Understanding the *in vitro* gastrointestinal transit tolerance of putative lactic acid bacteria isolated from Malaysian fermented foods.

Low Siew Jean

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School of Biosciences
University of Nottingham Malaysia

Abstract

Probiotics are defined as living microorganisms that when consumed in adequate amounts will exhibit health benefits to the host. Probiotics are known to play important roles in relief of lactose intolerance symptoms and improving gut health. However, till today the beneficial effect of probiotic bacterium is strain-specific, no single probiotic is able to resolve all gut health issues. Hence, the search for a stronger and more potent of probiotic bacterium is essential. Hence, the current study is aimed to isolate putative lactic acid bacteria from local fermented foods, namely *tempeh* and *tapai ubi*, and investigate the ability of these LABs to tolerate the upper gastrointestinal transit in an *in vitro* setting prior to future use as probiotics.

A total of 18 putative LABs were isolated from *tempeh* and *tapai ubi* using spread plate method with MRS selective medium. However only four isolates remained viable after frozen storage and was proceeded for subsequent studies. Three of the putative LABs were isolated from *tapai ubi* and designated as SU1, SU2, and SU8, and the putative LAB isolated from *tempeh* was designated as ST4. The identity of these four putative isolates were determined using 16s rRNA gene sequence analysis. SU1 and SU2 were identified as *Pediococcus acidilactici* with 80% and 87% similarity. SU8 was identified as *Pediococcus claussenii* with 82% similarity while ST4 was identified as *Leuconostoc mesenteroides* subsp. *mesenteroides* with 88% similarity.

After identification, the ability of these putative LAB isolates to survive the bile, gastric and small intestinal transits were evaluated in an *in vitro* setting. SU8, SU1

and SU2 were able to tolerate 0.5% of bile salt condition with growth ranging from 5.70 to 7.13 Log_{10}CFU/ml after 24h of incubation, except for ST4. ST4 was susceptible to 0.5 and 1.0% of bile salt. These putative LAB isolates were highly tolerant to the simulated gastric transit ranging from pH 2 to 4 and maintained a growth of more than 12 Log_{10}CFU/ml after 180 min of incubation. All four putative LAB isolates were able to tolerate the simulated small intestinal transit with addition of 0.3% bile salt and maintained a growth of more than 9 Log_{10}CFU/ml after 240 min of incubation. ST4 had the highest tolerance to the simulated small intestinal transit and therefore exhibited a putative potential as probiotics. Future study will need to be conducted to understand the health benefits exhibited by these putative LAB isolates for application as probiotics.

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Abbreviations

Common abbreviations

ANOVA	One-way analysis of variance
B.	*Bifidobacterium*
DMRT	Duncan multiple range test
DNA	Deoxyribonucleic acid
DNTP	deoxynucleotide triphosphates
E.	*Enterococcus*
EDTA	Ethylenediamine tetra-acetic acid
HCl	Hydrochloric acid
HE	Hepatic encephalopathy
IBS	Inflammatory Bowel Syndrome
L.	*Lactobacillus*
LAB	Lactic acid bacteria
Leu.	*Leuconostoc*
MHE	Minimal hepatic encephalopathy
MRS	De Man, Rogosa and Sharpe
NaOH	Sodium hydroxide

P.	*Pediococcus*
PCR	Polymerase chain reaction
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
Sacc.	*Saccharomyces*
spp.	*species pluralis*
subsp.	*Subspecies*

Units

%	percentage
°C	Degree Celsius
µl	Microliter
CFU/ml	Colony-forming unit per mL
G	gram
h	hour
M	Molar
m/v	Mass/volume
mg/ml	Milligram/milliliter
min	minute

ml	Milliliter
mM	Millimolar
s	second
U	micromol per minute
v/v	Volume/volume

Chapter 1 Introduction

1.1 Background of the study

Probiotics are living microorganisms, which when consumed in adequate amounts, will exhibit potential health benefit to the host (WHO, 2001). According to Food and Agriculture Organization (FAO) and World Health Organization (WHO), probiotics are living microorganism that play an important role in the digestive, immunological, and respiratory systems. Besides these, it may have positive effects towards alleviation of infectious diseases (FAO/WHO, 2006). Other than these health benefits, probiotics also play an important role in lowering the total cholesterol in the body, thus, reducing the risk of getting coronary heart disease (Miremadi, 2014; Chen, 2011).

Probiotics are mostly manufactured into probiotic drinks and the world-wide consumption of probiotic drinks have increased significantly throughout the year. According to Market Research Report (2020), the global probiotic drinks market has projected a significant growth rate of 8.44% from 2019 to 2024 with a market value of USD 45.3 billion by the end of the 2024. While the Yakult Honsha Co., Ltd. (2018) reported that the statistic of sales volume of probiotic drinks world-wide has increased significantly from 32,366 to 39,517 (thousands of bottles/day) from 2014 to 2018. Probiotic drinks market has the highest growth rate in Asia-Pacific during the forecast period due to the immense popularity of functional food and beverages of probiotics (Market Research Report, 2020).

According to Yakult Honsha Co., Ltd. (2018), the Asia region has the highest sale of yakult probiotic drinks compared to other countries. In Malaysia, probiotic supplements with *Lactobacillus* and *Bifidobacterium* were the most popular supplements in 2014 with the strongest annual growth rate of 15.4% among all supplements (Yap & Wan Nawawi, 2016).

Other than these commercial probiotic drinks and supplements, natural food products fermented by lactic acid bacteria (LAB) such as yogurt, kefir, kimchi and kombucha have been used as probiotics for their proposed health promoting properties. Several researchers have successfully isolated different types of LAB from Malaysian fermented food such as *tempoyak*, *tempeh*, *tapai ubi* (Khalil *et al*., 2018 Pisol *et al.*, 2015; Ramasamy *et al.*, 2012). *Tempeh* and *t*apai *u*bi were chose in this study for isolation of LAB because they are popular in Malaysia and other asia countries such as Indonesia and Philippines. Besides that, they rich in nutrients, tasty and affordable for everyone (Steinkraus, 2004; Babu *et al*., 2009).

In recent years, selected probiotic LAB strains have been thoroughly investigated for specific health effects such as *Lactobacillus rhamnosus GG, Lactobacillus acidophilus,* and *Bifidobacterium bifidum* (Ouwehand *et al*., 2002; de Roos & Katan, 2000). Properties like relief of lactose intolerance symptoms and shortening of rotavirus diarrhea by one or two days are now widely accepted as the health benefit for selected probiotic LABs (Crawford *et al*., 2017). Some areas, such as the treatment and prevention of atopy by probiotic LABs hold great promise. However, many proposed health effects still need additional investigation (Ouwehand

et al., 2002). Till today there is no single probiotic bacterium which able to resolve all the related health issues. Hence, this challenge has driven scientists and the author to continue to search for a stronger and more potent probiotic bacterium.

1.2 Aim and Specific Objectives

In the search of putative probiotics, the first step is to ensure that the probiotics are able to tolerate the gastrointestinal transit and remained viable when it reaches the small intestinal tract and colon to exert beneficial effects to the host. Hence, the current study is aimed to isolate putative lactic acid bacteria and investigate the probiotic potential of LAB isolated from local fermented foods, namely *tempeh* and *tapai ubi*, and investigate the ability of these LABs to tolerate the upper gastrointestinal transit in an *in vitro* setting.

The specific objectives were:

i) To isolate the putative probiotic lactic acid bacteria from local fermented foods.

ii) To identify the selected putative lactic acid bacteria using 16s rRNA sequence analysis.

iii) To investigate the simulated gastric transit tolerance of the selected putative lactic acid bacteria.

iv) To investigate the simulated small intestinal transit tolerance of the selected putative lactic acid bacteria.

Chapter 2 Literature review

2.1 Origin of Probiotics

2.1.1 History and definition of probiotics

Probiotics was originally described as 'substances produced by one protozoan which stimulated another' (Lily & Stillwell, 1965). Later on, it was described as 'an animal feed supplements which exert beneficial effect on the host animal by affecting the gut flora' (Parker, 1974). Then, the definition has revised by Fuller (1989) as 'a live microbial feed supplement which beneficially affects the host animal by improving the intestinal microbial balance'.

The history of probiotics started with fermented milks, that treated as medicine for body ailments that has been mentioned in Biblical scriptures in the early ages. According to Oberman (1985), Hippocrates prescribed sour milks as treatment for disorders of stomach and intestines. Then, in the beginning of the 20th century, the Russian bacteriologist Eli Metchnikoff (Pasteur Institute, France) was the first that gave a scientific explanation on the beneficial effects of lactic acid bacteria that present in fermented milk (Hughes & Hoover, 1991; O'Sullivan *et al*., 1992). In 1908, he implemented his 'longevity-without-aging' theory that explained that lactic acid caused the displacement of toxin producing that present in the intestine and resulting in prolonged lifespan (Lourens-Hattingh *et al*., 2001). Furthermore, Metchnikoff also proved that the growth and toxicity of anaerobic spore-forming bacteria was inhibited in large intestine after consuming products produced by lactic acid bacteria in sour milks (Lourens-Hattingh *et al*., 2001).

In 1920 to 1921, Rettger and his colleagues at Yale had shown the *Bulgarian bacillus* could not survive in the human gut and they used *L. acidophilus* instead (Rettger & Cheplin, 1921). This clinical trial gave good results in the patients with chronic constipation (Rettger *et al*., 1935).The interest of gut flora was raised in the second world war due to two factors: (a) the discovery of antibiotic growth promotion on animals that attempts to define the composition of the gut microflora through research; and (b) the improved techniques for raising germ-free animals (Fuller, 1992).

There were many studies about gut microflora and the used of lactic acid bacteria in food and the interest of probiotics continued. More recent studies have focused on the health benefits of the bacteria to the host and the survivability of the bacteria in the gastrointestinal tract (Lourens-Hattingh & Vijoen, 2001). This led to the changes and improvement for definition of probiotics. The most recent definition of probiotics was by The World Health Organization (2001) and defined as "living microorganisms which when apply in adequate amounts of health benefit on the host".

2.1.2 Source of probiotics

Probiotics are commonly found in fermented food and beverages such as milk, yogurt and cheese. The role of probiotics bacteria assists in milk is for preservation of the milk by generating lactic acid. As for yogurt and cheese, probiotics produced flavor compounds such as acetaldehyde, extracellular polysaccharides and other metabolites with organoleptic properties to the consumer (Parvez *et al*., 2006). The most common lactic acid bacterial use in world-wide are *Lactobacillus* and

Bifidobacteria (Chick *et al*., 2001; Van Hoorde *et al*., 2008; Ren *et al*., 2018).

Malaysia local fermented food such as *tempeh* and *tapai ubi* also contains probiotics. LABs that are usually found in *Tempeh are L. plantarum, Lactobacillus fermentum, Lactobacillus reuteri* and *Lactococcus lactis* (Feng *et al*., 2005; Huang *et al*., 2018). *Tempeh* is a soybean fermented product that is originally made by Central Javanese people through fermentation with *Rhizopus* species of fungi in the 1700s and is a world-wide accepted fermented product (Astuti *et al*., 2000). The production of *tempeh* was made by soaking and cooking to soften it and it taste mushroom or nutty odour. *Tempeh* can be fermented by different types of microorganism including moulds, yeasts, lactic acid bacteria and different species of gram-negative bacteria (Babu *et al*., 2009). Besides these, *Tempeh* provided staple diet to the consumers due to it being rich in protein, calcium, fibers, essential fatty acids and vitamin B-12 (Liem *et al*., 1977; Babu *et al*., 2009). *Tempeh* is consumed by a large population of consumers in Indonesia, Malaysia and other poor masses of countries as a source of proteins and calories, because it is a low-cost nutritious food is that affordable by all socio-economical groups (Babu *et al*., 2009).

Apart from *tempeh*, *tapai ubi* is another traditional fermented foods is that fermented using *Ragi* from Cassava (Djien, 1972). *Ragi* is a mixed culture of microorganisms used as a starter culture for *tapai ubi* fermentation (Djien, 1972). The production of the starter was by combining three groups of microorganisms such as mucoraceous fungi, yeasts and bacteria in the fermentation process (Hesseltine *et al*, 1988). The formation of *ragi* is always in the form of dry product, either as a

small flattened cake, a ball shape or as a white dusty powder (Hesseltine *et al*., 1988). As stated by Gandjar (2003), a well-prepared substrate and *ragi* will produce a good *tapai*. LABs that usually found in tapai ubi are *Lactobacillus brevis, L. plantarum, L. collinoides and Pediococcus* and *Enterobacteriaceae* (Chiang *et al*., 2006). After the fermentation, the flavor of tapai ubi is sweet, soft and juicy yet slightly alcoholic with a pleasant of aroma (Steinkraus, 2004). *Tapai ubi* is popular in Malaysia because it provided protein, thiamine content and folic acid to the consumer as dessert (Nor *et al*., 2008; Steinkraus, 2004).

2.2 Lactic acid bacteria as probiotics

The definition of 'fermentation' comes from a Latin word *fermentum* and it means boiling (Gogineni *et al*., 2013). The name originated by an observation of mixtures of crushed grapes that are kept in large vessels and produced bubbles while boiling. In ancient times, fermentation was used as preservation of food but the process of fermentation was carried out without any knowledge of microbial mechanisms (Chojnacka, 2010). Fermentation was established from ancient times in Egypt and the Middle East (McGovern, 2009). In 6,000 B.C., the earliest record of fermentation was in the Fertile Crescent region of lower Mesopotamia in between the Tigris and Euphrates rivers (Kiple & Ornelas, 2000). Then in 7,000 B.C, traditional Egyptian started to ferment milk products, Laban Rayeb and Laban Khad (Kosikowski & Mistry, 1997). Fast forward to 1941, the fermentation was introduced as using large scale of microorganism to produce product such as antibiotics, germ warfare, and lead to a huge increased of interested on microorganisms (Chojnacka, 2010).

In the year of 1980s, genetic engineering techniques was established to produce insulin (Chojnacka, 2010). The process of fermentation has been improving years by years. Nowadays, lactic acid fermentation was introduced and has been focused in word-wide especially in developing countries (Chelule *et al*., 2010). Most of the people preferred fermented food over unfermented food because of the longer preservation, texture and the colour of the food (Adriye & Laleye, 2003; Mosha & Vicent, 2004; Nout & Sarkar, 1999).

Besides this, lactic acid fermentation provide health benefits to consumers such as improved the nutritional value and digestibility of foods (Nout, 2009; Obiri-Danso *et al*., 1997), detoxification of mycotoxin in food (Mokoena *et al*., 2005; Chelule *et al*., 2010; Schnürer & Magnusson, 2005; Gourama & Bullerman, 1995; Dalié *et al*., 2010; Zinedine *et al*., 2005; Mokoena *et al*., 2006) and so on. Thus, fermented food has significantly increased the popularity as one of the main dietary components for developing world (Chelule *et al*., 2010).

The most important requirements for LAB is that it is non-invasive to the host and is safe to consume. According to Donohue & Salminen (1996), the *in vitro* study that test whether LABs such as *Lactobacillius, Lactococcus, Leuconostoc* and more will adhere to human intestinal cell lines to degrade the protective intestinal mucus intestinal cells has proved that the result was negative to the potential of LAB invading intestinal cells and damage the protective intestinal mucus. Besides that, two adhesion studies have been conducted to test the invasive properties of LAB by using Caco-2 cells (Coconnier *et al*., 1992; Elo *et al*., 1991). The results shown that

there were no invasive properties for LAB in this test. Intestinal mucus degradation is also considered as a marker of toxicity. As stated by Ruseler-van Embden *et al*. (1995), the study showed that probiotic strains such as *Lactobacillus GG, Lactobacillus acidophilus and Bifidobacterium bifidum* were not involved in mucosal degradation.

Furthermore, some of the clinical studies and animal studies have proven that LAB had an absence in toxicity (Donohue *et al*., 1993; Ishihara *et al*., 1985; Momose, 1979). Probiotics have been consumed in large quantities in this modern world. There are many LABs found as probiotics (Kongo, 2013) such as *L. rhamnosus, L. paracasei, L. fermentum, Enterococcus faecium, E. durans, L. plantarum, L. casei subsp. shirota, L. casei subps. immunitas, L. acidophilus subps. johnsonii* (Verdenelli *et al*., 2009; Kilic & Karahan, 2010; Kongo, 2013; Curto *et al*., 2011) and so on.

2.2.1 Probiotic characteristics of LAB

In order to ascertain the probiotic characteristics of LAB, a list of criterions must be fulfilled by the LAB. First and foremost, determination of the probiotic LAB strains of its taxonomic classification must be done (Wedajo, 2015). This gives an indication of the origin, habitat and physiology of the LAB strains. According to Moreilli (2007), LAB related with the habitats that are rich in nutrients, such as food products and plant materials. Then, several test for selection of probiotic must be done.

2.2.1.1 Acid and bile salt tolerance

LAB strains must be able to survive under acidic and bile conditions in human

gastrointestinal tract (Termont *et al*., 2006). It has to be tolerant to the stomach acid as low as pH 1.5 and the intestine with 2% concentration of bile salt (Dawson, 1998). Bile plays an important role as a biological detergent to emulsify lipids in the duodenum and also provided a strong antimicrobial activity that able to clutter the structure of cell membrane and lead to DNA destruction (Patel *et al*., 2012; Ruiz *et al*., 2013). There were several studies proved that LAB strains such as *Lactobacillus* and *Bifidobacterium* had resistance to acid and bile salt (Timmerman *et al*., 2007; Matto *et al*., 2004). Other than Lactobacillus and Bifidobacterium, *Weissella cibaria* from fermented vegetable-based food able to survive at pH 3.0 and tolerance against 0.3% oxgall bile salt (Ahn *et al*., 2013; Kim *et al*., 2018).

2.1.2 Cell viability

Cell viability is one of the important scientific criteria to classify the bacteria as probiotic (Amagase, 2008; Degnan, 2008; Vinderola *et al*., 2011). It is to determine the survivability of the cells by measuring the population of cells that survive through cell proliferation or different experimental conditions exposure (Riss *et al*., 2011). According to Ross *et al*. (2005), cell viability has been used for monitoring the corporation of probiotic and food production as a routine. It is an important assay for probiotic strains selection because to determine the ability of withstand stresses such as temperature, osmotic, solvent stress and freeze-drying for their progression to final from by manufacturing (Bron *et al*., 2011; Davis, 2014). A study of viability

of probiotic in Argentinian Fresco Cheese by Vinderola *et al*. (2000) showed that *Bifidobacterial* and *L. acidophilus* have high cell viability and able to survive after 60 days of refrigerated storage.

2.2.1.3 High antibacterial and antimicrobial activity

Besides this, LAB showed that it has antimicrobial substances against pathogen by producing the compound such as *lactolin* by *L. plantarum*, *lactobacillin* and *lactobrevin* by *L. brevis*, *acidophilin* and *lactocidin* by *L. acidophilus* and so on (Fuller, 1992) that will inhibit the growth or kill the pathogen (Desai, 2008). According to Campana *et al*. (2017), *L. case*i and *l. rhamnosus* showed that they have high antibacterial activity and other strains have moderate activity against the pathogens. There were other studies that proved that LAB strains provided antimicrobial activity (Servin, 2004; Marianelli *et al*., 2010). Lactic acid bacteria also produced hydrogen peroxide, diacetyl and bacteriocin as antimicrobial substances and against the foodborne pathogen and spoilage bacteria (Desai, 2008). As stated by Dave & Shah (1997), bacteriocin from the bacterium of the yoghurt able to against probiotic bacteria.

2.2.1.4 Adhesion ability

The capability of LAB to adhere to the intestinal mucosa is considered one of the criterions for probiotic. The adhesion of bacterial to intestinal surfaces is by hydrophobic interaction, then followed by specific cell wall components adhesion (H-addaji *et al*., 2015; Monteagudo-Mera *et al*., 2019). According to Pan *et al*. (2006)

has reported that there was a correlation between hydrophobicity and adhesion. Thus, some lactic acid bacteria contained surface proteins such as cell wall-anchored proteinases to enhance the hydrophobicity and adhesion (Muñoz-Provencio *et al*., 2012; Zhang *et al.*, 2015; Radziwill-Bienkowska *et al.*, 2017). Besides this, the presence of mucus-binding proteins in lactic acid bacteria cell wall such as *Bifidobacterium* and *Lactobacillus* able to enhance the process of adhesion of bacteria to intestine (Motherway *et al*., 2011; Piepenbrink & Sundberg, 2016; Reunanen *et al*., 2012; Toh *et al*., 2013). According to Rinkinen *et al*. (2000), the study observed that porcine *Enterococcus faecium* SF 273 able to adhere *in vitro* to canine intestinal mucus.

2.2.1.5 Immune response enhancement

Lactic acid bacteria able to enhance the immune response to the host. According to Matsuzaki & Chin (2000), *L. casei Shirota* strain has been injected into the mice and showed that there was a significantly increased in natural killer cells activity of mesenteric node cells and enhanced the innate immune response. In addition, there was a controlled clinical study to measure the enhancement of immune system in the elderly by consuming *B. lactis*. HN019 and *L. rhamnosus* HN001, and the result was positive towards the enhancement (Arunachalam *et al*., 2000; Gill *et al*., 2001; Sheih *et al*., 2001).

2.2.1.6 Cholesterol removal ability

High level of cholesterol is associated with cardiovascular diseases (CVD) and causes mortality in worldwide (Tomaro-Duchesneau *et al*., 2014). Several studies

have proven that probiotic bacteria could yield the level of cholesterol and low-density lipoprotein (LDL) cholesterol (Tomaro-Duchesneau *et al*., 2014; Sanders, 2000). Other than these, there was a *in vivo* male hamster study by Chiu *et al*. (2006) have proven that *Lactobacillus* strains such as *Lactobacillus paracasei subsp. paracasei*, *Lactobacillus plantarum* and *Lactobacillus acidophilus* successfully reduced the level of cholesterol in the blood and in the liver. According to Liong & Shah (2005), *lactobacilli* strains such as *L. acidophilus* and *L.casei* able to secret bile salt hydrolase that catalyzes the glycine- or taurine-conjugated bile salt hydrolysis into amino acid and free bile salts. Free bile salt was less soluble than conjugated bile salt and resulted a lower absorption in intestinal lumen. Thus, the deconjugation of bile salt has increased the amount of new bile salt to replace those have removed in the enterohepatic circulation, hence, the serum of cholesterol level was reduced. Besides that, probiotics such as *L. acidophilus* and *Bifidobacterium* strains able to assimilate some of the cholesterol from the medium by incorporating cholesterol into cell membrane during cell growth. Therefore, cholesterol that bind to the bacterial cell membrane would prevent absorption from the intestine into the blood (Liong & Shah, 2005a; Liong & Shah, 2005b; Watson & Preedy, 2015; Lye *et al*., 2009).

2.2.1.7 Thermotolerance

Probiotic such as *Lactobacillus* and *Bacillus subtilis* able to tolerant at mammalian gastric environment and temperature at 30°C and 37°C (Kim *et al*., 2019). However, the tolerance rate of *Lactobacillus* was declined while the temperature increases from 60°C due to the damage of the protective matrix in the

microbial cell wall (Mandal *et al*., 2006). Whereas for *Bacillus subtilis,* it has high survivability towards high temperature because it able to synthesize heat shock proteins when temperature increases (Schumann, 2003).

2.2.2 Health benefits of probiotics LAB

Probiotic bacteria contributed on a range of beneficial health effects to the host. In preparation of fermented food, inclusion of LAB could improve the quality, digestibility and bioavailability of nutrients in the food product (Parvez *et al*., 2006). LAB is also well-known to release different kind of nutrients such as folate by *Lactobacillus spp.* and *Lactococci spp.* (LeBlanc *et al*. 2007), vitamin B12 by *lactobacillus* of *L. reuteri* strain (Taranto *et al*., 2003), riboflavin by *L. fermentum* (Jayashree *et al*., 2010) and vitamin K by *Lactococcus, Lactobacillus, Enterococcus, Leuconostoc* and *Steptococcus* (Cooke *et al*., 2006; O'connor *et al*., 2005). According to Patel *et al*. (2012) and Novik *et al*. (2007), species of *Lactobacillus, Lactococcus, Pediococcus* and *Bifidobacterium* associated with fermented food produced carbohydrate degrading enzymes such as glucosidases, amylases and xylanases. However, there were reports that showed not all the probiotic bacteria attribute clinical effects to the host (Andersson *et al*., 2001). Thus, several studies from *in vitro*, animal and human studies have to be conducted to claim that the health benefits from certain probiotics is effective to the host.

2.2.2.1 Diarrhea treatment and prevention

There are numerous types of LAB that provided beneficial effect to diarrhea, travellers' diarrhea and diarrhea disease in young children that is caused by rotaviruses (Saavedra *et al*., 1994; Vanderhoof 2000). This included *Lactobacillus GG, Lactobacillus reuteri, Sacc. Boulardii, Bifidobacteria spp.,* and others (Duffy *et al*., 1994; Gorbach, 2000; Guandalini *et al*., 2000; Levy, 2000; Saavedra, 2000; Kyne and Kelly, 2001; Marteau *et al*., 2001; Benchimol & Mack, 2004). These probiotics decreased the viral shedding by increasing the secretary IgA in the immunological mechanism (Parvez *et al*., 2006). According to Desimone (1984) and O'Sullivan *et al*. (1992), probiotics can compete with invading viruses or bacteria pathogens that bind on epithelial cells and this might show an infection prevention effect to the individual. There were several studies that proven diarrhea was treated and prevented. According to Beck & Necheles (1961), *L. acidophilus* was effective towards diarrhea in humans and in gnotobiotic chicks that were inhabited with the *E. coli* pathogen (Watkins & Miller, 1983; Watkins *et al*., 1982).

Besides this, there was a study which determined the results of feeding a placebo calcium carbonate that contain tyophylized bacteria such as *Streptococcus thermophilus, Streptococcus lactis, Lactobacillus acidophilus, Lactobacillus bulgaricus* to infant and children who were associated with *E. coli*-mediated diarrhea and *Salmonella* and *Shigella* dysenteries in Yugosavia as treatment (Pearce & Hamilton, 1974; Tomic-Karovic & Fanjek, 1962; Zychowicz *et al*., 1974). The placebo calcium carbonate treatment had successfully recovered 66% in both groups of children, and

then all cases of dysenteries were eliminated by consuming *acidophilus* milk in long-term. Other than *L. acidophilus*, *Streptococcus faecium* SF 68 has been successfully used to alleviate diarrhea in animals (Underdahl *et al*., 1982) and humans, and also as an enteritis treatment in adult. The result was positive in all enteritis patients by observing the changes of the stool to normal stage after being administrated with *Streptococcus faecium* (Camarri *et al*., 1981; Lewenstein *et al*., 1979; Bellomo *et al*., 1980; Fernandes *et al*., 1987).

2.2.2.2 Inflammatory Bowel Syndrome disease

Inflammatory Bowel Syndrome (IBS) usually happens due to lactose and sucrose consumption by providing a nutritional source for pathogenic microbial population (Lin, 2004). These symptoms of IBS such as abdominal pain, flatulence, and irregular bowel movements (Santosa *et al*., 2006) happened collaboratively with the increase of the intestinal microbial population (Saarela *et al*., 2002; Lin, 2004).

There are reports which stated that an imbalance of intestinal bacteria, overgrowth of entero-adhesive and entero-hemorrhagic *Escherichia coli* and low levels of *Bifidobacterium*, might initiate the inflammation of these diseases (Saarela *et al*., 2002; Sartor, 1995; Rath, 2003). Probiotics was expected to decrease the pathogenic bacteria growth by lowering gut pH, providing barrier enhancement to prevent the invasion of pathogen and stimulating nonspecific and specific immune responses (Hart *et al*., 2003). As stated by Resta-Lenert & Barrett (2003), an *in vitro* study proven that probiotics might reduce the adhesion and invasion of epithelial cells.

Probiotcs such as *B. breve, B. bifidum and L. acidophillus* provided a beneficial effect in maintaining remission to ulcerative colitis by taking fermented milk as a complement (Cashman & Shanahan, 2003; Kato *et al*., 2004). Furthermore, as stated by Halpern *et al*. (1996) from a randomized, double-blind, crossover study, consuming *L. acidophilus* resulted an improvement of IBS from patients. Another study by Gade & Thorn (1989) showed an improvement on IBS patients who suffered for an average of 7 years by receiving *Enterococcus faecium*.

2.2.2.3 Colon Cancer prevention

Consuming probiotics could down regulate the activity of bacteria that have been associated with intestinal or colon cancer (Spanhaak *et al*., 1998; Ouwehand *et al*., 2002). Colon cancer was caused by the mutation of abnormal genes that control cell division when exposed to chemicals, namely carcinogens (Parvez *et al*., 2006). Carcinogens can be generated by metabolic activity of microbes that live in the gastrointestinal system (Santosa *et al*., 2006), such as enzymatic activity with enzymes β-glucuronidase, β-glucosidase, nitroreductase and so on (Santosa *et al*., 2006; Fernandes *et al*., 1987; Goldin *et al*., 1980; Goldin & Gorbach, 1984).

According to Sinha (1979), the study observed the significant decrease in fecal β-glucuronidase by ingesting *L. acidophilus*. Besides *L. acidophilus*, administration of *Lactobacillus casei* Shirota and *Bifidobacterium brev* also decreased of the β-glucuronidase activity (De Preter *et al*., 2008). There were other human intervention studies that showed LAB strains influenced the activity of nitroreductase and β-glucuronidase (Goldin *et al*., 1992; Benno & Mitsuoka, 1992; Bouhnik *et al*., 1996).

Moreover, the short-chain fatty acids such as butyrate produced by probiotics (Baghurst *et al*., 1996) lower down the pH of the digestive tract and prevent colon cancer (Malhotra, 1977; Segal *et al*., 1995). According to Kanauchi *et al*. (1999), the study showed that probiotic such as *Bifidobacterium* and *Eubacterium* have increased the production of butyrate. There were several studies that have proven that butyrate prevented colon cancer (Peng *et al*., 2009; Elamin *et al*., 2013; Roy *et al*., 2009;).

In addition, consumption of probiotic such as *lactobacilli* prevented the risk of developing tumors by binding to the mutagenic compound (Motta *et al*., 1991; Lidbeck *et al*., 1992; Murch, 2001; Isolauri, 2004). According Aso & Akazen (1992) and Aso *et al*. (1995), consumption of *L. casei* daily had delayed the recurrence of bladder tumor. There was an animal model study with 1,2-dimethylhydrazine (DMH)-induced colon cancer and showed that *Lactobacillus rhamnosus GG* has significantly reduced the incidence of colon tumors (Goldin *et al*., 1996).

2.2.2.4 Hepatic disease

Hepatic encephalopathy (HE) is a life-threatening liver disease in individuals which involves impairment of cognitive function (Sharma & Singh, 2016). According to Zhang *et al*. (2013), patients with minimal hepatic encephalopathy (MHE) were found to have high number of *streptococci* which is associated to cognitive impairment. Besides this, a study by Bajaj (2014) showed that patients with poor cognition associated with HE was found to have higher number of pathogens such as *Alcaligenaceae* and *Porphyromonadaceae* families.

Providing probiotic as a treatment has improved the imbalance in gut microbiota by decreasing the number of pathogenic bacteria and helped to improve endotoxemia, HE and liver functions (Liu *et al*., 2004). According to Bajaj *et al*. (2008), the reversal rate of MHE was significant higher in probiotic groups compared with the groups without treatment. There were several studies that showed consuming probiotics have positive effects on patient who has MHE and HE (Ziada *et al*., 2013; Sharma *et al*. 2008; Mittal *et al*. 2011; Mouli *et al*., 2014). The probiotics that have beneficial effect on disrupting the pathogenesis of HE included *Streptococcus thermophilus, Bifidobacteria, L. acidophilus, L.plantarum, L.casei, L. delbrueckii bulgaricus,* and *E. faecum* (Cunningham-Rundles *et al*., 2000; De Santis *et al*., 2000; Gorbach, 2000; Guslandi *et al*., 2000; Shanahan, 2001; Solga, 2003).

2.2.2.5 Hypertension

Probiotic bacteria play an important role in hypertension prevention. The development of hypertension was caused by inflammatory of endothelial dysfunction associated with the increased of systemic vascular resistance, commonly knowns as impaired endothelium dependent vasodilation due to imbalance between vasoconstrictors and vasodilators (Gomez-Guzman *et al*., 2015; Dinh *et al*., 2014). Consumption of probiotics inhibited the production of proinflammatory cytokines that will lead to the onset of hypertension (Matsuzaki *et al*., 2007; Arribas *et al*., 2008; Arribas *et al*., 2012). According to Gomez-Guzman *et al*. (2015), the study of probiotics treatment on hypertensive rats with *Lactobacillus fermentum* CECT5716 and *L. coryniformis* plus *L. gasseri* has improved the endothelial dysfunction and

resulted with reduction of vascular pro-inflammatory and pro-oxidative status. Besides this, a 3-weeks randomized, double-blind, placebo-controlled, parallel pilot study by Sharafedtinov *et al*. (2013) showed that subjects who consumed cheese with probiotics *Lactobacillus plantarum* have reduced blood pressure value compared to the control. According to Hata *et al* (1996), elderly patients who suffer with hypertension has consumed fermented milk with *Lactobacillus helyeticus* and *S. cerevisiae* experienced systolic and diastolic blood pressure reduction. Other than fermented milk, consuming powdered probiotic cell extracts also can lower the pressure of systolic and diastolic and heart rate (Sawada *et al*., 1990; Nakamura *et al*., 1995).

Chapter 3 Methodology

3.1 Isolation of putative lactic acid bacteria (LAB) from Malaysian fermented foods

Malaysian fermented foods such as fermented soybean curd *Tempeh* and fermented tapioca *Tapai Ubi* used in this study were purchased from the market and Tesco Cheras in Malaysia. Sample for each fermented foods was measured in 10g then put into a sterilized bag. After that, 90ml of sterile peptone buffered water was added into the sample and homogenized for 1 hour at room temperature. Then, 10-fold serial dilution (10^{-1} till 10^{-9}) was carried out by using peptone buffered water, in which 100 µl sample mixture (10^{0}) was pipetted into a sterilized Eppendorf tube that contained 900 µl of peptone buffered water. The 10^{-1} diluted sample was then mixed well by using vortex for 5s.

The steps above were repeated till obtained 10^{-9} diluted sample mixture. After that, 100 µl of each diluted sample was pipetted on MRS agar (de Man, Rogosa and Sharpe,1960) plate and spread well with sterile spreader. The agar plates were then incubated at 37°C for 48 hours. After incubation, 5 colonies on each food samples were picked and then inoculated into a sterile 10ml MRS broth, at 37°C for 24 hours. After that the cell morphology of the LAB culture was determined using Gram staining test. All assays were carried out in triplicate.

3.2 Maintenance of bacteria culture

All lactic acid bacteria cultures were subcultured twice and maintained in MRS broth and incubated at 37°C for 24 hours prior to assay. Bacteria cultures were stored in 20% (v/v) glycerol stock for long term storage. A total of 200 µl of sterile glycerol (100% v/v) was slowly pipetted into sterile Eppendorf tube. After that, 800 µl of revived culture was added into the Eppendorf tube that contained sterile glycerol, mixed well via vortex and stored in -20 or -80°C freezer.

3.3 Total viable plate count of bacteria

Total viable plate count was measured using Miles Misra method (Miles, Misra and Irwin, 1938) to quantify the number of bacteria present in a sample. A ten-fold serial dilution was carried out by pipetting 100 µl of active LAB culture into Eppendorf tube containing 900 µl of sterile peptone buffered water (dilution 10^{-1}). Then, this ten-fold serial dilution was repeated till dilution 10^{-9}. After that, 5 drops (10 µl /drop) of each diluted sample was pipetted onto MRS agar plate and allowed these drops to dissolve totally into the agar. All assays were conducted in triplicate. The agar plates were incubated at 37°C for 48 hours. The colonies formed were counted and the total viable plate count of bacteria present in a sample was determined using formula below:

$$\text{Total viable plate count (CFU/ml)} = \frac{\text{Number of colonies x Dilution factor (CFU)}}{\text{Sample volume plated (ml)}}$$

Notes: Dilution factor represent 10^1 to 10^9

3.4 Identification of the putative LAB via 16s rRNA sequence analysis

3.4.1 Extraction of DNA

In this step, Wizard Genomic DNA Purification Kit by 2020 Promega Corporation, USA was used. All the solutions were provided in the DNA purification kit.

1ml of a 24-h old putative LAB culture was added to a 1.5ml microcentrifuge tube. After that, the overnight culture was centrifuged at 13,000-16,000 x g for 2 minutes to pellet the cell, then the supernatant removed. The cells were resuspended thoroughly in 480 µl of 50mM EDTA. A total volume of 120 µl lysozyme buffer was added to the resuspended cell pellet, and gently pipetted to mix. The purpose of this pretreatment is to weaken the cell wall so that cell lysis can take place. Later on, the sample was incubated at 37°C for 30-60 minutes, then centrifuged for 2 minutes at 13,000-16,000 x g and the supernatant removed. Then, a total volume of 600 µl Nuclei Lysis solution was added and gently pipetted until the cells were resuspended. Afterwards, the mixture was incubated at 80°C for 5 minutes to lyse the cells, then cooled down at room temperature. A total volume of 3 µl RNase Solution was added to the cell lysate and inverted the tube for 2-5 times to mix well. Again, the mixture was incubated at 37°C for 15-60 minutes, then cooled down at room temperature. Following this, 200 µl of Protein Precipitation Solution was added to the RNase-treated cell lysate and vortexed vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution with the cell lysate. After that, the sample was incubated on ice for 5 minutes, and then centrifuged at 13,000-16,000 x g for 3

minutes. The supernatant that contained the DNA was transferred to a clean 1.5ml microcentrifuge tube that contained 600 µl of isopropanol at room temperature.

The supernatant that remained in the original tube that contained the protein pellet was neglected to avoid the contaminating the DNA solution with the precipitated protein. Furthermore, the supernatant that contained DNA was gently mixed by inversion until the thread-like strands of DNA form a visible mass, then centrifuged at 13,000-16,000 x g for 2 minutes. The supernatant was carefully poured off and the tube was drained on a clean absorbent paper. Then, a volume of 600 µl of room temperature 70% ethanol was added and the tube gently inverted for several times to wash the DNA pellet. The DNA pellet was centrifuged at 13,000-16,000 x g for 2 minutes and then the ethanol was carefully aspirated. The tube was drained on a clean absorbent paper again and the pellet was allowed to air-dry in a biosafety cabinet for 10-15 minutes. Last but not least, 30 µl of DNA rehydration Solution was added to the tube and rehydrated the DNA by incubating at 65°C for 1 hour. The solution was mixed by gently tapping the tube. Otherwise, the DNA was rehydrated by incubating the solution overnight at room temperature or at 4°C. The DNA was stored at 2-8°C.

3.4.2 Polymerase chain reaction

The extracted DNA was proceeded to amplification using Polymerase Chain Reaction (PCR). The universal primers used were the 27F forward primer: 5′-AGA GTT TGA TCM TGG CTC AG-3′ and 1492R reverse primer: 5′-GGT TAC CTT GTT ACG ACT T-3′ (AI *et al*., 2008). The PCR master mix for 6 reactions were prepared based

on Table 3.1 using the DreamTaq DNA polymerase kit (Thermo Fisher Scientific, USA). The reagents listed in the Table 3.1 were mixed in a sterile 1.5ml microcentrifuge tube. After mixing thoroughly, a total of 19 µl aliquot of the mix was poured into 200 µl of PCR tube. 1 µl of sample was added into each tube except for one tube which was added with 1 µl of distilled water to use it as the PCR no-template control. Then, the 200 µl PCR tubes were transferred into a thermal cycler (Bio-Rad Laboratories, Inc; USA): (i) Initial hot start activation or denaturation step at 95°C - 98°C for 5 minute; (ii) Denaturation step at 95°C for 20s and annealing at 60°C for 30s; (iii) Extension steps were performed at 72°C for 30 s; (iv) final extension at 72°C for 5 mins and (v) stored at 10°C till used. Lastly, 5 µl of the PCR products were analysed by 1.5% agarose gel electrophoresis stained with SYBR® safe DNA gel stain in 1X TBE buffer for 30 mins (Thermo Fisher Scientific, USA).

Table 3.1: Preparation of master mix for PCR

Reagent	Stock concentration	Final Concentration
DreamTaq Polymerase Buffer	10x	1x
DNTP Mix	2 mM	0.20 mM
Forward primer	10 mM	0.20 mM
Reverse primer	10 mM	0.20 mM
DreamTaq Polymerase	5 U/µl	1.5 U/µl
Magnesium Chloride	50 mM	3.5 mM
Sterile distilled water	0	0

3.4.3 16S rRNA gene sequence analysis

The PCR products for selected putative LAB isolates were contracted out to the Apical Scientific Sdn Bhd, Malaysia for 16s rRNA sequence analysis. Then, the sequences generated were aligned in the genetic sequences database at GenBank® (BLAST, National Center for Biotechnology Information) to verify the genus and species of the LAB isolates.

3.5 Determination of the probiotic properties of the selected putative LAB

3.5.1 Bile tolerance assay

In this assay, 200 µl of 24h-old putative LAB isolate ($1x10^8$ CFU/ml) was inoculated into 10ml MRS broth added with 0%, 0.3%, 0.5% and 1.0% (w/v) of bile salt. The putative LAB isolate was then incubated at 37°C for 24 hours and followed by determination of total viable count using Mile Misra Method. All tests were carried out in triplicate and MRS broth with 0% bile salt was used as control.

3.5.2 Simulated gastric and small intestinal transit tolerance assays.

3.5.2.1 Preparation of washed cell suspension

A total of 10ml of 24h-old putative LAB isolate (1x108 CFU/ml) was centrifuged at 8000xg for 10 minutes, at 4°C. Then, 10ml of 50mM K_2HPO_4, pH7 buffer was used to wash the cell pellet. The supernatant was discarded, and the cell pellet were reconstituted back to the volume of 10ml using the same buffer. The washed cell suspension was ready to use for gastric and small intestine transit assay.

3.5.2.2 Preparation of simulated gastric juice

A 0.3% (w/v) pepsin was mixed in sterile 0.5% (w/v) NaCl. After that, the mixture was adjusted to pH2, 3, 4 correspondingly with 5M of HCl or NaOH to make fresh simulated gastric juice.

3.5.2.3 *In vitro* gastric intestinal transit tolerance assay

The washed cell suspension with volume of 200 µl was inoculated into 0.3ml of NaCl (0.5% w/v) in a sterile microcentrifuge tube. After that, 1.0ml of simulated gastric juice with different pHs (pH 2, 3 and 4) were added into the mixture correspondingly. Further on, the mixtures were vortexed for 10s and incubated in 37°C water bath for 180 minutes. At intervals of 0, 60, 120, and 180 minutes of incubation, 100 µl of aliquot was withdrawn to determine the total viable count. All tests were conducted in triplicate.

3.5.2.4 Preparation of simulated small intestinal juice

Pancreatin (0.1% w/v) was suspended in sterile 0.5% (w/v) NaCl. The mixture was then added with and without 0.3% (w/v) bile salt. Then, the pH of the simulated small intestinal juices was adjusted to pH 8 by using 5M of NaOH.

3.5.2.5 *In vitro* small intestinal transit tolerance assay

The washed cell suspension (as in Section 3.5.2.1) with a volume of 200 µl was inoculated into 0.3ml of NaCl (0.5% w/v) in a sterile microcentrifuge tube. Then, 1.0ml of simulated small intestine juices (pH 8; with or without 0.3% w/v bile salt.) were added into the mixtures respectively. The mixtures were vortexed for 10s and incubated in 37°C water bath for 240 minutes. At interval of 0, 60, 120, 180, and 240 minutes of incubation, 100 µl of the aliquot were taken out to determine the total viable count. All tests were carried out in triplicate.

3.6 Statistical analysis

Data analysis was performed using IBM SPSS Statistics version 24.0 (SPSS Inc, Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Duncan multiple range test (DMRT) was performed to correlate the difference between individual variables in all experiments. A value of $P < 0.05$ was considered as being statistically significant. All assays were conducted in triplicate.

Chapter 4 Results

4.1 Isolation of putative lactic acid bacteria from Malaysian fermented foods

In this study, a total of 18 putative lactic acid bacteria (LAB) isolates were isolated from *Tempeh* and *Tapai Ubi*. Table 4.1 showed the cell morphology of each isolate determined via Gram stain test. All of the isolates were confirmed as Gram-positive coccus-shaped bacteria. Isolates from *Tempeh* were designated as ST1 till ST9, while isolates from *Tapai Ubi* were designated as SU1 till SU9. Although all 18 isolates were stored at -20 and -80°C freezer, but most of the isolates were failed to revive when subculture step was performed at 37°C for 24-48h. Only four isolates survived and remained active and viable throughout this project. Thus, these four putative isolates, namely ST4 from *Tempeh* and SU1, SU2 and SU8 from *Tapai Ub*i were selected from subsequent assays. Last but not least, several tests such as catalase activity test (Whittenbury, 1964), coagulase test (Zdolec et al., 2013) and more will perform in future to justify the property of lactic acid bacteria.

Table 4.1: Cell morphology of the putative lactic acid bacteria isolated from local fermented foods

LAB isolates	Fermented Foods	Gram stain reaction	Cell Shape	Designation
1	Tempeh	+	Coccus	ST1
2	Tempeh	+	Coccus	ST2
3	Tempeh	+	Coccus	ST3
4	**Tempeh**	**+**	**Coccus**	**ST4 #**
5	Tempeh	+	Coccus	ST5
6	Tempeh	+	Coccus	ST6
7	Tempeh	+	Coccus	ST7
8	Tempeh	+	Coccus	ST8
9	Tempeh	+	Coccus	ST9
10	**Tapai Ubi**	**+**	**Coccus**	**SU1 #**
11	**Tapai Ubi**	**+**	**Coccus**	**SU2 #**
12	Tapai Ubi	+	Coccus	SU3
13	Tapai Ubi	+	Coccus	SU4
14	Tapai Ubi	+	Coccus	SU5
15	Tapai Ubi	+	Coccus	SU6
16	Tapai Ubi	+	Coccus	SU7
17	**Tapai Ubi**	**+**	**Coccus**	**SU8 #**
18	Tapai Ubi	+	Coccus	SU9

Note: # This isolate was selected for subsequent assays.

4.2 Identification of the selected putative lactic acid bacteria via 16s rRNA sequence analysis

Table 4.2 showed the 16S rRNA sequence analysis of the putative LAB isolates of SU1, SU2, SU8 and ST4. SU1 and SU2 had 98.94% and 98.61% of similarity with *Pediococcus acidilactici* strain ZPA017. While, SU8 shared 85.58% of similarity with *Pediococcus claussenii* ATCC BAA-344, and ST4 shared 98.01% of similarity with *Leuconostoc mesenteroides subsp. Mesenteroides* ATCC8293. The sequences for these putative isolates were presented in Figure 4.1-4.4.

Table 4.2: Identities of putative isolate LABs via 16S rRNA gene sequence

LAB	Name	Query Cover	E value	Identity	Accession no.
SU1	*Pediococcus acidilactici,* strain ZPA017	80%	0	98.94%	NZ_CP015206.1
SU2	*Pediococcus acidilactici* strain ZPA017	87%	0	98.61%	NZ_CP015206.1
SU8	*Pediococcus claussenii* ATCC BAA-344	82%	0	85.58%	NC_016605.1
ST4	*Leuconostoc mesenteroides subsp. mesenteroides* ATCC 8293	88%	0	98.01%	NC_008531.1

Figure 4.1: Phylogenetic tree of SU1

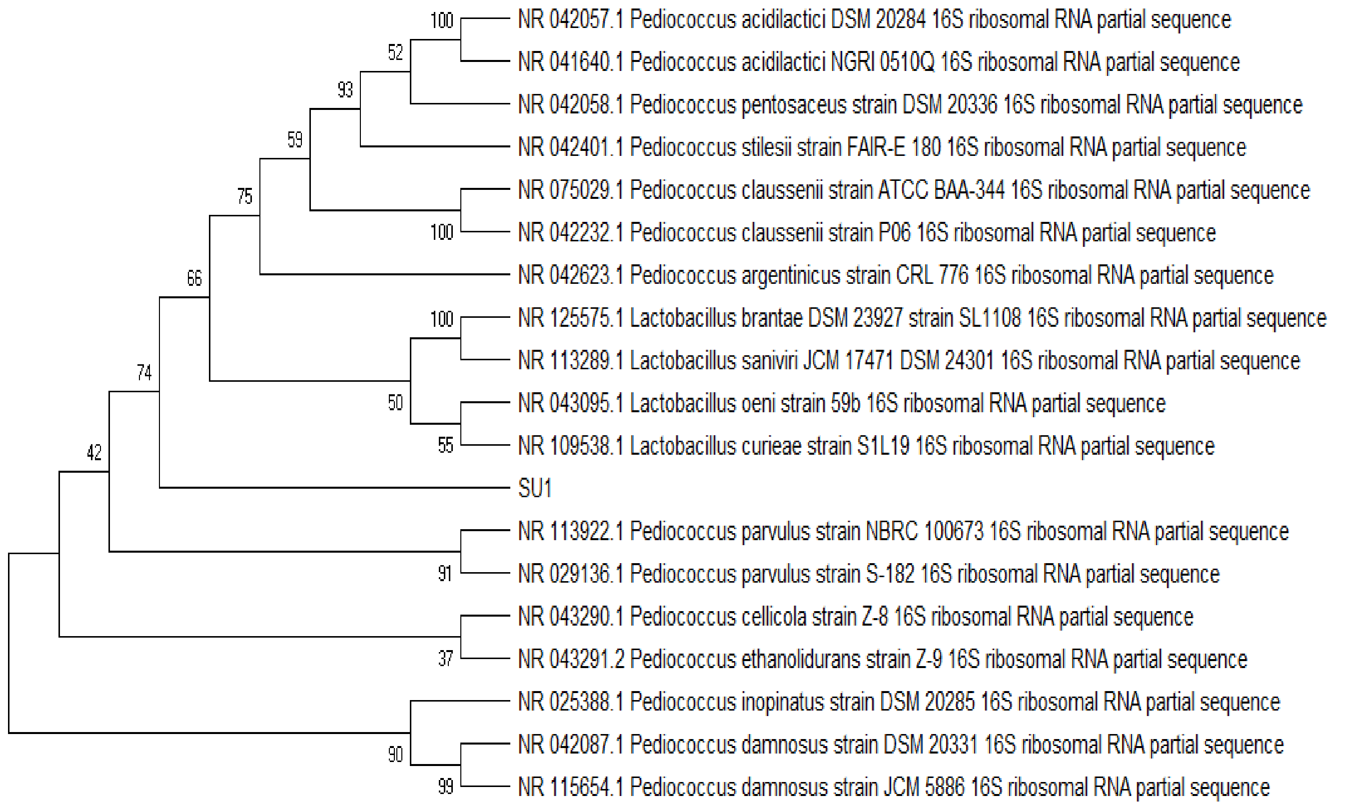


Figure 4.2: Phylogenetic tree of SU2

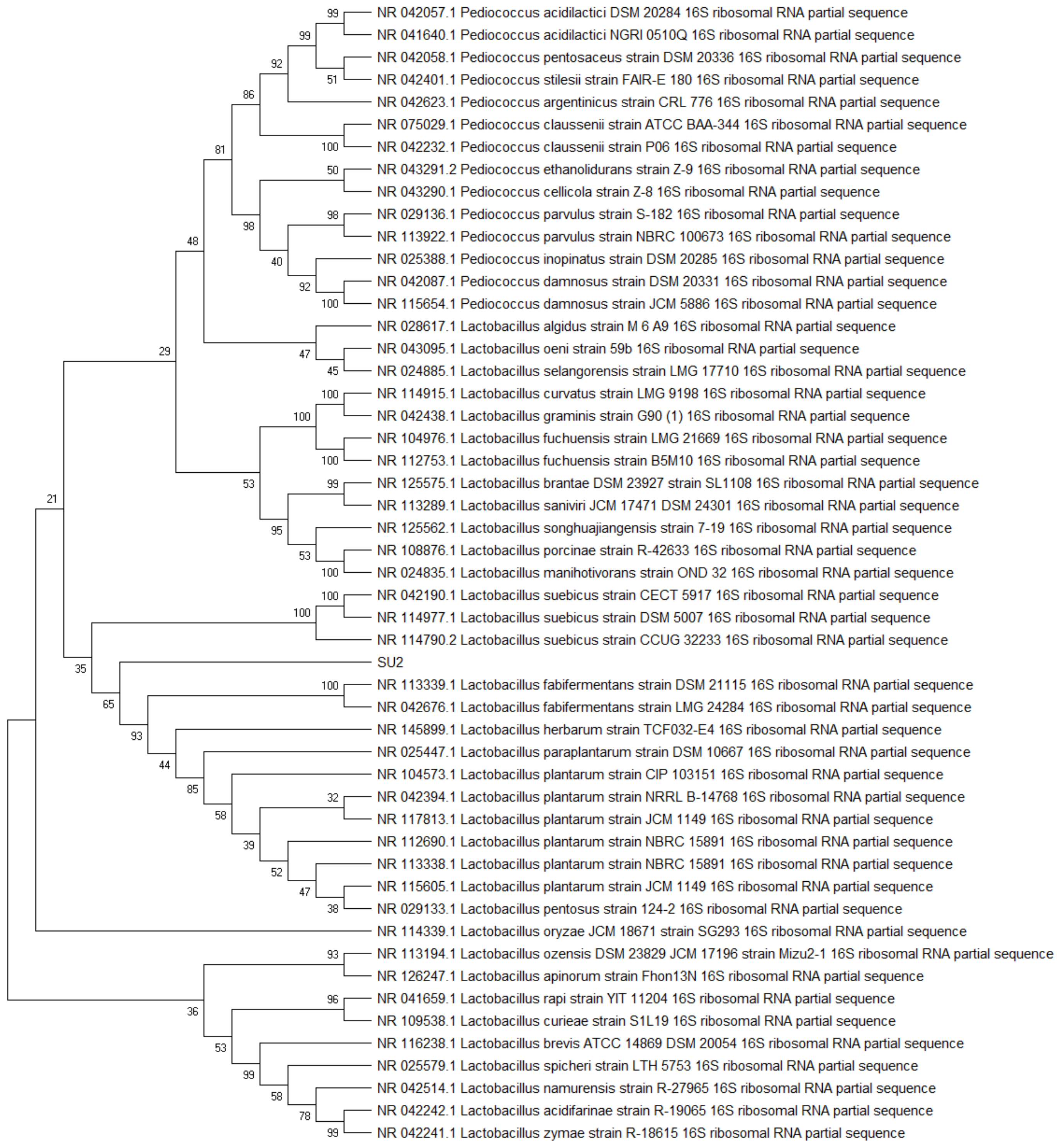


Figure 4.3: Phylogenetic tree of SU8

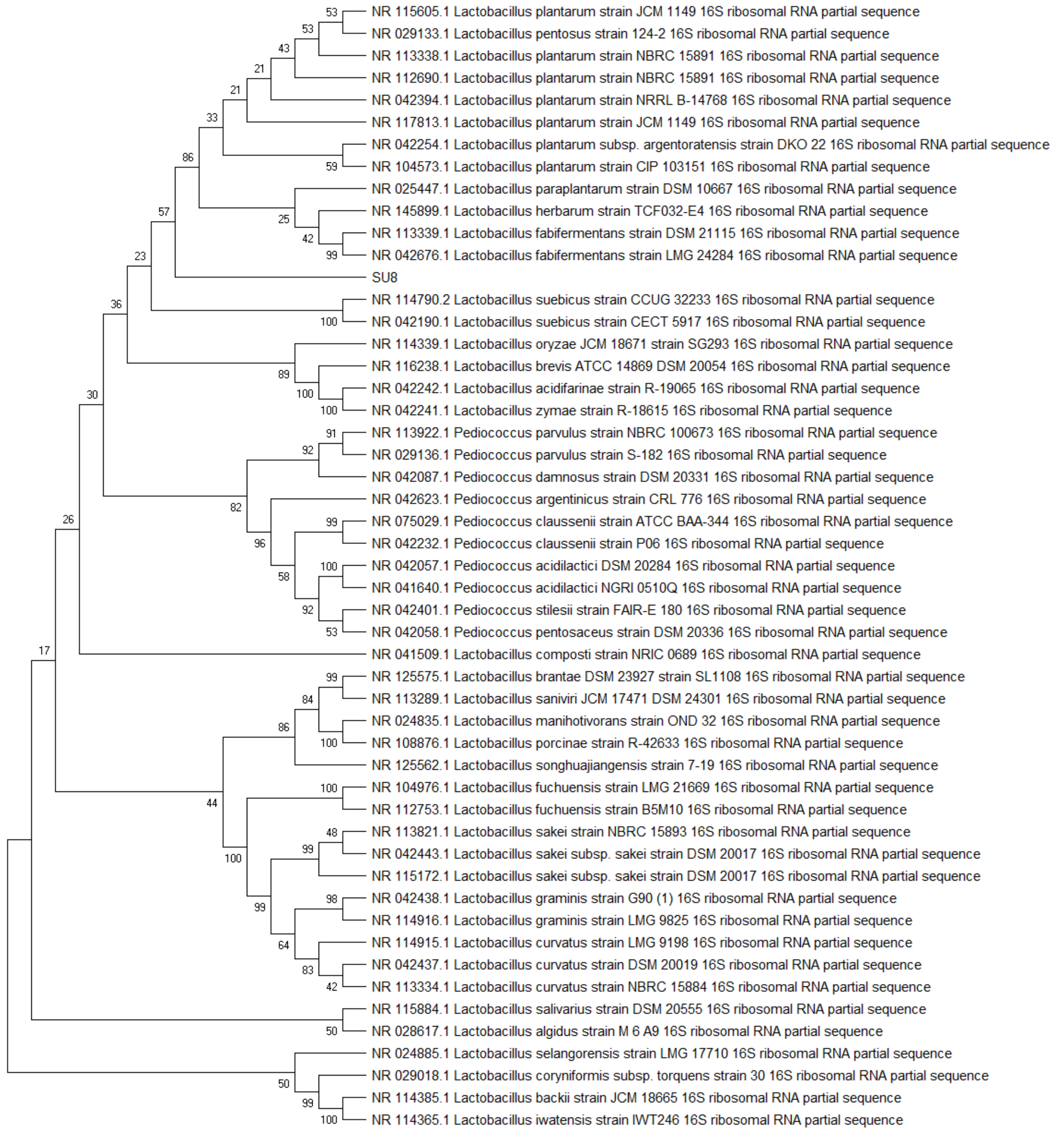
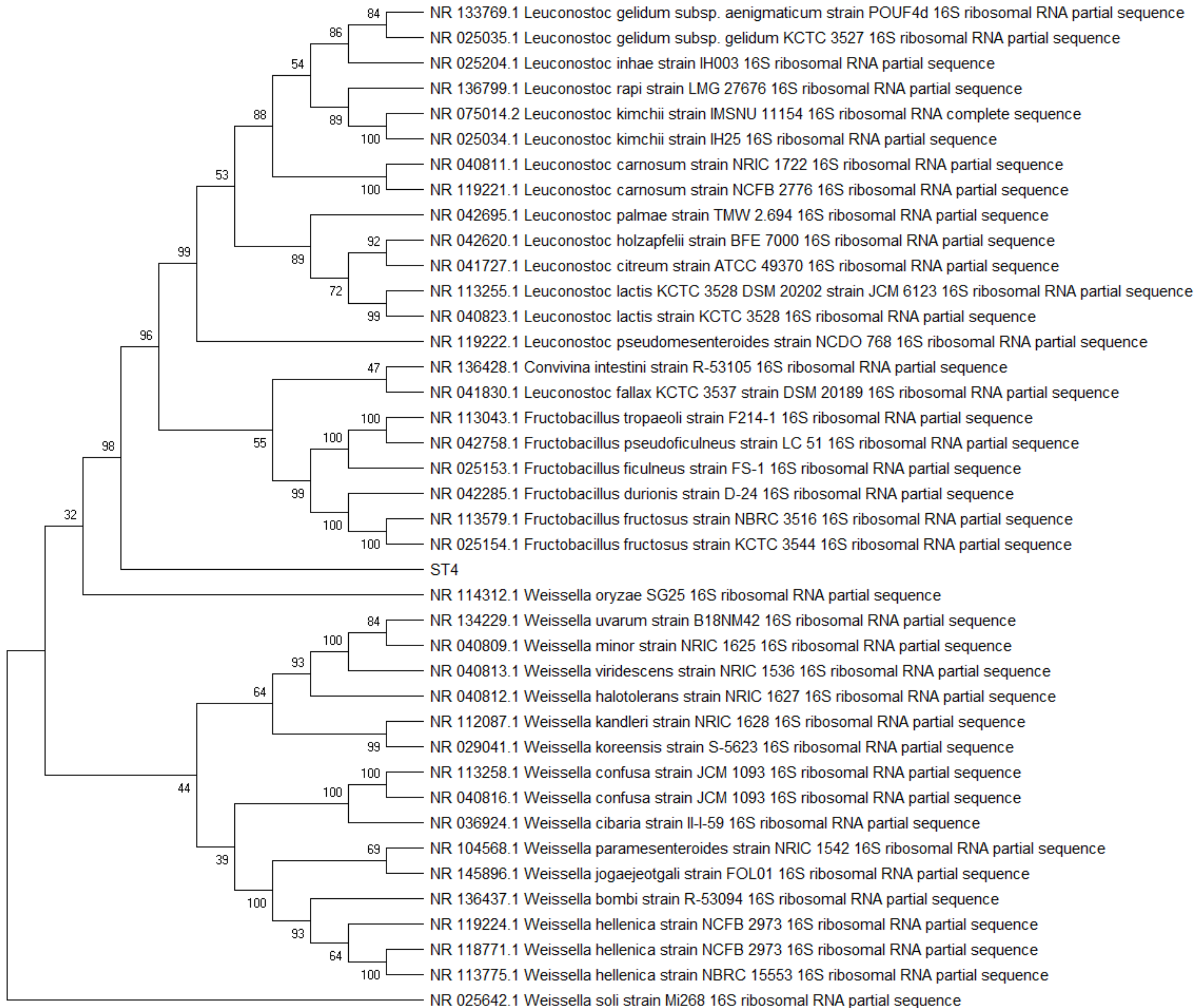


Figure 4.4: Phylogenetic tree of ST4



4.3 Determination of *in vitro* bile tolerance of the selected lactic acid bacteria

Table 4.3 showed the *in vitro* bile tolerance of the four putative LAB isolates (SU1, SU2, SU8 and ST4). The viable cell count for SU1 has the highest number of cells at 11.73 Log_{10} CFU/ml before adding the bile salt. After adding with 0.3% of bile salt, the viable cell count decreased by 2.08-fold. There was no significant different between the addition of 0.3% and 0.5% of bile salt. As for SU2 and SU8, the viable cell count started to decrease by 1.68-fold and 1.31-fold after the addition of 0.3% of bile salt. Interestingly, there was a significant increase of viable cell count by 1.33-fold and 1.1-fold when added with 0.5% of bile salt. However, no viable cell count was detected for SU1, SU2 and SU8 when 1.0% of bile salt was added. For ST4, the viable cell decreased by 1.84-fold after adding 0.3% of bile salt but there was no viable cell count detected from 0.5% to 1.0% of bile salt. Overall, SU8 showed the strongest tolerance to bile salt with highest viable cell count of 7.13 Log_{10} CFU/ml remained after incubation with 0.5% of bile salt. Whereas, ST4 showed the weakest tolerance to bile salt and failed to grow when incubated with 0.5% and 1.0% of bile salt.

Table 4.3: Effect of bile salt concentration on growth of putative LAB isolates

LAB	Bile salt%(w/v)			
	Viable cell count (Log_{10}CFU/ml) of LAB at different bile salt concentration			
	0% Bile	0.3% Bile	0.5% Bile	1.0% Bile
SU1	11.73 ± 0.20^{c}	5.64 ± 0.30^{b}	5.70 ± 0.26^{b}	0.00 ± 0.00^{a}
SU2	8.46 ± 0.41^{d}	5.01 ± 0.39^{b}	6.70 ± 0.02^{c}	0.00 ± 0.00^{a}
SU8	8.50 ± 0.12^{d}	6.48 ± 0.33^{b}	7.13 ± 0.22^{c}	0.00 ± 0.00^{a}
ST4	11.50 ± 0.31^{c}	6.25 ± 0.10^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Notes: Values are mean ± standard deviation, n=3

abcd within a row, values with different superscripts are significantly difference at $P<0.05$

4.4 Determination of the *in vitro* gastric intestinal transit tolerance of selected putative lactic acid bacteria

The simulated gastric juices were adjusted to pH 2, 3 and 4 respectively and each putative LAB isolate was incubated in the gastric juices and the viable cell count was measured at different time intervals (0, 60, 120, and 180 min) as shown in Table 4.4-4.7.

4.4.1 Comparison of the tolerance of each putative LAB isolate when incubated in simulated gastric juice.

Isolate SU1 was incubated in simulated gastric juices adjusted to pH 2-4 (Table 4.4). At pH2, the viable cell count decreased significantly after 1 hour of incubation from 12.21 to 10.97 Log_{10} CFU/ml. At 120 min, the viable cell counts were increased and there was no significant reduction and addition afterwards. As for pH3, there was a significant increase after 1 hour of incubation, however, there was no significant difference after 60 min incubation. Lastly for pH4, there was no significant addition nor reduction throughout the 3 hours incubation. Overall, isolate SU1 showed strong tolerance to gastric juices with no significant difference in viable cell count at 180 min when compared to the control at 0 min.

Table 4.4: Comparison of the tolerance of SU1 when incubated in stimulated gastric juices adjusted to different pH

Time (min)	Viable cell count (Log_{10}CFU/ml) of SU1 when incubated in pH adjusted gastric juices at different incubation time		
	pH2	pH3	pH4
0	12.21 ± 0.19^{b}	12.48 ± 0.16^{a}	12.49 ± 0.07^{a}
60	10.97 ± 1.36^{a}	12.76 ± 0.19^{b}	12.47 ± 0.24^{a}
120	12.26 ± 0.22^{b}	12.75 ± 0.13^{b}	12.60 ± 0.08^{a}
180	12.05 ± 0.02^{b}	12.56 ± $0.09^{a,b}$	12.59 ± 0.14^{a}

Notes: Values are mean ± standard deviation, n=3

ab within a column, values with different superscripts are significantly difference at $P<0.05$.

Isolate SU2 was incubated in simulated gastric juices adjusted to pH 2–4 (Table 4.5). At pH 2, the viable cell count started to decrease at 60 min but it was no significant difference compared to 0 min. However, there was a significant reduction of viable cell count at 120 min incubation, and also it was the least viable cell count (12.2 Log_{10} CFU/ml). Then, the viable cell count remained constant and no significant reduction nor addition of cells was observed. At pH3, there was an increase in viable cell count after 1hour of incubation but it was not significantly different.

However, at 180 min, the viable cell count increased significantly and also the highest amount of viable cell count was observed (12.60 Log_{10} CFU/ml). Lastly for pH4, there was a significant reduction of viable cell count after 1hour incubation from 12.86 Log_{10} CFU/ml to 12.68 Log_{10} CFU/ml, which also showed the highest amount of viable cell count to the lowest. After that, there was no significant addition or reduction at 120 min and 180 min of incubation. Overall, isolate SU2 showed strong tolerance to gastric juice adjusted to pH 4 with no significant difference in viable cell count at 180 min when compared to control at 0 min.

Table 4.5: Comparison of the tolerance of SU2 when incubated in stimulated gastric juice adjusted to different pH

Time (min)	Viable cell count (Log_{10}CFUml) of SU2 when incubated in pH adjusted gastric juices at different incubation time		
	pH2	pH3	pH4
0	12.42 ± 0.05^{c}	12.44 ± 0.02^{a}	12.86 ± 0.10^{b}
60	12.35 ± $0.15^{b,c}$	12.62 ± $0.24^{a,b}$	12.68 ± 0.09^{a}
120	12.20 ± 0.04^{a}	12.65 ± $0.10^{a,b}$	12.77 ± $0.06^{a,b}$
180	12.26 ± $0.09^{a,b}$	12.69 ± 0.07^{b}	12.74 ± $0.05^{a,b}$

Notes: Values are mean ± standard deviation, n=3

abc within a column, values with different superscripts are significantly difference at $P<0.05$.

Isolate SU8 was incubated in simulated gastric juices adjusted to pH 2–4 (Table 4.6). There was no significant different for viable cell counts at PH 2 and PH 3

throughout the 3hours incubation period. At PH 4, there was no significant reduction of viable cell counts after 1hour incubation, but after incubated another 1hour which is at 120 min, there was a significant reduction. At 180 min, there was a significant increase of viable cell count. Overall, isolate SU8 showed strong tolerance to gastric juices adjusted with pH 2 and 3 with no significant difference in viable cell count at 180 min when compared to control at 0 min.

Table 4.6: Comparison of the tolerance of SU8 when incubated in simulated gastric juices adjusted to different pH

Time (min)	Viable cell count (L_{og}10CFU/ml) of SU8 when incubated in pH adjusted gastric juices at different incubation time		
	pH2	pH3	pH4
0	12.35 ± 0.03^{a}	12.62 ± 0.12^{a}	12.76 ± 0.05^{c}
60	12.58 ± 0.14^{a}	12.53 ± 0.15^{a}	12.72 ± 0.09^{b,c}
120	12.55 ± 0.20^{a}	12.64 ± 0.10^{a}	12.39 ± 0.12^{a}
180	12.51 ± 0.13^{a}	12.64 ± 0.06^{a}	12.57 ± 0.12^{b}

Notes: Values are mean ± standard deviation, n=3

abc within a column, values with different superscripts are significantly difference at P<0.05.

Isolate ST4 was incubated in simulated gastric juices adjusted to pH 2–4 (Table 4.7). At PH2, there was a significant increase of viable cell count after 1hour incubation. After that, the amount of cells had decreased significantly but is not significantly different between the amount of cells at 0 min. Furthermore, there was

no significant reduction at 180 min of incubation. For PH 3, there was a significant increased of the viable cell count after 1 hour of incubation. However, there was no significant different of viable cell count from 120 min to 180 min. Lastly for PH 4, there was a significant reduction of viable cell after one hour incubation but there was no significant addition of viable cell for another one more hour incubation (120 min). However at 180 min, there was a significant decreased of viable cell count and also was the least amount of cells produced at PH 4 (12.64 Log10 CFU/ml). Overall, isolate ST4 showed strong tolerance to gastric juice adjusted to pH3 with significant increase in viable cell count when compare the growth at 180 min with control at 0 min.

Table 4.7: Comparison of the tolerance of ST4 when incubated in simulated gastric juices adjusted to different pH

Time (min)	Viable cell count (Log_{10}CFU/ml) of ST4 when incubated in pH adjusted gastric juices at different incubation time		
	pH2	pH3	pH4
0	12.31 ± 0.13[a]	12.34 ± 0.14[a]	12.78 ± 0.06[b]
60	12.61 ± 0.11[b]	12.62 ± 0.02[b]	12.61 ± 0.10[a]
120	12.34 ± 0.06[a]	12.54 ± 0.18[b]	12.71 ± 0.04[a,b]
180	12.28 ± 0.05[a]	12.54 ± 0.08[b]	12.64 ± 0.03[a]

Notes: Values are mean ± standard deviation, n=3

ab within a column, values with different superscripts are significantly difference at P<0.05.

4.5 Determination of the *in vitro* small intestinal transit tolerance of selected putative lactic acid bacteria

Each putative LAB isolate was incubated in the in the simulated small intestinal juice adjusted to pH 8 with and without addition of bile salt. The viable cell count was measured at different time intervals (0, 60, 120, 180 and 240 min) as shown in Table 4.8-4.11.

4.5.1 Comparison of the tolerance of each putative LAB isolate when incubated in simulated small intestinal juices

The addition of bile salt in the simulated small intestinal juice had impact on the viable cell count of SU1. The viable cell count was maintained for the first 60 min of incubation but then significantly decreased to 9.82 Log_{10} CFU/ml after 240 min of incubation. Interestingly an increment on viable cell count was also observed at 180 min of incubation then followed by a reduction at 240 min. However, when SU1 was incubated in small intestinal juice without addition of bile salt, the viable cell count was maintained for first 120 min with no reduction in growth, followed by 22.21% of reduction at 180 min and resulted with 8.30 Log_{10} CFU/ml at 240 min.

Table 4.8: Effect of simulated small intestinal juices on the growth of SU1

Time (min)	Viable cell count (Log_{10}CFU/ml) of SU1 at different incubation time	
	With bile salt	Without bile salt
0	10.27 ± 0.14^{c}	10.90 ± 0.16^{c}
60	$10.10 \pm 0.12^{b,c}$	10.53 ± 0.31^{c}
120	9.78 ± 0.21^{a}	10.67 ± 0.13^{c}
180	11.70 ± 0.32^{d}	9.77 ± 0.10^{b}
240	$9.82 \pm 0.17^{a,b}$	8.30 ± 0.50^{a}

Notes: Values are mean ± standard deviation, n=3

abcd within a column, values with different superscripts are significantly difference at $P<0.05$.

The addition of bile salt in the simulated small intestinal juice had no impact on the viable cell count of SU2. The viable cell count ranging from 11.40–12.00 Log_{10} CFU/ml was maintained throughout the 240 min of incubation. Interestingly when SU2 was incubated in small intestinal juice without addition of bile salt, the viable cell count was decreased significantly by 2.62% after 240 min of incubation.

Table 4.9 : Effect of simulated small intestinal juices on the growth of SU2

Time (min)	Viable cell count (Log_{10}CFU/ml) of SU2 at different incubation time	
	With bile salt	Without bile salt
0	11.98 ± 0.22^{a}	12.21 ± $0.11^{b,c}$
60	11.40 ± 0.36^{a}	12.08 ± 0.08^{b}
120	11.95 ± 0.50^{a}	12.31 ± 0.10^{c}
180	11.58 ± 0.40^{a}	12.10 ± 0.13^{b}
240	12.00 ± 0.34^{a}	11.89 ± 0.16^{a}

Notes: Values are mean ± standard deviation, n=3

abc within a column, values with different superscripts are significantly difference at $P<0.05$.

The addition of bile salt in the simulated small intestinal juice had impacted on the viable cell count of SU8. The viable cell count was maintained for the first 60 min of incubation but then significantly increased to 12.05 and 12.02 Log $_{10}$CFU/ml after 120 and 180 min of incubation, respectively. However, after 240 min of incubation, no significant difference was observed between the viable cell count at 0 and 240 min. However, when SU8 was incubated in small intestinal juice without addition of bile salt, the viable cell count was maintained for first 180 min with no reduction in growth, followed by 2.73% of growth reduction at 240 min. The tolerance of LAB isolates when incubated in simulated small intestinal juices were performed to 240 min because the transit time for small intestine was 4hours or more (Szarka & Camilleri, 2012).

Table 4.10: Effect of simulated small intestinal juices on the growth of SU8

Time (min)	Viable cell count (Log_{10}CFU/ml) of SU8 at different incubation time	
	With bile salt	Without bile salt
0	11.37 ± 0.34^{a}	12.01 ± $0.10^{b,c}$
60	11.53 ± 0.26^{a}	11.80 ± $0.16^{a,b}$
120	12.05 ± 0.13^{b}	12.03 ± $0.19^{b,c}$
180	12.02 ± 0.10^{b}	12.06 ± 0.20^{c}
240	11.73 ± $0.36^{a,b}$	11.73 ± 0.11^{a}

Notes: Values are mean ± standard deviation, n=3

abc within a column, values with different superscripts are significantly difference at P<0.05.

The addition of bile salt in the simulated small intestinal juice had no impact on the viable cell count of ST4. The viable cell count ranging from 12.33 – 12.51 Log_{10}CFU/ml was maintained throughout the 240 min of incubation. Interestingly when ST4 was incubated in small intestinal juice without addition of bile salt, the viable cell count was decreased significantly by 2.97% after 60 min of incubation and the growth was then maintained throughout the 240min of incubation.

Table 4.11: Effect of simulated small intestinal juices on the growth of ST4

Time (min)	Viable cell count (Log_{10}CFU/ml) of ST4 at different incubation time	
	With bile salt	Without bile salt
0	12.51 ± 0.20[a]	12.43 ± 0.12[b]
60	12.44 ± 0.10[a]	12.06 ± 0.35[a]
120	12.42 ± 0.14[a]	12.32 ± 0.11[a,b]
180	12.35 ± 0.27[a]	12.30 ± 0.09[a,b]
240	12.33 ± 0.14[a]	12.16 ± 0.10[a,b]

Notes: Values are mean ± standard deviation, n=3

ab within a column, values with different superscripts are significantly difference at P<0.05.

Chapter 5 Discussion

5.1 Isolation of putative lactic acid bacteria from fermented food

In this study, De Man, Rogosa and Sharpe (MRS) was used as a selective medium for isolation and cultivation of putative lactic acid bacteria (LAB) which is typically used to isolate species of *Lactobacillus, Streptococcus, Pediococcus* and *Leuconostoc*, from Malaysian fermented foods (Marshall, 1992). MRS medium was supplemented with nutrients which included peptone and beef extract that supplied carbon and nitrogen to lactic acid bacteria culture, yeast extract which provided vitamin D and dextrose from carbohydrate for energy source (Abbasiliasi *et al*., 2017). With the use of MRS medium, a total of eighteen of LAB were isolated from *Tempeh* and *Tapai Ubi*. All putative isolates showed positive Gram stain reaction and cells with coccus shaped.

These results suggested that the Gram-positive coccus shaped putative LABs isolated from *Tempeh* and *Tapai Ubi* could be from the genera of *Pediococcus* and *Leuconostoc.* This result was determined by DNA extraction, polymerase chain reaction (PCR) and 16S rRNA gene sequence analysis. There were several research reported that *Tempeh* and *Tapai Ubi* contained various type of putative LABs such as *Lactobacillus plantarum, Lactobacilli spp,* and *Lactococcus lactis* (Kormin *et al*., 2001; De Vuyst & Vandamme, 2012; Babu *et al*., 2009; Ooi, 2010; Loh *et al*., 2008).

The variation in the types of putative LAB obtained from *tempeh* and *tapai ubi* could be due to the changes of environment and fermentation conditions such as pH,

temperature, salinity or other relative ingredients which may affect the microbial population of fermented foods (Li *et al*., 2016; Giraffa, 2004). Unfortunately, in this study only four putative LAB isolates, namely SU1, SU2, SU8 from *Tapai Ubi* and ST4 from *Tempeh* remained viable after frozen storage, and hence these isolates were proceeded to subsequent assays.

5.2 Identification of putative lactic acid bacteria

According to Mora *et al*. (1997), the molecular identification of 16S rRNA sequence analysis is a powerful technique to determine the phylogenetic relationship of microorganism. Hence this analysis was adopted to identify the four putative LAB isolates (SU1, SU2, SU8 and ST4) from *Tempeh* and *Tapai Ubi*. In this analysis, SU1 and SU2 were identified as *Pediococcus acidilactici*, SU8 was identified as *Pediococcus claussenii*, and ST4 was identified as *Leuconostoc mesenteroides subsp. mesenteroides*. SU8 has only 85% of Pediococcus claussenii identity which is far lower than the cut-off for same genus (>94%), and also even much lower than the same species (<97%). Further verification on the identification will be done in future.

The genus of *Pediococcus* is the member of lactic acid bacteria family with characteristics such as coccus-shaped, gram-positive, non-mobile, non-spore formation, catalase-negative and anaerobic (Porto *et al*., 2017). There are seven *Pediococcus* species recognized which are P*. acidilactici, P. damnosus, Pediococcus inopinatus, P. parvulus, P. pentosaceus, Pediococcus urinaeequi* and *P. dextrinicus.* However, *P. dextrinicus* is considered a distantly related species compared to the others (Chaban *et al*., 2002). *Pediococcus* bacteria also produce pediocins which are

bacteriocins that have a wide range of bactericidal activity against the gram-positive bacteria and is used for food preservation (Biswas *et al*., 1991).

P. acidilactici and *P. claussenii* are usually used in beer fermentation but they can also be found in *Tapai Ub*i due to the low alcohol content in the fermentation process (Dobson *et al*., 2002). *P. acidilactici* is found in *Ragi*, a culture-mixed microorganism for *tapai* fermentation (Chiang *et al*., 2006). As for *P. claussenii*, it considered as a new species that is found in *Pediococcus* species by using phylogenic analysis (Chaban *et al*., 2002). Besides this, *P. claussenii* is commonly found in beer spoilage and it is known as a brewery contaminant (Pittet *et al*., 2012). It produces a slimy product, exopolysaccharide, which may be involved in forming biofilm in brewery. This bacterium contains the gene of *hor*A that is associated with spoilage; it causes the beer to spoil in 7 to 8 days (Haakensen *et al*., 2008).

Fermentation of *Tempeh* commonly uses the mould *Rhizopus oligosphorus* as the starter culture, but *Leuconostoc mesenteroides subsp. mesenteroides* bacteria also plays a huge part in this fermentation process (Pisol *et al*., 2015). It is a facultative anaerobic that is involved in a complex growth factor and amino acids. Besides this, it is immotile and its shape is cocci. According to Daba *et al*. (1991), *L. mesenteroids* has the ability to produce bacteriocin to against a well-known food-borne pathogen, *Listeria monocytogenes*.

5.3 Bile tolerance of selected putative lactic acid bacteria

Probiotics need to survive throughout the acid and bile conditions in the gastrointestinal tract in order to reach the small intestine and colon to provide health benefits to the host (Gibson *et al*., 2000). Bile salts are produced in the liver and made by bile acid that is conjugated with glycine or taurine (Bodewes *et al*., 2015). Bile salt is a toxic compound to bacteria. It is composed of lipids and fatty acid that interact with bacterial cell membranes to inhibit the growth and kill the bacteria. Besides this, bile salt is also involved in intracellular acidification which inhibits the proton motive force, as well as inhibit the transport of nutrients. Despite the toxicity of bile salt, some LABs produce bile salt hydrolase and is able to hydrolyze the bile salt and survive the harsh gut environment (Abbasiliasi *et al*., 2012).

Table 4.3 showed that the growth of the four putative LAB isolates reduced significantly after treatment with 0.3% of bile salt and was inhibited totally at 1.0% of bile salt. According to Liong & Shah (2005), their study showed that conjugated bile salt could inhibit *lactobacilli* strains. There was another study which showed that *Lactobacillus acidophilus* is able to survive under 1%, 2% and 3% of bile but the viable count decreased when the amount of bile increased (Pan *et al*., 2009). In addition, Shehata *et al*. (2016) and Jin *et al*. (1998) reported that some LABs such as *Lactobcoccus* and *Lactobacillus* were able to survive at 0.3% of bile salt for 3 hours of incubation.

In this study, the four putative LAB isolates were incubated for 24h and were still able to show tolerance to 0.3% of bile salt despite reduction in viable cell count

to a range of 5.01 – 6.48 Log_{10}CFU/mL. Several studies have proven that *Pediococcus* and *Leuconostoc* are able to tolerant below 0.3% of bile salt (Kobore *et al*., 2012; Sukumar & Ghosh, 2010; Zhang *et al*., 2013; Yi *et al*. 2017).

SU1, SU2 and SU8 continue to show tolerant 0.5% bile salt, except for ST4. All four putative isolates were susceptible to 1.0% bile. Current findings indicated that *P. claussenii* SU8, *P. acidilactici* SU1 and SU2 were able to tolerate 0.5% of bile salt condition, with SU8 showing the highest viability of 7.13 Log_{10}CFU/ml. Whereas, *L. mesenteroides sp. mesenteroides* ST4 was least tolerant to bile salt condition.

Similar observation was also reported by Sabir *et al*. (2010) where their study showed that *P. acidilactici* was able to survive at the concentration of 0.5% bile salt, but poor survival ability was recorded for *L. mesenteroides*. However, a contrasing study was reported by Mandal *et al*. (2009) where *P. acidilactici* was found to tolerant up to 2% of bile salt (20 mg/ml) after 24 hours overnight incubation. According to Surono (2003), *Leu. mesenteroides* had a poor survival rate in the presence of 0.3% of oxgall. In contrast, Benmechernene *et al*. (2013) and El-Jeni *et al*. (2016) reported that *L. mesenteroides* survived well with the concentration of 0.5%, 1% and 2% of bile salt for 4h incubation but the viability of the cells was reduced. Thus, these reports suggested that *L. mesenteroides* might be able to tolerate bile salt for 4 hours but not after 24 hours of incubation.

In this study, even though *Leu. mesenteroides* ST4 had poor survival rate at 0.5% of bile salt, it is possible that this isolate will still survive through the duodenum and proceed to the small intestinal tract because the human small intestinal tract

normally contain 0.3% of bile salt (Begley *et al*., 2006; Gilliland *et al*., 1984).

5.4 *In vitro* gastric transit tolerance of selected putative lactic acid bacteria

Human stomach produces gastric juice with a low pH between 2.5 to 3.5 (Holzapfel *et al*., 1998; Huang & Adams, 2004). The importance of testing gastric transit assay on probiotics is to understand the survivability of the probiotics in order to transmit the health benefit to the small intestine (Corcoran *et al*., 2005).

The current study showed that all four putative LAB isolates *P. claussenii* SU8, *P. acidilactici* SU1, SU2, and *Leu. mesenteroides* ST4 have high tolerance to the acidic environment in the stomach ranging from pH 2 to 4 and maintained a high viable cell count exceeding 12 Log_{10}CFU/ml. Similar findings were reported by other researches (Immerstand *et al*., 2010; Shukla *et al*., 2015; Seo *et al*., 2012).

According to Erkkilä & Petäjä (2000), *P. acidilactici* has the capacity of surviving under acidic condition from pH1 to 5 and 0.3% of bile salts. Guerra *et al*. (2007) also reported that, *P. acidilactici* was tolerant to pH 2,3,4 and 5 at the incubation of 4 hours. It also survived in the acidic condition slightly better than *Lactococcus lactis* and *Enterococcus faecium*. According to Immerstrand *et al*. (2010), *P. parvulus* survived a 4 hours exposure to the simulated gastric juice adjusted to pH 2.5 and pepsin at 37°C, but at pH 1.5, viability of the cell was reduced significantly.

Shukla *et al*. (2014) and Seo *et al*. (2012), reported that *L. mesenteroides* showed resistance to the simulated gastric juice at pH 3 and has a high survival rate. Zhang *et al*. (2013) and El-Jeni *et al*. (2016) showed that *L. lactis* and

L. mesenteroides were tolerant to pH 1.5 to 4.5.

5.5 *In vitro* small intestinal transit tolerance of putative lactic acid bacteria

The current study showed that all four putative LAB isolates *P. claussenii* SU8, *P. acidilactici* SU1, SU2, and *Leu. mesenteroides* ST4 have high tolerance to the alkaline environment in the small intestinal tract with the addition of 0.3% bile salt and maintained high viable cell count ranging from 9.82 to 12.33 Log_{10}CFU/ml after 240 min of incubation. Among the four putative isolates tested, ST4 showed the strongest tolerance and SU1 showed the lowest tolerance to the simulated small intestinal transit.

According to Prommadee *et al*. (2012), the study showed the *P. pentosaceus* was tolerant to the small intestinal condition which was pH8 with 0.3% of bile salt even though the viability was decreasing significantly within 4 hours. Besides this, there were several findings which proved that *Pediococcus* and *Leuconostoc* are tolerant to the small intestine condition (Kumar *et al*., 2020; Devi *et al*., 2015; Diana *et al*., 2015; Le & Yang, 2019).

In contrast, there was a finding by Tokatli *et al*. (2015) which showed that *P. ethanolidurans* strains were sensitive to simulated intestinal juice and gastric juice. In this study, ST4 had a better survivability under small intestine condition and thus showed the strongest tolerance in the small intestinal transit.

Surprisingly, when the bile salt was removed from the simulated small intestinal transit, SU1, SU2 and SU8 showed significant reduction of viable cell counts

ranging from 0.28 to 2.6 Log_{10}CFU/ml after 240 min of incubation. Only ST4 showed strong tolerance to the simulated small intestinal transit and with no significant different in viable cell count after 240 min of incubation. The underlying mechanism for this bile removal effect remains unknown and further study will need to be conducted.

Chapter 6 Conclusion and future recommendations

This study was conducted to search of putative probiotics from local fermented foods such as *Tempeh* and *Tapai Ubi*. A total of 18 putative LAB were isolated from *tempeh* and *tapai ubi* using MRS selective medium. However only four putative isolates remained viable after frozen storage. The putative LAB isolated from *Tapai Ubi* were designated as SU1, SU2, and SU8, and the putative LAB isolated from *Tempeh* was designated as ST4. The identity of these four putative isolates were determined using 16s rRNA gene sequence analysis. SU1 and SU2 were identified as *Pediococcus acidilactici*. SU8 was identified as *Pediococcus claussenii* and ST4 was identified as *Leuconostoc mesenteroides* subsp. *mesenteroides.* The ability of these four putative LABs to tolerate bile and gastric transit and remained viable when reaching the small intestinal tract of the host in an *in vitro* setting was determined. *P. claussenii* SU8, P*. acidilactici* SU1 and SU2 were able to tolerate 0.5% of bile salt condition, except for *L. mesenteroides* subsp. *mesenteroides* ST4. All four putative LABs were highly tolerant to the simulated gastric transit ranging from pH 2 to 4 and maintained a growth of more than 12 Log_{10}CFU/ml. All four putative LABs were able to tolerate the simulated small intestinal transit with addition of 0.3% bile salt. In conclusion, SU1, SU2 and SU8 have high tolerance to small intestinal transit and bile salt. However, ST4 was the least tolerant to bile salt as it cannot withstand more than 0.3% bile salt. This will be taking consideration on further investigation in future for more explanation.

In this study, the four putative LABs were isolated from Malaysian fermented food and all of them were able to tolerate the harsh gastrointestinal environments. In order to ascertain the probiotic potential of these four putative LABs, future *in vitro* and *in vivo* studies to evaluate the health benefits of putative LAB to the host will need to be conducted. *In vitro* studies such as determination of antimicrobial activity of the putative LAB in eliminating the pathogen in the host and maintain a balanced gut microflora; ability of putative LAB to assimilate cholesterol in serum; the mode of action of putative LAB to reduce gastrointestinal disease could be conducted. The putative LAB with significant health benefits could then proceed to in vivo animal trial to validate the probiotic potential.

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