, 3.4532)
LO	3	3.21000	0.01000	(3.18684, 3.23316)
RE	3	3.39667	0.01155	(3.37351, 3.41982)
WO	3	3.4500	0.0173	(3.4268, 3.4732)

Pooled StDev = 0.0184089

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

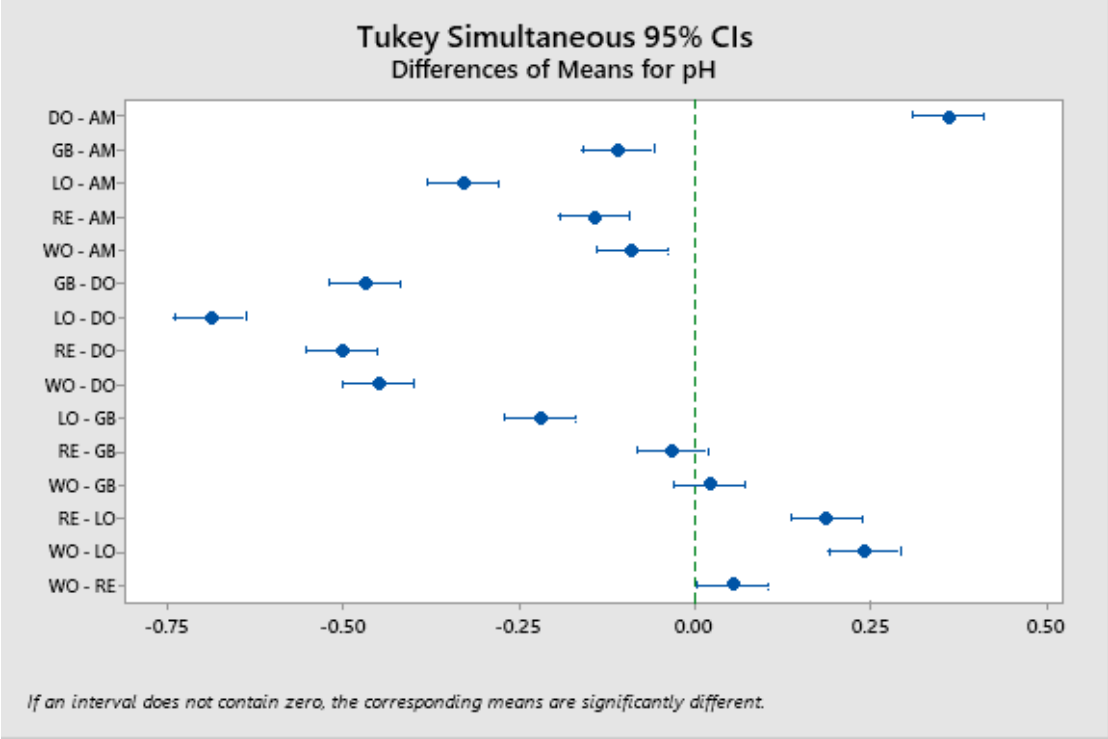
Sample	N	Mean	Grouping			
Daily Organic	3	3.90000	A			
Amplify	3	3.54000		B		
Wonder Drink	3	3.4500			C	
Good Buzz	3	3.4300			C	D
Remedy	3	3.39667				D
Lobros Feijoa	3	3.21000				E

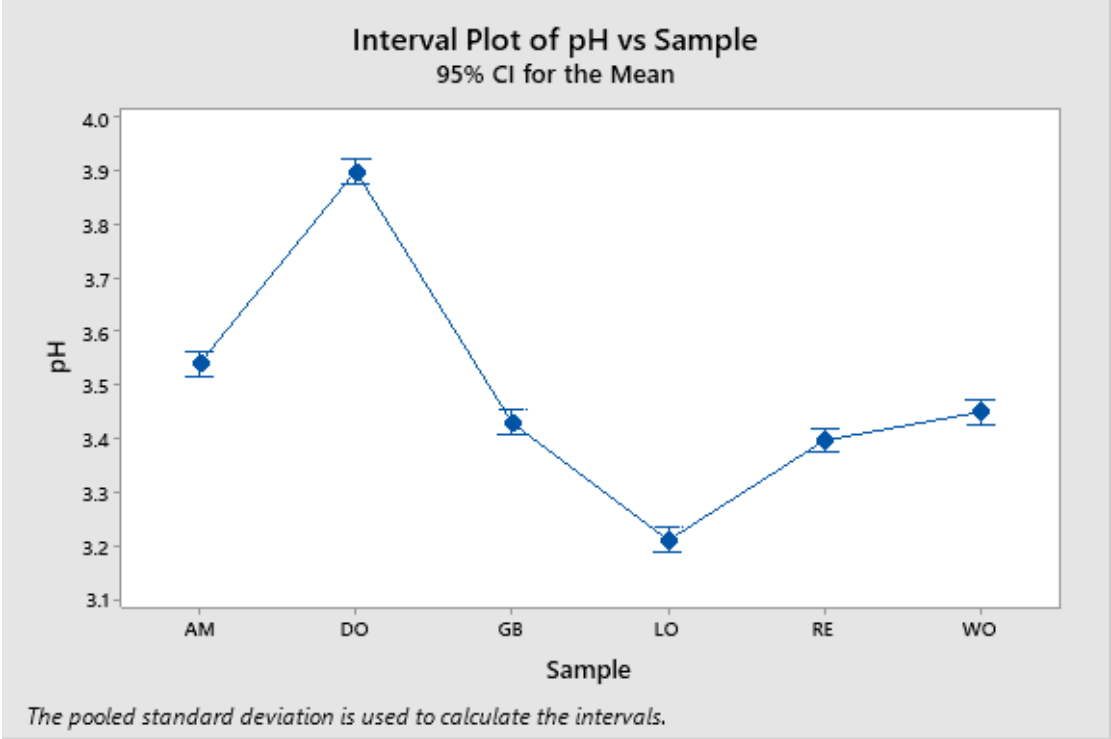
Means that do not share a letter are significantly different.

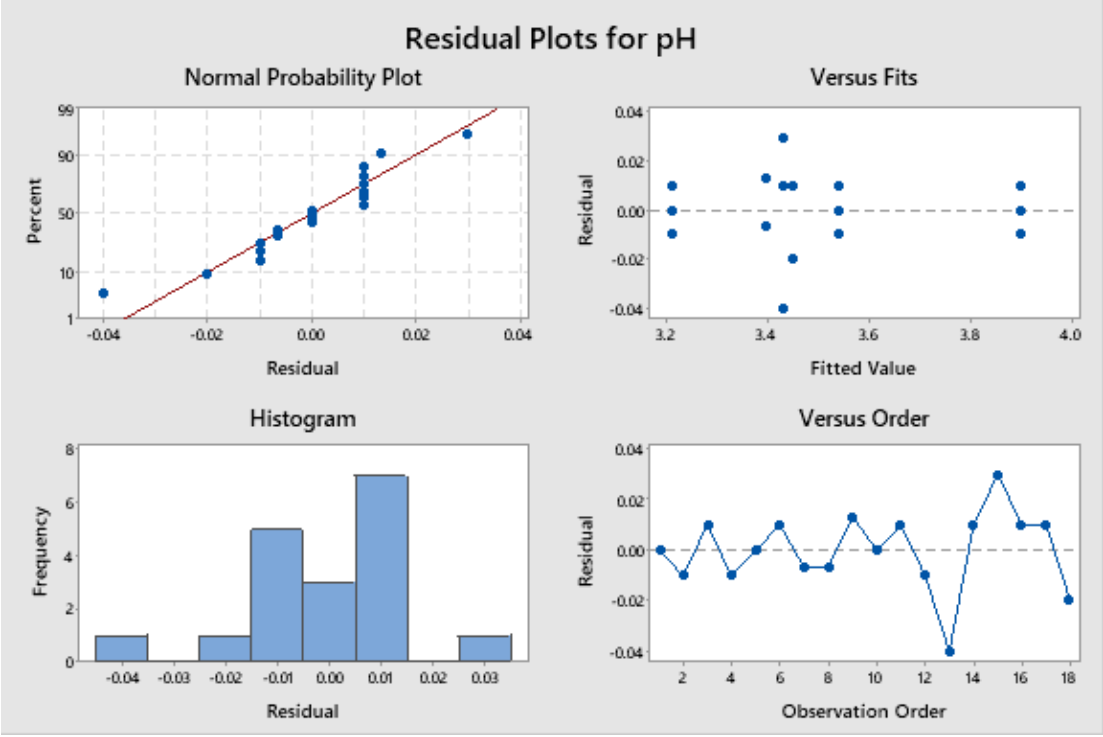
Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
DO - AM	0.3600	0.0150	(0.3095, 0.4105)	23.95	0.000
GB - AM	-0.1100	0.0150	(-0.1605, -0.0595)	-7.32	0.000
LO- AM	-0.3300	0.0150	(-0.3805, -0.2795)	-21.95	0.000
RE - AM	-0.1433	0.0150	(-0.1938, -0.0928)	-9.54	0.000
WO - AM	-0.0900	0.0150	(-0.1405, -0.0395)	-5.99	0.001
GB- DO	-0.4700	0.0150	(-0.5205, -0.4195)	-31.27	0.000
LO- DO	-0.6900	0.0150	(-0.7405, -0.6395)	-45.91	0.000
RE - DO	-0.5033	0.0150	(-0.5538, -0.4528)	-33.49	0.000
WO - DO	-0.4500	0.0150	(-0.5005, -0.3995)	-29.94	0.000
LO - GB	-0.2200	0.0150	(-0.2705, -0.1695)	-14.64	0.000
RE - GB	-0.0333	0.0150	(-0.0838, 0.0172)	-2.22	0.298
WO - GB	0.0200	0.0150	(-0.0305, 0.0705)	1.33	0.764
RE- LO	0.1867	0.0150	(0.1362, 0.2372)	12.42	0.000
WO - LO	0.2400	0.0150	(0.1895, 0.2905)	15.97	0.000
WO - RE	0.0533	0.0150	(0.0028, 0.1038)	3.55	0.036

Individual confidence level = 99.43%




Interval Plot of pH vs Sample
95% CI for the Mean
pH
4.0
3.9
3.8
3.7
3.6
3.5
3.4
3.3
3.2
3.1
AM
DO
GB
LO
RE
WO
Sample
The pooled standard deviation is used to calculate the intervals.


Residual Plots for pH
Normal Probability Plot
Percent
99
90
50
10
1
-0.04
-0.02
0.00
0.02
0.04
Residual
Versus Fits
Residual
0.04
0.02
0.00
-0.02
-0.04
3.2
3.4
3.6
3.8
4.0
Fitted Value
Histogram
Frequency
8
6
4
2
0
-0.04
-0.03
-0.02
-0.01
0.00
0.01
0.02
0.03
Residual
Versus Order
Residual
0.04
0.02
0.00
-0.02
-0.04
2
4
6
8
10
12
14
16
18
Observation Order

B.2 One-way ANOVA analysis: Titratable acidity results

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	5	0.009509	0.001902	1.23	0.353
Error	12	0.018531	0.001544		
Total	17	0.028041			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0392973	33.91%	6.38%	0.00%

Means

Sample	N	Mean	StDev	95% CI
AM	3	0.4751	0.0335	(0.4257, 0.5245)
DO	3	0.4337	0.0230	(0.3843, 0.4831)
GB	3	0.4250	0.0370	(0.3756, 0.4745)
LO	3	0.4824	0.0716	(0.4330, 0.5319)
RE	3	0.4651	0.0321	(0.4156, 0.5145)
WO	3	0.48243	0.00873	(0.43299, 0.53186)

Pooled StDev = 0.0392973

Grouping Information Using the Tukey Method and 95% Confidence

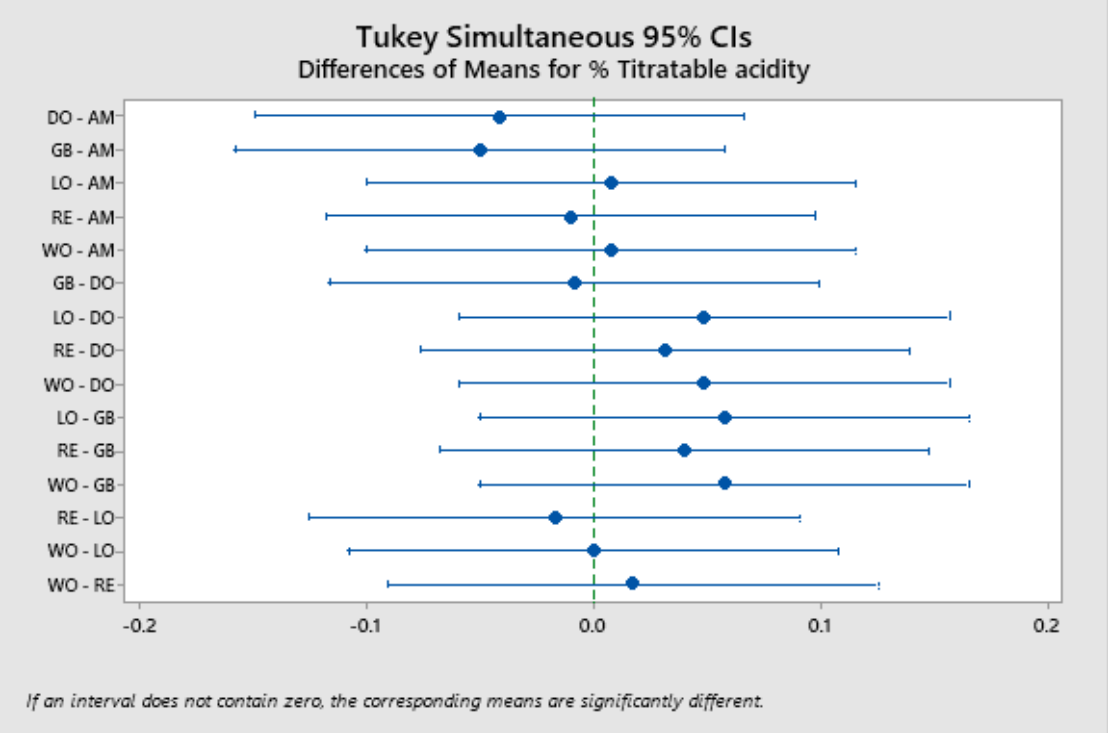
Sample	N	Mean	Grouping
WO	3	0.48243	A
LO	3	0.4824	A
AM	3	0.4751	A
RE	3	0.4651	A
DO	3	0.4337	A
GB	3	0.4250	A

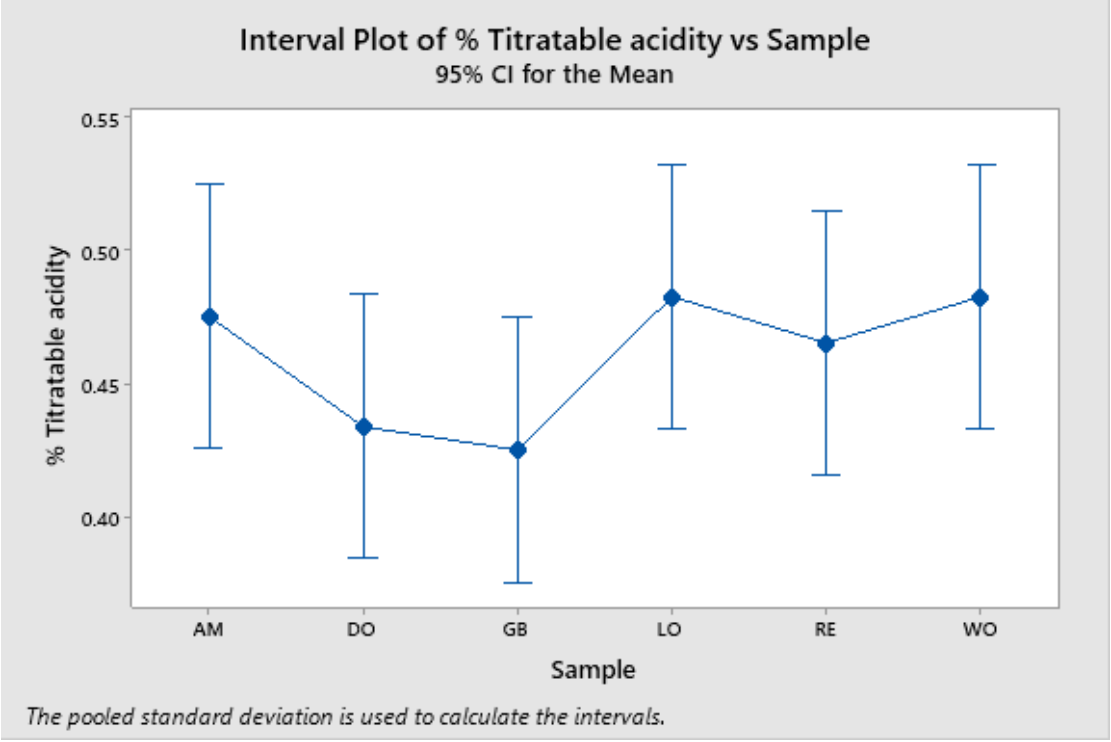
Means that do not share a letter are significantly different

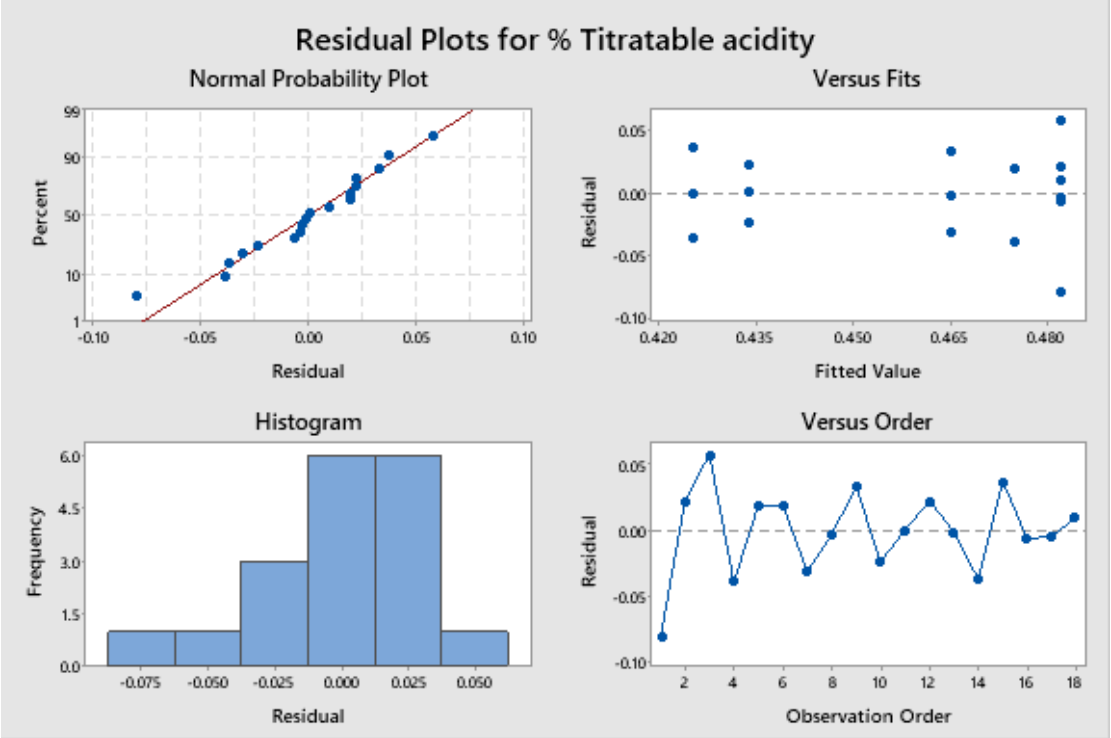
Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
DO - AM	-0.0414	0.0321	(-0.1491, 0.0664)	-1.29	0.785
GB - AM	-0.0500	0.0321	(-0.1578, 0.0577)	-1.56	0.637
LO - AM	0.0073	0.0321	(-0.1004, 0.1151)	0.23	1.000
RE - AM	-0.0100	0.0321	(-0.1178, 0.0978)	-0.31	0.999
WO - AM	0.0073	0.0321	(-0.1004, 0.1151)	0.23	1.000
GB - DO	-0.0087	0.0321	(-0.1164, 0.0991)	-0.27	1.000
LO - DO	0.0487	0.0321	(-0.0591, 0.1565)	1.52	0.660
RE - DO	0.0314	0.0321	(-0.0764, 0.1391)	0.98	0.917
WO - DO	0.0487	0.0321	(-0.0591, 0.1565)	1.52	0.660
LO - GB	0.0574	0.0321	(-0.0504, 0.1652)	1.79	0.507
RE - GB	0.0400	0.0321	(-0.0677, 0.1478)	1.25	0.806
WO - GB	0.0574	0.0321	(-0.0504, 0.1652)	1.79	0.507
RE - LO	-0.0173	0.0321	(-0.1251, 0.0904)	-0.54	0.993
WO - LO	0.0000	0.0321	(-0.1078, 0.1078)	0.00	1.000
WO - RE	0.0173	0.0321	(-0.0904, 0.1251)	0.54	0.993

Individual confidence level = 99.43%




Interval Plot of % Titratable acidity vs Sample
95% CI for the Mean
% Titratable acidity
0.55
0.50
0.45
0.40
AM
DO
GB
LO
RE
WO
Sample
The pooled standard deviation is used to calculate the intervals.


Residual Plots for % Titratable acidity
Normal Probability Plot
Percent
99
90
50
10
1
-0.10
-0.05
0.00
0.05
0.10
Residual
Versus Fits
Residual
0.05
0.00
-0.05
-0.10
0.420
0.435
0.450
0.465
0.480
Fitted Value
Histogram
Frequency
6.0
4.5
3.0
1.5
0.0
-0.075
-0.050
-0.025
0.000
0.025
0.050
Residual
Versus Order
Residual
0.05
0.00
-0.05
-0.10
2
4
6
8
10
12
14
16
18
Observation Order

B.3 One-way ANOVA analysis: Total soluble solid of samples

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	5	65.5511	13.1102	11799.20	0.000
Error	12	0.0133	0.0011		
Total	17	65.5644			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0333333	99.98%	99.97%	99.95%

Means

Sample	N	Mean	StDev	95% CI
AM	3	2.4667	0.0577	(2.4247, 2.5086)
DO	3	7.000	0.000	(6.958, 7.042)
GB	3	4.000	0.000	(3.958, 4.042)
LO	3	3.700	0.000	(3.658, 3.742)
RE	3	1.8667	0.0577	(1.8247, 1.9086)
WO	3	6.500	0.000	(6.458, 6.542)

Pooled StDev = 0.0333333

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

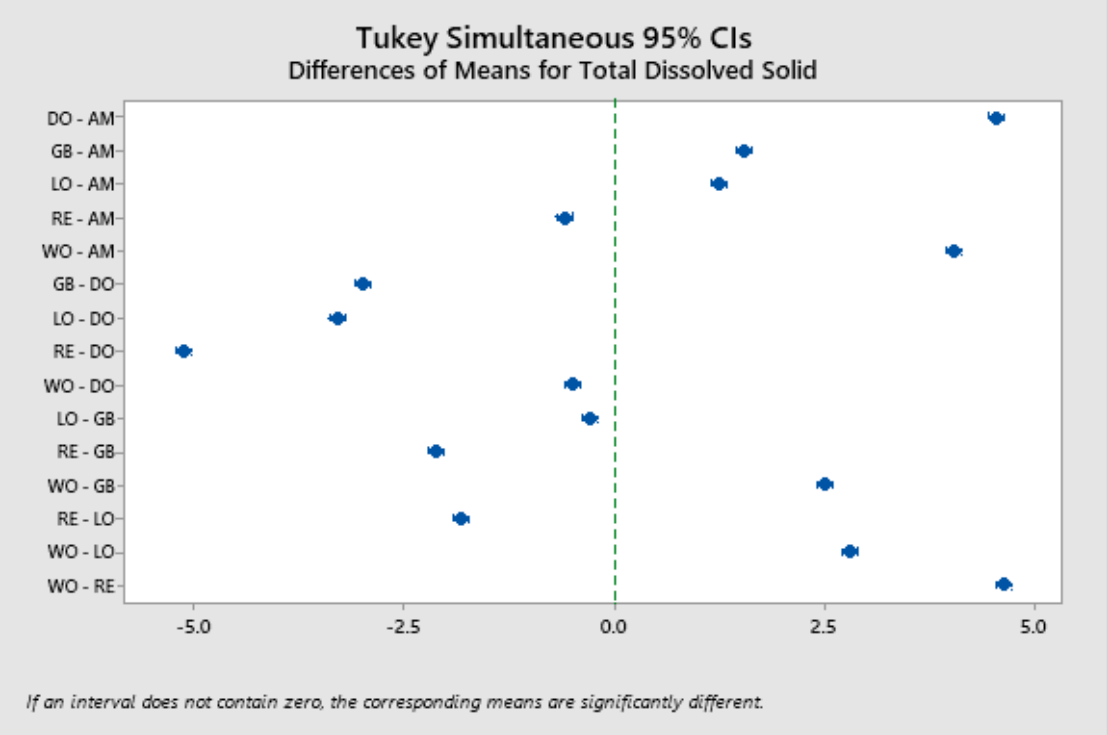
Sample	N	Mean	Grouping					
DO	3	7.000	A					
WO	3	6.500		B				
GB	3	4.000			C			
LO	3	3.700				D		
AM	3	2.4667					E	
RE	3	1.8667						F

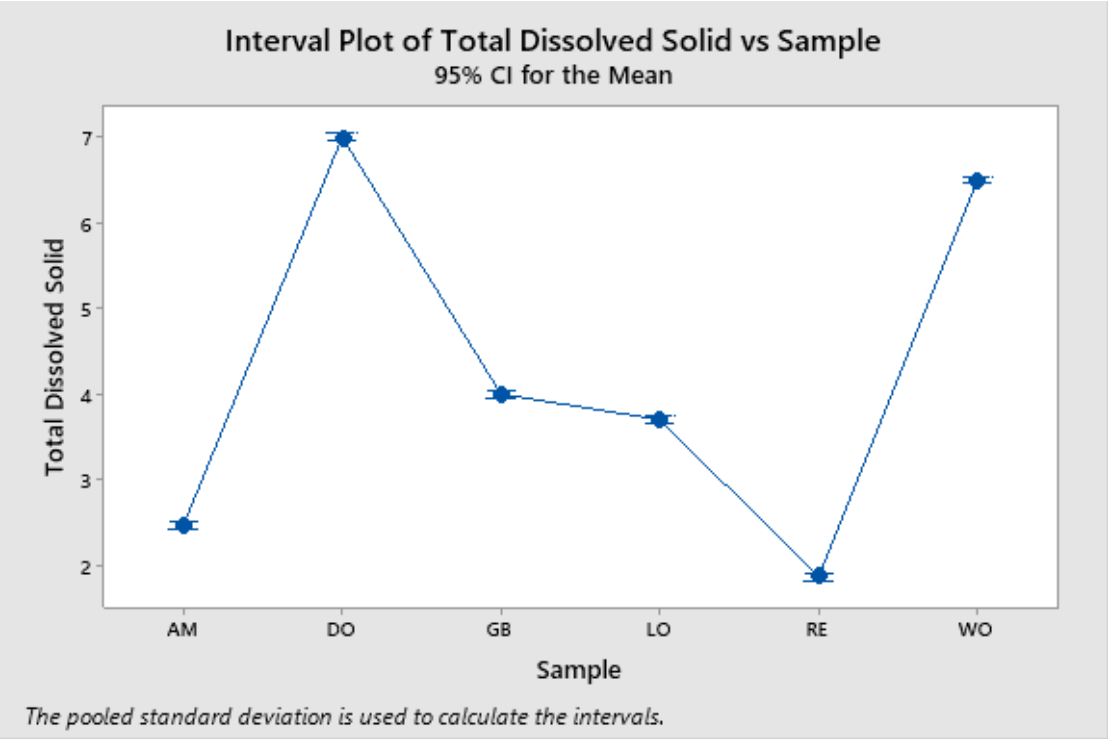
Means that do not share a letter are significantly different.

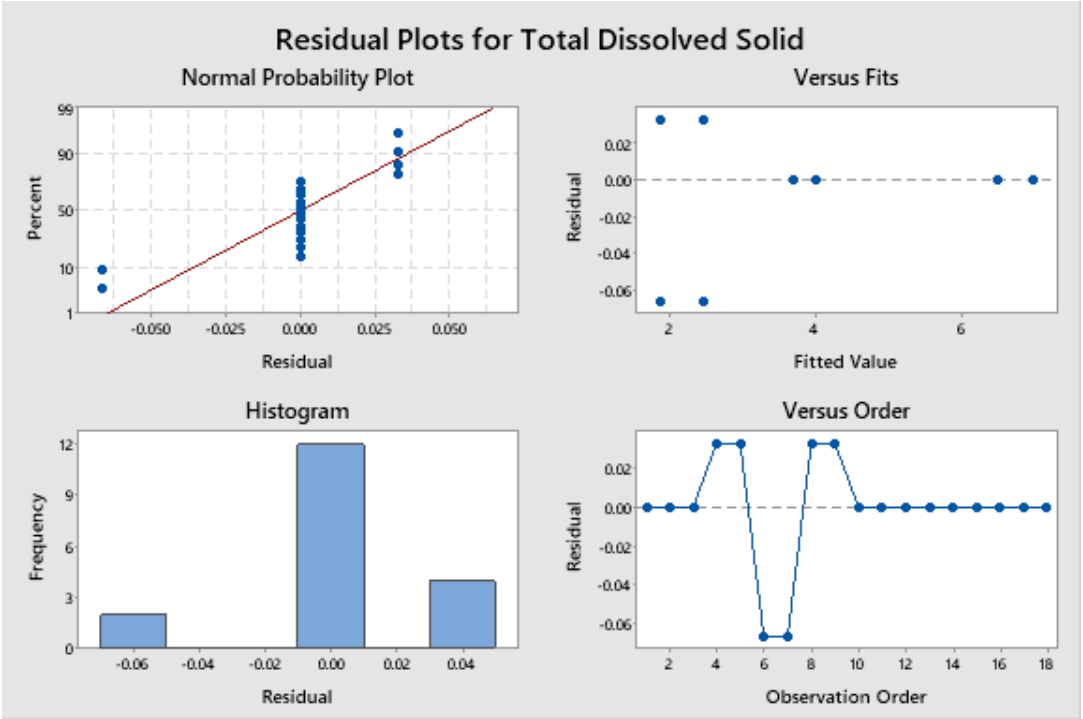
Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
DO - AM	4.5333	0.0272	(4.4419, 4.6247)	166.57	0.000
GB- AM	1.5333	0.0272	(1.4419, 1.6247)	56.34	0.000
LO - AM	1.2333	0.0272	(1.1419, 1.3247)	45.32	0.000
RE - AM	-0.6000	0.0272	(-0.6914, -0.5086)	-22.05	0.000
WO - AM	4.0333	0.0272	(3.9419, 4.1247)	148.19	0.000
GB - DO	-3.0000	0.0272	(-3.0914, -2.9086)	-110.23	0.000
LO - DO	-3.3000	0.0272	(-3.3914, -3.2086)	-121.25	0.000
RE - DO	-5.1333	0.0272	(-5.2247, -5.0419)	-188.61	0.000
WO - DO	-0.5000	0.0272	(-0.5914, -0.4086)	-18.37	0.000
LO-GB	-0.3000	0.0272	(-0.3914, -0.2086)	-11.02	0.000
RE-GB	-2.1333	0.0272	(-2.2247, -2.0419)	-78.38	0.000
WO-GB	2.5000	0.0272	(2.4086, 2.5914)	91.86	0.000
RE-LO	-1.8333	0.0272	(-1.9247, -1.7419)	-67.36	0.000
WO-LO	2.8000	0.0272	(2.7086, 2.8914)	102.88	0.000
WO-RE	4.6333	0.0272	(4.5419, 4.7247)	170.24	0.000

Individual confidence level = 99.43%




Interval Plot of Total Dissolved Solid vs Sample
95% CI for the Mean
Total Dissolved Solid
7
6
5
4
3
2
AM
DO
GB
LO
RE
WO
Sample
The pooled standard deviation is used to calculate the intervals.


Residual Plots for Total Dissolved Solid
Normal Probability Plot
Percent
99
90
50
10
1
-0.050
-0.025
0.000
0.025
0.050
Residual
Versus Fits
Residual
0.02
0.00
-0.02
-0.04
-0.06
2
4
6
Fitted Value
Histogram
Frequency
12
9
6
3
0
-0.06
-0.04
-0.02
0.00
0.02
0.04
Residual
Versus Order
Residual
0.02
0.00
-0.02
-0.04
-0.06
2
4
6
8
10
12
14
16
18
Observation Order

C. Composition of cultivate medium

Table C.1 Formulation of medium

Medium	Ingredients	Purpose
Glucose yeast extract peptone mannitol agar medium (GYPM)	20g/L D-glucose, 25g/L Mannitol, 5g/L yeast extract, 3g/L peptone, 12g/L bacteriological agar, 0.1g/L cycloheximide and 1ml/L pimaricin	Isolate the presumptive AAB
Glucose yeast extract agar medium (GY)	50g/L D-glucose, 10g/L yeast extract, and 15g/L bacteriological agar	Cultivate the presumptive AAB
Glucose yeast extract medium (GY)	50g/L D-glucose and 10g/L yeast extract	Cultivate the presumptive AAB and formation of γ-pyrone from D-glucose
Yeast extract glucose chloramphenicol agar (YGC)	5g/L yeast extract, 20g/L D-glucose, 0.1g/L chloramphenicol, 12 g/L bacteriological agar	Isolate the presumptive yeasts
Potato dextrose agar (PDA)	4g/L potato infusion solids, 20g/L dextrose, 15g/L bacteriological agar	Cultivate the presumptive yeasts
Yeast extract peptone dextrose broth (YPD)	10g/L yeast extract, 20g/L bacteriological peptone, 20g/L dextrose	Cultivate the presumptive yeasts
Glucose yeast extract calcium carbonate (GYC) agar	50g/L D-glucose, 10g/L yeast extract, 20g/L calcium carbonate, 15g/L bacteriological agar	Growth on different medium
0.35% (w/v) acetic acid medium	3.5 ml/L glacial acetic acid, 10 g/L yeast extract, 10g/L D-glucose	Growth on different medium
30% (w/v) D-glucose agar	300 g/L D-glucose, 10 g/L yeast extract, 15 g/L bacteriological agar	Growth on different medium
Methanol agar	8 ml/L methanol, 5 g/L yeast extract, 15g/L bacteriological agar.	Growth on different medium
Dextrose sorbitol mannitol	10g/L peptone, 3 g/L yeast extract, 15 g/L calcium lactate, 1 g/L D-sorbitol, 2g/L d-mannitol, 1g/L monopotassium phosphate, 0.02 g/L manganese sulfate monohydrate, 15g/L bacteriological agar,0.004g/L cycloheximide, 0.03g/L bromocresol purple,0.0295g/L brilliant green	Growth on different medium
Glutamate agar	10g/L glucose, 5g/L sodium glutamate, 1g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.1g/L KCl, 20g/L bacteriological agar	Growth on different medium
Carr agar	30g/L yeast extract, 0.022 g/L bromocresol purple, 20ml/L absolute ethanol, 20g/L bacteriological agar	Oxidation of ethanol and oxidation of acetic acid

Ethanol agar (2%,4%,6%,8% and 10%)	(20ml/L,40 ml/L, 60ml/L, 80ml/L, 100ml/L) absolute ethanol, 5g/L yeast extract, 20g/L bacteriological agar	Determine the alcoholic tolerance of AAB
SIM medium	20g/L tryptone, 6.1g/L meat extract, 0.2g/L ferrous ammonium sulphate, 0.2g/L sodium thiosulfate and 3.5g/L bacteriological agar	Determine the formation of H_2S, indole and motility of AAB
Gelatine medium	5g/L peptone, 3g/L meat extract and 120 g gelatine	Determine the gelatine hydrolysis ability of AAB
Acetate agar	15 g/L sodium acetate, 30 g/L yeast extract, 20 g/L bacteriological agar, and 0.022g/L bromothymol blue	Oxidation of acetate
Lactate agar	15g/L calcium acetate, 30 g/L yeast extract, 20 g/L bacteriological agar, and 0.022g/L bromothymol blue	Oxidation of lactate
HS medium	20g/L D-glucose, 5g/L peptone, 2.7g/L Na_2HPO_4, and 1.15 g/L citric acid	Production of cellulose
GYE agar	30g/L glycerol, 10 g/L yeast extract, and 20 g/L bacteriological agar	Ketogenesis from glycerol to DHA
Fructose yeast extract broth	50g/L fructose and 10g/L yeast extract	Formation of γ-pyrone from D-fructose
Glucose fermentation medium	10g/L D-glucose, 5g/L yeast extract, 10g/L peptone, and 0.02g/L bromocresol purple	Carbohydrate fermentation test
Mannitol fermentation medium	10g/L D-mannitol, 5g/L yeast extract, 10g/L peptone, and 0.02g/L bromocresol purple	Carbohydrate fermentation test
Glycerol fermentation medium	10g/L glycerol, 5g/L yeast extract, 10g/L peptone, and 0.02g/L bromocresol purple	Carbohydrate fermentation test
Nitrate peptone medium	10g/L peptone and 2g/L KNO_3	Nitrate reduction

Table C.2 Chemical reagents used in this study

Chemical reagent	Ingredients	Purpose
KHP	potassium hydrogen phthalate	Standardize 0.1N NaOH
Methylene blue	Methylene blue and sodium citrate dihydrate	Yeasts staining
6% (w/v) H_2O_2	Hydrogen peroxide	Catalase test
Kovac's reagent	4-(dimethyamino) benzaldehyde, hydrochloride, isoamylic alcohol	Formation of indole
Benedict solution	Copper sulphate pentahydrate; anhydrous sodium carbonate and sodium citrate	Production of DHA
(5% w/v) $FeCl_3$	Iron chloride	Formation of γ-pyrone
Reagent A	Sulfanilic acid and 5N acetic acid	Nitrate reduction
Reagent B	N,N-dimethyl-α-naphthylamine and 5N acetic acid	Nitrate reduction