Naturally Fermented Milk from Indonesia: A Study of Microbial Diversity and Probiotic Potency for the Potential Treatment of Intestinal Mucositis

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Abbreviations

ACE	Angiotensin I-converting Enzyme
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bsh	Bile salts hydrolase
Caco-2	Adenocarcinoma of the colon
CD	Crypt depth
CFS	Cell-free supernatant
cfu	Colony Forming Units
dATP	2-deoxy-adenosine-5-triphosphate
DC	Dendritic cells
dCTP	2-deoxy-cytidine-5-triphosphate
dGTP	2-deoxy-guanosine-5-triphosphate
DM	Dry matter
DNA	Deoxyribose nucleic acid
dNTPs	Deoxynucleotide triphosphates
DMEM	Dulbecco's modified Eagle Medium
dTTP	2-deoxy-thymin-5-triphosphate
EDTA	Ethylenediamine tetra acetic acid
FCS	Feta calf serum
5-FU	5-fluorouracil
GABA	Gamma-aminobutyric acid
H&E	Haematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic
IDZ	Inhibition diameter zones
IgA	Immunoglobulin A
IL	Interleukin
ITS	Intergenic transcribed spacer
LAB	Lactic acid bacteria
Lacl	*Lactococcus lactis* subsp. *lactis* SL3.34
Lp	*Lactobacillus plantarum* S1.30
LSD	Least significance difference

LSM	Lactic acid bacteria Susceptibility test Medium
MAPK	Mitogen-activated protein kinase
MilliQ H_2O	Milli-Q filtered deionized water
MPO	Myeloperoxidase
MRS	de Man, Rogosa and Sharpe
msa	Mannose-specific adhesion
MTX	Methotrexate
NCFS	Neutralized cell-free supernatant
NF-$_K$B	Nuclear factor-kappa B
NFM	Naturally fermented milk
NGS	Next generation sequencing
NSLAB	Non-lactic acid bacteria
OTU	Operational Taxonomy Unit
PBS	Phosphate-buffered saline
PD	Phylogenetic distance
*pln*A	*Plantaricin* A
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal RNA
ROS	Reactive oxygen species
SD	Standard deviation
SEM	Standard error of the mean
TAE	Tris-acetic EDTA
Taq	*Thermus aquaticus* DNA polymerase
TNF-α	Tumour Necrosis Factor-Alpha
Tris	Tris (hydroxymethyl) aminomethane
TSA	Tryptone soya agar
TSA	Tryptone soya agar
TSB	Tryptone soya broth
VH	Villus height
YEPD	Yeast extract peptone d-glucose
YMA	Yeast mannitol agar
YPD	Yeast peptone dextrose

Abstract

Naturally fermented milk (NFM) is prepared from fresh milk which is fermented spontaneously, without any inoculation of starter cultures. With increasing interest in novel dairy products, naturally fermented milks have become of interest to food microbiologists as a result of their potential as technologically important microorganisms. Dadih is a well-known naturally fermented milk product developed by local people in West Sumatra, Indonesia. This product is manufactured using unpasteurized buffalo milk which is then fermented spontaneously at ambient temperature. Dangke is prepared from heat-treated buffalo milk, and then processed enzymatically utilizing papain from papaya latex. Identification and characterization of the indigenous microbiota is essential for understanding how the microbial ecology impacts on the organoleptic, safety and potential health benefits of dadih and dangke. In addition, the presence of probiotic microorganisms in these products was also evaluated by investigating their potential to reduce the severity of chemotherapy-induced intestinal mucositis in rats. Probiotics have been defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Hill et al. 2014).

Lactic acid bacterial (LAB) groups detected using culture-dependent techniques in dadih were *Lactobacillus plantarum, Lactococcus lactis* subsp. *lactis*, and *Enterococcus faecium* (**Chapter 2**). Only one species of acetic acid bacteria was found, namely *Acetobacter orientalis,* while yeasts isolates were identified as *Saccharomyces cerevisiae*, *Candida metapsilosis* and *Kluyveromyces marxianus,* with *C. metapsilosis* as the principal yeast (**Chapter 3**). Other bacteria detected included *Klebsiella oxytoca*, *Klebsiella* sp. and *Bacillus pumilus.* Among these bacteria, *L. plantarum* was the most frequently isolated LAB from dadih, followed by *L. lactis* subsp. *lactis.*

Indigenous microbiota detected in dangke were relatively similar to dadih (**Chapter 2**). However, *E. faecium* and *B. pumilus* were not found in dangke. *Lactococcus lactis* subsp. *lactis* was the most predominant LAB, while *S. cerevisiae* was the most frequently isolated yeast (**Chapter 3**). Moreover, based on a culture-independent method (pyrosequencing), genus *Lactococcus* had the greatest relative abundance in dadih. Based on pyrosequencing results, a more diverse population of mesophilic LAB was found in dangke sourced from cow's milk; while family Enterobacteriaceae dominated dangke samples from buffalo milk.

Lactobacillus plantarum S1.30 isolated from dadih demonstrated probiotic properties which included tolerance to low pH and bile salts, antimicrobial activity and the presence of a bacteriocin regulating gene (plantaricin A) and *msa* and *bsh* genes, susceptibility to antibiotics and ability to adhere to Caco-2 cells (**Chapter 4**). From these probiotic features, only antimicrobial activity and the presence of *msa* and *bsh* genes were not demonstrated by *L. lactis* subsp. *lactis* SL3.34. However, from the pyrosequencing results, this strain was selected as the representative of the dominant genus/species in dadih.

The efficacy of probiotics evaluated in the present study was variable at treating 5-fluorouracil (5-FU)-induced intestinal damage *in vivo* (**Chapter 5**). The results suggested that *L. plantarum* S1.30 and *L. lactis* subsp. *lactis* SL3.34 could have beneficial effects through partially improving metabolic parameters such as water intake, urine output, food intake, and fecal output in 5-FU challenged rats. The severity of damage in the jejunum and ileum was also reduced following probiotic culture treatment.

In conclusion, this insight into the microbial composition of dadih and dangke will assist in the development of sustainable and technologically feasible starter cultures with probiotic properties. This information has the potential to enhance human health, food safety and food security from locally produced traditional fermented milk products.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Signed: Date: June 2017

Yoga Dwi Jatmiko

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Chapter One

General Introduction

1.1 Background Information

Consumption of fermented milk products has been popular in many countries due to their health benefits. Besides the nutrition content of the milk itself, the health benefits are also determined by the activity of living microorganisms) contained in the fermented milk (Buttriss 1997). The microorganisms are responsible for improving the shelf-life of the milk through increasing the acidity level and also development of desirable organoleptic characteristics, such as flavour and textures (Tamime 2002b). Interestingly, some of the microorganisms, known as probiotics, actively enhance human health through improving the balance of intestinal microbiota (Sánchez et al. 2009). Therefore, fermented milk products are considered to be functional foods (Shah 2007).

Fermented milk products were originally produced by spontaneous fermentation using wild starter cultures, so-called naturally fermented milk (NFM). Since the quality of final products is unpredictable, depending on the dominant microorganisms initially involved, the production of fermented milk has been optimized utilizing commercial starter cultures and controlled conditions (Ravyts, Vuyst & Leroy 2012). Yogurt is the most popular industrialized fermented milk product. Through industrial production, the types of fermented milk products have become more diverse with innovations such as the addition of probiotics, prebiotics and agents to enhance the organoleptic properties such as fresh fruits or even utilization of different milk types (Ravyts, Vuyst & Leroy 2012). Therefore, consumer preference has changed to these commercial products rather than NFM products (Lan et al. 2013).

In the era of industrialization of fermented milk product development, the issues of quality and safety have been thoroughly investigated. However, this approach could diminish diversity of indigenous microorganisms used and the organoleptic properties of NFMs (Garabal 2007; Lan et al. 2013; Ravyts, Vuyst & Leroy 2012). Some studies have shown that NFM products are potential bio-resources for the food technology industry, with starter cultures, probiotics, antimicrobial compounds and biocatalysts as examples of innovative

end-use (Lan et al. 2013; Mathara et al. 2008a; Mathara et al. 2008b; Parvez et al. 2006; Soomro & Masud 2012; Surono 2003; Surono et al. 2011). Moreover, the more diverse the indigenous microorganisms in the fermented milk, the better the results that could be expected in improving the balance and diversity of intestinal microbiota when such products are consumed. In this current review, a variety of NFM products will be explored and their therapeutic potential, in terms of probiotics, will be discussed.

1.2 Naturally Fermented Milk

Naturally fermented milk (NFM) is a popular fermented product made by rural communities in some developing countries. A part of traditional heritage, the highly nutritious content of milk is preserved through fermentation, and significantly, these products are important for therapeutic and social value (generating income) (Beukes, Bester & Mostert 2001). NFM is prepared from fresh milk which is fermented spontaneously without any inoculation of starter cultures (Narvhus & Gadaga 2003). The art of making NFM products has been handed down from one generation to the next. Basically, NFM products are prepared by using unpasteurized or pasteurized milk which is placed in a suitable container. After 2 – 3 days of incubation at an ambient temperature, the desirable texture and flavor of the product are achieved due to the role of the indigenous microbiota (Gran, Gadaga & Narvhus 2003; Robinson & Tamime 2007). NFM products are regarded as small-scale products because they are manufactured at the household level in a range of communities in some developing countries of Africa and Asia (Narvhus & Gadaga 2003). As milk has a high nutritional value, natural fermentation is the best way of enhancing the flavor and nutritional benefits of milk as well as extending its shelf-life (Robinson & Tamime 2007).

A range of NFM products can be found around the world, each with a slightly different composition. Many factors influence the unique characteristics of NFM products, such as source of milk used, climate conditions (temperature), pre-treatment of the milk (e.g. pasteurization), fermentation conditions (temperature and length of incubation), type of containers and environmental sanitation (hygiene) (Marsh et al. 2014; Wouters et al. 2002). As a result, the local name of products is distinct by regions (Robinson & Tamime 2007). However, in essence, NFM products have five principal similarities among them (Jatmiko, Barton & de Barros Lopes 2010). Firstly, the indigenous microbiota initiating fermentation originates from the raw milk and the environment, including the vessels and any equipment used. Consequently, a gradual selection of specific indigenous microbiota that was responsible for product stability and quality has occurred, which cannot easily be imitated by

modern dairy starter cultures (Gran, Gadaga & Narvhus 2003). Secondly, NFM products are manufactured by rural people with poor living conditions and limited facilities. Thirdly, a traditional and natural container is used, instead of plastic containers. Fourthly, the incubation condition is at ambient temperature for 2 – 3 days. Finally, the preparation of the products is under non-aseptic conditions, without any knowledge of good manufacturing practices.

The NFM products listed in Table 1 can be divided into two groups based on the fermentation technique, namely spontaneous fermentation and back-slopping. In spontaneous fermentation, the origin of indigenous microbiota is mainly from raw material (un-pasteurized milk and natural containers). The microbial succession is initially dominated by the growth of lactic acid bacteria followed by other microbes (yeast, mould and Gram-negative bacteria). In back-slopping, a small portion of a previous fermentation product is inoculated into the next batch of fermentation, which allows a more rapid fermentation process compared to spontaneous fermentation (Josephsen & Jespersen 2004). A range of NFM products are found in Asian and African regions, and Indonesia is one country that preserves the art of making fermented milk as part of traditional culture. Dadih and dangke are naturally fermented milk products from Indonesia, which will be discussed further.

1.2.1 Dadih

Dadih is a well-known naturally fermented milk product developed by local people in West Sumatra, Indonesia. This product is manufactured using unpasteurized buffalo milk and then fermented spontaneously (without starter addition) at an ambient temperature (Surono 2003). This product is categorized as a yogurt-like product and the texture is smooth, soft and creamy. This texture occurred due to higher fat content compared to common yogurt product (Yodoamijoyo et al. 1983). However, sometimes the consistency of dadih is more solid and relatively dry, affected by the milk solid and lipid content of the buffalo milk (Surono et al. 1984). In terms of raw material and the preparation process, dadih is similar to dahi (NFM from Bangladesh and India), but the container used is different. Dahi is made in earthenware pots (Rashid et al. 2007), while dadih utilizes bamboo tubes (Surono 2003). A study of the chemical composition of dadih showed the pH was approximately 4.1, water content 84.35%, protein 5.93%, fat 5.42%, carbohydrate 3.34% (Budijanto 2011; Yodoamijoyo et al. 1983). Protein and fat content of dadih was higher than western-type yogurt (Yodoamijoyo et al. 1983). The quality of buffalo milk influences the chemical composition in each batch of product.

The preparation process of dadih is in three main steps (Fig. 1). First, fresh buffalo milk is filtered and poured into a fresh bamboo tube. Second, the bamboo tube containing buffalo milk is covered with banana leaves, previously wilted by heating. Lastly, incubation is conducted at room temperature for at least overnight (Suryono 2003). The shelf-life of dadih is three days at an ambient temperature; beyond this day, the dadih surface will turn yellow and undesirable microorganisms such as fungi will grow (Hasbullah 2012).

Figure 1 Dadih manufacturing process

1.Milking the buffalo; 2. Filtration; 3. Pouring; 4. Capping; 5. Incubation (at least 24 h); 6. Final products. Adapted from Jatmiko, Barton & de Barros Lopes (2010).

A recent study showed that indigenous microbiota involved in the fermentation of dadih included three groups of microorganisms, namely LAB, acetic acid bacteria and yeasts (Jatmiko, Barton & de Barros Lopes 2010) (Table 1). In this investigation, the microbiota was isolated using a culture-dependent method and successfully has provided new information regarding microbial diversity in dadih. *Lactobacillus paracasei* was frequently isolated from dadih samples, and also the finding of previously undetected bacteria, namely *Leuconostoc pseudomesenteroides* and *Acetobacter cerevisiae* (acetic acid becteria). Additionally, the detection of yeasts *Pichia jadinii* and *Candida stellimalicola* complemented the information on yeasts in dadih (Jatmiko, Barton & de Barros Lopes 2010; Jatmiko, de Barros Lopes & Barton 2012). These finding add substantially to our understanding that there is variation in the microbial composition of NFM products such as dadih and therefore, further exploration using culture-independent techniques is warranted.

Dadih is believed to have therapeutic benefits for human health. Besides its nutritional content, this beneficial health effect might be associated with the role of probiotics. The presence of probiotics in dadih and its health properties have been explored either *in vitro* or *in vivo*. Anti-mutagenic properties against mutagenic foods (terasi and tauco) have been reported in probiotic candidates from dadih such as *Leuconostoc mesenteroides* R-51 (Pato 2003a) and *Enterococcus faecium* IS-27526 (Surono et al. 2009). Another health property is lowering the level of cholesterol in rats (hypocholesterolemic effect), which was expressed by *Lactococcus lactis* subsp. *lactis* IS-10285 (Pato et al. 2004). Moreover, a contribution of probiotic candidates from dadih in reducing health risk caused by toxic mycrocystins was demonstrated by two strains of *Lactobacillus plantarum* IS-10506 and IS-20506 (Surono et al. 2008). The ability of indigenous LAB to inhibit pathogens such as *Escherichia coli* O157:H7 and adherence to the mucus layer and Caco-2 cells has also been reported for *Lactococcus lactis* IS-16183 and *Lactobacillus rhamnosus* IS-7257 (Dharmawan, Surono & Kun 2006). A current finding showed that the administration of probiotic *Enterococcus faecium* IS-27526, supplemented into UHT low-fat milk to pre-school children could modulate the immune response by increasing total serum IgA as well as bodyweight (Surono et al. 2011). Thus, probiotic candidates derived from dadih have been successfully characterized and applied to benefit human health. However, the application of dadih probiotics for treating gastrointestinal diseases has not been performed. Future studies on this topic are therefore recommended.

1.2.2 Dangke

Another important NFM product of Indonesia is dangke, which originated from Enrekang Regency, Province of South Sulawesi. This product is prepared from heat-treated buffalo milk, and then processed enzymatically utilizing papain from papaya latex (Razak et al. 2009). Thus, Surono et al. (1984) classified dangke as a cheese-like product. The manufacturing process of dangke is not as simple as dadih (Fig. 2). To begin, freshly drawn buffalo milk is heated until boiling. After cooling to about 90^{o}C, the papaya (*Carica papaya*) latex from the sliced leaves and the unripe/ young fruits is added to the warm buffalo milk. Papaya latex contains a papain enzyme which plays an important role in coagulating the milk proteins. The amount of the papaya latex added should not be excessive because a strong bitter taste can result. When the milk containing the papaya latex is stirred gently, a separation between curd (solid) and whey (liquid) occurs. The curd is filtered and poured into a coconut shell, prepared with a small hole at the bottom for draining off the whey. Furthermore, the curd is pressed to extract the excess whey. Finally, the curd with an arch shape is detached from the coconut shell and wrapped with banana leaves, and kept at room temperature until ready for consumption. The curd, usually, is immersed in a salt solution overnight, before wrapping to extend the shelf-life (Surono et al. 1984). Dangke is the only cheese-like product using the coagulant derived from higher plants, and also using heat-treated milk. It has been assumed that heat treatments associated with the protein coagulation contribute to the taste of the product. Surono et al. (1984) confirmed that the higher the temperature (~ 90^{o}C), the greater activity of milk clotting, whilst the proteolytic activity decreased. As a result, a strong bitter taste is not developed in dangke.

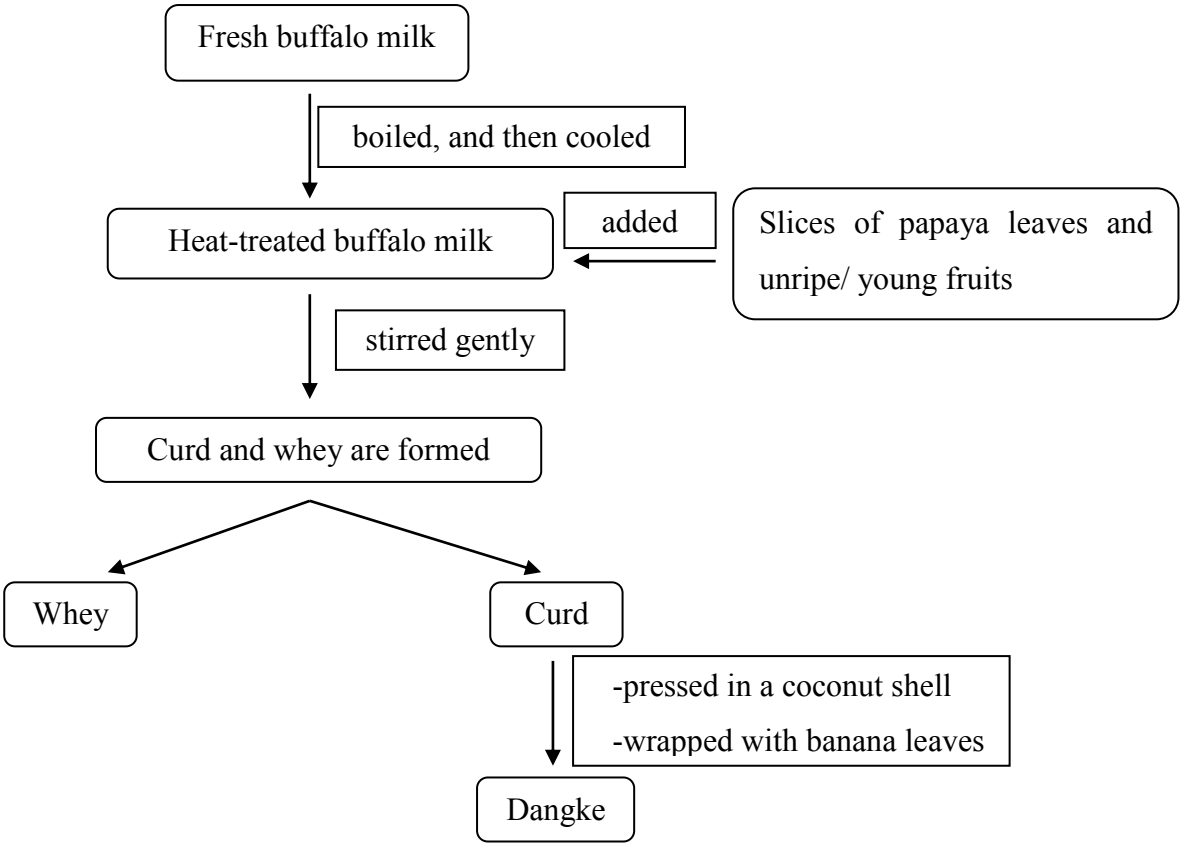

Figure 2 The manufacturing process of dangke

Figure 3 Dangke final product

The availability of scientific information on microbial diversity of dangke is very limited to date. The only published article available reported that three indigenous LAB have been identified, namely *Lactobacillus plantarum* DU15*, Enterococcus faecium* DU55 and *Leuconostoc mesenteroides* DU02 (Razak et al. 2009). Interestingly, *Enterococcus faecium* DU55 was able to inhibit the growth of *Salmonella typhimurium* FNCC 0050 as a result of bacteriocin activity. In fermentation conditions, the bacteriocin production reached a high concentration after 42 hours of incubation at 30^{o}C in MRS medium. However, this study did include any characterization of the bacteriocin, and the antimicrobial substance produced was considered a bacteriocin-like inhibitory substance. The implication of this study is the possibility that probiotic candidates could be isolated from dangke, since antimicrobial-producing LAB was found and antimicrobial production is one of the selection criteria for probiotics.

Table 1 Naturally fermented milk products with identified microbiota and probiotics

No	Products	Raw materials	Country	Microbiota	Bacteriocin producers	Probiotic strains	Ref.
1.	Airag/ Chigee	Mare's milk	Mongolia	*Lactococcus garvieae*, *Lactococcus lactis* subsp. *lactis*, *Streptococcus parauberis*, *Enterococcus faecium*, *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides*	*Enterococcus durans*	-	(An, Adachi & Ogawa 2004; Batdorj et al. 2006)
2.	Amasi and mafi	Cow's milk	South Africa, Zimbabwe	*L. lactis* subsp. *lactis*, *Leu. mesenteroides* subsp. *dextranicum, Leu. citreum*, *Leu. lactis*, *L. delbrueckii* subsp. *lactis* and *L. plantarum*	-	-	(Beukes, Bester & Mostert 2001; Todorov et al. 2007)
3.	Amasi/ hodzeko/ mukaka wakakora	Cow's milk	Zimbabwe	LAB *(L. helveticus, L. plantarum, L. delbrueckii* subsp. *lactis, L.casei* subsp. *casei and L. casei* subsp. *pseudoplantarum)* Yeasts (*Saccharomyces cerevisiae, Candida lusitaniae, C. colliculosa, S.*	*L. plantarum* AMA-K	-	(Feresu & Muzondo 1990; Gadaga, Mutukumira & Narvhus 2000; Todorov et

				dairenensis, Dekera bruxillensis, C. lipolytica, C. tropicalis)			al. 2007)
4.	Dadih/ dadiah (yoghurt-like)	Buffalo milk	West Sumatra, Indonesia	LAB (*Streptococcus faecalis* subsp. *liquefaciens*, *S. cremoris*, *S. lactis* subsp. *diacetylactis*, *S. lactis*, *Leuconostoc paramesenteroides*, *L. casei* subsp. *casei* and *L. casei* subsp. *rhamnosus, L. paramesenteroides*, *L. rhamnosus*, *L. plantarum, L. paracasei, L. pseudomesenteroides, L. lactis* subsp. *lactis*); yeasts (*Endomyces lactis, Pichia jadinii, Candida stellimalicola*), others (*Micrococcus varians*, *Staphylococcus saprophyticus, Bacillus cereus* var. *mycoides* and *Acetobacter cerevisiae*)	*L. lactis* subsp. *lactis* (IS-10285 & IS-16183), *L. brevis* IS-26958 and *L. casei* IS-7257	*L. plantarum* IS-10506, *E. faecium*IS-27526	(Collado et al. 2007b; Hasono, Wardojo & Otani 1989; Jatmiko, Barton & de Barros Lopes 2010; Jatmiko, de Barros Lopes & Barton 2012; Surono 2003; Surono et al. 2011; Zakaria et al. 1998)
5.	Dahi (yoghurt-like)	Cow's and buffalo milk or mixture	India, Pakistan	*Streptococcus bovis*, *L. fermentum*, *L. delbrueckii* ssp. *bulgaricus, L. delbrueckii* ssp. *lactis*, *E. faecium, S. thermophilus*, *Leu. mesenteroides* ssp. *mesenteroides, Leu. mesenteroides* ssp. *dextranicum*, *L. lactis* ssp. *lactis, L. raffinolactis* and *Pediococcus pentosaceus*	*Streptococcus bovis* J2 40-2, *L. lactis* CM1	*L. acidophilus* LA 02, *L. delbrueckii* subsp. *bulgaricus* M3 40-3	(Harun-ur-Rashid et al. 2007; Harun-ur-Rashid et al. 2009; Mitra, Chakrabartty & Biswas 2007, 2010; Rashid et al. 2007; Soomro & Masud 2012)
6.	Dangke (cheese-like)	Buffalo milk	Makassar, Indonesia	*L. plantarum* DU15, *Enterococcus faecium* DU55 *and Leu. mesenteroides* DU02	*Enterococcus faecium* DU55	-	(Razak et al. 2009)
7.	Kule naoto	Cow's milk	Maasai, Kenya	*L. plantarum, L. fermentum, L. paracasei and*	-	*L. acidophilus,*	(Mathara et al. 2008a;

				L. acidophilus		*L. fermentum*	Mathara et al. 2004)
8.	Kurut	Yak milk	Qinghai, China	LAB (*L. plantarum, L. acidophilus, L. casei, L. fermentum, L. brevis, L. minor, L.* curvatus), and yeasts	-	*L. acidophilus* E2*, L. casei* G12	(Zhang et al. 2008b; Zhang et al. 2011)
9.	Raïb	Cow's milk	Morocco	*L. lactis, Enterococcus faecium, E. faecalis*	*Lactococcus lactis, E. faecium, E. faecalis*	-	(Elotmani et al. 2002)
10.	Rob	Cow's milk	Sudan	LAB (*L. fermentum, L. acidophilus, L. lactis* and *Streptococcus salivarius*); yeasts (*Saccharomyces cerevisiae* and *Candida kefyr*)	-	*L. lactis, L. delbrecukii, E. faecium*	(Abdelgadir et al. 2001; Mohammed Salih, Hassan & El Sanousi 2011)
11.	Sethemi	Cow's milk	South Africa	LAB (lactobacilli, leuconostocs and lactococci), yeasts (*Debaryomyces hansenii, Saccharomyces cerevisiae, Cryptococcus curvatus, Cryptococcus humicola and Kluyveromyces marxianus*)	-	-	(Kebede et al. 2007)
12.	Shubat	Camel milk	China	LAB (*L. sakei, E. faecium, L. helveticus, Leu. lactis, E. faecalis, L. brevis* and *Weissella hellenica),* yeasts (*Kluyveromyces marxianus, Kazahtan uiosporus* and *Candida ethanolica).*	-	-	(Rahman et al. 2009)
13.	Suusac	Camel milk	Kenya	LAB (*L. curvatus, L. plantarum, L. salivarius, L. raffinolactis* and *Leu. mesenteroides* subsp. *mesenteroides*); yeasts (*Candida krusei, Geotrichum penicillatum* and *Rhodotorula mucilaginosa*)	-	-	(Lore, Mbugua & Wangoh 2005)

14.	Tarag (yoghurt-like)	Cow's milk / camel milk/ yak milk/ goat milk	Mongolia	LAB (*L. delbrueckii* subsp. *bulgaricus, L. helveticus*, and *S. thermophilus*), yeasts (*Kluyveromyces marxianus, Saccharomyces cerevisiae, Issatchenkia orientalis*, and *Kazachstania unispora*)	-	*L. plantarum, L. paracasei*	(Takeda et al. 2011; Watanabe et al. 2008)
15.	Gioddu	Sheep or goat milk	Sardinia	*L. paracasei, L. plantarum* and *L. reuteri*	*L. paracasei* Olb46As2, Olb46As3, and Olo45As2	*L. reuteri, L. paracasei, L. plantarum*	(Ortu et al. 2007)
16.	Jben	Cow's or goat milk	Morocco	*L. lactis*	*L. lactis* CCMM/IAV/BK2	-	(Benkerroum et al. 2000)
17.	Sameel	Sheep/ goat/ cow/ camel milk	Saudi Arabia	LAB (*L. plantarum, L. pentosus, L. lactis* ssp *lactis, L. brevis, L. salivarius, L. paracasei* ssp *paracasei*) Yeast (*C. lusitania, Cryptococcus laurentii, S. cerevisiae, C. kefy*)	-	-	(Al-Otaibi 2012)
18.	Koumiss	Mare's milk	Central Asia (Mongolia, China)	LAB (*L. casei, L. fermentum, L. plantarum, L. acidophilus, L. helventicus*) Yeast (*C. pararugosa, Dekkera anomala, Geotricum sp., Issatchekia orientalis, Kazachstania unispora, Kluyveromyces marxianus, Pichia deserticola, P. fermentans, P. manshurica, P. membranaefaciens, S. cerevisiae, Torulaspora delbruekii*).	*L. plantarum* IMAU10116, *Lb. fermentum* SM-7	*L. casei* Zhang, *L. fermentum* SM-7	(Pan, Zeng & Yan 2011; Wang et al. 2011a; Zhang et al. 2010a)
19.	Nunu	Cow's milk		LAB (*L. fermentum, L. plantarum, Leu. mesenteroides, L.*		-	(Akabanda et al. 2012)

				helveticus, E. faecium, E. italicus, Weisella confusa, Lactococcus spp.) Yeast (*C. parapsilosis*, *C. rugosa, C. tropicalis, Galactomyces geotrichum, P. kudriavzenii, S. cerevisiae*)			
20.	Chhu	Caw's milk, yak milk	India (Sikkim Himalaya)	LAB (*L. alimentarius, L. farciminis, L. salivarius, L. bifermentans, L. brevis, L. lactis* subsp. *cremoris*) Yeast (*S. crataegensis, C. castelli*)	-	-	(Dewan & Tamang 2006)

As illustrated in Table 1, all the NFM products have unique characteristics influenced by their local culture and climates. The main dissimilarities are preparation techniques, containers and types of milk. Among spontaneous fermented milks, dadih and dangke are relatively different from other products. Fresh plant materials are always used in the production of dadih and dangke as a vessel (bamboo tube) and coagulant (papaya latex), respectively. Kule naoto, a fermented milk from Kenya, also uses plant materials as its container, the dried fruit of *Lagenaria siceraria* (Mathara et al. 2004). All plants are colonized by microbial endophytes (Hardoim et al. 2015), some of which have a beneficial effect on the development of the fermentation process.

The presence of lactic acid bacteria in both bamboo and papaya has been examined. Lactic acid bacteria have been isolated from raw bamboo tender shoots, and they were present in fermented bamboo shoot products (Tamang et al. 2008). Lactic acid bacteria were also found in ripe papaya, which was used in papaya pickle (Zhang et al. 2014b). Furthermore, *Lactobacillus plantarum* isolated from papaya was able to produce bacteriocins (Todorov et al. 2011). Another study has also reported that papaya shoot tips are colonized by some Gram-negative genera (*Pantoea, Enterobacter, Brevundimonas, Sphingomonas, Methylobacterium,* and *Agrobacterium*) and Gram-positive bacteria (*Microbacterium* and *Bacillus*). These bacteria can become contaminants in micropropagation of papaya shoot tips (Thomas

et al. 2007). On the one hand, these findings suggested that a bacterial community is present in fresh bamboo tubes and papaya and provide benefits for fermentation; on the other hand, some undesirable plant organisms could become a potential threat to food safety.

1.3 Characterization of Probiotic Properties

Promising probiotic microorganisms must be selected on the basis of certain criteria, and many *in vitro* tests are performed when screening for probiotic candidates. The main widely-used selection criteria for potential probiotics are adhesion to gut epithelial tissue, resistance to gastric acidity and bile toxicity, production of antimicrobial substances, and ability to modulate the immune response (Bermudez-Brito et al. 2012; Dunne et al. 2001; Fontana et al. 2013; Saarela et al. 2000; Shewale et al. 2014).

1.3.1 Adhesion ability

The ability of prospective probiotic organisms to adhere to the host intestinal mucosa is an important characteristic. The adherence capacity is considered a prerequisite for other beneficial health effects, including extended duration of colonization, capacity to modulate immune response, repair of damaged mucosa and competition with pathogens (Ouwehand, Salminen & Isolauri 2002). To date, several *in vitro* models utilized in evaluating the adhesion of microorganisms have been developed, namely tissue cell culture (intestinal epithelial cells), intestinal mucus and whole tissue (Ouwehand & Salminen 2003). Among these models, the tissue culture cells (Caco-2, HT-29 and HT-29 MTX cell lines) and intestinal mucus (isolated from faeces or resected tissue) are the most widely implemented (Ouwehand & Salminen 2003; Ouwehand et al. 2002). However, both models have shortcomings in that; for example the cell culture model does not take account of the possibility of adhesion to the mucus layer covering the epithelial cells in the intestine. In fact, the mucus layer as a border between the intestinal contents and the underlying epithelium also plays a major role in the microbiota habitat. Surface proteins secreted by probiotics termed sortase-dependent proteins have been shown to facilitate adhesion to both mucus and epithelial cells (Gibson, Bowen & Keefe 2008; Zwielehner et al. 2011). However, the results with an *in vitro* model are highly variable, affected by method and strain used (Fontana et al. 2013).

1.3.2 Tolerance to gastric acidity

The viability of a potential probiotic organism that reaches the intestinal tract indicates that the microorganism is tolerant of gastric juice in the stomach. To assess the survival of potential probiotics in the gastric environment, *in vitro* methods have been commonly used including the use of MRS medium adjusted with hydrochloric acid (HCl) to pH values of 2.0 and 3.5 (Danese, Sans & Fiocchi 2004; Xavier & Podolsky 2007), and simulated gastric juice (Danese, Sans & Fiocchi 2004). The latter is limited by the availability of suitable materials (Charteris et al. 1998b). The survival of Gram-positive bacteria, particularly lactic acid bacteria in a low-pH environment is attributed to the increase of membrane-bound F1F0-ATPase activity, which causes the extrusion of protons from the cytoplasm, facilitated by the proton motive force. As a result, the intracellular pH value is maintained near neutral (Cotter & Hill 2003).

1.3.3 Tolerance to bile salts

Another important criterion for the assessment of probiotic strains is that the potential probiotic must tolerate bile salts in the intestine. The physiological function of bile for vertebrates is assisting in fat digestion by acting as a biological detergent that emulsifies and stabilises lipids. As a consequence, bile is also able to disrupt bacterial membrane integrity (antimicrobial activity) due to this detergent property (Begley, Gahan & Hill 2005). The bile salt tolerance in probiotics is apparently contributed by bile salt hydrolase (BSH) activity, which is a detoxification enzyme active against the conjugated bile salts (Patel et al. 2010). It is also believed that BSH activity is associated with a beneficial health effect of probiotics which is their cholesterol lowering potential (Patel et al. 2010).

In an experimental situation, the survival of potential probiotic strains can be evaluated by either assessing the BSH activity or growing the presumptive strains in bile-containing medium. The activity of BSH can be detected indirectly by measuring amino acid (taurine or glycine) released from the conjugated bile acid (Bustos et al. 2012). Furthermore, a test plate has been developed for a rapid screening of BSH activity using differential media containing taurodeoxycholic, taurocholic, or taurochenodeoxycholic acids (Dashkevicz & Feighner 1989). In order to evaluate the survival of probiotic candidates, “oxgall” derived from bovine bile is commonly

added to MRS medium to a final concentration of between 0.3% and 7.5% (Dunne et al. 2001; Morelli 2007). However, the characteristic of bovine bile is not as good as porcine bile, in terms of inhibitory activity and the similarity with human bile (Begley, Gahan & Hill 2005).

1.3.4 Antimicrobial activity against pathogens

In addition, to survival in the harsh environment of the gut (acidic pH, and the presence of bile salts), antimicrobial action against pathogens is an important contributor to probiotic properties to protect the gastrointestinal tract from microbial infection. The antimicrobial compounds produced by potential probiotics, especially from lactic acid bacteria, are categorized into two groups, as follows: proteinaceous substances (bacteriocins) and non-proteinaceous substances (organic acids, hydrogen peroxide, carbon dioxide, diacetyl and reuterin) (Dunne et al. 2001; Reis et al. 2012; Shewale et al. 2014). Bacteriocins have been more extensively studied compared to the other antimicrobial substances, as their contribution to the therapeutic effect of probiotics in the gastrointestinal tract (GIT) has been the focus of many studies (Bowen & Keefe 2008; Corr et al. 2007; Cursino et al. 2006; Dobson et al. 2012; Gillor, Etzion & Riley 2008; Šmajs et al. 2010), as well as their use as food biopreservatives (Galvez et al. 2008). Dobson et al. (2012) provided important information on the positive effects of bacteriocins produced by probiotics within GIT. They proposed three mechanisms of bacteriocin action in the GIT; facilitation of probiotic colonization, inhibition of pathogen invasion and modulation of the immune system. However, screening to date has focused on the search for probiotics active against pathogens. As one aspect of probiotic characterization, the presence of bacteriocins can be detected *in vitro* by assessing the inhibition of probiotic produced extracellular substances against indicator microorganisms in agar medium. Methods include the double layer method, the well-diffusion method, the cross-streak method, and the disc-diffusion method (Kesarcodi-Watson et al. 2008). It is recommended that an *in vivo* study should be carried out routinely to investigate bacteriocin production. However, in some cases, the efficacy of bacteriocin activity resulting from *in vitro* studies is inconsistent with the *in vivo* studies (Dobson et al. 2012). Multiple factors can influence the *in vivo* assay, such as the survival of the strain, the specific activity of the bacteriocin, the dosing regimen, the animal model, and the target organism (Fontana et al. 2013).

1.3.5 Immune response modulation

The ability of probiotics to modulate immune responses is considered an important trait in contributing to host defence mechanisms (Isolauri et al. 2001). The immunomodulatory effect of probiotics might occur when the adhesive microorganism (live probiotic) or dead organism (its fragments or metabolite products) interact with the intestinal epithelial cells, especially gut-associated lymphoid tissue (Collins, Thornton & Sullivan 1998; Hardy et al. 2013; Ouwehand et al. 1999). The reinforcement of host defences includes modulation of dentritic cells/ natural killer cell interactions, maintaining the T-helper cell response, limiting the inflammatory response, and the secretion of polymeric IgA (Tsai, Cheng & Pan 2012). Probiotic strains with immunomodulatory potential can be assayed by co-culturing the probiotic candidates with immune cells, such as human monocyte derived dendritic cells (DCs), human peripheral blood mononuclear cells (PBMCs) and mouse bone marrow derived DCs. Interleukin-10 (IL-10) to IL-12 ratio is typically measured to evaluate pro- and anti-inflammatory properties (Hardy et al. 2013).

1.4 Health Properties of Naturally Fermented Milk

Milk, which constitutes a highly nutritive component of the healthy human diet, is perishable and susceptible to contamination. A detailed description of bioactive components in milk has been reviewed by Mills et al (2011). Milk contains certain vitamins, specific proteins, bioactive peptides, oligosaccharides, and organic acids. Natural fermentation of milk is principally aimed at extending the shelf-life of the product (Sánchez et al. 2009). The indigenous microbiota play an important role in the fermentation process by producing metabolite products, such as organic acids, amino acids, bioactive peptides, and enzymes (Divya et al. 2012). Accordingly, the value added to milk is increased, and the NFM products are, in turn categorized as functional foods (Ebringer, Ferenčík & Krajčovič 2008). The predicate of a functional food is also strengthened by the presence of probiotics as the main contributor to the health properties of the NFM products (Parvez et al. 2006). For this reason, the research into NFM products is significantly increased by exploring the role of their microbiota in traditional fermented milk products from many regions.

The benefits of NFM products are mainly attributable to the microbial flora which are not only responsible for the organoleptic development, but also play an important role in therapeutic activity in improvement of digestion properties, activity against diarrhoea and their antimicrobial properties. Therefore, the benefits of fermented products are classified into two areas, namely nutritional and food safety benefits (Farnworth 2004). In nutritional benefits, the role of microbial enzymes is predominant. The enzymes are able to transform substrates so that they become more digestible. For instance, lactase (galactosidase) enzyme in fermented milk products can reduce the lactose intolerance problem in humans (Farnworth 2004). Amino acids and vitamins are two important health products generated by microbes, which provide an additional value from fermented products (Khurana & Kanawjia 2007). In addition, in order to improve the nutritional value of fermented milk products, genomic and proteomic techniques can be applied to accurately define the function of enzymes for specific nutraceutical applications (Beermann & Hartung 2012). In fact, the NFM products are a potential functional food source because they contain useful substances which require further exploration (Bayarsaikhan et al. 2011).

The nutritional composition of NFM products has been widely investigated worldwide. Tarag, a traditional fermented goat milk from Mongolia, has higher nutritional value when compared to yoghurt. The beneficial constituents are casein, lactoferin, serum, albumin, β-lactoglobulin and α-lactalbumin (Zhang et al. 2009b). A similar composition has been reported for kurut (naturally fermented yak milk) from Qinghai, China (Zhang et al. 2008b). Dahi (naturally fermented buffalo milk) from India and Bangladesh contained protein at 22.5 % DM (dry matter), fat 24.5 % DM and carbohydrate 48.2 % DM (Tamang et al. 2012). Although the nutritional information available was not specifically related to the functional properties, dahi is considered to assist in amelioration of intestinal diseases, such as constipation, diarrhoea and dysentery (Rashid et al. 2007).

Furthermore, milk contains bioactive peptides (El-Salam & El-Shibiny 2013). Angiotensin I-converting enzyme (ACE) inhibitor has been intensively studied recently as a bioactive peptide. The active peptide is known as an anti-hypertensive peptide because its activity can reduce peripheral blood pressure by inhibiting ACE activity (Wang et al. 2011c). Koumiss, a traditional fermented mare's milk, has been reported to be rich in ACE-inhibitory peptides (Chen et al. 2010). Besides ACE-

inhibitory activity, NFM from Tibet (yak and cattle milk) had a higher concentration of γ-aminobutyric acid (GABA), which was also suitable for hypertension treatment (Sun et al. 2009).

The second benefit of fermented products is food safety. Lactic acid bacteria are the microbial group commonly dominating in NFM products (Narvhus & Gadaga 2003; Tamime 2002a). This group of bacteria can produce some metabolic products, such as organic acids, hydrogen peroxide, carbon dioxide, diacetyl and bacteriocins which have an antibiosis effect against undesirable microorganisms (Farnworth 2004). These compounds are able to inhibit the growth of some spoilage and pathogenic microbes, such as coliforms and enterobacteriaceae members. These microbes may be found in significant numbers, depending on the level of hygiene employed during preparation (Gran et al. 2003; Marsh et al. 2014). Moreover, antimicrobial substances, especially bacteriocins, have been applied as a food preservatives as well as an alternative to antibiotics for treating infectious diseases (Galvez et al. 2008; Hassan et al. 2012).

In addition, the use of probiotic from fermented milks is a promising tool in reducing exposure to harmful food components, such as mycotoxins, cyanobacterial toxins, pesticides and heavy metals. The risk of this exposure is more likely to occur in developing countries via food and water (Sybesma, Kort & Lee 2015). The ability of lactic acid bacteria to counter these toxins has been demonstrated. Viable *L. plantarum* IS-20506 isolated from dadih efficiently reduced microcystin-LR (dose of 100 μg/L) contamination by 65% with 1% (w/v) glucose supplementation after 20 h incubation at 37º C (Surono et al. 2008). Consumption of fermented milk containing *Lactobacillus casei* Shirota can reduce the risk of aflatoxin exposure via dietary intake (Mohd Redzwan et al. 2016). Furthermore, LAB strains from fermented camel milk and shubat reduced lead (Pb) concentration as observed in a study in cavies (Akhmetsadykova et al. 2012). Since NFM products are prepared under poor hygienic conditions, the incidence of heavy metal contamination from raw materials, equipment used and containers is more likely to occur. A preliminary study of Suturovic´ et al. (2014) reported that two of five fermented milk samples contained cadmium and lead with the concentrations above the acceptable limit. Therefore, good manufacturing practices should be applied during preparation of NFM products to improve the safety level.

1.5 Naturally Fermented Milk Products Improve the Diversity of Intestinal Microbiota

Human intestinal microbiota plays an essential role in disease and health. The existence of intestinal microbiota contributes to not only a negative impact (source of infection) but also a positive effect (protection against diseases and maintenance of intestinal function) (Walsh et al. 2014). A healthy intestine is indicated by a greater richness and diversity of bacterial species in the human intestines (Hollister, Gao & Versalovic 2014). Increasing the number of beneficial microbiota in the intestines is the purpose of probiotic supplementation. According to Walsh et al. (2014) bifidobacteria and lactobacilli are the important components of beneficial intestinal microbiota, so that they are mainly used as food ingredients and supplements. As functional foods, fermented milk products containing dairy starter and probiotics contribute to the modulation of the gut microbiome, especially increasing the presence of beneficial microbiota (Veiga et al. 2014).

Functional properties of NFM products are not only shown by the presence of the important compounds but also the existence of viable probiotics. As mentioned earlier, LAB and yeast or combinations of these are responsible for the natural fermentation of milk (Narvhus & Gadaga 2003). There has been much discussion about LAB as probiotics (Naidu, Bidlack & Clemens 1999). However, probiotic microorganisms are not restricted to the LAB such as *Lactobacillus, Leuconostoc* and *Streptococcus*. Probiotic properties are also demonstrated by yeasts (e.g. *Saccharomyces* strains) from fermented food products (Dixit, Gandhi & Chauhan 2009). Therefore, there is a growing interest in further investigating probiotics from fermented milk products, even from non-dairy products (Giraffa 2012; Rivera-Espinoza & Gallardo-Navarro 2008).

Many commercial probiotic strains (bifidobacteria and lactobacilli) generally originate as enteric flora. Interestingly, studies have shown that microorganisms isolated from NFM products also have probiotic characteristics (Table 1). Among these probiotics, only *Lactobacillus casei* Zhang from koumiss from China has been well-characterized and patented (Zhang et al. 2010a). Apart from its probiotic properties, this organism demonstrated other beneficial health effects, namely immunomodulation (Ya et al. 2008), antioxidative effects (Zhang et al. 2010b), and antihypertensive activity (Wang et al. 2011b). In addition, another probiotic derived

from koumiss, *Lactobacillus fermentum* SM-7 was able to reduce cholesterol levels both *in vitro* and *in vivo* in a mouse model (Pan, Zeng & Yan 2011). In clinical studies, a positive effect of *Enterococcus faecium* IS-27526 (a probiotic from dadih, a naturally fermented buffalo milk of Indonesia) towards humoral immune response and bodyweight increase in pre-school children was observed using a randomized double-blind placebo-controlled study (Surono et al. 2011). Thus there is evidence that NFM products could be a rich source of probiotics.

Dairy products are the most suitable matrices for a probiotic carrier as well as the natural habitat of these organisms (Sanders & Marco 2010). The main reason is that milk and its derivative products provide an environment with a high nutritive value required for growth of wild-type probiotics, especially LAB (Giraffa 2012). In NFM manufacture, the indigenous LAB occupies a central role in spontaneous acidification of the raw milk in the absence of added commercial starter cultures (Wouters et al. 2002). Due to their nutritional content, NFM products are considered to be a complex ecosystem with a variety of metabolic activities produced by LAB and non-starter LAB (NSLAB) during fermentation. This condition leads to the indigenous LAB withstanding competition from other microorganisms by producing antimicrobial substances and other supporting compounds (Leroy & Vuyst 2004). The presence of bacteriocins (antimicrobial peptides) has been reported from a number of NFM products as we can see from Table 1. Furthermore, NFM products are a richer source of other important nutrients or beneficial microbiota compared to conventional yoghurt as shown by the product Tarag. Zhang, et al. (2009a) highlighted that Tarag had a higher content of casein, lactoferrin, serum albumin, β-lactoglobulin, α-lactalbumin, vitamins and minerals; and contained a larger number of LAB and yeasts than yoghurt. Hence, the presence of probiotic candidates in NFM products is required to increase the availability of probiotics with high survival rate in the GIT (Sanders & Marco 2010).

1.6 Intestinal Mucositis

Mucositis is a term used to describe mucosal injury caused by cytotoxic effects of radiotherapy and chemotherapy treatment for malignancy (van Vliet et al. 2010). Keefe (2007) introduced another term for mucositis, 'alimentary mucositis', which comprises both oral and gastrointestinal mucositis. In the case of intestinal mucositis, this disease is characterized by both inflammation and loss of epithelial cell integrity

(ulceration) throughout the entire gastrointestinal tract, with damage particularly severe in the oral mucosa and small intestine (Smith et al. 2008; Yazbeck & Howarth 2009). Common clinical indications of mucositis include dehydration, malnutrition, nausea, vomiting, abdominal pain, bloating and diarrhoea (Mauger et al. 2007). These systemic effects are initiated by increased cell apoptosis causing impairment to the intestinal mucosal barrier. Consequently, nutrient and fluid intake are disrupted and secondary infection is inevitable (Prisciandaro et al. 2011).

The pathogenesis of mucositis is considered to be complex, involving a dynamic series of biological events in the mucosa, including initiation, primary damage response, signal amplification, ulceration and healing (Sonis 2004). The characteristics of each stage have been explained by Sonis (2004). The initiation stage is characterized by injury to target cells, including basal epithelium and submucosal cells, DNA damage and formation of reactive oxygen species (ROS). The presence of ROS and DNA or non-DNA damage activate subsequence stages of mucositis development, which is the primary damage response. In this second stage, the transcription factors (p53 and nuclear factor – κB) are activated. Nuclear factor – κB (NF- κB) is able to regulate up to 200 genes which are responsible for the mucosal damage. At the same time, production of pro-inflammatory cytokines (TNF-α, IL-β and IL-6) are increased leading to the loss of epithelial basal-cells. The death of epithelial basal-cells potentially initiates a destructive signal, which is the next stage in pathogenesis. Pro-inflammatory cytokines, especially TNF-α promote mitogen-activated protein kinase (MAPK) signalling as well as sphingomyelinase. These molecules increase the level of pro-apoptotic signals in the mucosal tissue, which in turn lead to the development of ulceration. Ulceration as the fourth stage is characterized by the loss of mucosal integrity (protective barrier) causing the invasion of enteropathogenic bacteria into the submucosa and colonization of the ulcer. During this phase, microbial products are produced which induce the production of other pro-inflammatory cytokines. Healing is the final phase when the cancer therapy is discontinued. At this phase, proliferation and differentiation of replacement cells commences. The rate of healing varies depending on the type of cancer, chemotherapy drugs used, dosage and duration of treatment.

Damage to the intestinal mucosa has serious consequences and leads to changes in the composition of the intestinal microbiota (dysbiosis) (Prisciandaro et al. 2011). This dysbiosis is likely to contribute to the severity of intestinal mucositis (van

Vliet et al. 2010). Evidence of this theory is shown in a study conducted by van Vliet et al. (2009). Their study indicated that anaerobic bacteria play a pivotal role in maintaining intestinal mucosal integrity. During chemotherapy treatment, the density of anaerobic bacteria decreased, whilst the number of potentially pathogenic aerobic enterococci increased (van Vliet et al. 2009). Furthermore, *Clostridium difficile* and *Enterococcus faecium* have been found to predominate in fecal samples of patients with chemotherapy-induced mucositis compared to healthy controls (Zwielehner et al. 2011).

The relationship existing between GIT microbes and the mammalian host indicates that modulation of the gut microbiota may affect host health (Sánchez et al. 2009). This symbiotic relationship has enabled the maintenance of immune homeostasis in the gut. The mucosal immune system in the intestinal epithelial tract is crucial for maintenance of innate and adaptive immunity (Jarchum & Pamer 2011). A change of balance in the commensal microbiota balance in the GIT could disrupt intestinal epithelial cells and the immune response, which in turn could initiate inflammatory intestinal disorders.

1.7 Rationale for Use of Probiotics for Treating Intestinal Mucositis

A disturbance of homeostasis of the gut microbiota, epithelial cell disruption, inflammation, microbial infection, and immune response dysfunction are acknowledged as contributory factors to the development of intestinal mucositis (Howarth & Wang 2013). Effective therapeutic strategies should be considered in order to improve the balance of gut microbiota, increase the ability of the microbiota to suppress inflammatory response, increase the epithelial cell integrity, and increase immune function. The use of probiotics and their products has been shown to improve intestinal health through these modes of action (van Vliet et al. 2010).

Successful application of probiotics in treating chemotherapy-induced intestinal mucositis has not been demonstrated yet. For example, potential beneficial effects of *Lactobacillus fermentum* BR11, *Lactobacillus rhamnosus* GG, and *Bifidobacterium lactis* BB12 were not demonstrated in treating chemotherapy-damaged small intestine mucositis (Mauger et al. 2007). The investigators recommended further study using a higher dosage, timing variation, and mixture of probiotic cultures. Thus probiotic supernatants may be useful as adjunctive therapy for intestinal mucositis. Supernatants of *Lactobacillus fermentum* BR11 and *E. coli*

Nissle 1917 showed a partial beneficial effect in preventing intestinal damage due to a 5-fluorouracil (5-FU) regimen (Prisciandaro et al. 2011). Therefore, further studies are essential to explore novel probiotic candidates from potential sources such as NFM products.

Beneficial outcomes attributed to probiotics are strain- and indication-specific (Girardin & Seidman 2011). Specific probiotic strains are likely effective to a specific disease indication due to the genetic variation within probiotic species (Aktas 2015; Girardin & Seidman 2011; Sánchez et al. 2017). For example, variation in cell-surface component within *Lactobacillus casei* strains may be contribute to variation in adhesion properties and immune response modulation (Aktas 2015). A wider range of prospective probiotic candidates are therefore urgently required, especially some specific for intestinal mucositis. Although the most commonly used probiotics include intestinal strains of *Lactobacillus* and *Bifidobacterium* species, other intestinal microbes or microbes from other ecological niches may also have a beneficial role in human health (Sánchez et al. 2009). Therefore, NFM products hold a key position as a rich resource for probiotics with potential health-improving properties.

The relationship between GIT microbes and the mammalian host indicate that the modulation of gut microbiota may affect host health (Sánchez et al. 2009). This symbiotic relationship has enabled the maintenance of immune homeostasis in the gut. The mucosal immune system in the intestinal tract is essential for maintenance of innate and adaptive immunity (Jarchum & Pamer 2011). A change of balance in the commensal microbiota balance in the GIT could disrupt intestinal epithelial cells and the immune response, which in turn could initiate inflammatory intestinal disorders. In the current study, prospective probiotics derived from two Indonesian NFM products (dadih and dangke) were utilized to reduce the severity of intestinal mucositis.

1.8 Aims of this Study

The general aim of this study was to obtain probiotic candidates with the potential to reduce the severity of intestinal mucositis. The specific objectives of this project were to:

- assess the microbial diversity of the fermented milk products,
- isolate indigenous probiotic candidates from Indonesian NFM products,

- characterize the probiotic properties using *in vitro* assays and molecular techniques,
- evaluate the capacity for the selected probiotic candidates to reduce the severity of intestinal mucositis in a rat model.

Chapter Two

Culture-Dependent and -Independent Analysis of Microbial Communities Associated with Dadih and Dangke

2.1 Introduction

With increasing interest in novel dairy products, naturally fermented milks have become of interest to food microbiologists as a result of their great potential phenotypic and genetic microbial diversity (Ghiamati et al. 2016; Tamang, Watanabe & Holzapfel 2016; Wouters et al. 2002). The main problems with naturally fermented milk products are difficulties in maintaining consistency and quality, due to the traditional production technique, together with the presence of undefined fermentative microorganisms. As a consequence, it is imperative to select appropriate microbial strains as starter cultures with specific properties, such as enhancing the organoleptic properties, assuring the bioavailability of nutrients, meeting food safety requirements and providing health benefits to humans (Tamang et al. 2016). However, producing fermented products with the use of starter cultures may lead to reduced microbial diversity and organoleptic variation (Ghiamati et al. 2016). Assessing the diversity and the succession of microorganisms involved in the fermentation process is required for a better understanding of its microbial community and an attempt to improve quality and safety.

Dadih is an Indonesian spontaneously fermented buffalo milk product and categorized as a yogurt-like product. After pouring fresh buffalo milk into fresh bamboo tubes, which are then capped with banana leaves, the fresh buffalo milk is allowed to ferment for 2 to 3 days at ambient temperature ranging from 25-30^{o}C until the desired properties are achieved (Surono 2003). Dadih has received considerable attention with respect to its beneficial effects on human health, especially its bacterial strains. These include probiotic effects (Dharmawan, Surono & Kun 2006; Surono 2003; Surono et al. 2011) in addition to anti-mutagenic (Pato 2003a; Surono et al.

2009), anti-pathogenic (Collado et al. 2007a), hypocholesterolemic (Pato et al. 2004), and anti-cancer properties (Pato 2003b).

Dangke is a traditional cheese-like product popularly produced and consumed by the community in Enrekang Regency, Province of Makassar, Indonesia. In contrast to a typical cheese-making process, either fresh buffalo or cow's milk is firstly heated or boiled, then coagulated using papain from papaya (*Carica papaya*) latex until curd and whey are produced. The curd is pressed, and then soaked in a saline solution overnight before wrapping with banana leaves (Razak et al. 2009; Surono et al. 1984). The final product of dangke is characterized by a white curd and an elastic texture. At ambient temperature, the shelf-life of this product is three days. The production of dangke occurs in a small-scale facility, employing artisanal methods of production (Surono 2015).

Culture-dependent methods have been widely applied to bacterial community analysis of dadih (Hosono, Wardojo & Otani 1989; Surono 2003; Yodoamijoyo et al. 1983; Zakaria et al. 1998), although this has yielded limited information. Studies on the bacterial composition of dangke have been undertaken as well, but the strain identification has only involved biochemical and phenotypic assays. The predominant lactic acid bacteria found in dangke with antimicrobial activities were *Lactobacillus plantarum, Enterococcus faecium* and *Leuconostoc mesenteroides*. From those isolates, *Enterococcus faecium* strain DU55 produced bacteriocin active against *Salmonella typhimurium* (Razak et al. 2009). Besides being time-consuming and laborious, the presence of uncultivable microorganisms has become evident. To the best of my knowledge, no study has been conducted to investigate the bacterial communities in dadih and dangke using both culture-dependent and culture-independent based approaches. The work described in this chapter addresses the characterization and identification of indigenous lactic acid bacteria in dadih and dangke. Pyrosequencing based on 16s rRNA gene sequences was applied to analyse bacterial communities in dadih and dangke samples.

2.2 Materials and Methods

2.2.1 Sample collections

Dadih samples were collected from two different areas (Solok Regency and Gadut, Agam Regency) in West Sumatra Province, Indonesia. In this study, only

dadih samples from Solok Regency were collected at different stages of the fermentation process. The samples were taken aseptically to the laboratory and kept on ice (below 5^{o}C). Microbial isolation was conducted on arrival at the laboratory.

Four dangke samples (samples referred to as A, B, C, and D) were sampled from Enrekang Regency, Makassar Province, Indonesia. Buffalo milk was used for dangke A, B, and C, while dangke D was made from cow's milk. Samples were transported in a cooling box within 48 h to Department of Biology, University of Brawijaya, Indonesia. The samples were kept under refrigeration (4^{o}C) until the next day when microbiological analyses were conducted.

Acidity level of each sample was measured by suspending 10 g of sample into 20 mL of sterile distilled water and mixing thoroughly using a vortex mixer (Model VM-2000, DS Instruments, Inc., Taiwan). The pH level was measured using a pH meter (model 3205, Jenway, UK).

2.2.2 Isolation of strains

Ten grams of each sample were homogenized in 90 ml of a sterile saline solution (0.75% w/v NaCl) using a vortex mixer for 2 min. The samples were further diluted in a 10-fold serial dilution and 100-µl aliquots were spread-plated onto different media – MRS agar and M17 agar (all from Oxoid Ltd., Basingstoke, UK). The plates were incubated at 37^{o}C for 48 hours under aerobic conditions. Ten colonies per plate were picked randomly from the agar plates of the highest dilutions. After picking, the strains were sent to Adelaide, Australia for further characterization. In Adelaide, they were repeatedly streaked out to check for purity using their preferred media and incubated at 37^{o}C with 5% of CO_2. Stock cultures of the isolates were stored in their preferred media containing 15% glycerol (Merck, Darmstadt, Germany) at -80^{o}C.

Besides the dadih products, a possibility of microbiota living in the bamboo tubes was also assessed. The bamboo tubes were filled with 10 ml of a sterile saline solution (0.75% w/v NaCl) and the solution mixed using a sterile spoon. The saline was collected and processed as described above.

2.2.3 Phenotypic characterization

Phenotypic characterization conducted in this study was by Gram reaction, colony and cell morphology, oxidase test (Sigma-Aldrich, St. Louis, US), and catalase test (Sigma-Aldrich, St. Louis, US). The catalase test was performed as follows. A small amount of the culture was smeared on a clean microscope slide. Then, one drop of hydrogen peroxide (3%, v/v) was added to the culture. A positive result was indicated by the formation of bubbles. The positive control for Gram stains was *Lactobacillus casei*, and the negative control was *Escherichia coli,* while the positive control for the catalase test was *Staphylococcus aureus*, and the negative control was *Lactobacillus casei*.

2.2.4 Genotypic characterization

Genotypic characterization of the isolates was conducted by PCR and then analysed with RFLP. The target genes were in the region of the chromosome between the 16S and 23S rRNA genes, termed the intergenic transcribed spacer (ITS). The oligonucleotide primers used (Table 2) were 23S/p10 (position 456-474 of 23S rRNA) and tAla (position tRNA gene) (Rachman et al. 2003). Total DNA was extracted by a simple lysis method (Bell, Paton & Turnidge 1998). Isolates were cultivated on MRS agar (Oxoid) overnight and then a single colony was picked and placed into a sterile Eppendorf tube containing 200 µl of sterile milliQ water. Next, the bacterial suspension was homogenized using a vortex mixer to obtain a light turbid suspension. The bacterial suspension was heated on the heating block at 95^{o}C for 20 minutes. Then, the sample was centrifuged at 10,188 x g for 5 minutes at room temperature (Eppendorf Centrifuge 5415 C, Brinkmann Instruments, Inc. NY, US). The supernatants (170 ml) were removed and transferred into a sterile Eppendorf tube ready to be used as a DNA template. The DNA templates were stored at -20^{o}C. The PCR reactions were carried out using a Mycycler thermal cycle (Bio-Rad Laboratories, Hercules, CA, USA). The master mix was prepared in 25 µl of final volume containing 5 µl of 5x PCR buffer, 2.5 µl of 25 mM $MgCl_2$, 2.5 µl of 2.5 mM dNTPs (2 mM each dATP, dCTP, dGTP and dTTP), 0.25 µl of *Taq* polymerase (5 U/µl) and 3.75 µl of 2 µM each primer. The volume of DNA template was 2.5 µl. The PCR amplification conditions are listed in Table 2. ITS-PCR products obtained were separated using electrophoresis at room temperature on horizontal 1.5% (w/v) agarose

gels. Gels containing 5% (v/v) of Ethidium Bromide (EtBr) were run in a tank with 1 × TAE buffer as the running buffer for at least an hour at 100 V. ITS-PCR products (7 µl) were dispensed into the wells. The result of electrophoresis was visualized using the Gel Doc system (Bio-Rad Laboratories, Hercules, CA, USA).

A total volume of 10.2 µl was used for RFLP analysis which comprised 9 µl of amplicons (previous ITS-PCR products), 1 µl of buffer (composition dependent on the enzymes), and 0.2 µl of restriction enzymes (HinfI and HindIII, New England BioLabs Inc., MA, US). The mixture was incubated at 37^{o}C for 24 h. Following this incubation, 5 µl of the RFLP product added with 2 µl of loading dye were dispensed into the wells of the 1.5% (w/v) agarose gels containing 5% (v/v) of ethidium bromide (EtBr), and then electrophoresed in 1 × TAE buffer for 1 h. After separation, the gel result was visualized using the Gel Doc system (Bio-Rad Laboratories, Hercules, CA, USA). The sizes of the DNA fragments were estimated by comparing their relative mobility with 100 bp of molecular weight ladder (New England BioLabs Inc., MA, US).

2.2.5 Molecular identification

In order to confirm the results provided by PCR ITS-RFLP, 16S rRNA sequencing was also carried out. The primer design was based on a conserved region between the V1 and V2 variable regions in 16S rRNA and a product of approximately 500 bp in size (Kullen et al. 2000). The PCR reactions were carried out using Mycycler thermal cycle (Bio-Rad Laboratories, Hercules, CA, USA). The master mix was prepared in 25 µl volume containing 5 µl of 5x PCR buffer, 1.5 µl of 25 mM $MgCl_2$, 2.5 µl of 2.5 mM dNTPs (2 mM each dATP, dCTP, dGTP and dTTP), 0.25 µl of *Taq* polymerase (5 U/µl) and 3.75 µl of 2 µM each primer. The volume of DNA template was 2.5 µl. The PCR amplification conditions are listed in Table 3. The result of PCR amplification was visualized using agarose gel electrophoresis. PCR products were purified for sequencing by using UltraClean PCR clean-up kit (MO BIO Laboratories Inc, Solena Beach, CA, USA.) according to the manufacturer's instructions.

2.2.6 DNA sequencing analysis

Sequencing of the DNA products was performed using Big Dye Terminator v3.1 cycle sequencing ready reactions (Applied Biosystem, Foster City, CA, USA) at

the DNA Sequencing Facility, Flinders Medical Centre, Bedford Park, South Australia. The nucleotide sequences were edited using BioEdit software (version 7.0.9.0, Ibis Bioscience, Carlsbad, CA). Homology searches were performed using Basic Local Alignment Search Tool (BLAST) at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/). A phylogenetic tree derived from partial 16s rRNA sequence data was constructed by using Geneious® 9.1.7 (Biomatters Ltd., New Zealand) and then visualized using FigTree v1.4.2 (Institute of Evolutionary Biology, University of Edinburgh).

2.2.7 Pyrosequencing analysis

Total DNA was isolated from dadih samples using a PowerFood® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, California) according to the manufacturer's protocol. DNA concentrations were calculated using the NanoDrop Lite Spectrophotometer (Thermoscientific, Australia) as per manufacturer's instructions. Samples were diluted with MilliQ water to a final concentration of 10 ng/µL and used as template in subsequent barcoded PCR. The DNA was then sent to the Australian Genomic Research Facility (AGRF) in Brisbane for pyrosequencing on a Titanium GS-FLX (454 Life Sciences/ Roche FLX). Samples for 454-amplicon pyrosequencing were amplified using a barcoded universal 16s rRNA gene (variable region V1/V3: 27F (AGAGTTTGATCMTGGCTCAG) – 519R (GWATTACCGCGGCKGCTG) target). Denoised sequences were analysed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline software (version 1.8.0) to obtain a summary from phylum to genus level (Caporaso et al. 2010).

Table 2 List of primers used in this study

Primer*	Target	Oligonucleotide sequence (5'-3')	Annealing T (°C)	Amplicon size (bp)	References
23S/p10-R tAla-F	ITS region	f 'CCT TTC CCT CAC GGT ACT G' r 'TAG CTC AGC TGG GAG AGC'	56	-	(Rachman et al. 2003)
plb16-F mlb16-R	16S rRNA (V1 and V2 regions)	f 'AGA GTT TGA TCC TGG CTC AG' r 'GGC TGC TGG CAC GTA GTT AG'	52	~500	(Kullen et al. 2000)

*F, forward; R, reverse

Table 3 PCR amplification condition used for the characterization and identification of LAB isolated from dadih

Primer*	PCR amplification condition**	References
23S/p10-R tAla-F	94°C- 1m, 56°C- 1m, 72°C- 1m (35 cycles)	(Rachman et al. 2003)
plb16-F mlb16-R	94°C- 1m, 52°C- 1m, 72°C- 1m (35 cycles)	(Kullen et al. 2000)

*F, forward; R, reverse

** Initial denaturation step at 94°C for 5 minutes and final extension step at 72°C for 5 minutes

2.3 Results

2.3.1 Enumeration and LAB isolation

In the present study, two sources of dadih from different regions (Solok Regency and Gadut, Agam Regency) in West Sumatra Province, Indonesia were used. In particular, the dadih samples from Solok Regency were on consecutive days from day 1 of fermentation until day 3 as well as from its source raw buffalo milk sample. The viable counts of the LAB group present in the raw buffalo milk and dadih products are shown in Table 4. LAB counts on MRS agar for dadih samples (final product/ day 3) from Solok and Gadut were 6.22±0.83 log cfu/g and 7.42±0.05 log cfu/g, respectively. Total counts of LAB from M17 agar from dadih products from Solok and Gadut were 6.91±0.74 log cfu/g and 7.73±0.42 log cfu/g, with pH values relatively similar (4.65 and 4.85, respectively). LAB counts in dadih product (day 3) from Gadut were higher than in dadih Solok (day 3).

To investigate the LAB community succession during dadih fermentation, a culture-dependent technique was applied. On MRS agar, surprisingly, LAB was not detected in raw buffalo milk, but after fermentation commenced, from day 1 until day 3, the LAB count was 7.99±0.1 log cfu/g, 7.39±0.01 log cfu/g, and 6.22±0.83 log cfu/g, respectively. By using M17 agar, the LAB count in raw buffalo milk, dadih Solok from day 1 until day 3 was 6.67±1.17 log cfu/g, 8.04±0.09 log cfu/g, 7.99±0.18 log cfu/g, and 6.91±0.74 log cfu/g, respectively. The pH value of raw buffalo milk was about 6.92, and then decreased to 4.65 at day 3 of fermentation. Over the three days of fermentation, the decrease in pH values of dadih was inversely correlated with the increase in the bacterial abundance. LAB was also detected in the inner part of the bamboo (container). The total of LAB on MRS and M17 agar was 4.58±0.13 log cfu/g and 4.74±0.27 log cfu/g, respectively.

Four samples of dangke were collected from different producers in Enrekang District. LAB counts on MRS agar ranged from 4.16±0.41 log cfu/g (sample dangke B) to 7.46±0.03 log cfu/g (sample dangke C) (Table 4). The total LAB on M17 agar varied in the range of 7.21±0.28 log cfu/g (sample dangke C) to 8.23±0.11 log cfu/g (sample dangke A). Acidity level of dangke samples ranged from 4.08 (sample dangke B) to 5.14 (sample dangke D), with the highest pH value was dangke made from cow's milk (dangke D).

Table 4 Lactic acid bacterial count of dadih and dangke samples

Samples	Milk type	Location	pH value	Colony number (log cfu/mL or g)	
				MRS	M17
Raw milk	Buffalo milk	Solok	6.92	ND*	6.67±1.17
Dadih day 1	Buffalo milk	Solok	5.02	7.99±0.11	8.04±0.09
Dadih day 2	Buffalo milk	Solok	4.65	7.39±0.01	7.99±0.18
Dadih day 3	Buffalo milk	Solok	4.65	6.22±0.83	6.91±0.74
Bamboo	-	Solok	ND*	4.58±0.13	4.74±0.27
Dadih day 3	Buffalo milk	Gadut	4.85	7.42±0.05	7.73±0.42
Dangke A	Buffalo milk	Enrekang	4.09	6.34±0.03	8.23±0.11
Dangke B	Buffalo milk	Enrekang	4.08	4.16±0.41	7.89±0.14
Dangke C	Buffalo milk	Enrekang	4.26	7.46±0.03	7.21±0.28
Dangke D	Cow's milk	Enrekang	5.14	7.39±0.02	7.56±0.17

*ND = not detected

2.3.2 Characterization and identification of isolates

Eighty-one bacterial isolates were collected from both dadih and dangke samples; among them, 58 isolates (47 were isolated from dadih and 11 isolated from dangke) were characterized as Gram-positive and catalase-negative microorganisms, so that they were considered as presumptive LAB. The majority of isolates were cocci (34 in total of which 24 were isolated from dadih and 10 from dangke), and the rest were rod-shaped bacteria (24 in total of which 23 were isolated from dadih and one from dangke). A total of 23 isolates were non-LAB; 15 isolates were negative Gram and eight isolates were Gram-positive. Numbers of presumptive LAB and non-LAB isolates as well as their phenotypic characterization obtained from each sample are depicted in Appendix 1. Based on phenotypic characteristics, the 81 bacterial isolates were assigned to seven groups. In validation of the phenotypic characterization, the isolates were also clustered in seven groups based on electrophoretic profiles obtained by RFLP of PCR product targeted 16S-23S rRNA spacer region (ITS) (Table 5, Fig. 4, 5 and 6). Group II was divided into two sub-groups due to different ITS-PCR RFLP

results. The representative isolates for each group/sub-group were identified by 16S rRNA gene sequencing then confirmed by species-specific amplification, including for two related species (group I), namely *L. plantarum* and *L. pentosus*. This taxonomic differentiation supported *L. plantarum* as the species name with PCR product size of 220 bp (Fig. 7).

Table 5 Grouping of bacteria based on ITS-PCR RFLP results and identification by 16S rRNA gene sequencing

Groups	Number of isolates (%)		Cell-shaped	ITS-PCR (bp)	RFLP		BLAST analysis (%)	Identification (16S rRNA gene sequencing)	
	Dadih	Dangke			HinfI	Hind III		Closely similar to	Accession Number
I	23 (37)	1 (5)	Rod	700	90, 200, 440	700	100	*Lactobacillus plantarum* strain AN66	LC21362
IIA	15 (24)	9 (48)	Coccus	700	50, 200	700	100	*Lactococcus lactis* subsp *lactis* strain A12	LT599049
IIB	7 (11)	1 (5)	Coccus	700	80, 190, 220	220, 480	100	*Lactococcus lactis* subsp *lactis* strain A12	LT599049
III	2 (3)	0	Coccus	700	80, 150, 500	200, 500	99	*Enterococcus faecium* strain ISPA FRP2	KC510246
IV	1 (2)	1 (5)	Rod	700	200, 500	700	100	*Acetobacter orientalis* strain S30-3	HM217982
V	1 (2)	1 (5)	Rod	800	800	250, 500	98	*Klebsiella oxytoca* strain BR37	KC593550
VI	5 (8)	6 (32)	Rod	700	50, 190, 220	700	99	*Klebsiella* sp. clone JXSH1-86 (uncultured)	JX535203
VII	8 (13)	0	Rod	700	80, 170, 200	280, 400	100	*Bacillus pumilus* strain YKCM-AS-2B	LC010659
Total	62	19							

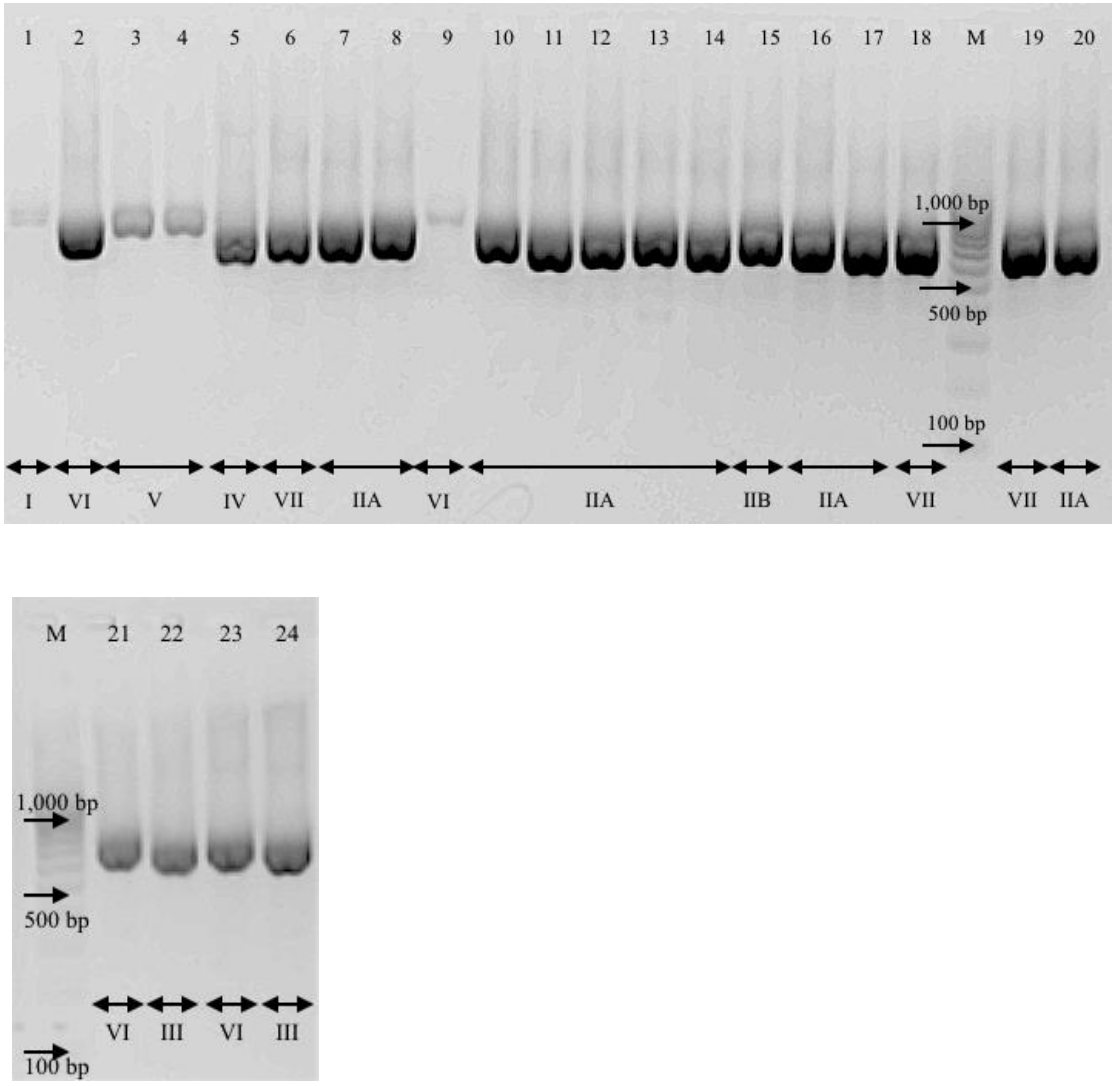

Figure 4 PCR amplification of 16S-23S rRNA gene (ITS) region

Lane M: 100 bp DNA marker; lane 1: *Lb. plantarum* SL0.17; lane 2: *Klebsiella* sp. D1.15; lane 3: *Klebsiella oxytoca* SL1.12; lane 4: *Klebsiella oxytoca* D3.10; lane 5: Acetobacter cerevisiae D2.25; lane 6: *B. pumilus* SL1.6; lane 7: *L. lactis* subsp. *lactis* D2.17; lane 8: *L. lactis* subsp. *lactis* D1.1; lane 9: *Klebsiella* sp. D1.9; lane 10: *L. lactis* subsp. *lactis* SL1.17; lane 11: *L. lactis* subsp. *lactis* D2.20; lane 12: *L. lactis* subsp. *lactis* Sl0.10; lane 13: *L. lactis* subsp. *lactis* SL3.27; lane 14: *L. lactis* subsp. *lactis* D2.15; lane 15: *L. lactis* subsp. *lactis* SL0.15; lane 16: *L. lactis* subsp. *lactis* SL0.11; lane 17: *L. lactis* subsp. *lactis* SL3.22; lane 18: *B. pumilus* SL1.7; lane 19: *B. pumilus* SL2.14; lane 20: *L. lactis* subsp. *lactis* D1.7; lane 21: *Klebsiella* sp. GD.15; lane 22: *E. faecium* SL3.26; lane 23: *Klebsiella* sp. GD.14 and lane 24: *E. faecium* GD.12

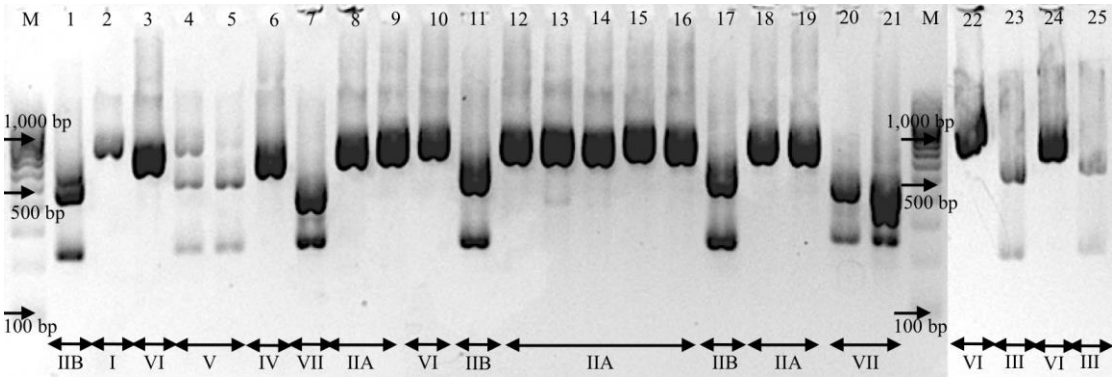

Figure 5 Gel electrophoresis of PCR-amplified 16S-23S rRNA (ITS) region digested with HindII

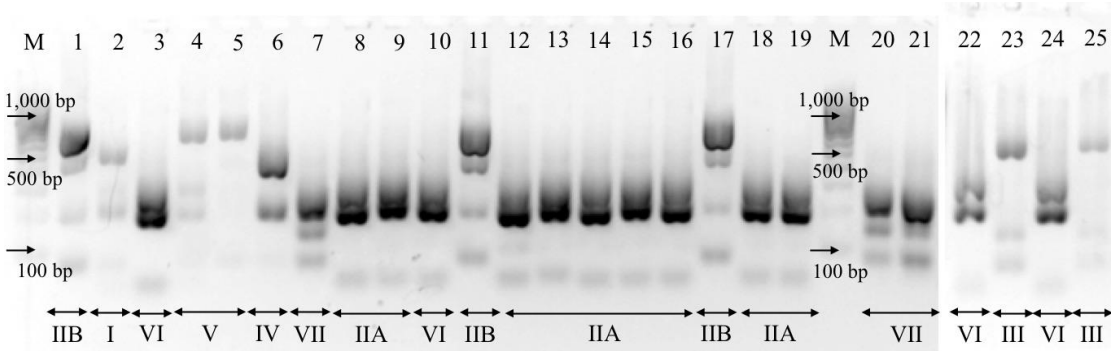

Figure 6 Gel electrophoresis of PCR-amplified 16S-23S rRNA (ITS) region digested with HinfI

Lane M: 100 bp DNA marker; lane 1: *L.lactis* subsp. *lactis* D3.11; lane 2: *Lb. plantarum* SL0.17; lane 3: *Klebsiella* sp. *D1.15*; lane 4: *Klebsiella oxytoca* SL1.12; lane 5: *Klebsiella oxytoca* D3.10; lane 6: Acetobacter cerevisiae D2.25; lane 7: *B. pumilus* SL1.6; lane 8: *L. lactis* subsp. *lactis* D2.17; lane 9: *L. lactis* subsp. *lactis* D1.1; lane 10: *Klebsiella* sp. D1.9; lane 11: *L. lactis* subsp. *lactis* SL1.17; lane 12: *L. lactis* subsp. *lactis* D2.20; lane 13: *L. lactis* subsp. *lactis* Sl0.10; lane 14: *L. lactis* subsp. *lactis* SL3.27; lane 15: *L. lactis* subsp. *lactis* D2.15; lane 16: *L. lactis* subsp. *lactis* SL0.15; lane 17: *L. lactis* subsp. *lactis* SL0.11; lane 18: *L. lactis* subsp. *lactis* SL3.22; lane 19: *L. lactis* subsp. *lactis* SL3.24; lane 20: *B. pumilus* SL1.7; lane 21 : *B. pumilus* SL2.14; lane 22: *Klebsiella* sp. GD.15; lane 23: *E. faecium* SL3.26; lane 24: *Klebsiella* sp. GD.14 and lane 25: *E. faecium* GD.12

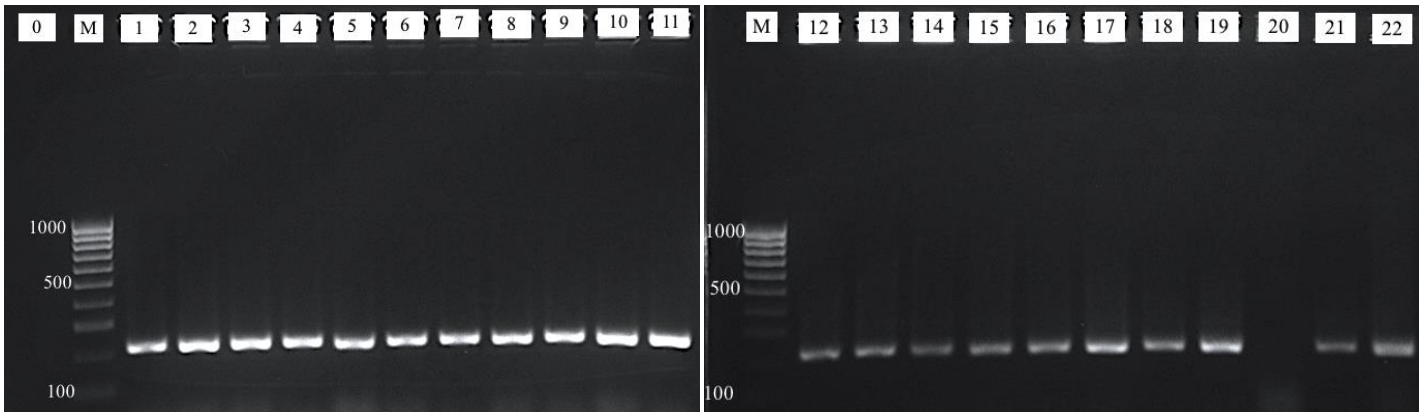

Figure 7 Agarose gel electrophoresis of *L. plantarum* using species-specific primers (220 bp)

Lane M: 100 bp DNA marker; lane 0: sterile milliQ H_2O; lane 1: *L. plantarum* S130; lane 2: *L. plantarum* GD1; lane 3: *L. plantarum* SL22; lane 4: *L. plantarum* SL017; lane 5: SL010; lane 6: SL34; lane 7: *L. plantarum* GD3; lane 8: *L. plantarum* SL27; lane 9: *L. plantarum* SL31*;* lane 10: *L. plantarum* GD2; lane 11: *L. plantarum* GD4; lane 12: *L. plantarum* GD12; lane 13: *L. plantarum* SL32; lane 14: *L. plantarum* SL35*;* lane 15: *L. plantarum* LS33; lane 16: *L. plantarum* SL37; lane 17: *L. plantarum* SL36*;* lane 18: *L. plantarum* SL38; lane 19: *L. plantarum* GD5; lane 20: *L. rhamnosus* GG; lane 21: *L. plantarum* GD6; lane 21: *L. plantarum* ATCC 14917

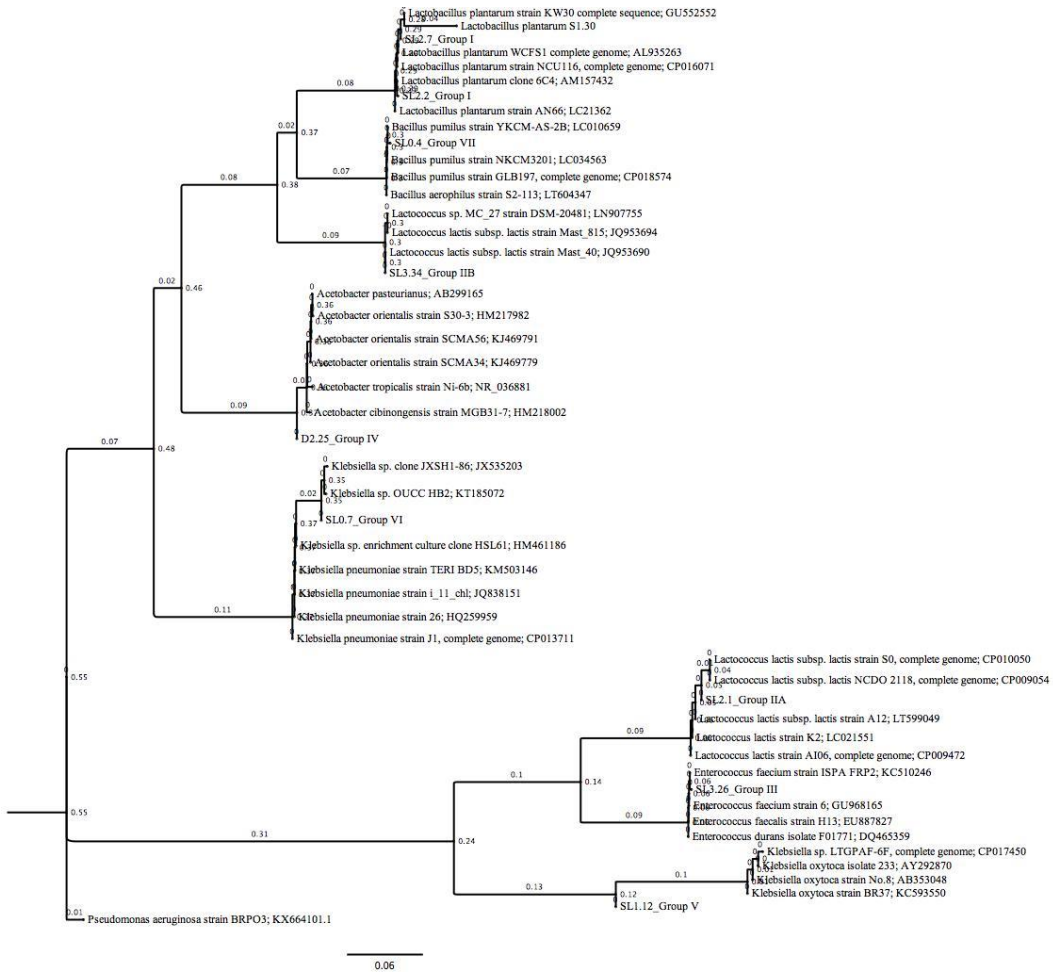

Figure 8 Neighbor-joining tree showing the phylogenetic relationships among bacterial isolates from dadih and dangke samples and the type isolates based on 16S rRNA gene sequences. *Pseudomonas* (*P.*) *aeruginosa* was used as an outgroup.

Based on these analyses, the 81 isolates were classified as belonging to six genera (*Lactobacillus, Lactococcus, Enterococcus, Acetobacter, Klebsiella* and *Bacillus*), and to seven species (Table 5): *L. plantarum* (24 isolates, group I), *L. lactis* subsp. *lactis* (24 isolates: sub-group IIA, eight isolates: sub-group IIB), *E. faecium* (2 isolates, group III), *A. orientalis* (2 isolates, group IV), *K. oxytoca* (2 isolates, group V), *Klebsiella* sp. (11 isolates, group VI) and *B. pumilus* (8 isolates, group VII). As observed in Fig. 8, the phylogram has 8 clades and each represents the group of organisms, which were similar to the molecular characterization (PCR-RFLP). *Lactococcus lactis* subsp. *lactis* appeared to be grouped separately. Among all these isolates, the two largest groups were *L. lactis* subsp. *lactis* (39.5% of all isolates) and

L. plantarum (29.6% of all isolates) (Table 5). Two species that were found only from dadih samples were *E. faecium* and *Bacillus pumilus*.

2.3.3 Pyrosequencing data

The V1-V3 variable region of the 16S rRNA gene was amplified from five dadih and three dangke samples, and a total of 96,887 sequence reads were obtained (Table 6). The read number for each sample varied from 6,892 (Dadih Gadut day 3) to 20,120 (Dangke buffalo milk B), with the average being 12,110.87. A total of 91,324 sequence reads passed the quality check and barcode-sequence tag sorting; 11,161 sequence reads (12.22% of filtered sequence reads) were detected as chimeras and therefore removed. The sequence reads clustered into 211 operational taxonomy units, with an average of 26.37 per sample. The bacterial sequences identified were phylogenetically classified into phylum and genus level.

Table 6 Pyrosequencing data and alpha diversity scores of dadih and dangke samples

Samples	Unfiltered sequence	Filtered passed sequence	Chimeric sequence number (Percentage)	Observed OTUs	PD whole tree	Shannon indices (H')
Raw buffalo milk	16329	15658	2082 (13.30)	6	8.14	1.06
Dadih Solok day 1	14732	13930	1654 (11.87)	38	28.53	4.65
Dadih Solok day 2	8494	7957	695 (8.73)	17	10.90	2.53
Dadih Solok day 3	7304	6680	340 (5.19)	17	17.53	4.30
Dadih Gadut day 3	6892	6202	601 (9.69)	29	23.10	4.98
Sub-total for dadih	53751	50427	5372 (10.65)	107 (21.4)		
Dangke cow's milk	9378	8922	2664 (29.86)	36	27.35	5.78
Dangke buffalo milk A	13638	12898	425 (3.29)	34	22.36	4.10
Dangke buffalo milk B	20120	19077	2700 (14.15)	34	34.28	5.67
Sub-total for dangke	43136	40897	5789 (14.15)	104 (34.7)		
Total	**96887**	**91324**	**11161 (12.22)**	**211 (26.37)**		

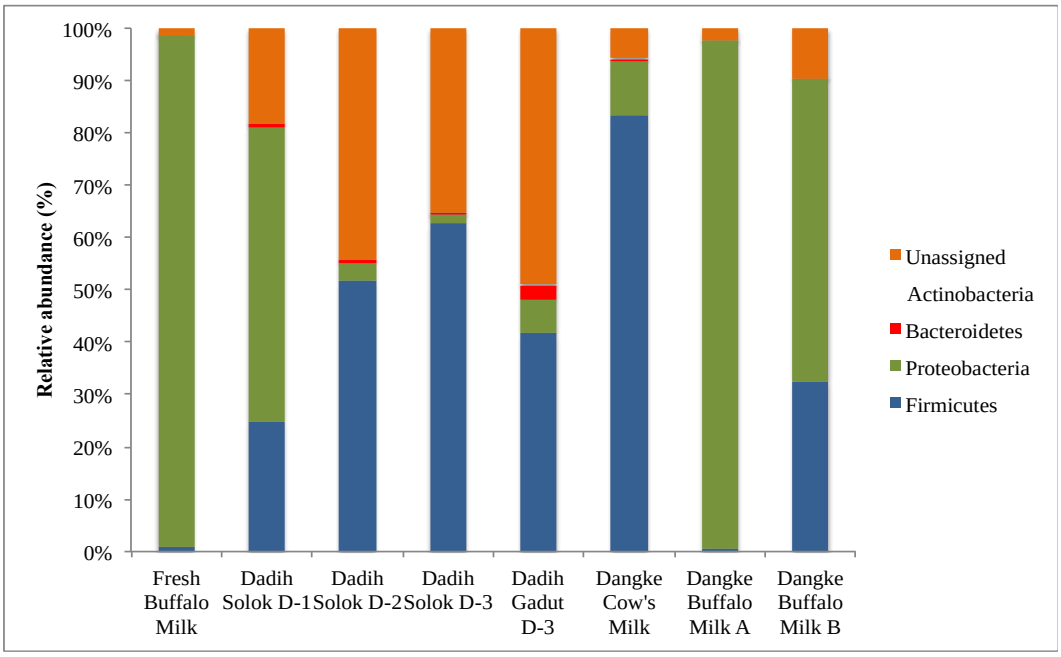

Figure 9 Relative abundances of bacteria at the phylum level of dadih and dangke products

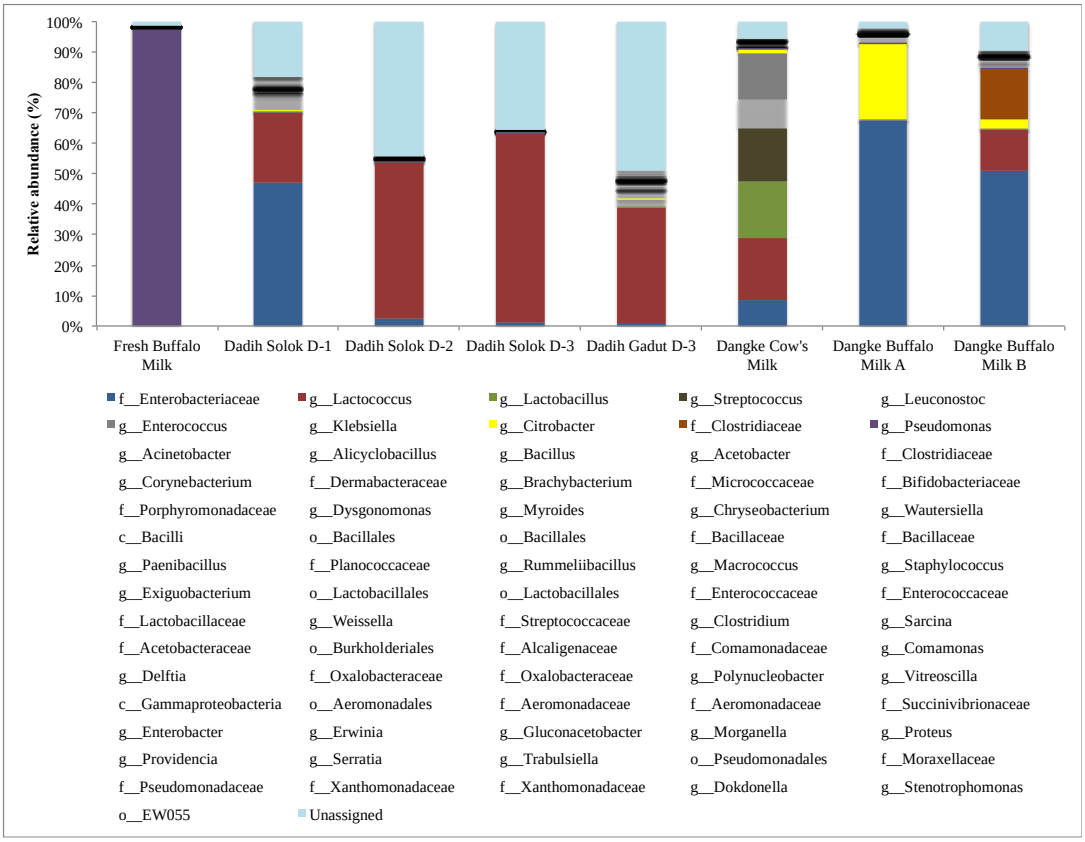

Figure 10 Relative abundances of bacteria at the genus level of dadih and dangke products

2.3.4 Composition of bacterial communities

Composition at the phylum and genus level is shown in Fig. 9 and 10. A total of four phyla were found in both dadih and dangke samples. At the phylum level, bacterial communities were dominated by Firmicutes (1.01-62.62% in dadih; 0.4-83.38% in dangke), Proteobacteria (1.72-97.72% in dadih; 6.51-97.29% in dangke), Bacteroidetes (0.25-2.69% in dadih; 0.45% in dangke cow's milk) and Actinobacteria (0.01-0.16% in dadih; 0.01-0.04% in dangke) (Fig. 9). From those phyla were assigned to 37 families (22 for dadih; 15 for dangke) and 37 genera (18 for dadih; 19 for dangke). In dadih, *Lactococcus* (22.84-62.22% of sequences) was the predominant genus, followed by Enterobacteriaceae (0.53-47.25% of sequences) and *Acinetobacter* (0.28-4.25% of sequences, Fig. 10). The abundance of *Lactococcus* throughout the fermentation stages increased substantially, with a concomitant decrease in the number of sequences belonging to the Enterobacteriaceae family. While, *Acinetobacter* in dadih decreased on day 2, and then slightly increased on day 3. Besides phylum Actinobacteria, some genus only found in dadih Gadut day 3 compared to dadih Sianok day 3 (with relative abundance more than 1%) were *Leuconostoc, Myroides,* and *Stenotrophomonas.*

Bacteria in dangke prepared from cow's milk were more diverse than those is dangke from buffalo milk. At the family level, Enterobacteriaceae (9.7-92.5%), Enterococcaceae (0.1-15.5%), Moraxellaceae (0.1-4.1%) and Pseudomonadaceae (0.1-0.3%) were found in all dangke samples. At the genus level, the OTU analysis identified six genera (*Lactococcus*, 20.48%*; Lactobacillus,* 18.57%*; Streptococcus,* 17.17%*; Enterococcus,* 15.42%*; Leuconostoc,* 9.48%; and unclassified genera belonging to the Enterobacteriaceae family, 8.53%) that would represent the core components of dangke sourced from cow's milk (Fig. 10). The Enterobacteriaceae family was the predominant taxa in dangke derived from buffalo milk (dangke A: 67.4% and B: 51.04%). *Enterococcus* (0.14% of sequences) was the only lactic acid bacteria detected in dangke A; meanwhile *Lactococcus* (13.36% of sequences) and *Enterococcus* (0.5% of sequences) were identified in dangke B. As mentioned in the dependent-culture analysis, besides LAB members (*Lactococcus, Lactobacillus* and *Enterococcus*), genus of *Acetobacter* (0.02-0.72% in dadih and 0.08% in dangke B)*, Klebsiella* (0.07% in dadih and 0.11-0.19% in dangke) and *Bacillus* (0.03-1.01% in

dadih and 0.01-0.59% in dangke) were also detected through the pyrosequencing method.

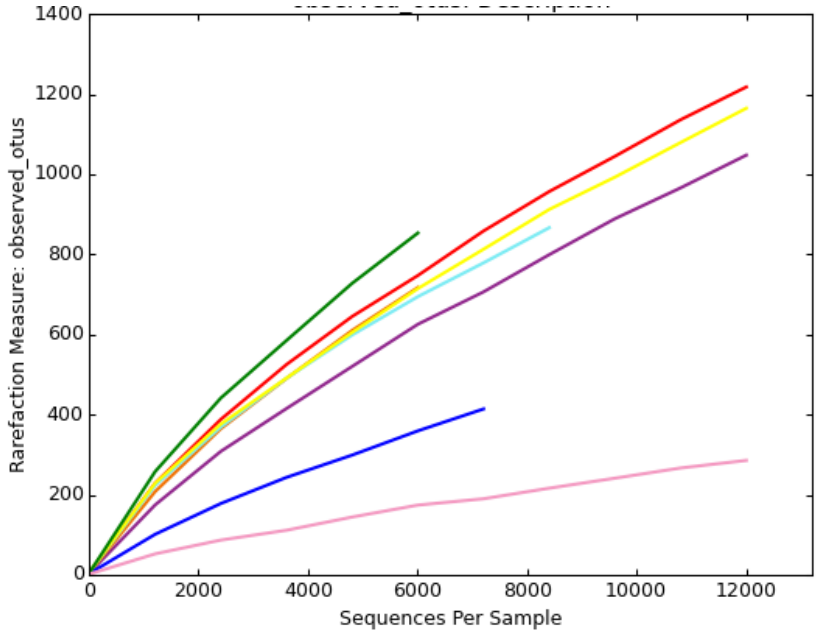

Figure 11 Rarefaction curves of dadih and dangke samples

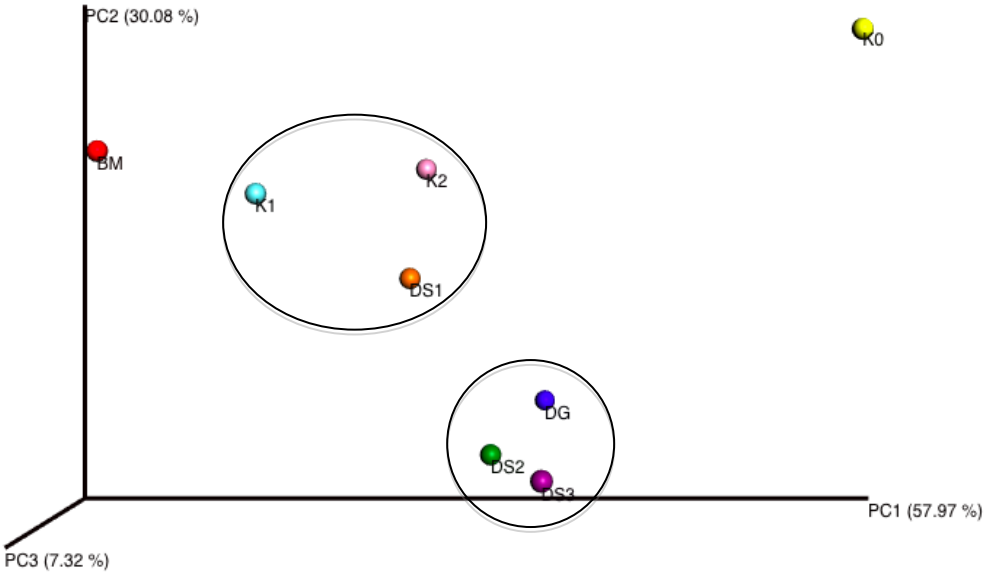

Figure 12 Principal coordinates analysis (weighted UniFrac) of dadih and dangke samples

BM: fresh buffalo milk; DG: dadih Gadut day 3; DS1-3: dadih Sianok day 1-3; K0: dangke cow's milk; K1: dangke buffalo milk A; K2: dangke buffalo milk B.

2.3.5 Bacterial diversity

Rarefaction curves based on observed OTUs of each sample as presented in Fig. 11 indicating the sequence coverage and the diversity in the samples (alpha diversity). Raw buffalo milk and dadih Sianok day 2 samples showed a saturated curve, meaning that it has good sequence coverage. The raw buffalo milk sample curve was represented by the lowest curve, demonstrating that the bacterial diversity was the lowest diversity value compared to the other samples. This is supported with the lowest value of Shannon indices among dadih samples (H': 1.06, Table 6). While, other samples (dadih and dangke) were unsaturated, indicating that the microbial survey could not cover all the taxonomic diversity present in those samples. Among dangke samples, dangke from cow's milk has the highest Shannon index (H': 5.78), followed by dangke B (H': 5.67) and dangke B (H': 4.10). Bacterial diversity (Shannon indices) between dadih Sianok and Gadut (day 3) showed that dadih from Gadut (H': 4.98) had a more diverse flora than that of dadih from Sianok (H': 4.30).

Bacterial diversity between communities (beta diversity) based on weighted UniFrac (quantitative/ OTU abundance) was shown in Fig. 12. Dadih Gadut, dadih Sianok and dangke represented different community/ different sampling sites. The closer the dot positions the more similar the bacterial relative abundance between communities. Dadih from Gadut, dadih Sianok day 2 and 3 were clustered into one group. While, dadih Sianok day 1, dangke A and B were grouped into another group. Dangke from cow's milk and fresh buffalo milk were separated from other samples.

2.4 Discussion

2.4.1 Bacterial communities in dadih and dangke

An integrated approach combining culture-dependent and -independent methods has been implemented to investigate bacterial communities in dadih and dangke. Based on the culture-dependent method confirmed with 16s rRNA gene sequencing, two bacterial groups were detected namely LAB (three genera and species) and non-LAB (three genera and four species). Species of *L. plantarum*, *L. lactis* subsp. *lactis* and *E. faecium* were present in dadih and have been reported in previous studies (Jatmiko, Barton & de Barros Lopes 2010; Surono et al. 2011; Zakaria et al. 1998). The prevalence of non-LAB is likely to be underestimated when using MRS agar as LAB isolation medium. Although MRS medium is designed for

isolation of LAB, other bacteria will also grow. Therefore, this media is considered as semi-selective media due to its nutrient-rich ingredients (Jans et al. 2012; Kim, Fung & Kang 2001). In this present study, MRS agar without addition of indicator substances was used to isolate LAB; as a consequence, besides LAB, *Acetobacter orientalis, Klebsiella oxytoca, Klebsiella* sp. and *Bacillus pumilus* could grow on it, albeit in lower proportions (from dadih).

Similar results were also found with dangke samples. However, *E. faecium* (LAB) and *B. pumilus* (non-LAB) were not detected in dangke. Meanwhile the other species detected in dadih were also present in dangke (Table 5). Only *L. plantarum* has been reported in previous study of dangke (Nur, Hafsan & Wahdiniar 2015; Razak et al. 2009). The remaining species have not been reported before. The predominant species in final products of dadih (day 3) was *L. plantarum* (31%) followed by *L. lactis* subsp. *lactis* (21%); while *L. lactis* subsp. *lactis* (53%) was the most abundant LAB found in dangke. In previous study of dadih, *L. lactis* subsp. *lactis* (13%) was also observed as second dominant species after *L. paracasei* (81%) (Jatmiko, Barton & de Barros Lopes 2010).

2.4.2 Molecular characterization

Molecular characterization of isolated bacteria was performed using PCR-RFLP targeted ITS region (tRNA). The result of phenotypic characterization was in agreement with the molecular characterization, resulted seven groups, except for group II (Table 5). As a member of group II, *L. lactis* subsp. *lactis* was devided into two sub-groups owing to different RFLP profiles. The combination of phenotypic characterization and 16s rRNA gene sequence could not discriminate this group. The RFLP pattern of *L. lactis* subsp. *lactis* was different from the previous study (Jatmiko, Barton & de Barros Lopes 2010). Genetic variation within species of *L. lactis* subsp. *lactis* has been observed which was affected by its source, either from dairy or non-dairy environments (Dhaisne et al. 2013; Passerini et al. 2010). However, in depth phenotypic characterization involving metabolic activities, such as lipolysis, proteolysis and glycolysis was a highly recommended method to discriminate genetically closely related strains of *L. lactis* subsp. *lactis* when genetic methods have failed (Dhaisne et al. 2013). RFLP analysis of the targeted ITS region provides better information as a simple method to distinguish this strain, instead of using MLST and PFGE analyses.

2.4.3 Structure and dynamics of microbial community

Pyrosequencing serves as culture-independent approach and has been used in recent studies to assess the structure of microbial communities in NFM products (Jayashree et al. 2013; Liu et al. 2015a; Liu et al. 2015b; Liu et al. 2015c; Oki et al. 2014; van Hijum, Vaughan & Vogel 2013). This technique was used to observe bacterial succession in dadih fermentation, from raw buffalo milk until final product at day 3. The most abundant genus in raw buffalo milk was *Pseudomonas* (97.6% of sequence), and then this genus was not detected again after fermentation started. *Pseudomonas* as a member of psychrotrophic group of bacteria is commonly found in raw milk causing spoilage of dairy products (Uraz & Citak 1998). Acidic conditions and a microaerophilic environment provide undesirable conditions for *Pseudomonas* growth. Members of family Enterobacteriaceae were detected after 24 h fermentation of dadih, and this group predominated. Evidence for this comes from the isolation of *Klebsiella oxytoca* and *Klebsiella* sp. on day 1 of fermentation. The presence of this bacterial group indicates unhygienic conditions and contamination from either fecal material or the dairy farm environment, such as water, equipment, plant materials and dirt (Martin et al. 2016). *Klebsiella* sp. (group VI) was phenotypically closely related to *Klebsiella pneumonia* because its colony texture was mucoid (Appendix 1). A mucoid phenotype is associated with *Klebsiella pneumonia* (Yu et al. 2007).

Mesophilic LAB, namely genus *Lactococcus* was the most abundant bacterial group during the fermentation period from day 2 to 3. The presence of this genus was in agreement with culture-dependent technique represented by *L. lactis* subsp. *lactis*. This bacteria, which has a long history of safe use in the fermented milk products may originate from multiple sources, both dairy and the non-dairy environment, particularly plant materials (Ainsworth et al. 2014; Cavanagh, Fitzgerald & McAuliffe 2015). In the case of dadih, *L. lactis* subsp. *lactis* may be derived from the buffalo milk, bamboo tubes or banana leaf. Adaptation processes contribute to the prevalence of genetic variation in *L. lactis* subsp. *lactis* strains (Cavanagh, Fitzgerald & McAuliffe 2015). Therefore, in this present study, *L. lactis* subsp. *lactis* was divided into two sub-groups based on their RFLP patterns.

The third bacterial group contributing to fermentation in dadih was acetic acid bacteria. Two genera were detected using the pyrosequencing method in a relatively low abundance, namely genera *Acinetobacter* and *Acetobacter.* The use of a culture

method successfully isolated *Acetobacter orientalis*. The role of acetic acid bacteria in fermented products has been intensively investigated in kefir. Principally, these bacteria contributed to consuming or oxidising ethanol produced by yeasts (Martínez-Torres et al. 2017). In previous study, *Acetobacter cerevisiae* was isolated from dadih in low numbers as well (Jatmiko, Barton & de Barros Lopes 2010).

Dangke can be made from either cow's milk or buffalo milk, depending on the milk availability. Although the manufacture technique is similar, dangke made from cow's milk has a higher microbial diversity than buffalo milk. Based on pyrosequencing results, a more diverse population of mesophilic LAB was found in dangke from cow's milk; while family Enterobacteriaceae dominated two dangke samples from buffalo milk. Factors affecting this different microbial diversity could be different feeding habits of cows and buffalos, length of time the milk is pre-treated (heating), duration of milk storage and nutritional value (fats, protein and sugars) of the milk. Differences in microbial communities in a fermented milk products have been associated with the diet of the animals (Giello et al. 2017). Traditionally, in Indonesia, the cow is kept in a pen with nutritious feeds provided and occasionally the cow is shepherded in a grass field. Whereas, buffalo are kept out of doors and graze on fresh grass only. Since the production process for dangke is not standardized yet, the length of time milk is heated is dependent on the producer's experience. Moreover, milk pre-treatment, either pasteurization or heating, should be conducted not more than 24 h after milk collection (Li et al. 2016). Furthermore, the nutritional value of buffalo milk is higher than cow's milk, especially casein content. The casein content allows the acidification of buffalo milk to occur relatively more slowly than with cow's milk (Yang et al. 2013). As a consequence, the growth of Gram-negative bacteria is faster than LAB growth rate, allowing family Enterobacteriaceae to dominate in dangke from buffalo milk.

2.5 Conclusion

This study focused on understanding the bacterial resources and systematic analysis of bacterial composition in naturally fermented buffalo milk, dadih and dangke by ITS-PCR RFLP, 16S rRNA gene analysis and pyrosequencing. Three predominant groups of bacteria were detected during fermentation using these methods; namely lactic acid bacteria (*Lactococcus lactis* subsp. *lactis*), *Enterobacteriaceae* (*Klebsiella* sp.) and acetic acid bacteria (*Acetobacter orientalis*).

The other lactic acid bacteria also detected were *Lactobacillus plantarum* and *Enterococcus faecium.* This bacterial community analysis indicates that the role of lactic acid bacteria is important not only in developing the product's qualities but also in improving the product's safety. The results suggest the need to study more samples from different households and geographical regions to obtain a wider range of samples of dadih and dangke which may be more representative of these products.

Chapter 3

Evaluation of Yeast Diversity in Dadih and Dangke by ITS-PCR RFLP

3.1 Introduction

Besides lactic acid bacteria (LAB), yeasts also play an essential role in naturally fermented milk products, and their diversity and important role have been the subject of intensive studies (Abdelgadir et al. 2001; Bai et al. 2010; Gadaga, Mutukumira & Narvhus 2000; Kebede et al. 2007; Lore, Mbugua & Wangoh 2005; Rahman et al. 2009; Zhang et al. 2014a; Zhang et al. 2008a; Zhang et al. 2008c). Yeasts that have been recovered from naturally fermented milk (NFM) products are vary in numbers and originally come from a wider range of sources, such as the milk, containers, equipment used and the processing environment (eg airborne contamination) (Viljoen 2001). Common yeast genera used in food industry are *Candida, Saccharomyces* and *Kluyveromyces* (Diosma et al. 2014). There are fewer yeasts in fermented milk products compared with bacterial (LAB) numbers; whereas yeasts outnumber bacteria in alcoholic fermentation products and play a primary role in the fermentation (Deak 2009). However, as secondary microbiota, the presence of yeasts in fermented milk products is inevitable, and can be both undesirable and desirable (Caplice & Fitzgerald 1999).

The occurrence of yeasts in NFM products is common and they play an essential role in determining product quality and safety. The important contribution of indigenous yeast to NFM products as noted by Narvhus & Gadaga (2003) is in development of texture and flavor. In airag, koumiss and kefir, CO_2 produced by yeasts positively affects the aroma and flavor of final products (Mu, Yang & Yuan 2012; Narvhus & Gadaga 2003). Besides stimulating the growth of starter microorganisms through production of amino acids and vitamins, enzymatic activities (lipolytic and proteolytic) of yeasts play a substantial role in the development of

desired taste, texture and flavor (Jakobsen & Narvhus 1996; Narvhus & Gadaga 2003). In terms of safety, some yeasts also produce natural killer factors that can inhibit the growth of undesired microorganisms (Jakobsen & Narvhus 1996; Viljoen 2001). Additionally, *Saccharomyces cerevisiae* was able to reduce the concentration of aflatoxin M1 in a fermented milk (Karazhiyan et al. 2016). A complex interaction between yeasts and other microbiota especially LAB bring advantages to the quality and safety of final products.

Nowadays, the study of the ecology of yeasts in NFM products is of increasing interest. Particular yeast species are used not only as starter cultures for controlled fermentation processes, but also because of their benefits to human health, such as probiotic properties (Diosma et al. 2014; Pedersen et al. 2012). However to date *Saccharomyces cerevisiae* var. *boulardii* is the only yeast probiotic that has proven clinical efficacy (Sharif et al. 2016). Therefore, interest in identification and characterization of yeasts from many NFM products is high. In order to obtain novel potential non-LAB organisms, the current work was conducted to isolate and identify the dominant yeast species contributing to fermentation of the dadih and dangke products. Yeast isolates were fingerprinted using the PCR-RFLP method and their identity confirmed using gene sequencing of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers (ITS) regions.

3.2 Methods

3.2.1 Collection of samples

Two dadih samples were collected from two rural areas of Solok Regency and Gadut (Agam Regency), respectively, in West Sumatra Province, Indonesia. To evaluate the microbial succession, samples from three consecutive days of fermentation were used for the dadih samples collected form Solok Regency. Meanwhile, dangke samples were also collected from rural areas in Enrekang Regency, Makassar Province, Indonesia. Four different household producers were sampled (identified as samples A, B, C, and D). Buffalo milk were used in dangke A, B and C; while dangke D used cow's milk. All the samples were placed in sterile 100 mL propylene tubes. The samples were kept at 4-6^{o}C and transported in a cool box within two days to Microbiology laboratory, Department of Biology, University of

Brawijaya, Indonesia. The pH of each sample was measured at the laboratory using a calibrated pH-meter (model 3205, Jenway, UK).

3.2.2 Isolation and enumeration of yeasts

Ten grams of fermented milk samples (dadih and dangke) was transferred aseptically into 90 mL saline water (0.75% w/v NaCl) and mixed thoroughly. Further tenfold serial dilution (10^{-1}-10^{-6}) was made for each sample and 0.1 mL of appropriate dilution was spread on YMA (yeast mannitol agar) acidified to pH 3.5 with 1 N HCl. As well as this medium, MRS agar pH 6.2±0.2 (Oxoid Ltd., Basingstoke, UK) and M17 agar pH 6.9±0.2 (Oxoid Ltd., Basingstoke, UK) were also used. All the plates then were incubated aerobically at 37^{o}C for 48 hours. A similar technique was applied to assess the presence of yeasts in bamboo. A fresh bamboo tube was filled with 10 ml of a sterile saline solution (0.75% w/v NaCl) and the solution mixed thoroughly using a sterile spoon and cultured on media as described above. Representative colonies were picked randomly from the plates where the number of colonies fell between 30 and 300. Duplicate plates of each dilution were prepared and the numbers of yeasts detected was calculated as an average value. All isolates were transferred to Adelaide, Australia for further characterization. After arriving, yeast colonies were checked for purity by streaking twice on YEPD agar (1% yeast extract, 1% peptone, and 2% of D-glucose). Stock cultures of the isolates were preserved in their preferred medium containing 15% glycerol (Merck, Darmstadt, Germany) and stored at -80^{o}C until further analysis.

3.2.3 Phenotypic characterization

The phenotypic characterization of yeast from YPD agar supplemented with 2% of D-glucose was conducted based on cell morphology and colony characteristics, such as shape, size, margin, elevation, surface, texture and colour. The catalase and oxidase tests (Sigma-Aldrich, St. Louis, US) were also assessed as part of biochemical characterization.

3.2.4 Genotypic characterization

A total of 37 yeast isolates were grouped into clusters based on the band pattern of RFLP analysis of the internal transcribed spacer (ITS) region. The oligonucleotide primers used in this study were ITS 1 (5’

TCCGTAGGTGAACCTGCG G 3') and ITS 4 (5' TCCTCCGCT TATTGATATGC 3') (Granchi et al. 1999). A single colony of yeast isolate was used as a DNA template by using a sterile tooth pick to sample the colony and place the material into a PCR tube. To denature the yeast cell wall, the colony in the PCR tubes was heated in a Microwave oven (Panasonic) for one minute. After this, 25 µl of reaction mixture for PCR were dispensed into the tubes. The DNA amplification was carried out using a Mycycler thermal cycle (Bio-Rad Laboratories, Hercules, CA, USA) with the following components: 5 µl of 5x PCR buffer, 1.25 µl of 50 mM $MgCl_2$, 2.5 µl of 2.5 mM dNTPs (2 mM each dATP, dCTP, dGTP and dTTP), 0.25 µl of *Taq* polymerase (5 U/µl) (Promega, Madison, Wis. USA) and 1.5 µl of a 5 µM concentration of each primer. The PCR conditions consisted of 35 cycles (denaturation step at 94^{o}C for 1 min, annealing step at 53^{o}C for 1 min, and elongation step at 72^{o}C for 1 min), initial denaturation step at 94^{o}C for 5 min and additional cycle at 72^{o}C for 5 min as a final extension.

A total volume of 10.2 µl was used for RFLP analysis which comprised 9 µl of amplicons, 1 µl of buffer (composition dependent on the enzymes), and 0.2 µl of restriction enzymes. HinfI and HaeIII enzymes were used for digesting yeasts (New England BioLabs Inc., MA, US). The mixture was incubated at the optimum temperature for the enzyme activity for 24 h. Following this incubation, 5 µl of the RFLP product added with 2 µl of loading dye were dispensed into the wells of the 1.5% (w/v) agarose gels containing 5% (v/v) of ethidium bromide (EtBr), and then electrophoresed in 1 × TAE buffer for 1 h. Gel image was visualized with the Gel Doc system (Bio-Rad Laboratories, Hercules, CA, USA). The sizes of the DNA fragments were estimated by comparing their relative mobility with 100 bp of molecular weight ladder (size range: 100-1,000 bp, New England BioLabs Inc.).

3.2.5 Molecular identification and DNA sequencing analysis

The PCR products from the targeted ITS region were purified for sequencing by using UltraClean PCR clean-up kit (Mo Bio Laboratories Inc, Solena Beach, CA, USA.) according to the protocol described by the manufacturer. The DNA sequence was determined using the Big Dye Terminator v3.1 cycle sequencing ready reactions (Applied Biosystem, Foster City, CA, USA) at the DNA Sequencing Facility, Flinders Medical Centre, Bedford Park, South Australia. The nucleotide sequences were aligned and used for the analysis of sequence similarity through Basic Local

Alignment Search Tool–BLAST (http://www.ncbi.nlm.nih.gov/blast*)* performed using the Gen-Bank database (The National Centre for Biotechnology Information-NCBI). A phylogenetic tree based on neighbor-joining with likelihood model (Tamura-Nei) was constructed using Geneious® 9.1.7 (Biomatters Ltd., New Zealand) and then visualized using FigTree v1.4.2 (Institute of Evolutionary Biology, University of Edinburgh).

3.3 Results

3.3.1 Predominant yeasts in dadih and dangke

Table 7 Enumeration of yeast from the YMA medium

Samples	Milk type	Location	pH value	Colony number (log cfu/mL or g)
Raw milk	Buffalo milk	Solok	6.92	7.35±0.24
Dadih Sianok day 1	Buffalo milk	Solok	5.02	8.08±0.00
Dadih Sianok day 2	Buffalo milk	Solok	4.65	6.85±0.05
Dadih Sianok day 3	Buffalo milk	Solok	4.65	6.99±0.42
Bamboo	-	Solok	ND*	4,19±0.42
Dadih Gadut day 3	Buffalo milk	Gadut	4.85	8.05±0.00
Dangke A	Buffalo milk	Enrekang	4.09	7.29±0.23
Dangke B	Buffalo milk	Enrekang	4.08	7.14±0.47
Dangke C	Buffalo milk	Enrekang	4.26	3.75±0.05
Dangke D	Cow's milk	Enrekang	5.14	7.18±0.29

The viable counts of the yeast from raw buffalo milk, dadih and dangke products, and their pH values are shown in Table 7. The initial count of yeast for the raw buffalo milk sample was 7.35±0.24 log cfu/mL, and then the yeast count gradually increased after 24 h of fermentation (8.08±0.00 24 log cfu/g). A decreased yeast count was observed at day 2 (6.85±0.05 log cfu/g), and slightly increased again at day 3 (6.99±0.42 log cfu/g). Decreasing pH value was also observed from 6.92 (raw buffalo milk) to 4.65 (dadih Sianok day 2 and 3). The viable yeast count of dadih from Gadut (day 3 of fermentation) was higher (8.05±0.00 log cfu/g) than dadih from

Sianok (6.99±0.42 log cfu/g), while the pH value was relatively similar (4.65 and 4.85, respectively).

The viable yeast counts of dangke A, B and D was relatively similar, which were 7.29±0.23 log cfu/g, 7.14±0.47 log cfu/g and 7.18±0.29 log cfu/g, respectively, with pH values ranged from 4.08-5.14. The viable count of dangke C was the lowest number (3.75±0.05 log cfu/g) and a pH value of 4.26.

In the first stage of yeast identification, phenotypic characterization (morphological observation, cell-shape and biochemical tests) was similar for all yeast isolates as shown in Appendix 2. All the isolates appeared to be ovoid-shaped, catalase-positive and oxidase-positive.

3.3.2 Molecular characterization and identification

Table 8 Grouping of yeasts based on ITS-PCR RFLP and identification by the 5.8S rRNA gene and ITS region gene sequencing

Groups	Number of isolates		ITS-PCR (bp)	RFLP		Similarity (%)	Identification (gene sequencing)	
	Dadih	Dangke		HinfI	HaeIII		Closely similar to	Accession Number
I	5	13	800	50, 110, 310	150, 180, 230, 320	90	*Saccharomyces cerevisiae* isolate KDLYH4-1	KF710035
II	10	0	500	80, 260	300, 500	100	*Candida metapsilosis* CBS 2916	KY102207
III	2	9	750	80, 120, 185, 240	80, 600	100	*Kluyveromyces marxianus* CBS 1557	KY103803
Total	17	22						

A total of 39 yeast strains (17 strains from dadih and 22 strains from dangke) were characterized and identified using PCR amplification of the 5.8S rRNA gene and ITS region (ITS1 and ITS2) combined with RFLP and gene sequencing. The PCR products of all yeast strains showed a high length variation in this region for the different groups: 800 bp for group I, 500 bp for group II, and 750 bp for group III (Table 8). The restriction analysis results were consistent with cellular and colony appearance, which differentiated the yeast isolates into three groups as well (Table 8, Fig. 13A). The restriction enzymes *Hinf*I (Fig. 14) and *Hae*III (Fig. 15) successfully digested the amplicons and confirmed the presence of three different groups (species) indicates that the use of *Hae*III and *Hinf*I to digest the ITS-PCR products could provide a reproducible result. Based on DNA sequencing analysis, the three species were identified as *Saccharomyces cerevisiae, Candida metapsilosis* and *Kluyveromyces marxianus*. However, nucleotide homology of group I in the GenBank database was only 90% of similarity level, which was close to *Saccharomyces*

cerevisiae KDLYH4-1 (accession number: KF710035). In this case, yeast isolated from baker's yeast was used as a reference strain. Both amplicon and RFLP fragments (digested using HaeIII) showed a similar size and profiles (Fig. 13B) confirming that they are corresponded to each other. A phylogenetic tree was also constructed to establish the genetic relationship between the representative yeasts and the type strains or submitted strains on GenBank database (Fig. 16).

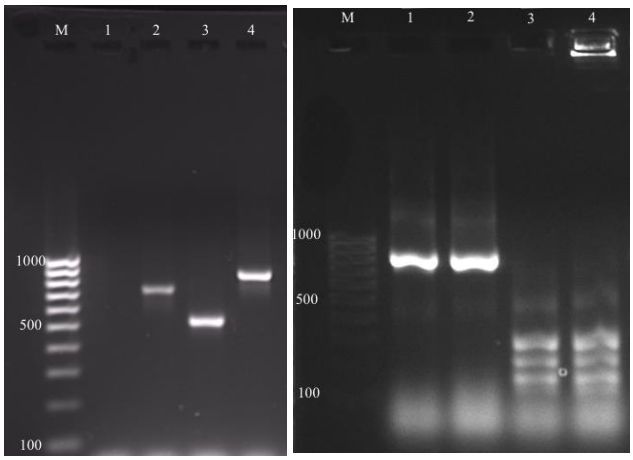

Figure 13 PCR amplification of the 5.8S rRNA gene and ITS region of selected yeasts (A) & Confirmation of ITS-PCR RFLP analysis between *Saccharomyces cerevisiae* SL1.1 and *Saccharomyces cerevisiae* isolated from baker's yeast (B).

A) Lane M: 100 bp DNA marker; lane 1: H_2O; lane 2: *Kluyveromyces marxianus* SL2.13; lane 3: *Candida metapsilosis* B.1; lane 4: *Saccharomyces cerevisiae* SL1.1; B) lane 1 and 3: *Saccharomyces cerevisiae* SL1.1; lane 2 and 4: *Saccharomyces cerevisiae* (baker's yeast).

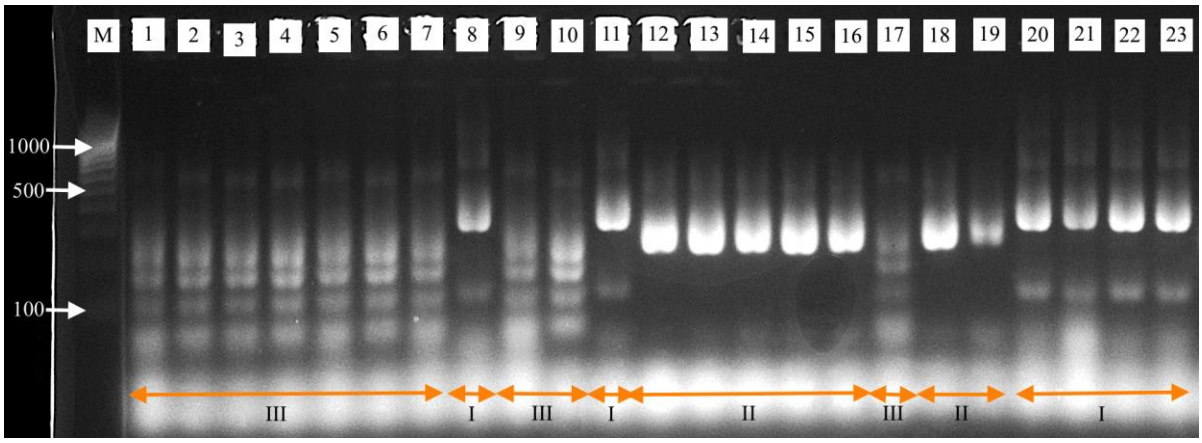

Figure 14 Gel electrophoresis of PCR-amplified 5.8S rRNA gene and ITS region digested with Hinf1

Lane M: 100 bp DNA marker; lane 1: *Kluyveromyces marxianus* D2.26; lane 2: *Kluyveromyces marxianus* D2.24; lane 3: *Kluyveromyces marxianus* D2.23; lane 4: *Kluyveromyces marxianus* D2.22;

lane 5: *Kluyveromyces marxianus* D2.21; lane 6: *Kluyveromyces marxianus* DC.16; lane 7: *Kluyveromyces marxianus* DC.15; lane 8: *Saccharomyces cerevisiae* DC.14; lane 9: *Kluyveromyces marxianus* DC.12; lane 10: *Kluyveromyces marxianus* DC.11; lane 11: *Saccharomyces cerevisiae* DC.10; lane 12: *Candida metapsilosis* GD.21; lane 13: *Candida metapsilosis* GD.20; lane 14: *Candida metapsilosis* GD.19; lane 15: *Candida metapsilosis* GD.18; lane 16: *Candida metapsilosis* GD.16; lane 17: *Kluyveromyces marxianus* SL2.13; lane 18: *Candida metapsilosis* SL0.14; lane 19: *Candida metapsilosis* SL0.12; lane 20: *Saccharomyces cerevisiae* DC.13; lane 21: *Saccharomyces cerevisiae* DC.8; lane 22: *Saccharomyces cerevisiae* DC.7; lane 23: *Saccharomyces cerevisiae* D2.2.

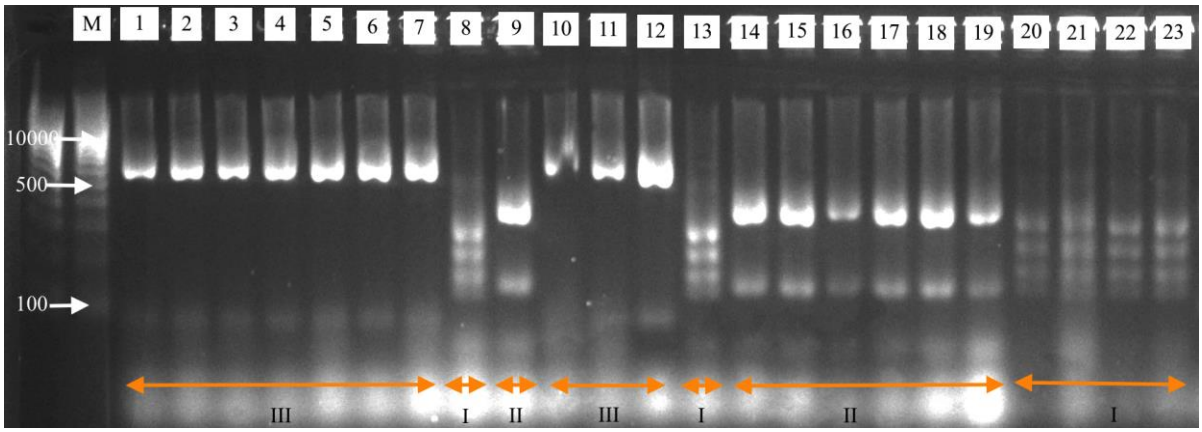

Figure 15 Gel electrophoresis of PCR-amplified 5.8S rRNA gene and ITS region digested with HaeIII

Lane M: 100 bp DNA marker; lane 1: *Kluyveromyces marxianus* B.8; lane 2: *Kluyveromyces marxianus* D2.24; lane 3: *Kluyveromyces marxianus* D2.23; lane 4: *Kluyveromyces marxianus* D2.22; lane 5: *Kluyveromyces marxianus* D2.21; lane 6: *Kluyveromyces marxianus* DC.16; lane 7: *Kluyveromyces marxianus* DC.15; lane 8: *Saccharomyces cerevisiae* DC.14; lane 9: *Candida metapsilosis* GD.15; lane 10: *Kluyveromyces marxianus* SL2.13; lane 11: *Kluyveromyces marxianus* DC.12; lane 12: *Kluyveromyces marxianus* DC.11; lane 13: *Saccharomyces cerevisiae* DC.10; lane 14: *Candida metapsilosis* GD.21; lane 15: *Candida metapsilosis* GD.20; lane 16: *Candida metapsilosis* GD.19; lane 17: *Candida metapsilosis* GD.18; lane 18: *Candida metapsilosis* SL0.14; lane 19: *Candida metapsilosis* SL0.12; lane 20: *Saccharomyces cerevisiae* DC.13; lane 21: *Saccharomyces cerevisiae* DC.8; lane 22: *Saccharomyces cerevisiae* DC.7; lane 23: *Saccharomyces cerevisiae* D2.2.

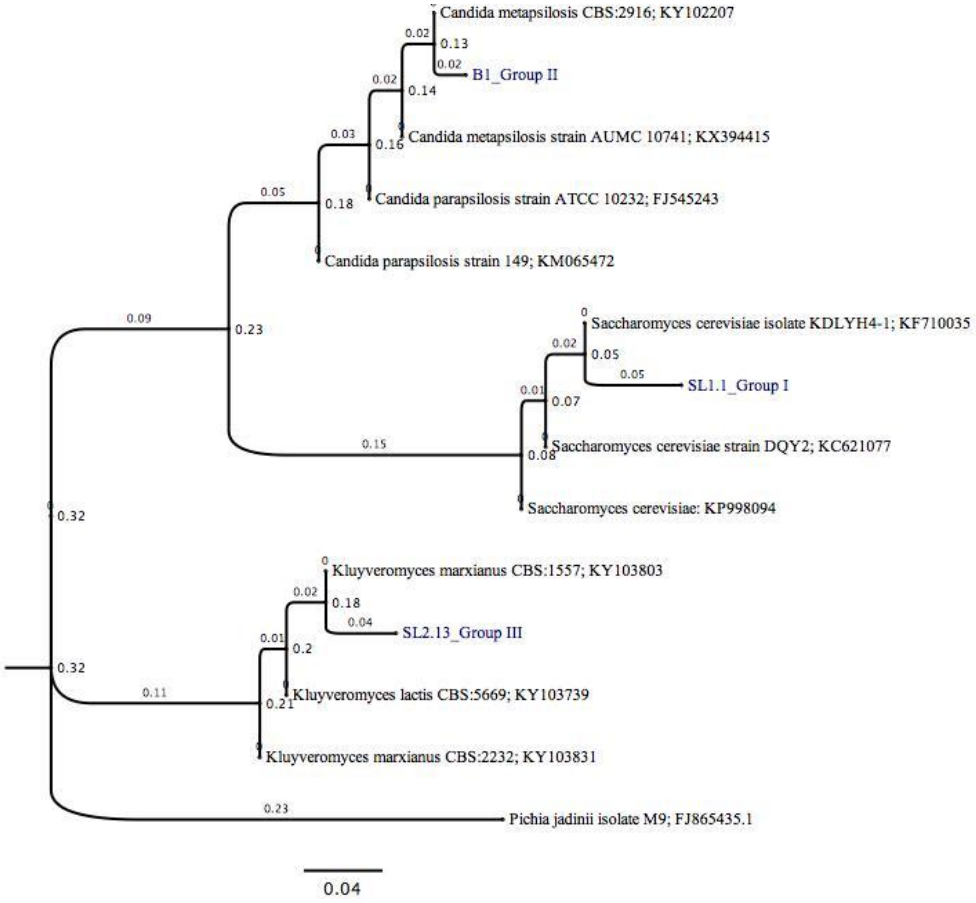

Figure 16 Neighbor-joining tree showing the phylogenetic relationships among yeast isolates from dadih and dangke samples and the type isolates based on ITS1, 5.8S rRNA gene and ITS2 sequences. *Pichia* (*P.*) *jadinii* was used as an outgroup

Saccharomyces cerevisiae and *Kluyveromyces marxianus* were found in both dadih and dangke samples. Unfortunately, yeasts were not detected in dangke samples A and C. *Saccharomyces cerevisiae* was the dominant strain in dangke, while *Candida metapsilosis* was found frequently in dadih (Appendix 6). Interestingly, the identity of yeasts (two isolates) in the inner part of the bamboo was found to be *C. metapsilosis* B.1 and *K. marxianus* B.8 (Appendix 5).

3.4 Discussion

Naturally fermented milk is a unique ecological niche in which only well-adapted species are able to grow and interact synergistically with each other. In Chapter 2, mesophilic LAB and non-LAB in dadih and dangke have been evaluated. In this present study, the structure and dynamics of yeasts involving in these products was also investigated using a culture-dependent approach. Concerning yeast enumeration, some literature reports that the number of yeasts in some NFM products usually ranges from 2 to 7 log cfu/mL or g (Akabanda et al. 2013; Ghiamati et al. 2016; Lore, Mbugua & Wangoh 2005; Mathara et al. 2004), substantially in line with the data obtained from this present study. However, viable count for yeasts in dadih from Gadut and dadih Sianok day 1 reached 8 log cfu/g. The reason was the abundance of mesophilic LAB (genus *Lactococcus*) at this period was lower as seen from the pyrosequencing result. The data obtained at present study was in agreement with previous study (Jatmiko, Barton & de Barros Lopes 2010), in which microbiota in dadih from Gadut was dominated by yeasts instead of LAB. This decrease was affected by the increase of LAB counts due to lactic acid production (Gul et al. 2015).

The most predominant yeast in dadih was *C. metapsilosis,* while *S. cerevisiae* predominated in dangke followed by *K. marxianus*. Meanwhile, *C. metapsilosis* was only present in dadih. The occurrence of yeasts in dadih has been reported before with different species, namely *Candida stelimalicola* and *Pichia jadinii* (Jatmiko, de Barros Lopes & Barton 2012). Yeasts in dangke have also been reported, namely *Candida* sp., *Saccharomyces* sp., *Geotrichum* sp. and *Rhodotorula* sp., with *Candida* sp. as the dominant species (Syah 2012). *Saccharomyces cerevisiae* is the most frequently reported yeast in a range of NFM products, such as fermented goat milk from Tajikistan (Qvirist et al. 2016), fermented milk matsoni from Georgia and Armenia (Bokulich et al. 2015), Tarak from Korea (Jung et al. 2015), Tibetan kefir grains (Lu et al. 2014), Sameel milk from Saudi Arabia (Al-Otaibi 2012), NFM from Tibetan Plateau of China (Bai et al. 2010), Nunu from Ghana (Akabanda et al. 2013), Amabere amaruranu from Kenya (Nyambane et al. 2014), yak milk dreg from Tibet (Yang et al. 2014) and Shubat from Kazakhstan (Akhmetsadykova et al. 2013). *Kluyveromyces marxianus* was also identified in some of those NFM products, apart from Tarak, Nunu, Sameel milk, amabere amaruranu and fermented milk products from Tibetan Plateau of China. Moreover, this yeast species was also reported in

Koumiss (Mu, Yang & Yuan 2012). Interestingly, *C. metapsilosis* has not been reported in any NFM products before, thus this is first record from NFM products.

In relation to RFLP analysis of the 5.8S-ITS region, the RFLP profile of *K. marxianus* was consistent with previous research (Qvirist et al. 2016; Rai et al. 2016). The RFLP profile of *S. cerevisiae* in this present study compared with those reported in previous studies exhibited a slightly different pattern (Esteve-Zarzoso et al. 1999; Qvirist et al. 2016). Digestion of HaeIII produced identical pattern, but HinfI gave a completely different pattern. As a result, the strain of *S. cerevisiae* in this study was considered to be different from the strains covered in the database. However, this strain displayed similar RFLP pattern with Baker's yeast as shown Fig. 1B, although only one restriction enzyme used (HaeIII). In contrast, the database for RFLP profiles of the 5.8S-ITS region for *C. metapsilosis* has been not documented so far. The available RFLP profile targeted a different region, the D1/D2 domain (Tantirungkij, Nasanit & Limtong 2015). These authors also reported that *C. metapsilosis* grouped as endophytic yeast. This report was in agreement with the result of present study, in which *C. metapsilosis* and *K. marxianus* also found in bamboo tubes.

3.5 Conclusion

Three yeast species were obtained from dadih and dangke samples, namely *S. cerevisiae, C. metapsilosis* and *K. marxianus*, with *C. metapsilosis* as the predominant yeast in dadih and *S. cerevisiae* in dangke. Although the viable count of yeasts can be determined from isolation medium, the yeast isolates derived from this study may not be fully representative of the full yeast flora of dadih and dangke. The total picture of the microbes involved in the fermentation processes of these products is still unclear. Further investigations are needed to shed light on microbial dynamics, and diversity analysis of more dadih and dangke samples. This would contribute to a better understanding of the fermentation process since the changes in the abundance and type of microbiota during the fermentation process play a pivotal role in the quality of the final products.

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Chapter 4

Screening and Characterization of Lactic Acid Bacteria Isolated from Dadih and Dangke for Potential Use as Probiotic

4.1 Introduction

Increased awareness of consumers towards health promoting foods containing probiotic microorganisms (functional foods) has resulted in increasing popularity of fermented food products (Fadda et al. 2017; Liu & Han 2015). Nowadays, probiotics have been developed in many different product forms. Apart from pharmaceutical products containing both single- or multi-strains, probiotic organisms are added into non-dairy products as such as cereal based, fruit and vegetables based and soy based foods, and other products including meat and fish (Kumar, Vijayendra & Reddy 2015; Shori 2016). As a result, the affordability of probiotic fortified products is becoming a concern in some developing countries (Sybesma, Kort & Lee 2015). Therefore, traditional fermented milk products mainly produced in rural areas are offering potential affordable functional products with probiotic attributes as well as a source of income generation to the local people (Sybesma, Kort & Lee 2015).

Common probiotic vehicles are still yogurt and fermented milk products, as the acid environment provides favourable conditions for the survival of the probiotics (Liu & Han 2015; Lourens-Hattingh & Viljoen 2001). Traditional fermented milk products are considered to be good probiotic carriers for maintaining the probiotics' viability and functionality (Kandylis et al. 2016). Additionally, locally produced well-adapted probiotics are better suited to the local people (Sybesma, Kort & Lee 2015). Exploration of interesting strains with probiotic properties from fermented milk products is increasing (Angmo et al. 2016). Many naturally fermented milk products from developing countries offer a rich source of potential probiotics such as

Lactobacillus casei Zhang from koumiss (Zhang et al. 2010a), *Lactobacillus casei* LC2W from a NFM product of Inner Mongolia (Guo, Wu & Ye 2009), *Lactobacillus acidophilus* LaVK2 and *Bifidobacterium bifidum* BbVK3 from dahi (Shandilya et al. 2016) and *Lactobacillus plantarum* from kule naoto (Mathara et al. 2008b).

Dadih and dangke are categorized as naturally fermented milk products from Indonesia. Dadih contains a large number of unique probiotic microorganisms; mainly belonging to the genera *Lactobacillus* and *Enterococcus* (Collado et al. 2007b; Surono 2003; Surono et al. 2011; Surono et al. 2009). To our knowledge, limited information is available on probiotics from dangke. By using biochemical tests and partial probiotic characterization (low pH tolerance), *Lactobacillus fermentum* and *Lactobacillus plantarum* isolated from dangke exhibited incomplete probiotic properties (Nur, Hafsan & Wahdiniar 2015). Hence, in present study, to examine the probiotic properties of indigenous isolated LAB from dadih and dangke, some *in vitro* tests were implemented, including acid–bile salt tolerance; their capability to adhere to intestinal cell line Caco-2 cells; inhibition of pathogenic bacteria; and sensitivity to antibiotics. In addition to these assays, the detection of bile salt hydrolase and mannose-specific adhesin genes in their genome as probiotic marker genes was also performed.

4.2 Methods

4.2.1 Screening of acid- and bile-resistant strains and determination of acid tolerance

The representative of LAB strains (20 strains of *L. plantarum*, 29 strains of *L. lactis* subsp. *lactis* and one strain each of *E. faecium* and *L. plantarum* S1.30 were further examined in acid and bile resistance tests. *Lactobacillus plantarum* S1.30 was included in this probiotic screening, because this dadih strain exhibited a potent antimicrobial activity and has a bacteriocin precursor gene in its genome (Jatmiko, Barton & de Barros Lopes 2010). The pH tolerance test was conducted in 96 round-bottom microwell plates (Nunclon, Roskilde, Denmark) according to du Toit et al. (1998), with slight modifications. Medium (MRS broth) was adjusted to pH values 2, 3, 4, 5 and 6, respectively, using 1 N HCl (Merck, Germany) or 1 N NaOH (Merck). 180 µl of MRS broth with adjusted pH was inoculated with 20 µl of the LAB cultures

into microwell plates. The plates were incubated at 37°C in an EnSpire Alpha Plate Reader 2300-001A (Perkin Elmer) and shaken for 3 min before the absorbance was determined. The absorbance value (620 nm) against incubation time was plotted, and the acid tolerance of each selected LAB was calculated based on the time required for the absorbance value to increase by 0.3 units. Growth in MRS without pH change (initial pH, 6.2) served as negative control.

The bile tolerance of LAB was conducted essentially as for the acid tolerance. The test media used were MRS broth and MRS broth supplemented with three bile concentrations (0,3, 0,5 and 1,0% of oxgall, w/v) (Sigma-Aldrich, St. Louis, MO, USA). The bile tolerance of each selected LAB was calculated based on the time required for the absorbance value to increase by 0.3 units (Ramasamy et al. 2012). Growth in MRS broth without bile served as negative control. All experiments were conducted in duplicate.

4.2.2 Molecular detection of *msa* and *bsh* genes

The *L. plantarum* strains identified on the basis of species-specific primers for *L. plantarum* group were tested by PCR to detect the presence of *bsh* and *msa* genes encoding for the bile salts hydrolase (*bsh*) and the mannose-specific adhesin (*msa*). Oligonucleotide primers and its PCR conditions were chosen according to Zago et al. (2011). Forward and reverse primers sequences for the detection of bsh gene were 5'-CGTATCCAAGTGCTCATGGTTTAA-3' and 5'-ATGTGTACTGCCATAACTTATCAATCTT-3', respectively. Forward and reverse primer sequences for the detection of msa gene were 5'-GCTATTATGGGGATTACGTTG-3' and 5'-CTGTCTTGACAATAGCCATATA-3', respectively. The expected PCR products lengths were of 919 bp (for *bsh*) and 1,740 bp (for *msa*). PCR amplifications were performed in 25 µl volumes in a Mycycler thermal cycle (Bio-Rad Laboratories, Hercules, CA, USA) and were set as follows: pre-denaturation for 4 min at 94°C; followed by 30 cycles; denaturation for 30 s at 94°C, annealing for 30 s at 64°C (*bsh*) or 52°C (*msa*), and extension for 1 min at 72°C. The final extension of 7 min at 72°C was added. The PCR products were separated on a 1.5 % agarose gel in Tris-Acetate EDTA buffer (TAE: 40 mM Tris-Acetate, 1 mM EDTA, pH 8.0), stained with ethidium bromide, and visualized under

UV light using the Gel Doc system (Bio-Rad Laboratories, Hercules, CA, USA).

4.2.3 Antimicrobial activity and bacteriocin detection

The well-diffusion assay method was performed according to Schillinger & Lucke (1989) with a slight modification. The LAB cultures were prepared as follows: The LAB isolates from frozen stocks were subcultured into 15 ml of MRS broth and incubated at 37^{o}C under 5% CO_2 atmosphere. After an overnight incubation, 10% (v/v) of the cultures were transferred into 15 ml of fresh MRS broth and incubated with similar condition for 24 h.

At the same time, the indicator bacteria (Gram-positive and Gram-negative pathogenic bacteria) (Table 9) were also prepared in the same way, except for the incubation conditions, which was under aerobic conditions (without 5% CO_2). 100-µl of the indicator bacteria was added to 20 ml of semi solid TSA (1%, w/v) at about 45^{o}C and mixed gently. Subsequently, the semi solid suspensions seeded with the indicator bacteria were poured into sterile Petri dishes. After solidification, wells (6 mm in diameter) were made in the lawn of the hardened agar using a sterile metal cork borer. Aliquots of the overnight test bacteria (80 µl) were dispensed into the wells. The culture suspensions in the well were allowed to diffuse into the agar for 24 hours at -4^{o}C. Afterward, the plates were incubated at the optimum temperature of growth of the indicator bacteria. A clear inhibition zone resulted if the LAB cultures produced antimicrobial substances. The diameter of clear zones was measured including the diameter of the wells. This experiment was conducted in duplicate.

The detection of bacteriocins was conducted using the well-diffusion assay as explained by Zhou et al (2014) with a minor modification. In this assay, a cell-free supernatant (CFS) of putative bacteriocin-producing LAB was used. The CFS was obtained by centrifuging the overnight cultures (15 ml) in a Sigma 3-16K centrifuge at 5,444 x g for 20 minutes at 4^{o}C. Afterwards, the supernatant was adjusted to pH 6.5 with NaOH (5 M) to exclude the organic acid activity. Next, the neutralized-CFS (NCFS) was filtered through a filter membrane (pore size 0.22 µm) (PES membrane, Millex® GP, Millipore, Carrigtwohill Co, Cork, Ireland). This sterile neutralized supernatant (80 µl) was dispensed into the wells (6 mm in diameter) on agar plates seeded with the indicator bacteria (*Lactobacillus sakei* ATCC 15521 and *Listeria*

monocytogenes). After incubation at 37ºC for 24 h, the diameters of clear zones were measured indicating the result of bacteriocin-like inhibitory substance activity.

A PCR-based method was used to detect *pln*A, a gene associated with bacteriocin activity using specific primers (Remiger, Ehrmann & Vogel 1996). The LAB strains used were those showing the greatest activity in inhibiting the growth of both Gram-positive and Gram-negative bacteria in agar well-diffusion test even though they were negative for bacteriocin production in the cell-free supernatant tests. The PCR products were separated on a 1.5% agarose gel in Tris-Acetate EDTA buffer (TAE: 40 mM Tris-Acetate, 1 mM EDTA, pH 8.0), stained with ethidium bromide, and visualized under UV light using the Gel Doc system (Bio-Rad Laboratories, Hercules, CA, USA).

Table 9 Bacterial strains used in this study as indicator bacteria

Strains	**Collection code**	**Characteristic**	**Source**
Salmonella typhimurium P135	3-1 (99-5503917)	Gram negative - multiple antibiotic resistance	University of South Australia
Salmonella sofia	1-31 (99-5503892)	Gram negative - multiple antibiotic resistance	University of South Australia
Escherichia coli	99363-1	Gram negative - multiple antibiotic resistance	University of South Australia
Escherichia coli	99386-3	Gram negative - multiple antibiotic resistance	University of South Australia
Bacillus cereus	Strain 33	Gram positive pathogen	University of South Australia
Enterococcus faecalis ATCC 29212	-	Gram positive pathogen	University of South Australia
Listeria monocytogenes	Strain 1	Gram positive pathogen	University of South Australia
Staphylococcus aureus ATCC 25923	-	Gram positive pathogen	University of South Australia
Streptococcus pyogenes ATCC 10389	-	Gram positive pathogen	University of South Australia

Lactobacillus sakei ATCC 15521	-	Bacteriocin sensitive indicator (non-bacteriocin producer)	American Type Culture Collection

4.2.4 Antibiotic resistance

Antibiotic resistance was first screened by the agar disk diffusion method to determine resistance profiles against seven antibiotics (ampicillin, clindamycin, streptomycin, vancomycin, chloramphenicol, erythromycin, and tetracycline) belonging to the clinically most relevant antibiotic classes. Bacterial suspensions with a turbidity equivalent to McFarland Standard 1 (approx 3.0 x 10^8 cfu/mL) were swabbed onto Lactic acid bacteria Susceptibility test Medium (LSM) agar plates with a sterile cotton swab (Klare et al. 2005). Antibiotic disks containing 10 µg ampicillin, 2 µg clindamycin, 300 µg streptomycin, 15 µg erythromycin, 30 µg vancomycin, 30 µg chloramphenicol and 30 µg tetracycline (Oxoid) were placed on the plates. Inhibition Diameter Zones (IDZ), including the diameter of the 6 mm disks, were measured after 24 h of incubation at 37°C under 5% CO_2 atmosphere. Strains were recorded as resistant, moderately susceptible or susceptible based on IDZ according to Charteris et al. (1998a).

4.2.5 Adhesion to Caco-2 cells

The method described by Minelli et al (2004) was followed in this study with a slight modification. Caco-2 cells (2 x 10^5 cells) were grown in 24 flat bottom well plates (2 cm^2 per well) with Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) containing 1% (v/v) non-essential amino acids, 10 mmol/L sodium bicarbonate, 25 mmol/L 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 100 U/mL penicillin/streptomycin, and 20% (v/v) fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MO, USA) incubated at 37°C in a humidified atmosphere containing 5%, v/v, CO_2. The culture medium (1 mL) was changed every two days. Caco-2 cells were used after 19 days of growth, when fully differentiated. On the day of the experiment the cells were washed three times with DMEM without antibiotics.

Aliquots (5 ml) of overnight LAB strains were pelleted, washed (twice with Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, St. Louis, MO, USA), resuspended in DPBS and adjusted to an OD_{600nm} of 1 corresponds to ~1 x 10^8

cfu/mL prior to adding (1 mL) into each well containing a Caco-2 monolayer. Plates were incubated for 1 h at 37°C under 5% CO_2 atmosphere and washed three times with DPBS (1 mL /well) to remove unbound bacteria. Adhered LAB cells were then detached using 1 ml of DPBS containing 1% (v/v) Triton X-100 solution for 15 min with agitation. The bacterial cell suspensions were diluted in DPBS and plated onto MRS agar to enumerate adhered bacteria.

4.2.6 Statistical analysis

All data are expressed as mean values ± standard error of the mean (SEM). Statistical comparisons were made by one-way ANOVA with Tukey's post hoc test using SPSS version 22.0 for Windows (SPSS Inc. Chicago, IL). For all analyses, $P < 0.05$ was considered statistically significant.

4.3 Results

4.3.1 Screening of acid- and bile-resistant strains and determination of acid tolerance

The effect of acid conditions on the viability of *L. plantarum* (20 strains) is shown in Table 10. The growth rate for all strains in acidic MRS broth was slower than in MRS broth with pH close to neutral. Strains of *L. plantarum* fell into two groups based on their tolerance to low pH: slightly acidic media (pH 5-6) and acidic media (pH 2-4). Of the 20 strains, 35% of *L. plantarum* strains tolerated slightly acidic media; while only 5% of strains showed higher tolerance to more acidic media, namely *L. plantarum* SL2.7. The delay time for this strain was -18 min, which means the time required for this culture to increase the absorbance by 0.3 units at 620 nm was faster than the control medium. The remaining strains were not tolerant to pH 2-4, shown by their relatively slow growth (>18 h) for increasing the absorbance by 0.3.

Table 10 Acid tolerance of *Lactobacillus plantarum* in MRS broth (pH 2–6.2) measured by the time taken to reach absorbance 0.3 units at 620 nm

Strain	Time* (h) MRS pH 2.0	d^t (min)	Time* (h) MRS pH 3.0	d^t (min)	Time* (h) MRS pH 4.0	d^t (min)	Time* (h) MRS pH 5.0	d^t (min)	Time* (h) MRS pH 6.0	d^t (min)	Time* (h) control MRS pH 6.2
S1.30	>18	n.a	>18	n.a	>18	n.a	**6.1**	66	5.2	12	5.2
GD.1	>18	n.a	>18	n.a	>18	n.a	**7.6**	144	4.8	-18	5.3
SL2.2	>18	n.a	>18	n.a	>18	n.a	**16.7**	678	5.7	18	5.4
SL0.17	>18	n.a	>18	n.a	>18	n.a	>18	n.a	7.6	78	6.3
SL2.10	>18	n.a	>18	n.a	>18	n.a	>18	n.a	8.0	60	7.0
SL3.4	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18
GD.3	>18	n.a	>18	n.a	>18	n.a	>18	n.a	7.4	36	6.8
SL2.7	**>18**	**n.a**	**8.0**	**-18**	**21.2**	**774**	**8.8**	**30**	**7.9**	**-24**	**8.3**
SL3.1	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18
GD.2	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18
GD.4	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18
GD.12	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18
SL3.2	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18
SL3.5	>18	n.a	>18	n.a	>18	n.a	**4.6**	36	4.0	0	4.0
SL3.3	>18	n.a	>18	n.a	>18	n.a	**9.4**	36	8.3	-30	8.8
SL3.7	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18
SL3.6	>18	n.a	>18	n.a	>18	n.a	**5.3**	132	3.3	6	3.2
SL3.8	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18
GD.5	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18
GD.6	>18	n.a	>18	n.a	>18	n.a	**10.3**	-18	9.8	-42	10.6

*Time (h) required to increase absorbance by 0.3 units at 620 nm in each medium.
[t] Delay of growth between the control culture and low pH culture in minutes.
n.a: not available

On the other hand, different results were shown when the 20 strains of *L. plantarum* were cultivated in MRS broth supplemented with oxgall (0.3%, 0.5% and 1%). Although supplementation of bile (oxgall) in the medium also inhibited the growth rate of the tested strains, the number of tolerant strains was higher than when they were grown in acidic condition. The higher the concentration of the oxgall, the longer the delay observed. Among the tolerant strains (S1.30, GD.1, SL2.7, SL3.5 and

SL3.6), *Lactobacillus plantarum* S1.30 and *L. plantarum* GD.1 were the most tolerant strains against 1% of oxgall, which had the delay time of 210 min. When considering the other oxgall concentration (0.3% and 0.5%), *L. plantarum* S1.30 exhibited a lower delay time (lag phase) compared to other tolerant strains.

Table 11 Bile tolerance of *Lactobacillus plantarum* in MRS broth measured by the time taken to reach absorbance of 0.3 units at 620 nm

Strain	Time* (h) MRS bile 0.3	d^t (min)	Time* (h) MRS bile 0.5	d^t (min)	Time* (h) MRS bile 1.0	d^t (min)	Time* (h) control MRS pH 6.2
S1.30	**8.2**	**180**	**8.0**	**168**	**8.7**	**210**	**5.2**
GD.1	**10**	**288**	**11**	**342**	**8.7**	**210**	**5.3**
SL2.2	>18	n.a	3.5	-114	>18	n.a	5.4
SL0.17	>18	n.a	9.5	198	>18	n.a	6.3
SL2.10	>18	n.a	>18	n.a	>18	n.a	7.0
SL3.4	>18	n.a	>18	n.a	>18	n.a	>18
GD.3	>18	n.a	>18	n.a	>18	n.a	6.8
SL2.7	**11.6**	**198**	**13.7**	**324**	**20.8**	**762**	**8.3**
SL3.1	>18	n.a	>18	n.a	>18	n.a	>18
GD.2	>18	n.a	>18	n.a	>18	n.a	>18
GD.4	>18	n.a	>18	n.a	>18	n.a	>18
GD.12	>18	n.a	>18	n.a	>18	n.a	>18
SL3.2	>18	n.a	>18	n.a	>18	n.a	>18
SL3.5	**5.9**	**114**	**5.9**	**114**	**8.1**	**246**	**4.0**
SL3.3	>18	n.a	>18	n.a	>18	n.a	8.8
SL3.7	>18	n.a	>18	n.a	>18	n.a	>18
SL3.6	**5.1**	**114**	**5.1**	**114**	**6.9**	**228**	**3.2**
SL3.8	>18	n.a	>18	n.a	>18	n.a	>18
GD.5	>18	n.a	>18	n.a	>18	n.a	>18
GD.6	>18	n.a	>18	n.a	>18	n.a	10.6

*Time (h) required to increase absorbance by 0.3 units at 620 nm in each medium.
[t] Delay of growth between the control culture and low pH culture in minutes.
n.a: not available

To confirm whether these five strains of *L. plantarum* tolerant to 1% of oxgall can survive in MRS broth at pH 2, the selected strains were grown in MRS broth at

pH 2 for 3 h and then the survival rate (%) was calculated by dividing the number of viable cell before and after incubation (Table 12). The highest survival rate was *L. plantarum* SL2.7 with the survival rate of 94.9%.

Table 12 Survival rates of five strains of *L. plantarum* in MRS broth at pH 2 (n=2, x±SEM)

Strains	Tolerance to MRS broth at pH 2 (log cfu/mL)		Survival rate (%)
	0 h	3 h	
S1.30	8.42±0.01	6.38±0.10	75.7
GD.1	8.28±0.00	7.12±0.12	85.9
SL2.7	8.28±0.01	7.85±0.10	94.9
SL3.5	8.39±0.02	7.78±0.18	92.7
SL3.6	8.31±0.01	7.61±0.10	91.5

By measuring the delay time of 29 strains of *L. lactis* subsp. *lactis* and one strain of *E. faecium* in M17 medium at pH ranges from 2-6.2, a number of strains of *L. lactis* subsp. *lactis* (SL3.34, SL1.9, D1.1, SL0.15, DC.6, SL1.18 and SL3.27) showed a reduced delay time when pH increased from 5 to 6.2 (Table 13). These strains were sensitive to low pH (below pH 5). After these selected strains were grown in M17 broth at pH 2 for 3 h, only two strains of *L. lactis* subsp. *lactis* (SL3.34 and SL3.27) were able to survive in the low pH, with survival rates of 66.3% and 51.3%, respectively (Table 14). This indicates that *L. lactis* subsp. *lactis* SL3.34 demonstrated a better tolerance to low pH than other strains.

Table 13 Acid tolerance of *Lactococcus lactis* subsp. *lactis* and *Enterococcus faecium* in M17 broth (pH 2–6.9) measured by the time taken to reach absorbance 0.3 units at 620 nm

Strain	Time* (h) pH 2.0	d^t (min)	Time* (h) pH 3.0	d^t (min)	Time* (h) pH 4.0	d^t (min)	Time* (h) pH 5.0	d^t (min)	Time* (h) pH 6.0	d^t (min)	Time* (h) control pH 6.2
SL3.34	**>18**	**n.a**	**>18**	**n.a**	**>18**	**n.a**	**7.8**	**198**	**3**	**48**	**4.5**
SL1.9	>18	n.a	>18	n.a	>18	n.a	>18	n.a	7.6	78	6.3
D2.15	>18	n.a	>18	n.a	>18	n.a	>18	n.a	7.5	42	6.8
SL0.3	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18	n.a	9.2
SL1.9	>18	n.a	>18	n.a	>18	n.a	14	612	6.0	132	3.8
SL3.32	>18	n.a	>18	n.a	>18	n.a	2.8	42	2.2	0	2.2
D1.1	**>18**	**n.a**	**>18**	**n.a**	**>18**	**n.a**	**12.2**	**480**	**4.5**	**18**	**4.2**
SL1.16	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18
SL0.11	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18
D2.17	>18	n.a	>18	n.a	>18	n.a	>18	n.a	5.7	138	3.3
SL0.15	**>18**	**n.a**	**>18**	**n.a**	**>18**	**n.a**	**14.2**	**570**	**6.5**	**108**	**4.7**
D1.9	>18	n.a	>18	n.a	>18	n.a	>18	n.a	3.6	48	2.8
SL2.15	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18	n.a	13.1
SL3.16	>18	n.a	>18	n.a	>18	n.a	>18	n.a	6.7	108	4.8
DC.6	**>18**	**n.a**	**>18**	**n.a**	**>18**	**n.a**	**6.7**	**282**	**2.5**	**24**	**2.1**
D3.6	>18	n.a	>18	n.a	>18	n.a	>18	n.a	2.7	36	2.1
SL3.19	>18	n.a	>18	n.a	>18	n.a	>18	n.a	2.7	24	2.3
D1.2	>18	n.a	>18	n.a	>18	n.a	>18	n.a	5.3	138	2.9
D1.7	>18	n.a	>18	n.a	>18	n.a	>18	n.a	7.3	96	5.8
SL3.14	>18	n.a	>18	n.a	>18	n.a	>18	n.a	2.6	24	2.2
SL1.18	>18	n.a	>18	n.a	>18	n.a	>18	n.a	6.7	114	4.8
SL0.10	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18
SL3.24	>18	n.a	>18	n.a	>18	n.a	>18	n.a	2.7	30	2.2
SL3.31	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18
SL3.27	>18	n.a	>18	n.a	>18	n.a	>18	n.a	6.8	108	5.0
SL2.14	>18	n.a	>18	n.a	>18	n.a	>18	n.a	6.3	30	5.8
D2.9	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18
SL1.17	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18

D1.8	>18	n.a	>18	n.a	>18	n.a	>18	n.a	4.7	78	3.4
E. faecium SL3.26	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18	n.a	>18

*Time (h) required to increase absorbance by 0.3 units at 620 nm in each medium.
[t] Delay of growth between the control culture and low pH culture in minutes.
n.a: not available

Table 14 Survival rate of seven strains of *Lactococcus lactis* subsp. *lactis* in M17 broth at pH 2 (n=2, x±SEM)

Strains	Tolerance to M17 broth at pH 2 (log cfu/mL)		Survival rate (%)
	0 h	3 h	
SL3.34	7.98±0.07	5.72±0.12	66.3
SL1.9	8.22±0.05	0	0
D1.1	7.29±0.03	0	0
SL0.15	8.23±0.06	0	0
DC.6	8.15±0.04	0	0
SL1.18	8.23±0.04	0	0
SL3.27	8.22±0.04	4.89±0.11	51.3

On the other hand, the presence of bile tolerance in *Lactococcus lactis* subsp. *lactis* was greater than acid tolerance (Table 15). Sixteen strains (Table 15) of *Lactococcus lactis* subsp. *lactis* exhibited tolerance to 1%, 0.5% and 0.3% of oxgall. *Enterococcus faecium* was sensitive to these three concentrations of oxgall. Unfortunately, strains of SL3.34 and SL1.9 were sensitive to these oxgall concentrations. However, *L. lactis* subsp. *lactis* strain SL3.34 was still regarded as a potential probiotic candidate due to its greater survival rate at pH 2 pending further probiotic characterization with antibiotic susceptibility and adhesion to Caco-2 cells assessments.

Table 15 Bile tolerance of *Lactococcus lactis* subsp. *lactis* and *Enterococcus faecium* in M17 broth measured by the time taken to reach absorbance of 0.3 units at 620 nm

Strain	Time* (h) M17 bile 0.3	d^t (min)	Time* (h) M17 bile 0.5	d^t (min)	Time* (h) M17 bile 1.0	d^t (min)	Time* (h) control M17 pH
SL3.34	>18	n.a	>18	n.a	>18	n.a	4.5
SL1.9	>18	n.a	8.8	162	>18	n.a	6.3
D2.15	>18	n.a	>18	n.a	>18	n.a	6.8
SL0.3	>18	n.a	>18	n.a	>18	n.a	9.2
SL1.9	**3.5**	**-18**	**3.2**	**-42**	**3.3**	**-30**	**3.8**
SL3.32	2.2	0.0	2.0	-12	>18	n.a	2.2
D1.1	**7.8**	**222**	**6.2**	**120**	**7.8**	**222**	**4.2**
SL1.16	>18	n.a	>18	n.a	>18	n.a	>18
SL0.11	>18	n.a	>18	n.a	>18	n.a	>18
D2.17	**3.0**	**-24**	**2.8**	**-30**	**2.8**	**-30**	**3.3**
SL0.15	**4.8**	**12**	**3.5**	**72**	**4.3**	**18**	**4.7**
D1.9	**2.8**	**6.0**	**2.8**	**0.0**	**3.1**	**18**	**2.8**
SL2.15	>18	n.a	>18	n.a	>18	n.a	13.1
SL3.16	**6.4**	**96**	**5.3**	**30**	**9.3**	**270**	**4.8**
DC.6	**2.4**	**18**	**2.1**	**0.0**	**2.2**	**6.0**	**2.1**
D3.6	**1.8**	**-18**	**1.8**	**-18**	**2.0**	**-6.0**	**2.1**
SL3.19	**2.0**	**-18**	**1.8**	**-24**	**1.9**	**-18**	**2.3**
D1.2	**2.7**	**-18**	**2.8**	**-12**	**2.9**	**0.0**	**2.9**
D1.7	**6.3**	**-30**	**5.3**	**-30**	**9.4**	**222**	**5.8**
SL3.14	**2.0**	**-12**	**1.8**	**-18**	**1.9**	**-18**	**2.2**
SL1.18	**4.8**	**0.0**	**4.2**	**-36**	**5.2**	**24**	**4.8**
SL0.10	>18	n.a	>18	n.a	>18	n.a	>18
SL3.24	**2.2**	**0.0**	**2.0**	**-12**	**2.2**	**0.0**	**2.2**
SL3.31	>18	n.a	>18	n.a	>18	n.a	>18
SL3.27	**4.8**	**-12**	**4.3**	**-42**	**8.6**	**216**	**5.0**
SL2.14	>18	n.a	>18	n.a	>18	n.a	5.8
D2.9	>18	n.a	>18	n.a	>18	n.a	>18
SL1.17	>18	n.a	>18	n.a	>18	n.a	>18
D1.8	**3.8**	**18**	**2.8**	**-36**	**2.9**	**-30**	**3.4**

E. faecium SL3.26	>18	n.a	>18	n.a	>18	n.a	>18

*Time (h) required to increase absorbance by 0.3 units at 620 nm in each medium.
[t] Delay of growth between the control culture and low pH culture in minutes.
n.a: not available

4.3.2 Molecular detection of *msa* and *bsh* genes

All strains of *L. plantarum* tested (20 strains) as well as the *L. plantarum* type strain ATCC 14917^T showed the expected band size for both *msa* (1,740 bp) and *bsh* (919 bp) genes (Fig. 17). Thus, all strains met the requirement to be considered as probiotic cultures due to the presence of this probiotic marker.

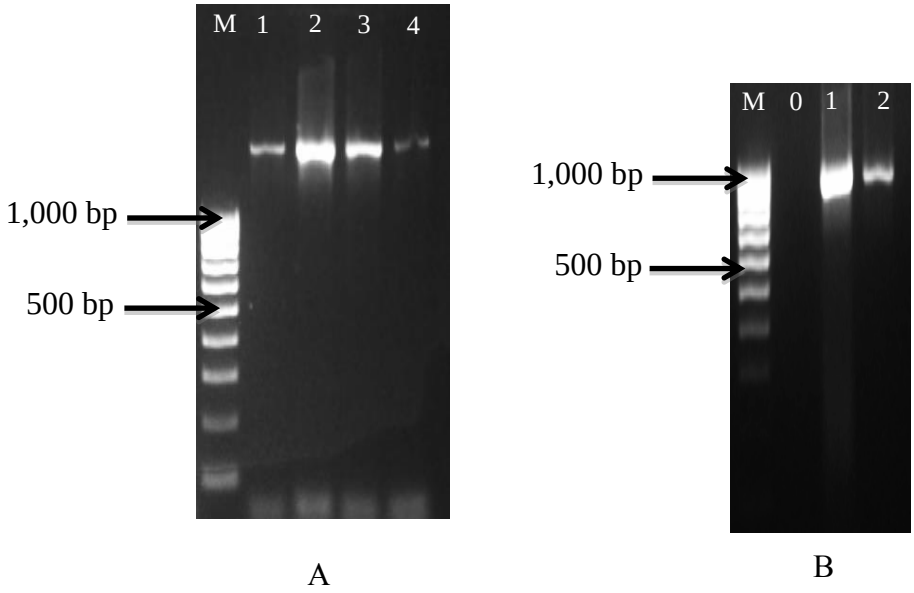


Figure 17 Representation of amplification products of *msa* gene – 1,740 bp (A) and bsh gene – 919 bp (B)

Lane M: 100 bp DNA marker; lane 0: sterile milliQ H_2O; lane 1: *L. plantarum* S1.30; lane 2: *L. plantarum* S2.7; lane 3: *L. plantarum* S2.5

4.3.3 Antimicrobial activity and bacteriocin detection

All strains of *L. plantarum* isolated from dadih and dangke were evaluated their antimicrobial activity against a range of pathogenic bacteria either Gram-negative or Gram-positive (Appendix 7). The indicator bacteria used in this study as listed in Table 1. The tested bacteria were not able to inhibit two indicator bacteria

used, namely *B. cereus* and *S. aureus* ATCC 25923. In general, Gram-positive bacteria were more sensitive than Gram-negative bacteria as shown by the sizes of the inhibition zones. The Gram-positive bacteria targeted were *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* and *Streptococcus pyogenes* ATCC 10389. By assessment of these indicator bacteria, the largest inhibition zones were exhibited by *L. plantarum* S1.30 and *L. plantarum* ATCC 14941^{T}. However, these antimicrobial producers also demonstrated an antagonistic activity against *E. coli* strain 99364-1 and 99386-3.

LAB can produce some antimicrobial substances for their defence and survival strategy against other microbes in their ecological niche. One of antimicrobial substances that has recently emerged as important in the food industry are bacteriocins. In this study, the presence of bacteriocin from all strains of *L. plantarum* was assessed through an agar-based method and a PCR-based method. Detection of bacteriocin performed by agar well diffusion assay failed to reveal the activity of any bacteriocin (data not shown). Thus, a PCR-based technique was applied to detect the presence of a specific gene for regulating bacteriocin activity especially for *L. plantarum*, that is plantaricin A (*pln*A). The PCR amplification resulted 450 bp of DNA fragment from all strains of *L. plantarum* (Fig. 18).

Figure 18 Amplification products of plantaricin A (450 bp) from representative's strains of *Lactobacillus plantarum*

Lane M: 100 bp DNA marker; lane 1: *L. plantarum* S1.30; lane 2: *L. plantarum* ATCC 14941^{T}, lane 3: *L. plantarum* SL3.5; lane 4: *L. plantarum* SL3.6; lane 5: *L. plantarum* SL3.7; lane 6: *L. plantarum* SL3.8; lane 7: *L. plantarum* SL2.4; lane 8: *L. plantarum* SL2.6; lane 9: *L. plantarum* SL2.9; lane 10: *L. plantarum* SL0.17; lane 11: *L. plantarum* GD.1; lane 12: *L. plantarum* GD.2

4.3.4 Antibiotic resistance and adhesion to Caco-2 cells

The result of antibiotic susceptibility testing of six strains of *L. plantarum,* two type strains (*L. plantarum* ATCC 14917^{T} and *L. rhamnosus* GG), and one strain of *Lactococcus lactis* subsp. *lactis* using disk diffusion agar are listed in Table 16. In general, all the *L. plantarum* strains and the reference strains were sensitive to tested antibiotics, except to vancomycin. While, *Lactococcus lactis* subsp. *lactis* strain SL3.34 was sensitive to all antibiotics used.

The strains that showed a tolerance to low pH, bile salts, sensitivity to almost all of tested antibiotics and additional probiotic features (the presence of *msa* and *bsh* genes) were further analysed for their ability to adhere to Caco-2 cells. *Lactobacillus plantarum* strains S1.30, SL2.7 and SL3.5 were selected for the adhesion test. Strains of SL2.7 and SL3.5 were selected because they have a greater survival rate at pH 2. While, strain S1.30 was also chosen because this strain exhibited a potent antimicrobial activity compared to other strains (Appendix 7), although it has a moderate survival rate at low pH. The highest adhesion ability has been shown by *L. plantarum* strain S1.30 (82.24±0.14%), followed by strain SL2.7 (80.26±0.39%), *Lactobacillus rhamnosus* GG (78.89±0.64%) and strain SL3.5 (78.34±0.16%). Meanwhile, *Lactococcus lactis* subsp. *lactis* strain SL3.34 and SL2.1 exhibited adhesion to Caco-2 cells as much as 73.94±1.26% and 73.44±1.51%, respectively. Therefore, *L. plantarum* strain S1.30 and *Lactococcus lactis* subsp. *lactis* SL3.34 were the best probiotic candidates representing each genus.

Table 16 Antibiotic resistance and adhesion percentage on Caco-2 cells of selected strains of *Lactobacillus plantarum* and *Lactococcus lactis* subsp. *lactis*

Strains	Inhibition zone diameter (mm)							% adhesion on Caco-2
	AMP (10 µg)	CMN (2 µg)	HLS (300 µg)	ERY (15 µg)	VAN (30 µg)	CHL (30 µg)	TET (30 µg)	
L. plantarum S1.30	45	30	23	37	-	40	30	82.24±0.14[b]
L. plantarum SL2.7	45	30	25	38	-	40	30	80.26±0.39[b]
L. plantarum SL3.5	45	30	25	38	-	40	30	78.34±0.16[b]
L. plantarum GD1	45	30	25	37	-	40	30	n.d
L. plantarum SL2.2	43	30	24	37	-	40	30	n.d
L. plantarum SL3.6	45	30	25	38	-	40	30	n.d
L. plantarum ATCC 14917	46	24	30	34	-	40	30	n.d
L. rhamnosus GG	41	36	35	43	-	44	44	78.89±0.64[b]
L. lactis SL3.34	40	30	48	62	60	56	34	73.94±1.26[a]
L. lactis SL2.1	n.d	n.d	n.d	n.d	n.d	n.d	n.d	73.44±1.51[a]

*Ampicillin- AMP (≤19 R, >23 S), Clindamycin-CMN (≤14 R, >20 S), Streptomycin-HLS (≤8 R, >15 S), Erythromycin-ERY (≤13 R, >19 S), Vancomycin-VAN (≤14 R, >18 S), Chloramphenicol-CHL (≤12 R, >18 S), Tetracycline-TET (≤14 R, >18 S) (Babot et al. 2014)

% adhesion on Caco-2 cells was expressed as percentage of the viable bacteria (log cfu/ml) compared to their initial population.

Data of adhesion test are expressed as mean (%) ± SEM. Different letters are significantly different ($P < 0.05$)

n.d: not determined

4.4 Discussion

Screening for new potential LAB probiotics from dadih and dangke includes an assessment of their resistance to low pH and bile salts, antimicrobial activity, antibiotic resistance and adhesion to Caco-2 cells. The presence of probiotic marker namely *bsh* and *msa* genes in species of *L. plantarum* were also elucidated to confirm their probiotic properties. Tolerance to low pH and resistance to high concentrations of bile salt was used as the preliminary selection testing of the LAB isolates. Ability to tolerate exposure to these conditions allows the probiotic organism to pass unharmed through the upper gastrointestinal tract (Bull et al. 2013). The best strain to survive under low pH was *L. plantarum* SL2.7, while strain S1.30 showed the greatest growth in media containing high concentrations of bile salt. In case of *L. lactis* subsp. *lactis* strains, four strains were tolerant to slightly acidic media (pH 5-6) with strain SL3.34 demonstrating the highest survival rate. Strain SL3.34 had a moderate survival rate (66.3%) for 2 h at pH 2; however, this strain was not resistant to a high concentration of bile salt. Interestingly, the number of *L. lactis* subsp. *lactis* isolates able to resist to bile salt was much greater than that of *L. plantarum* strains. These observations are in agreement with a previous study (Surono 2003). In fact, only a small proportion of LAB isolated from fermented products has a good tolerance to low pH and bile salts (*in vitro*), as compared to LAB strains obtained from the gastrointestinal tract of animals or humans (Kizerwetter-Świda & Binek 2016).

The presence of the *msa* and *bsh* genes, encoding for mannose-specific adhesion and bile salts hydrolase, in *L. plantarum* strains indicates mucosal adhesion properties, and resistance to toxic bile salts in the GIT and is associated with the ability to reduce blood cholesterol concentration, respectively (Patel et al. 2010; Turchi et al. 2013). All *L. plantarum* strains demonstrated these probiotic markers, indicating that the strains could be expected to be resistant to bile salts and able to colonize intestinal epithelial cells. Although all examined strains carried the *bsh* gene, only five strains were resistant to a high concentration of bile salt. This could be due to sequence variation in the *bsh* gene in *L. plantarum* (Zago et al. 2011).

As desirable attributes, a probiotic strain should demonstrate antimicrobial activity against a range of pathogenic bacteria. In this present study, some potential LAB strains exhibiting a good survival rate towards low pH and bile salt were evaluated. The strain with the highest potential to produce antimicrobial substances

was *L. plantarum* S1.30. Antibacterial activity of this strain was previously elucidated (Jatmiko, Barton & de Barros Lopes 2010), and it was included in this current study due to its potential antimicrobial activity. Since bacteriocin detection using agar-based method failed, the presence of the plantaricin A (*pln*A), gene responsible for bacteriocin production, was used and the gene was detected in all *L. plantarum* strains through a PCR-based method. This result was in agreement with previous studies that organic acids, particularly lactic acid were the main key player in the antagonistic activity (Argyri et al. 2013; Zhang et al. 2016). *Lactobacillus plantarum* S1.30 has a bacteriostatic effect against *L. sakei* ATCC 15521 and *L. monocytogenes in vitro* as a result of increasing the acidity level (Jatmiko, Barton & de Barros Lopes 2010). The failure of bacteriocin detection using conventional techniques probably relates to unsuitable culture medium conditions (pH, temperature and ingredients) and the regulatory mechanism of bacteriocin biosynthesis which is associated with the quorum sensing mechanism in these bacteria (Heng et al. 2007; Rojo-Bezares et al. 2007).

Application of probiotics harbouring antibiotic resistant genes may lead to horizontal gene transfer to the intestinal microbiota, which contains both commensal and pathogenic organisms. Thus it is essential to assess the susceptibility of examined strains to a range of antibiotics. In this work, all of tested strains were sensitive to all selected antibiotics; with the exception of a *Lactobacillus* strain that was resistant to vancomycin. Lactobacilli are characterized as naturally resistant to vancomycin (Sharma et al. 2014). The vancomycin resistance is chromosomal encoded and not transferable to other microbial species (Argyri et al. 2013). Therefore, all strains showing a good survival rate against low pH and bile salt (Table 16) were appropriate for further probiotic characterization. In contrast, strains of *L. plantarum* DH1 and *Pediococcus acidilactici* DH7 isolated from dadih showed low levels of resistance to chloramphenicol, up to 5 µg/mL (Sukmarini et al. 2014).

The adhesion capacity of potential probiotic candidates using Caco-2 cell culture is an *in vitro* model for adhesion to the intestinal epithelium. The adhesion ability may be linked to competitive exclusion of pathogens and the immunomodulatory system (Ortu et al. 2007). The most adhesive strain was *L. plantarum* S1.30 (82%) representing genus *Lactobacillus*, while *L. lactis* subsp. *lactis* SL3.34 (74%) was the best strain in the genus *Lactococcus.* The adhesion ability of *L. plantarum* S1.30 was strengthened with the presence of *msa* gene in its genome. The

adhesion capability of *Lactobacillus* is dependent on the isolation source; the adhesion will be higher if the strains are derived from human faeces or from buffalo milk, rather than from cheese (Duary et al. 2011). Kaushik et al. (2009) have previously characterized *L. plantarum* Lp 9 isolated from raw buffalo milk. In addition to probiotic parameters utilized in this current study, *L. plantarum* Lp9 also demonstrated a good hydrophobicity, cellular autoaggregation, the presence of the gene for mucus-binding and fibronectin-binding protein and antioxidative activity (Kaushik et al. 2009). Therefore, *L. plantarum* S1.30 is a potential candidate to be used for further *in vivo* tests.

Among members of genus *Lactobacillus*, *L. plantarum* is the most versatile microorganism and is widely distributed in diverse ecological niches such as plants/ vegetables, meat, fish, dairy products and mammalian gastrointestinal tract (Martino et al. 2015; Oguntoyinbo et al. 2016). Consequently, *L. plantarum* exhibiting probiotic properties have been detected and characterized in many NFM products (Angmo et al. 2016; Collado et al. 2007b; Ortu et al. 2007; Pinto et al. 2006; Takeda et al. 2011). Meanwhile, *L. lactis* subsp. *lactis* derived from NFM products has also been previously shown to demonstrate probiotic characteristics (Dharmawan, Surono & Kun 2006; Harun-ur-Rashid et al. 2007; Mohammed Salih, Hassan & El Sanousi 2011; Surono 2003). However, the prevalence of *L. lactis* subsp. *lactis* with probiotic properties in NFM products is less frequent than *L. plantarum*. Considerable variation among presumptive probiotic candidates was normally observed when characterization of probiotic properties was performed. This variability may be associated with variation in their genome sequences and ecological niches. Therefore, probiotic selection is urgently required to obtain the best candidate.

4.5 Conclusion

The results presented in this study indicate that *L. plantarum* S1.30 and *L. lactis* subsp. *lactis* SL3.34 isolated from dadih possess probiotic traits (*in vitro*) and therefore could be considered appropriate probiotic candidates for further *in vivo* examination. *Lactobacillus plantarum* S1.30 representing genus *Lactobacillus* demonstrated some probiotic properties such as tolerance to low pH and bile salts, anbtimicrobial activity and the presence of a bacteriocin regulating gene (plantaricin A) and *msa* and *bsh* genes, susceptibily to antibiotics and ability to adhere to Caco-2 cells. From these probiotic features, only antimicrobial activity and the presence of

msa and *bsh* gene were not demonstrated by *L. lactis* subsp. *lactis* SL3.34. However, this strain was selected as the representative of the dominant genus/ species in dadih, particularly as seen from pyrosequencing result (indicating this species is an apparent key player in the dadih fermentation). However, further studies are needed on the evaluation of additional important probiotic traits such as antioxidant activity, assimilation of cholesterol, anti-inflammatory properties as well as lysozyme resistance.

Chapter Five

Effects of Probiotics Isolated from Dadih on Intestinal Mucositis Induced by 5-Fluorouracil in Rats

5.1 Introduction

Several human diseases have been associated with the abundance and diversity of the gastrointestinal microbiota (Huttenhower et al. 2012; Mimee, Citorik & Lu 2016). These diseases include metabolic disorders such as obesity, immune-based conditions such as inflammatory bowel disease, allergies and autoimmunity, and disorders of the gut-brain axis; for example anxiety-related behavior and autism spectrum disorders (Belkaid & Hand 2014; Gérard 2016). Development of microbiome therapeutics, especially using probiotics targeting modulation of the intestinal microbiota, has increased in recent years (Mimee, Citorik & Lu 2016). It is generally accepted that administration of probiotics in adequate amounts confers a human health benefit either for prevention or remediation against some intestinal diseases (Saad et al. 2012). An example is intestinal mucositis, which is a common side effect of anticancer chemotherapy. Intestinal mucositis can result from treatment with a range of different chemotherapy drugs such as irinotecan, afatinib, doxorubicin, cyclophosphamide, methotrexate and 5-fluorouracil (5-FU) (Fijlstra et al. 2015; Flórez et al. 2016; Howarth et al. 2006; Ribeiro et al. 2016).

In addition to gut mucosal barrier injury leading to malabsorption of nutrients and a high risk of infections, creating a dysbiosis in the intestinal microbiota is another detrimental effect of cancer chemotherapy (Tang et al. 2016). von Bũtzingslõwen et al. (2003) observed that after administration of 5-FU in female Lewis rats, the composition of Gram-negative bacteria became greater than that of the Gram-positive organisms. Likewise, in methotrexate (MTX)-treated rats, a significant decrease in absolute number of bacterial groups was reported, namely anaerobic bacteria (*Clostridium* cluster XIVa, ruminococci, eubacteria, bifidobacteria, and *Clostridium ramnosum*) and aerobic bacteria (lactobacilli and streptococci) (Fijlstra et

al. 2015). However, the number of *Bacteroides, Enterobacteriaceae* and enterococci, as potentially enteropathogenic bacteria, was relatively increased (Fijlstra et al. 2015). These findings suggest that alteration in intestinal microbiota composition plays a role in the pathogenesis of intestinal mucositis. As a result, restoring normal intestinal microbiota composition is a potential approach to reduce the severity of intestinal mucositis.

Probiotic administration may therefore have a beneficial effect in reducing the toxicity of anticancer therapy, especially using commercial and/or established probiotics; either single- or multi-strain (Mego et al. 2016). A number of these probiotics have been applied in chemotherapy-induced intestinal mucositis rodent models including VSL#3 (Bowen et al. 2007), *Streptococcus thermophilus* TH-4 (Whitford et al. 2009), *Saccharomyces boulardii* (Justino et al. 2014), *Escherichia coli* Nissle 1917 (Wang et al. 2014a), *Lcr35-Antibiophilus*® and *LaBi-Infloran*® (Yeung et al. 2015), *Lactobacillus acidophilus* (Justino et al. 2015), and *Bifidobacterium infantis* (Yuan et al. 2015). However, only limited studies have successfully been implementing probiotics isolated from fermented products. For instance, application of multi-strain probiotics DM#1 from Chinese fermented food (*Bifidobacteria breve* DM8310, *Lactobacillus acidophilus* DM8302, *Lactobacillus casei* DM8121, and *Streptococcus thermophilus* DM8309) in 5-FU-induced intestinal mucositis in rats (Tang et al. 2016). Furthermore, *Lactobacillus plantarum* NCU116 from pickled vegetables demonstrated improvements in both the intestinal microbiota and metabolic activity (Xie et al. 2016). Probiotic health benefits are likely to be strain-specific (Saad et al. 2012); accordingly, further studies are required to explore the therapeutic potential of probiotics derived from naturally fermented milk products.

A newly identified probiotic termed *Lactobacillus plantarum* S130, has been obtained from dadih, a naturally fermented buffalo milk product from Indonesia. This strain exhibits properties as a potential probiotic candidate, such as antimicrobial activity (Jatmiko, Barton & de Barros Lopes 2010), adhesion ability, tolerance to acidity and bile salts (as described in Chapter 4). However, *in vivo* testing of this strain has yet to be performed. Therefore, the present study sought to investigate the effects of *L. plantarum* S130 (Lp) on intestinal mucositis induced by 5-FU in rats. Besides this strain, *Lactococcus lactis* subsp. *lactis* SL334 (Lacl), the most predominant bacteria from dadih was included to represent the dadih microbiota more broadly.

5.2 Methods

5.2.1 Animal studies

Female dark agouti rats (101 - 146g; 12 rats per cage) were acclimatized for 3 days before being transferred to metabolism cages (Tecniplast, Exton, PA, USA) and housed individually at room temperature with a light:dark cycle of 12h. All rats were given *ad libitum* access to food (standard 18% casein-based diet (Tomas et al. 1991)) and water. All animal studies were conducted in compliance with the Australian Code of Practice for the Care and Use of Animals, and were approved by the Animal Ethics Committee of the University of Adelaide (Approval number: S-2014-088A).

Rats were randomly assigned to six groups (n=8/group; average weight 121 to 122 g/group): Group 1: saline + water, Group 2: saline + *L. plantarum* S1.30, Group 3: saline + *L. lactis* subsp. *lactis* SL3.34, Group 4: 5-FU + water, Group 5: 5-FU + *L. plantarum* S1.30, Group 6: 5-FU + *L. lactis* subsp. *lactis* SL3.34. Water, *L. plantarum* S1.30 or *L. lactis* subsp. *lactis* SL3.34 (1 mL) was administered once daily via orogastric gavage from days 0 to 8. The probiotic cultures (*L. plantarum* S1.30-Lp and *L. lactis* subsp. *lactis* SL3.34-Lacl) were grown in MRS broth for 16-18 h at 37^{o}C in the presence of 5% CO_2 to achieve 2 x 10^9 cfu/ml. On day 5, all rats were intraperitoneally injected with a single dose of saline or the chemotherapeutic agent, 5-fluorouracil (5-FU, Mayne Pharma Pty Ltd, Mulgrave, Victoria, Australia; 150 mg/kg). Body weight, food and water intake, and fecal and urine output were monitored and measured daily.

5.2.2 Tissue collection

On day 8, rats were humanely killed by CO_2 asphyxiation followed by cervical dislocation. The gastrointestinal tract was removed, and the stomach, small intestine and colon separated out, measured, emptied of contents and weighed. Two centimetre sections of the small intestinal tract (jejunum and ileum) were removed and placed in tissue embedding cassettes (CAS-LID-01, Techno Plas, Adelaide, SA, Australia) and placed in 10% buffered formalin (HT501128, Sigma-Aldrich, Castle Hill, NSW, Australia) for histological analysis. Additionally, segments (4 cm) directly adjacent to the corresponding histological samples were collected and snap-frozen in liquid nitrogen for biochemical analysis. Biochemical samples were stored at -80°C until prepared for analysis by homogenization in 10 mmol/L phosphate buffer.

Gastrointestinal sections including stomach, duodenum, remaining small intestine, caecum and colon were weighted. The remaining visceral organs including kidneys, heart, liver, spleen, thymus and lungs were weighed and discarded (Mashtoub, Tran & Howarth 2013; Wang et al. 2017).

5.2.3 Histological analysis and intestinal damage severity scoring

Small intestinal samples (jejunum and ileum) were transferred from 10% buffered formalin to 70% ethanol 24 h post-collection. Specimen preparations and histological evaluation followed the process previously reported by Mashtoub, Tran & Howarth (2013). Jejunum and ileum specimens were embedded in paraffin and then sectioned (4 μm). The sections were then stained with haematoxylin and eosin (H&E). Measurements of villus height (VH) and crypt depth (CD) were determined by the mean value of 40 well-orientated villi and crypts per small intestine section per rat.

Damage severity of intestinal sections was assessed using a semi quantitative analysis based on parameters including villus fusion and stunting (villus and crypt ratio), enterocyte disruption, reduction in goblet cell numbers, reduction in mitotic figures, crypt cell disruption, crypt abscess formation, lymphocytic and polymorphonuclear infiltration, and thickening/oedema of the submucosa and muscularis externa. All analyses were performed in a blinded fashion, using an Olympus CX-41 light microscope (Tokyo, Japan) with Olympus digital camera UC30 (Tokyo, Japan) (Howarth et al. 1996).

5.2.4 Biochemical analysis: Myeloperoxidase (MPO) activity

Neutrophil infiltration in the jejunum and ileum was measured by MPO assay, which is an indication of acute inflammation. Sample preparations and data analysis were performed by methods described in Mashtoub, Tran and Howarth (2013). Intestine tissues (4 cm) were firstly homogenized, aliquoted and stored at -80°C. Homogenized samples were centrifuged at 13,000 g for 12 min, supernatants was then discarded. The pellets were then re-suspended in 0.5% hexadecyltrimethyl ammonium bromide buffer (200 μL; Sigma Chemicals, Castle Hill, NSW, Australia). Following by vortexing for 2 min and centrifuging at 13,000 g for 2 min, samples (50 μL) were added to a 96 well followed by reaction reagent (200 μL). The reaction reagent was made with 4.2 mg of O-dianisidine dihydrochloride reagent, 12.5 μL H_2O_2, 2.5 mL potassium phosphate buffer (pH 6.0) and 22.5 mL distilled water (sufficient amount

for one 96 well plate). Absorbance was measured at 450 nm at 1 min intervals for 15 min with a spectrophotometer. Data were expressed as units of MPO per gram of tissue.

5.2.5 Statistical analyses

Statistical comparisons were conducted suing SPSS version 22.0 for Windows (SPSS Inc., Chicago, IL, USA). All data sets were tested for normality of distribution using skewness and kurtosis values. Water intake, urine output data sets were Log_{10} transformed to fit normal distribution. Body weight, food intake, log_{10} transformed water intake, fecal output, log_{10} transformed urine output were analysed using a repeated measures analysis of variance (ANOVA) pair-wise comparisons *post-hoc* test adjusted with LSD to compared the differences both among groups and within groups prior to (days 0-5) and post (days 6-7) saline or 5-FU injection. Visceral organ weights (heart, liver, spleen, thymus, lung, left and right kidneys), gastroinstestinal organ weights (small intestine, stomach, duodenum and caecum), intestinal organ lengths (small intestine and colon), VH and CD and MPO activity were analysed using a one-way ANOVA and LSD *post-hoc* test to compare differences among groups. All parametric data were expressed as means with their standard errors. Differences were considered statistically significant at $P < 0.05$.

5.3 Results

5.3.1 Daily metabolic data

A significant body weight gain was observed daily on the days prior to saline or 5-FU injection (days 0-5) ($P < 0.001$), without a significant difference among treatment groups (Fig. 19, $P > 0.05$). Similar effect was also noted that following saline injection, body weight was increased gradually on days 6-8, without significant differences among groups ($P > 0.05$).

Following 5-FU injection, body weight was decreased significantly on days 6-8, as follows 3.8%, 4.2% and 3.6%, respectively, compared to saline controls ($P < 0.05$). However, on day 9, the average of body weight in 5-FU treated rats started to increase slightly compared to the previous day (average 0.5% or 0.57 g/rat).

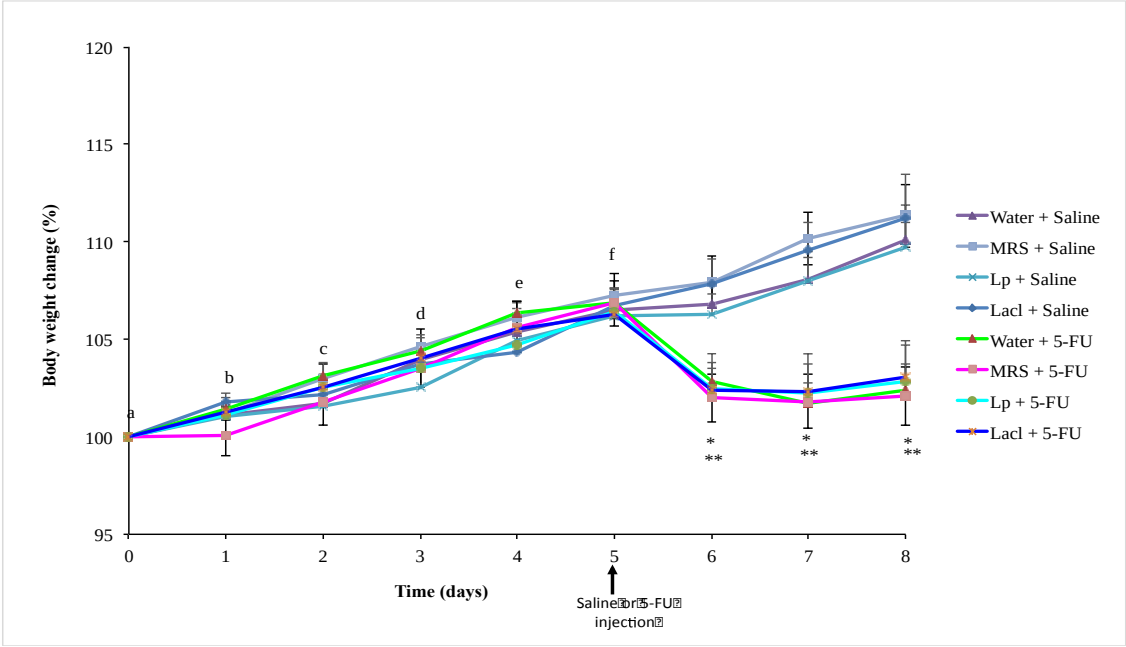


Figure 19 Daily body weight change of saline or 5-FU injected rats on day 5, gavaged daily with 1 mL of water, MRS broth, Lp or Lacl.

Data are expressed as mean ± standard error of the mean. Bar values not sharing the same letter are significantly different from days 0 to 5. *$P < 0.05$ compared to MRS + saline, ** $P < 0.05$ compared to MRS + saline; Lp: *L. plantarum*, S1.30: Lacl, *L. lactis* subsp. *lactis* SL3.34.

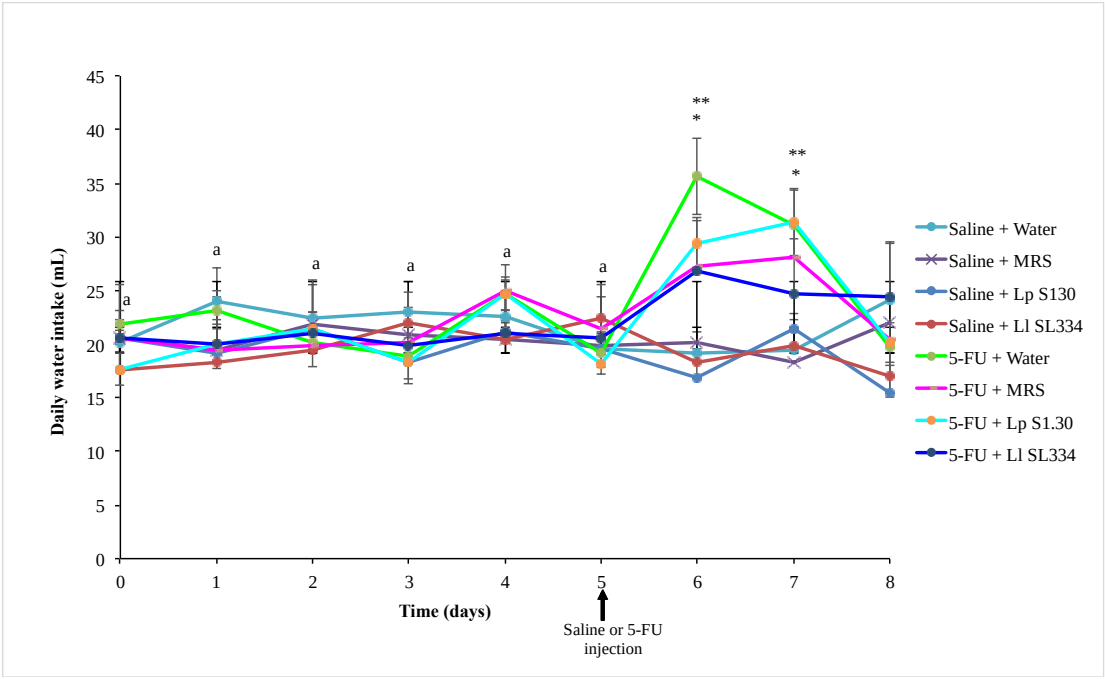


Figure 20 Daily water intake of saline or 5-FU injected rats on day 5, gavaged daily with 1 mL of water, MRS broth, Lp or Lacl.

Data are expressed as mean ± standard error of the mean. Bar values not sharing the same letter are significantly different from days 0 to 5. *$P < 0.05$ compared to Water + saline, ** $P < 0.05$ compared to MRS + saline; Lp: *L. plantarum*, S1.30: Lacl, *L. lactis* subsp. *lactis* SL3.34.

On days 0-5, daily water intake of all rats was fluctuated with no significant differences among groups or between days (Fig. 20, $P > 0.05$). In saline treated rats, this pattern was continued until day 9. Administration of probiotic cultures (Lp and Lacl) did not affect the water intake of saline treated rats.

On the other hand, a significant increase in water intake was observed in 5-FU treated rats receiving water, MRS broth, Lp and Lacl cultures compared to saline treated rats receiving water or MRS broth ($P < 0.05$) on days 6-7. On day 9, water intake in 5-FU treatment rats was improved similar to saline treatment.

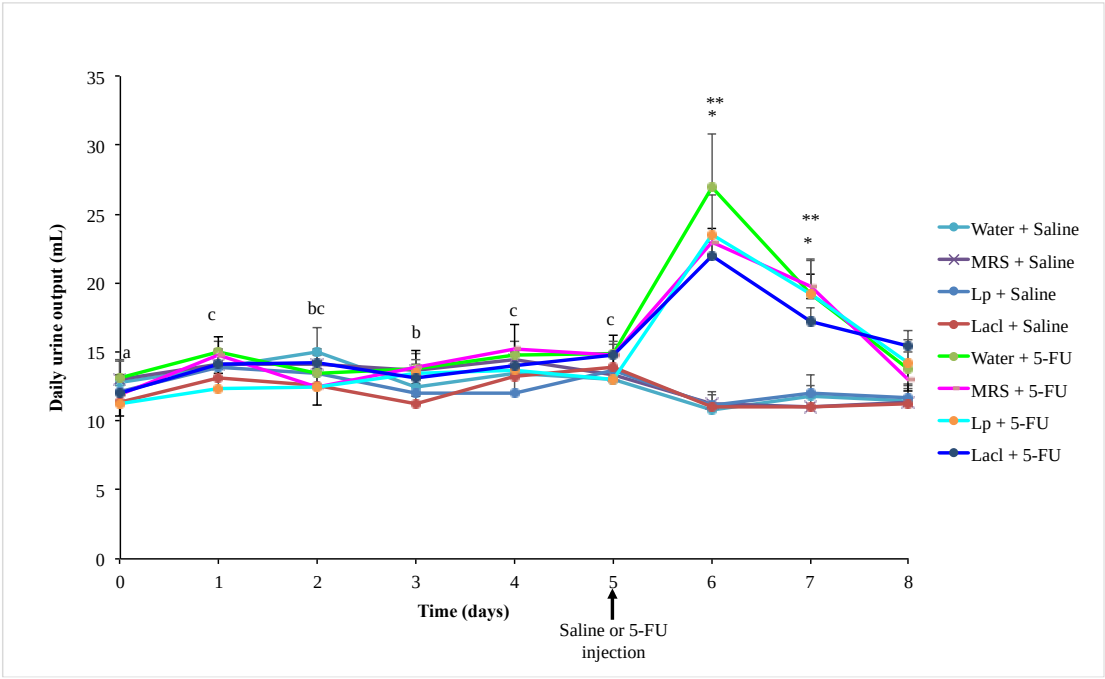


Figure 21 Urine output of saline or 5-FU injected rats on day 5, gavaged daily with 1 mL of water, MRS broth, Lp or Lacl.

Data are expressed as mean ± standard error of the mean. Bar values not sharing the same letter are significantly different from days 0 to 5. *$P < 0.05$ compared to Water + saline, ** $P < 0.05$ compared to MRS + saline; Lp: *L. plantarum*, S1.30: Lacl, *L. lactis* subsp. *lactis* SL3.34.

Before saline or 5-FU was injected (days 0-5), a significant increase in urine output began from day 2 and remained stable until day 5 (Fig. 21, $P < 0.05$). No significant differences were observed among treatment groups on days 0-5. A similar pattern was also noted on days 6-8, especially in the saline treated rats.

In 5-FU injected rats, on day 6, 5-FU injection led to an increase in urine output compared to saline control (saline or MRS broth) ($P < 0.05$). Although urine output was decreasing on day 7, a significant difference in urine output was still observed compared to saline control; returning to normal levels on day 8 (Fig. 21).

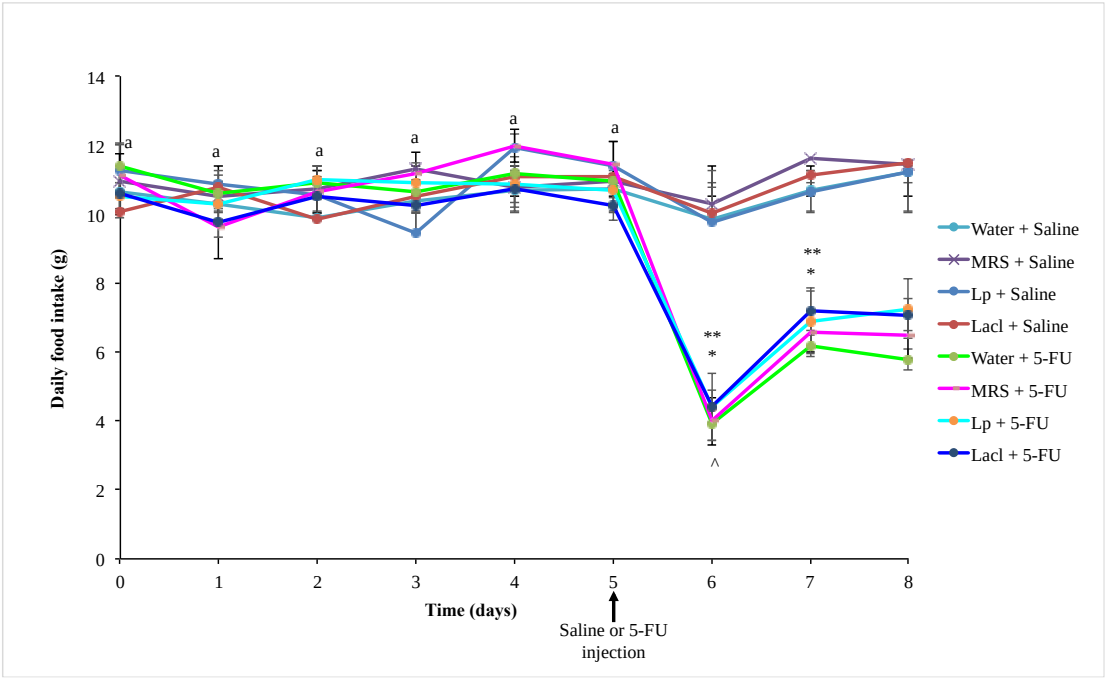


Figure 22 Food intake of saline or 5-FU injected rats on day 5, gavaged daily with 1 mL of water, MRS broth, Lp or Lacl.

Data are expressed as mean ± standard error of the mean. Bar values not sharing the same letter are significantly different from days 0 to 5. *$P < 0.05$ compared to Water + saline, ** $P < 0.05$ compared to MRS + saline, ^ $P < 0.05$ compared to 5-FU + Lacl; Lp: *L. plantarum*, S1.30: Lacl, *L. lactis* subsp. *lactis* SL3.34.

During the period before saline or 5-FU injection (day 0-5), food intake was not changed significantly ($P > 0.05$), with no significant differences among groups (Fig. 22). No change was observed in feed consumption in the post-saline injection period ($P > 0.05$).

Daily food consumption decreased significantly after 5-FU injection (Fig. 22). Indeed, mean food intake decreased by more than half (61.4%) on day 6, with significant differences compared to saline controls (saline or MRS broth) ($P < 0.05$). On day 7, food consumption increased (38% in average), but was still significantly different compared to saline controls ($P < 0.05$). Interestingly, rats treated with Lacl cultures had the greatest improvement in food intake compared to the other 5-FU treated rats, with no significant difference between Lacl + saline and Lp + saline treated rats ($P > 0.05$). Normal food consumption was restored on day 8 with no significant differences compared to saline treatment.

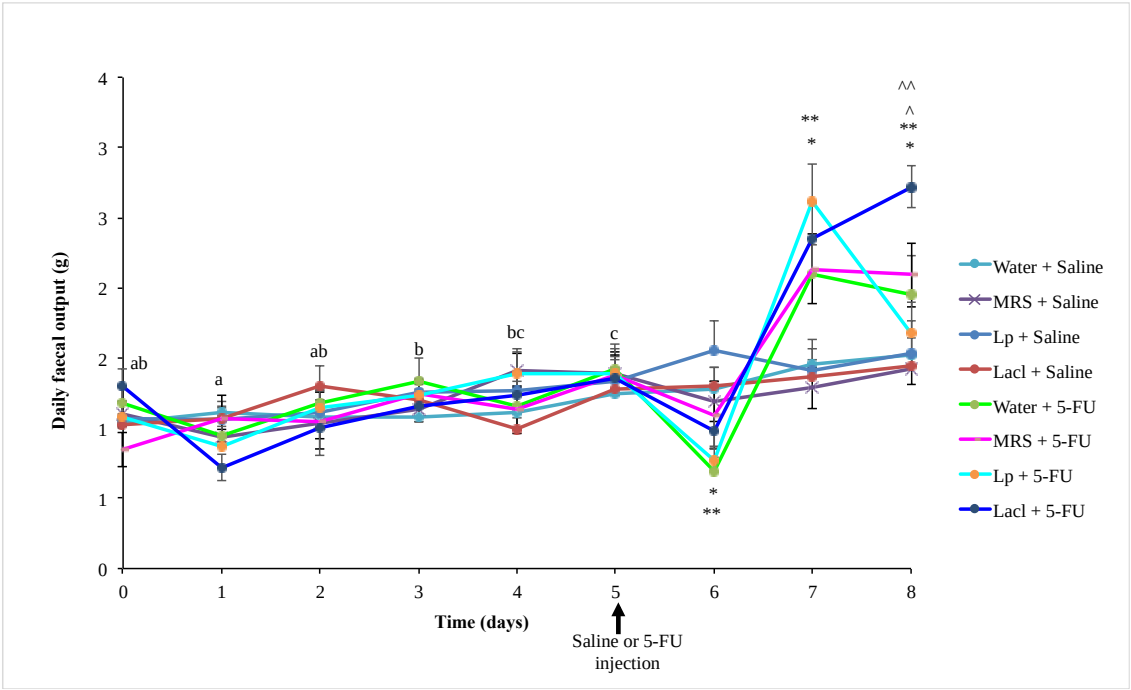


Figure 23 Fecal output of saline or 5-FU injected rats on day 5, gavaged daily with 1 mL of water, MRS broth, Lp or Lacl.

Data are expressed as mean ± standard error of the mean. Bar values not sharing the same letter are significantly different from days 0 to 5. *$P < 0.05$ compared to Water + saline, ** $P < 0.05$ compared to MRS + saline, ^ $P < 0.05$ compared to 5-FU + Lacl, ^^ $P < 0.05$ compared to 5-FU + water & 5-FU + MRS; Lp: *L. plantarum*, S1.30: Lacl, *L. lactis* subsp. *lactis* SL3.34.

Prior to saline or 5-FU injection (days 0-5), fecal output was only minimally affected prior to day 5. No significant differences were observed between consecutive days or among groups (Fig. 23, $P > 0.05$). Following saline injection, all rats, including those gavaged with MRS broth, Lp or Lacl cultures, increased their fecal output form day 6 to 8, with no significant differences among groups.

The injection of 5-FU reduced the fecal output on day 6 compared to the previous day (Fig. 23). In this group, fecal output in rats receiving water and Lp cultures was reduced significantly compared to normal saline controls (water and MRS broth) ($P < 0.05$), whereas rats treated with MRS broth or Lacl cultures resulted in a similar fecal output compared to normal saline controls ($P > 0.05$). Otherwise, on day 7, fecal output in all rats injected with 5-FU was significantly increased compared with normal saline controls ($P < 0.05$). On day 8, fecal output in rats treated with Lacl cultures remained significantly increased, and plateaued for rats receiving MRS broth, compared to saline controls ($P < 0.05$). However, rats treated with Lp or water

normalized fecal output compared to saline controls, on day 8. Interestingly, the effect of Lp in normalizing fecal output was greater than rats treated with water.

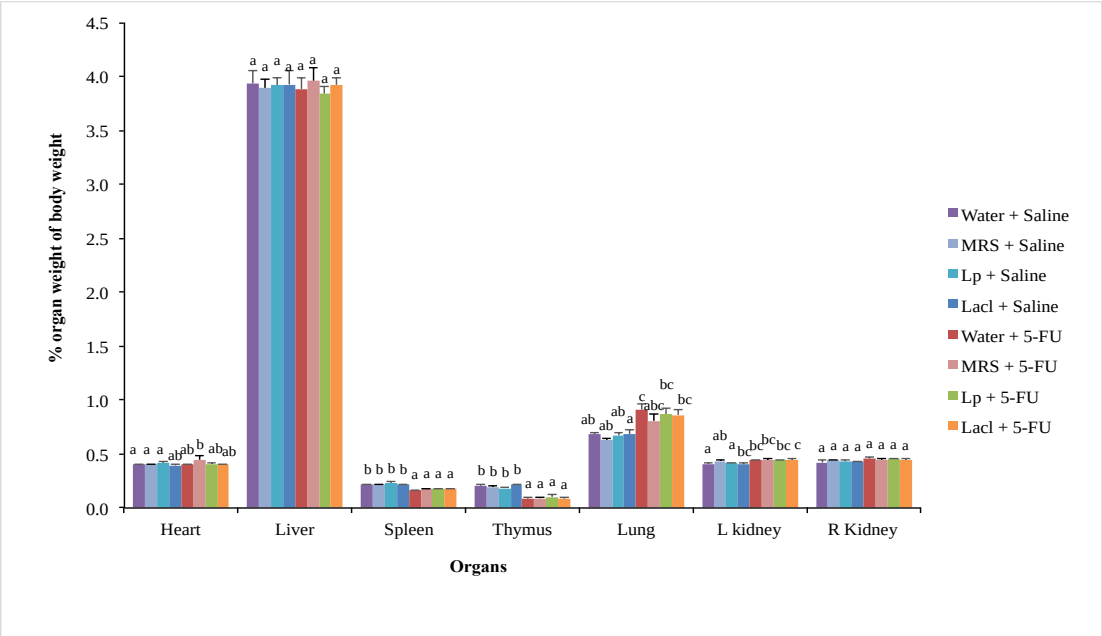


Figure 24 Proportional visceral organ weights on day 8.

Data are expressed as mean of proportional visceral organ weight (%) ± standard error of the mean. Bar values not sharing the same letter are significantly different ($P < 0.05$).

Visceral organ weight percentage is the proportion of visceral organ weight compared to total body weight (Fig. 24). From seven visceral organs (heart, liver, spleen, thymus, lung, left and right kidney) only two organs (liver and right kidney) showed no significant differences between saline and 5-FU treated rats. 5-FU treatment significantly affected the spleen and thymus ($P < 0.05$), with decreasing percentages of 21% and 55%, respectively. In 5-FU treated rats, rats treated with water had a significantly increased proportional lung weight compared to saline controls (34% average, $P < 0.05$). Rats receiving Lacl cultures had a significantly increased left kidney proportional weight compared with saline control (water or MRS broth), and no significant difference within group and counterpart control (Lacl + saline).

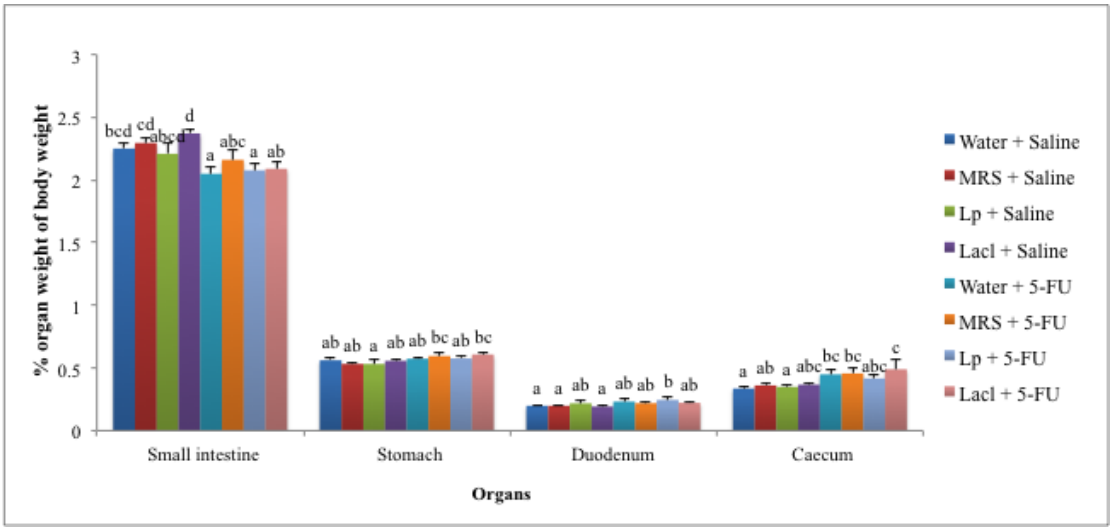


Figure 25 Proportional gastrointestinal organ weights on day 8.

Data are expressed as mean of proportional visceral organ weight (%) ± standard error of the mean. Bar values not sharing the same letter are significantly different ($P < 0.05$).

In general, gastrointestinal weights among groups, both saline and 5-FU injected rats, were not significantly different (Fig. 25). However, a significant difference was observed in small intestine weight. Rats receiving water and Lacl cultures reduced the organ weight by 10% and 8%, respectively compared to saline controls ($P < 0.05$). Similarly, duodenum weight was significantly increased in rats receiving Lp cultures compared to saline controls. Rats receiving Lacl, water and MRS broth increased caecum weight significantly compared with the water + saline group. Administration of Lp partially reduced caecum weight, although the difference was not statistically significant ($P > 0.05$). Meanwhile, in 5-FU injected rats, administration of probiotic cultures, MRS medium and water did not affect the stomach weight compared with saline controls (water and MRS broth).

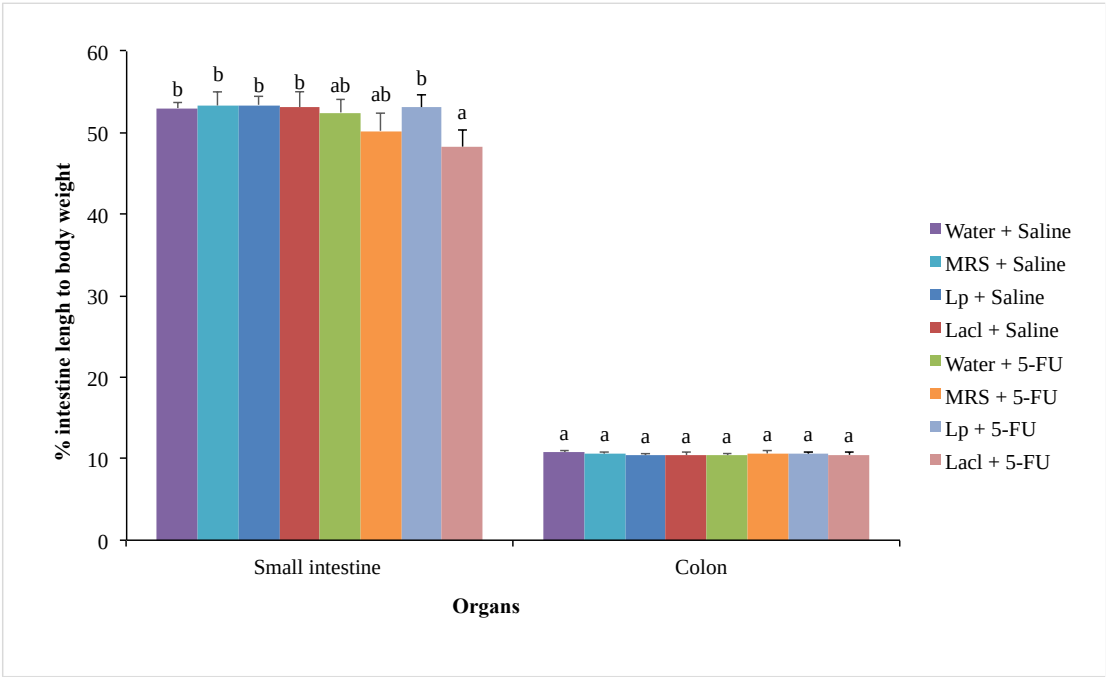


Figure 26 Proportional intestinal organ lengths collected on day 8.

Data are expressed as mean of proportional visceral organ weight (%) ± standard error of the mean. Bar values not sharing the same letter are significantly different ($P < 0.05$).

5-FU injection did not significantly affect length of the small intestine and colon, compared to saline injected rats (Fig. 26). In 5-FU treated rats, rats receiving Lacl cultures had a significantly decreased small intestine length ($P < 0.05$). Lp cultures did not increase intestinal organ length.

5.3.2 Intestinal damage severity (IDS) scoring

In general, an increase in disease severity score in 5-FU injected rats was observed compared to saline treatment groups (Table 17). Jejunal and ileal histological disease severity scores were significantly increased in 5-FU treated controls compared to saline controls ($P < 0.05$). Disease severity score of 5-FU treated rats was significantly reduced in both the jejunum and ileum after receiving Lp and Lacl cultures compared to 5-FU control ($P < 0.05$). In the jejunum, administration of MRS broth also significantly decreased disease severity compared with 5-FU control ($P < 0.05$).

Table 17 Disease severity index in the jejunum and ileum between saline and 5-FU treatment groups

	Saline injection				**5-FU injection**			
	Water	**MRS**	**Lp**	**Lacl**	**Water**	**MRS**	**Lp**	**Lacl**
Jejunum	6 (5-7.5)	5.5 (5-8)	7.75 (6-8)*	7 (6-7.5)	17.75 (15-20)**	11.5 (7-17)^^	10.5 (6-16)^^	12 (8.5-14.5)^^
Ileum	5.75 (5-7)	7 (5.5-9)	6.25 (6-7)	6 (5-7)	17.5 (13-20.5)**	16.25 (10-19)	13 (6-15.5)^^	12.75 (6-16.5)^

** indicates $p<0.05$ or * indicates $p<0.01$compared to Water + Saline
^^ indicates $p<0.05$ or ^ indicates $p<0.01$compared to Water + 5-FU
Lp: *L. plantarum*, S1.30: Lacl, *L. lactis* subsp. *lactis* SL3.34

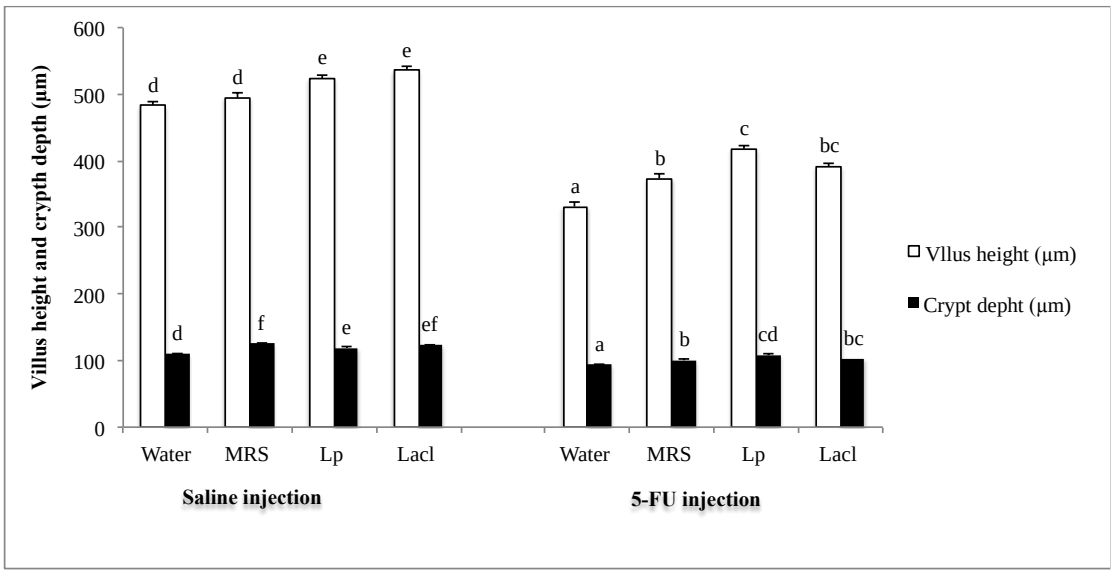


Figure 27 Villus height (VH) and crypt depth (CD) measurements in the rat jejunum for each group on day 8.

Data are expressed as mean (length; µm) ± standard error of the mean. Bar values not sharing the same letter are significantly different ($P < 0.05$); Lp: *L. plantarum*, S1.30: Lacl, *L. lactis* subsp. *lactis* SL3.34.

In the rat jejunum, villus height and crypt depth of 5-FU treated rats was reduced compared with saline treatment (Fig. 27, $P < 0.05$). Administration of Lp and Lacl cultures to saline injected rats contributed to significantly lengthened VH and CD compared with water + saline treatment. Also, rats receiving Lp and Lacl cultures exhibited lengthened VH compared with MRS broth + saline (6% and 8.2%, respectively, $P < 0.05$). Similarly, VH and CD were significantly increased following administration of Lp and Lacl cultures to 5-FU treated rats compared with 5-FU control ($P < 0.05$). Interestingly, administration of MRS broth also significantly lengthened both VH and CD compared to 5-FU control (12.7% and 6.7%, respectively, $P < 0.05$). Administration of different probiotic cultures (Lp and Lacl) either to saline or 5-FU-injected rats equally increased VH and CD in the jejunum.

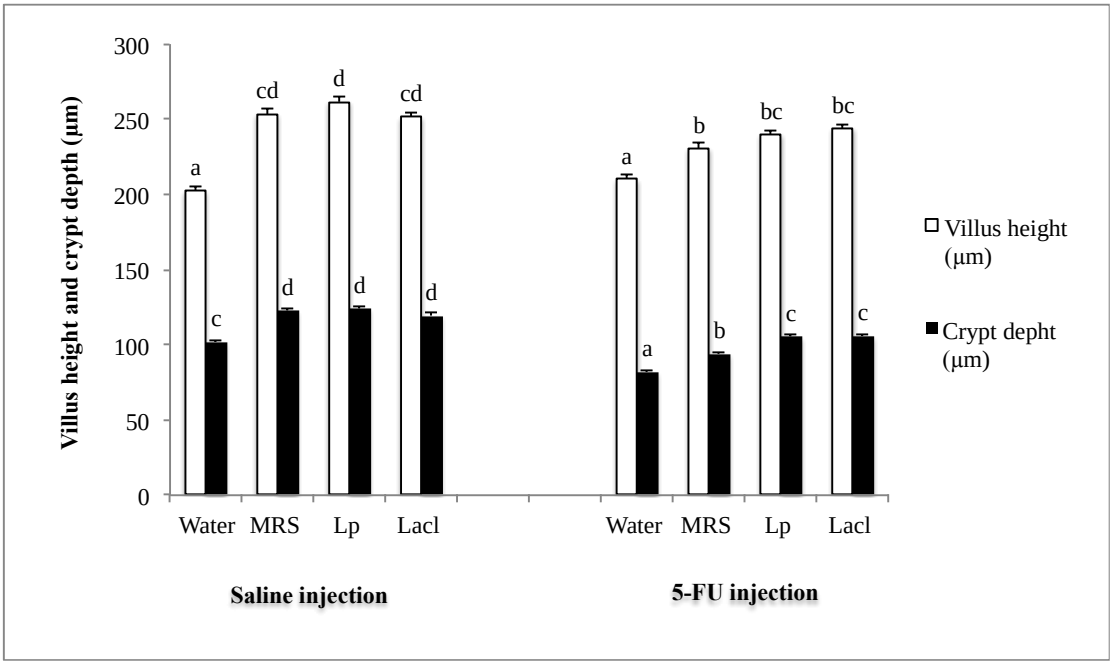


Figure 28 Villus height (VH) and crypt depth (CD) measurements in the rat ileum for each group on day 8.

Data are expressed as mean (length; µm) ± standard error of the mean. Bar values not sharing the same letter are significantly different ($P < 0.05$); Lp: *L. plantarum*, S1.30: Lacl, *L. lactis* subsp. *lactis* SL3.34.

Villus height and crypt depth in the ileum of saline injected rats and 5-FU-treatment groups were increased significantly following administration of MRS broth, Lp and Lacl cultures (Fig. 28, $P < 0.05$). Administration of MRS broth elongated VH and CD in both groups compared with saline and 5-FU treatment. This was also observed with the two-probiotic cultures. In saline injected rats, rats receiving Lp and Lacl cultures significantly elongated both VH (29% and 19%, respectively) and CD (22% and 14%, respectively) compared to saline control (water) ($P < 0.05$). Likewise, in 5-FU treated rats, rats administered LP and Lacl had significantly lengthened VH (14% and 16%, respectively) and CD (28% and 29%, respectively) ($P < 0.05$). No significant differences were detected among rats receiving MRS broth, Lp and Lacl cultures, except in CD of 5-FU treated rats. However, the effect of probiotic administration was equal on VH and CD in the ileum.

5.3.3 Myeloperoxidase (MPO) activity

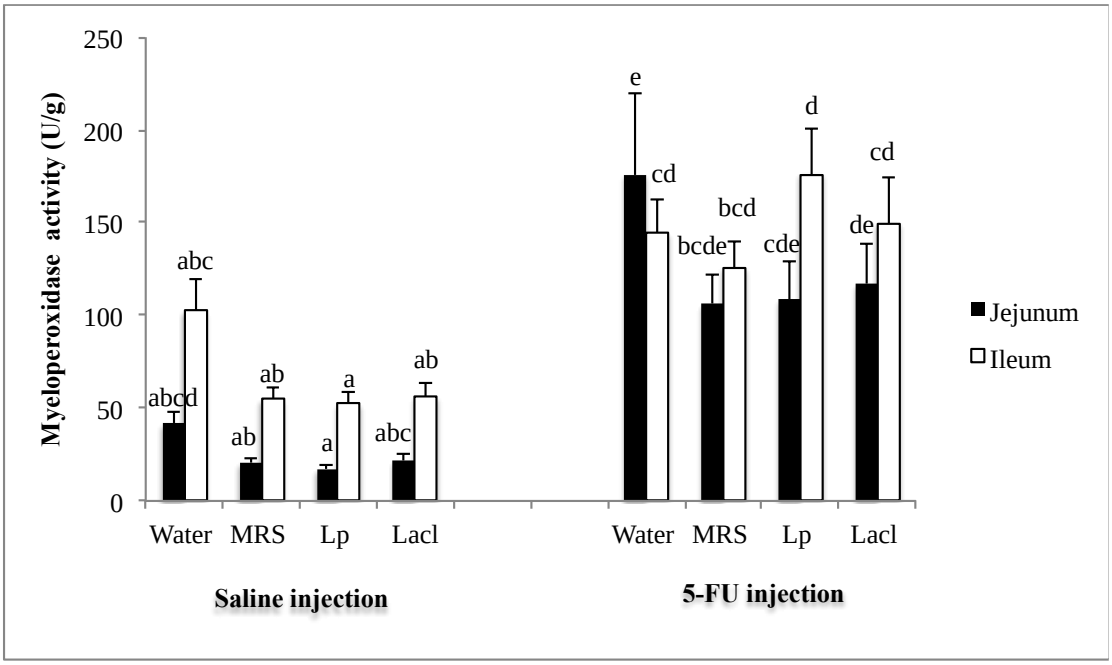


Figure 29 Myeloperoxidase activity in the jejunum and ileum on day 8.

Data are expressed as mean of myeloperoxidase activity (units per gram; U/g) ± standard error of the mean. Bar values not sharing the same letter are significantly different ($P < 0.05$); Lp: *L. plantarum*, S1.30: Lacl, *L. lactis* subsp. *lactis* SL3.34.

Myeloperoxidase activity in the jejunum and ileum of saline injected rats and 5-FU treated rats revealed no significant differences among treatments (Fig. 29, $P > 0.05$). In saline treated rats, rats receiving MRS broth, Lp and Lacl partially reduced MPO activity in the jejunum and ileum compared to saline controls. This effect was also demonstrated in the jejunum, where the MPO activity of 5-FU injected rats treated with MRS broth, Lp and Lacl was lower than the water + 5-FU control. On the other hand, only MRS broth in the ileum decreased MPO activity of 5-FU treated rats.

5.4 Discussion

The present study evaluated the potential for probiotic candidates isolated from dadih to ameliorate 5-FU-induced intestinal damage *in vivo*. Previous studies have shown that probiotics have been able to exhibit therapeutic benefits for the treatment of 5-FU-induced intestinal mucositis (Bowen et al. 2007; Justino et al. 2014; Justino et al. 2015; Wang et al. 2014a; Whitford et al. 2009; Yeung et al. 2015; Yuan et al. 2015). The current study demonstrated that probiotics Lp and Lacl improved some metabolic parameters following 5-FU administration. These metabolic parameters were water intake, urine output, food intake and fecal output. Decreased colon length and gastrointestinal organ

weights (small intestine, increasing duodenum, caecum and stomach) are characteristics observed in mucositis. These symptoms were partially prevented by administration of *L. plantarum* S1.30 and *L. lactis* subsp. *lactis* SL3.34 cultures. The thymus and spleen are important immune system organs which are impacted by 5-FU (immunosuppressive drug) (Itoh et al. 1994). Decreased thymus and spleen weights were observed, consistent with previous studies (Prisciandaro 2012; Wang et al. 2017; Wright et al. 2009). The effects on the thymus were most pronounced with the weight reduction likely attributed to apoptosis and deletion of thymocytes by 5-FU (Eichhorst et al. 2001).

A protective effect from probiotic bacteria (Lp and Lacl) was observed in histological damage analysis and MPO levels. In the context of histological damage, represented as disease severity score, Lp and Lacl were able to decrease disease severity in the jejunum and ileum. This improvement was evidenced by an increased villus height and crypt depth in the jejunum and ileum. Although the minor reduction in MPO levels was not statistically significant, the administration of both probiotic cultures (Lp and Lacl) reduced MPO levels in the jejunum. In contrast, improvement of MPO levels in the ileum was not evident after administration of live bacteria (Lp and Lacl). *Lactobacillus plantarum* has been reported to express immunomodulatory properties. A study in a mouse colitis model showed that *L. plantarum* Lp91 successfully reduced the pro-inflammatory cytokine expression and increased the production of anti-inflammatory cytokines through up regulated anti-inflammation markers, namely COX1, IL-4 and IL-6 (Duary et al. 2012). In a clinical trial, oral consumption of *L. plantarum* P-8 was able to modulate gut microbiota balance, and elevate fecal levels of secretory immunoglobulin A (SIgA), total bile acids (TBAs) and short-chain fatty acids (SCFAs) (Wang et al. 2014b). Fermented soymilk containing *L. lactis* subsp. *lactis* S-SU2 ameliorated inflammation in mice with inflammatory bowel disease, which was likely affected by LPS-stimulated nitric oxide secretion (Kawahara et al. 2015). Similar anti-inflammatory activity was also demonstrated by *L. lactis* subsp. *lactis* BF3 (Kuda et al. 2014). Anti-inflammatory activity of the probiotics in the current study is warranted to further investigate its immunological mechanisms.

The beneficial effect of MRS medium in the treatment of 5-FU-induced rats was evidenced by the reduction of severity damage in the jejunum and ileum. Partial reduction of MPO levels by MRS medium was also apparent. An anti-inflammatory effect derived from MRS medium has been reported previously (Martín et al. 2014; Martín et al. 2015; Prisciandaro 2012). MRS medium comprises a high concentration of porcine neutrophil

elastase inhibitor, serpin B1 (Serpin Proteinase Inhibitor). This compound has anti-inflammatory properties (Martín et al. 2015). Anti-inflammatory mechanisms of serpin B1 containing neutrophil elastase inhibitor have been reported by inhibiting the production of elastase protein during inflammation (Urdaci & Sánchez 2009). However, serpin in MRS medium could be denatured after autoclaving although the medium component could still influence the host immunological condition; therefore it should not be ignored (Pot 2009). Glutamine and glucose are ingredients in MRS medium and are thought to exert a beneficial effect towards metabolic disorders in the host (Prisciandaro 2012). The positive effects of glutamine and glucose have been associated with maintaining epithelial cell integrity by stimulating cell proliferation (Prisciandaro 2012), and the increasing level of anti-apoptotic proteins (Bcl-2 and Bcl-X_L) and to protect enterocytes from LPS-induced apoptosis and barrier defects (Yu et al. 2005), respectively.

In addition to injury of the intestinal mucosal barrier, the pathogenesis of chemotherapy-induced intestinal mucositis also involves shifting the composition of the intestinal microbiota (Tang et al. 2016). The exact mechanisms by which probiotics exert therapeutic effects to alleviate intestinal mucositis remain unknown. However, possible mechanisms include reduction of proinflammatory cytokine secretion and gene expression, improvement of barrier function, maintenance of mucin secretion, prevention of epithelial cell apoptosis and normalization of intestinal homeostasis (Oh et al. 2017). In the current study, the results of the animal trial showed that *L. plantarum* S1.30 and *L. lactis* subsp. *lactis* SL3.34 partially ameliorated the severity of intestinal mucositis by improving metabolic activities and protecting the intestine from damage.

5.5 Conclusion

Variable efficacy of probiotics evaluated in the present study was evident at treating 5-FU-induced damaged *in vivo*. The results suggest that *L. plantarum* S1.30 and *L. lactis* subsp. *lactis* SL3.34 may have potential beneficial effects through partially improving metabolic parameters such as water intake, urine output, food intake, and fecal output in 5-FU challenged rats. The severity of damage in the jejunum and ileum was also improved following probiotic culture treatment. In order to better investigate the therapeutic potential of these probiotics, further studies are required to assess utilization of different dosing regimens and concentrations of *L. plantarum* S1.30 and *L. lactis* subsp. *lactis* SL3.34. In addition, evaluation and characterization of soluble compounds secreted by these probiotics could also be incorporated in future studies. Identification and characterization of the components of probiotic growth should also be further investigated.

Chapter 6

General Discussion and Summary

6.1 Introduction

Naturally fermented milk products (NFM), often referred to as functional foods, are still produced in many regions of the world, even though this is an ancient form of milk preservation. Microorganism action during the fermentation process improves nutritional content (vitamins, conjugated linoleic acid, bioactive peptides, exopolysaccharides, gamma-aminobutyric acid and oligosaccharides) of the products, which are known for their health-promoting attributes (Fernández et al. 2015). The indigenous microorganisms in these products also contribute to either organoleptic or physicochemical characteristics, as well as impacting on human health via their probiotic properties. Traditional fermented milk products are regarded as important nutrient sources for local/ethnic people in some developing countries. Although NFM products are generally considered safe, contamination with spoilage and pathogenic bacteria becomes a threat for product safety and quality (Mufandaedza et al. 2006). Hence, improvements in NFM product safety and quality are imperative to preserve NFM integrity among industrial fermented milk products. Furthermore, evaluating probiotic microorganisms in these products is also essential in order to identify novel probiotic candidates to raise their economic and health value.

The microbial composition in traditional fermented milk products is variable, depending on the production process, sample type and the locality (Zhong et al. 2016). In the current study, NFM products from Indonesia, namely dadih and dangke, were studied. The purposes of this study were determined as: 1) to assess the microbial diversity of the fermented milk products, 2) to isolate indigenous probiotic candidates from Indonesian NFM products, 3) to characterize the probiotic properties using *in vitro* assays and molecular techniques, and 4) to evaluate the capacity for the selected probiotic candidates to reduce the severity of intestinal mucositis in a rat model.

6.2 Microbial Diversity and Dynamics in Dadih and Dangke

The present study was designed to be an ecological study of the microbiota of dadih and dangke using culture-dependent and -independent approaches. This study was the first

to utilize pyrosequencing to analyse the microbial structure and dynamics of dadih and dangke samples. A culture-dependent method was used to support the culture-independent method results as well as to identify potential strains for further applications, either as starter cultures or for probiotic characterization. In general, three microbial groups were identified in dadih and dangke using a combination of these methods, namely lactic acid bacteria (LAB), yeasts and acetic acid bacteria (**Chapters 2 and 3**). However, acetic acid bacteria are only occasionally found in NFM products, including dadih and dangke.

In dadih, members of the lactic acid bacterial group detected using culture-dependent technique were *Lactobacillus plantarum, Lactococcus lactis* subsp. *lactis*, and *Enterococcus faecium.* Only one species of acetic acid bacteria was found, namely *Acetobacter orientalis* whilst yeast isolates were identified as *Saccharomyces cerevisiae*, *Candida metapsilosis* and *Kluyveromyces marxianus,* with *C. metapsilosis* the principal yeast found in dadih. Other bacteria, such as *Klebsiella oxytoca*, *Klebsiella* sp. and *Bacillus pumilus* were also detected. Among these bacteria, *L. plantarum* was the most frequently isolated LAB from dadih, followed by *L. lactis* subsp. *lactis*.

The indigenous microbiota in dangke was relatively similar to dadih, with the exception that *E. faecium* and *B. pumilus* were not found in dangke. *Lactococcus lactis* subsp. *lactis* was the most predominant LAB in dangke, while *S. cerevisiae* was the most frequently isolated yeast. However, based on a culture-independent method (pyrosequencing), genus *Lactococcus* revealed the greatest relative abundance in dadih. The type of milk utilized in dangke production affected microbiota composition. For example, *Enterobacteriaceae* were predominant in dangke from buffalo milk, whereas genus *Lactococcus* dominated dangke prepared from cows' milk.

Coexistence of LAB and yeasts has been observed in many traditional fermented milk products (Abdelgadir et al. 2001; Akabanda et al. 2013; Gadaga, Mutukumira & Narvhus 2000; Kebede et al. 2007; Lore, Mbugua & Wangoh 2005; Panda et al. 2016; Rahman et al. 2009; Zhang et al. 2008c). Interaction among the microbial consortia provides benefits to product development. These include the production of desirable product characteristics through a variety of metabolic mechanisms and increasing resistance against bacteriophage attack as compared to single or dual strain cultures (Smid & Lacroix 2013). Lactic acid bacteria play a fundamental role in acidifying the milk and producing antimicrobial substances such as bacteriocins and organic acids. These mechanisms facilitate product shelf-life and safety. Moreover, LAB also determine product aroma as a secondary functionality (Smid & Kleerebezem 2014). Meanwhile, the role of yeasts is associated with flavour development through lipolytic and proteolytic enzymatic

activities (Jakobsen & Narvhus 1996; Narvhus & Gadaga 2003). Although acetic acid bacterium *A. orientalis* was detected at low levels in dadih and dangke it has an important role. Besides utilizing ethanol produced by LAB and yeasts, *A. orientalis* is characterized by its ability to oxidize acetate and lactate to CO_2 and H_2O (Lisdiyanti et al. 2001). The role of acetic acid bacteria is related to development of flavour and texture of the final products (Ongol & Asano 2009). The interaction between LAB, yeasts and acetic acid bacteria has been well studied in kefir (Altay et al. 2013), mashita from Uganda (Ongol & Asano 2009) and cocoa bean fermentation (De Vuyst & Weckx 2016). Therefore, it is important to isolate acetic acid bacteria using a suitable medium. The biological impact of the specific interaction between LAB, yeasts and acetic acid bacteria in dadih and dangke requires further investigation, especially their role in developing organoleptic characteristics.

The dynamic nature of dadih fermentation and microbial succession pattern over the course of 3-day fermentations was elucidated using a metagenomic sequencing approach (**Chapter 2**). The raw buffalo milk was dominated by psychrotrophic bacteria, namely genus *Pseudomonas*. However, during the early stages of fermentation, this genus was absent and replaced with family Enterobacteriaceae and genus *Lactococcus.* Subsequently, *Lactococcus* became more prevalent in the latter stages (days 2 and 3). A significant reduction of pathogenic contaminants indicated an improvement in food safety as a result of acidification of the milk and antimicrobial activity performed by *Lactococcus* and other LAB members (Smid & Kleerebezem 2014). A microaerophilic environment also plays a role as a stressor to non-LAB. This result suggested that dadih would be ready to be consumed safely after fermentation was achieved on day 2, as generally applied by the local people. In contrast, dangke is ready to be consumed even without fermentation. Metagenomic analysis detected the presence of family *Enterobacteriaceae* in dangke made from buffalo milk. Even though pre-treatment (heating) of milk and salt addition are important elements of dangke production, hygienic conditions must be a concern during manufacturing. In addition, as dangke characteristics are similar to cheese, a greater fermentation period is highly recommended to eliminate undesirable microorganisms, similar to that used in common cheese production.

Fermentation products allow well-adapted microorganisms to contribute to product quality and safety. As a result, microbial diversity is generally lower than that of other ecosystems, such as soil and the mammalian gut (van Hijum, Vaughan & Vogel 2013). Pyrosequencing generates less sequence depth than the latest next-generation sequence (NGS) technology, such as Illumina paired-end read technology. Therefore, in future, the

use of more advanced NGS technology is recommended to gain greater insight into microbial ecology and taxonomy, as well as the functional potential of the microbiota, including bacteria and moulds (yeasts) in dadih and dangke. Furthermore, increased sample numbers of NFM products from different geographical regions is also suggested to obtain more representative data.

The presence of probiotic strains in fermented milk products is another health-promoting attribute. In the current study, probiotic candidates were derived from dadih (**Chapter 4**). This indicated that dadih was an effective probiotic carrier compared to dangke. Since the LAB number in dadih was more than 10^6 cfu/g or 6 log cfu/g, the minimum potential therapeutic effect of probiotics was likely achieved. The recommended probiotic concentration is approximately 6- to 7-log cfu/g of food to offer optimum health benefits (Lourens-Hattingh & Viljoen 2001). Dairy products have generally used a probiotic vehicle, such as yogurt, kefir and cheese. Dadih is considered to be a yogurt-like product. Apart from the fermentation conditions and post-production factors (transport and storage conditions), physicochemical factors (fat and protein content, type of protein, sugar and pH), the presence of food ingredients (stabilizer, sweetener and flavor agents), and added value ingredients (vitamins and bioactive peptides) in the food matrix are important considerations to maintain probiotic functionality (Coman et al. 2012). Among these factors, only dadih matrix has the physicochemical parameters to support probiotic performance (viability and efficacy). Moreover, according to its sensory profile (taste, aroma, texture and flavour), dadih is acceptable for consumption by all age groups, and is suitable to be mixed with fruits or other foods. In comparison, dangke is also supposed to be a suitable probiotic carrier. However, sometimes dangke produces a strong bitter taste as a result of the papaya latex being added to excess (Surono et al. 1984). This influences product acceptance based on the sensory profile (taste). As consistency during manufacturing process is essential, standardization of the process is required.

6.3 Probiotics Isolated from Dadih Partially Improve Parameters of 5-Fluorouracil-Induced Intestinal Mucositis

Probiotics isolated from dadih and dangke have been assessed via *in vitro* studies (**Chapter 4**), and revealed *L. plantarum* S1.30 to be the optimal probiotic candidate. Besides this bacterial strain, *L. lactis* subsp. *lactis* SL3.34 was also applied in the *in vivo* study as the most abundant *Lactococcus* genus identified in the pyrosequencing study. These bacteria were isolated from dadih. The *in vivo* study was designed to determine the

potential for the probiotics to reduce the severity of 5-FU-induced intestinal mucositis in rats (**Chapter 5**). In this chapter, probiotic-based therapies (*L. plantarum* S1.30 and *L. lactis* subsp. *lactis*) were partially effective in ameliorating indicators of intestinal damage including metabolic parameters (water intake, urine output, food intake and fecal output) and maintaining colon length and gastrointestinal organ weights (small intestine, duodenum, caecum and stomach). Additionally, these probiotic cultures also reduced mucositis disease severity in the jejunum and ileum by increasing villus height and crypt depth, compared with 5-FU treated control rats. The positive effects of the probiotics were likely to be associated with either live bacteria or probiotic factors derived from the supernatants (Prisciandaro et al. 2011). In future studies, probiotic supernatants from NFM should be studied to determine their potential efficacy against intestinal injury. In addition, evaluation of microorganisms, either in fecal or intestinal contents, is essential to achieve an overview of probiotic effects on gut microbiota modulation. Overall, probiotics from dadih, either singly or as a mixture, require further exploration to determine their potential to maintain bowel health and protect against intestinal disease.

Beneficial effects of the probiotic growth media were observed on the intestinal cells. Constituents of MRS broth such as porcine neutrophil elastase inhibitor (serpin B1) (Martín et al. 2015) as well as glutamine and glucose (Prisciandaro 2012) were most likely to be responsible for the observed effect (reduction of damage severity in the jejunum and ileum). Anti-inflammatory mechanisms of serpin B1 containing neutrophil elastase inhibitor have been reported by inhibiting the production of elastase protein during inflammation (Urdaci & Sánchez 2009). Meanwhile, the protective effect of glutamine has been associated with maintaining epithelial cell integrity by stimulating cell proliferation (Prisciandaro 2012). MRS media contains a high level of glucose (Oxoid 2017). In addition, high glucose concentrations have been reported to increase the level of anti-apoptotic proteins (Bcl-2 and Bcl-X_L) and to protect enterocytes from LPS-induced apoptosis and barrier defects (Yu et al. 2005). In order to determine the contribution of MRS broth to the effects on intestinal mucositis further studies employing other media, saline and water are indicated.

The use of NFM products containing probiotics for intestinal mucositis treatment is not recommended, since injury of the intestinal mucosal barrier leads to increased vulnerability to infection. This study showed that potential harmful bacteria in NFM products are still detectable, although in an insignificant number. The preferred approach would be to use the selected probiotics in pure cultures, which could then be incorporated in an appropriate carrier in order to increase viability across the digestive tract. However,

consumption of NFM products for maintaining human health is suggested, as long as optimal manufacturing practices and food safety standards are maintained.

6.4 Summary

The existence of NFM products as a potential source of technologically important microorganisms for sustainability of food technology and health-promoting agents is important. LAB and yeast play a significant role in product quality and safety. In the current study, the pyrosequencing method was used successfully to address food safety concerns in consuming NFM products such as dadih and dangke. New knowledge on the composition of indigenous microorganisms during fermentation has increased the confidence level for consuming NFM products, especially dadih. This study also increases our understanding of the important contribution of hygiene and environmental sanitation during the production of NFM products. These are critical features to eliminate the presence of undesirable microorganisms, based on lessons learnt from dangke. Probiotics from dadih are considered to be potential candidates to reduce the severity of chemotherapy-induced intestinal mucositis. Further studies to determine the potential efficacy of these probiotics against other intestinal disorders are therefore warranted. Thus, dadih is considered to be the preferred product to obtain benefits including nutritional value, digestibility, therapeutic benefits, and safety against pathogens.

Appendices

Appendix 1 Molecular and phenotypic characterization of bacterial isolates from dadih and dangke samples

Isolate number			ITS	RFLP		Cell-shaped	Gram staining	Catalase test	Oxidase test	Colony morphology							
Bamboo	Dadih	Dangke	(bp)	HinfI	HindIII					Shape	Size (mm)	Margin	Elevation	Surface	Texture	Colour	Mucoid
0	23	1	700	90, 200, 440	700	Rod	+	-	-	Round	1-3	Entire	Raised	Glistening	Moist	Opaque	No
2	13	9	700	50, 200	700	Coccus	+	-	+	Round	1-3	Entire	Raised	Glistening	Moist	Opaque	No
0	7	1	700	80, 190, 220	220, 480	Coccus	+	-	+	Round	1-3	Entire	Raised	Glistening	Moist	Opaque	No
0	2	0	700	80, 150, 500	200, 500	Coccus	+	-	-	Round	< 1	Entire	Convex	Dull	Moist	Opaque	No
0	1	1	700	200, 500	700	Rod	-	+	+	Round	< 1	Entire	Raised	Dull	Moist	Opaque	No
0	1	1	700	800	250, 500	Rod	-	-	-	Round	< 1	Entire	Convex	Glistening	Moist	Opaque	No
0	5	6	700	50, 190, 220	700	Rod	-	-	+	Round	1-3	Entire	Raised	Glistening	Viscous	Opaque	Yes
1	7	0	800	80, 170, 200	280, 400	Rod	+	+	+	Round	1-3	Entire	Raised	Wrinkled	Dry	Opaque	No
3	59	19															

*Group I: *L. plantarum*; group IIA&B: *L. lactis* subsp *lactis*; group III: *E. faecium*; group IV: *A. orientalis*; group V: *K. oxytoca*; group VI: *Klebsiella* sp.; group VII: *B. pumilus*

Appendix 2 Molecular and phenotypic characterization of yeast isolates from dadih and dangke samples

Isolate number			ITS (bp)	RFLP		Cell-shaped	Catalase test	Oxidase test	Colony morphology							
Bamboo	Dadih	Dangke		HinfI	HindIII				Shape	Size (mm)	Margin	Elevation	Surface	Texture	Colour	Mucoid
0	5	13	800	50, 110, 310	150, 180, 230, 320	Ellipsoidal	+	+	Round	1-3	Entire	Raised	Dull	Butyrous	Opaque	No
1	7	0	500	80, 260	300, 500	Ellipsoidal	+	+	Round	1-3	Entire	Raised	Dull	Butyrous	Opaque	No
1	3	9	750	80, 120, 185, 240	80, 600	Ellipsoidal	+	+	Round	1-3	Entire	Raised	Dull	Butyrous	Opaque	No
2	15	22														

*Group I: *S. cerevisiae*; group II: *C. metapsilosis*; group III: *K. marxianus*

Appendix 3 Bacterial composition in dadih samples

Group*	Fresh buffalo milk		Solok Day 1		Solok Day 2		Solok Day 3		Gadut Day 3		Bamboo		Total
	Number	Isolate	Number	Isolate	Number	Isolate	Number	Isolate	Number	Isolate	Number	Isolate	
I	1	SL0.17	0		7	SL2.2, SL2.4, SL2.5, SL2.6, SL2.7, SL2.9, SL2.10	9	SL3.1, SL3.2, SL3.3, SL3.4, SL3.5, SL3.6, SL3.7, SL3.8, SL3.10	6	GD.1, GD.2, GD.3, GD.4, GD.5, GD.6	0		23
IIA	3	SL0.3, SL0.10, SL0.15	1	SL1.9	3	SL2.1, SL2.13, SL2.15	6	SL3.14, SL3.16, SL3.19, SL3.22, SL3.24, SL3.27,	0		2	B.5, B.6	15
IIB	1	SL0.11	3	SL1.16, SL1.17, SL1.19	1	SL2.14	2	SL3.31, SL3.34			0		7
III	0		0		0		1	SL3.26	1	GD.12	0		2
IV	0		0		0		1	SL3.28	0		0		1
V	0		1	SL1.12	0		0		0		0		1
VI	2	SL0.6, SL0.7	1	SL1.18	0		0		2	GD.14, GD.15	0		5
VII	2	SL0.4, SL0.5	4	SL1.6, SL1.7, SL1.8, SL1.14	0		0		1	GD.13	1	B.3	8

*Group I: *L. plantarum*; group IIA&B: *L. lactis* subsp *lactis*; group III: *E. faecium*; group IV: *A. orientalis*; group V: *K. oxytoca*; group VI: *Klebsiella* sp.; group VII: *B. pumilus*

Appendix 4 Bacterial composition in dangke samples

Group*	Dangke buffalo milk A		Dangke buffalo milk B		Dangke buffalo milk C		Dangke-Cow's milk		Total
	Number	Isolate	Number	Isolate	Number	Isolate	Number	Isolate	
I	0		0		1	D3.17	0		1
IIA	3	D1.1, D1.2, D1.7	4	D2.9, D2.15, D2.17, D2.20	1	D3.6	1	DC.6	9
IIB	0		0		1	D3.11	0		1
III	0		0		0		0		0
IV	0		1	D2.25	0		0		1
V	0		0		1	D3.10	0		1
VI	4	D1.8, D1.9, D1.10, D1.15	0		2	D3.1, D3.14	0		6
VII	0		0		0		0		0
Total	7		5		6		1		19

*Group I: *L. plantarum*; group IIA&B: *L. lactis* subsp *lactis*; group III: *E. faecium*; group IV: *A. orientalis*; group V: *K. oxytoca*; group VI: *Klebsiella* sp.; group VII: *B. pumilus*

Appendix 5 Yeast composition in dadih samples

Group*	Fresh buffalo milk		Solok Day 1		Solok Day 2		Solok Day 3		Gadut Day 3		Bamboo		Total
	Number	Isolate	Number	Isolate	Number	Isolate	Number	Isolate	Number	Isolate	Number	Isolate	
I	0		5	SL1.1, SL1.2, SL1.3, SL1.4, SL1.5	0		0		0		0		5
II	2	SL0.12, SL0.14	0		0		1	SL3.12	6	GD.16, GD.17, GD.18, GD.19, GD.20, GD.21	1	B.1	10
III	0		0		1	SL2.13	0		0		1	B.8	2
Total	2		5		1		1		6		2		17

*Group I: *S. cerevisiae*; group II: *C. metapsilosis*; group III: *K. marxianus*

Appendix 6 Yeast composition in dangke samples

Group*	Dangke buffalo milk A		Dangke buffalo milk B		Dangke buffalo milk C		Dangke-Cow's milk		Total
	Number	Isolate	Number	Isolate	Number	Isolate	Number	Isolate	
I	0		6	D2.1, D2.2, D2.3, D2.6, D2.10, D2.13	0		7	DC.1, DC.3, DC, 7, DC.8, DC.10, DC.13, DC.14	13
II	0		0		0		0		0
III	0		5	D2.21, D2.22, D2.23, D2.24, D2.26	0		4	DC.11, DC.12, DC.15, DC.16	9
Total	0		11		0		11		22

*Group I: *S. cerevisiae*; group II: *C. metapsilosis*; group III: *K. marxianus*

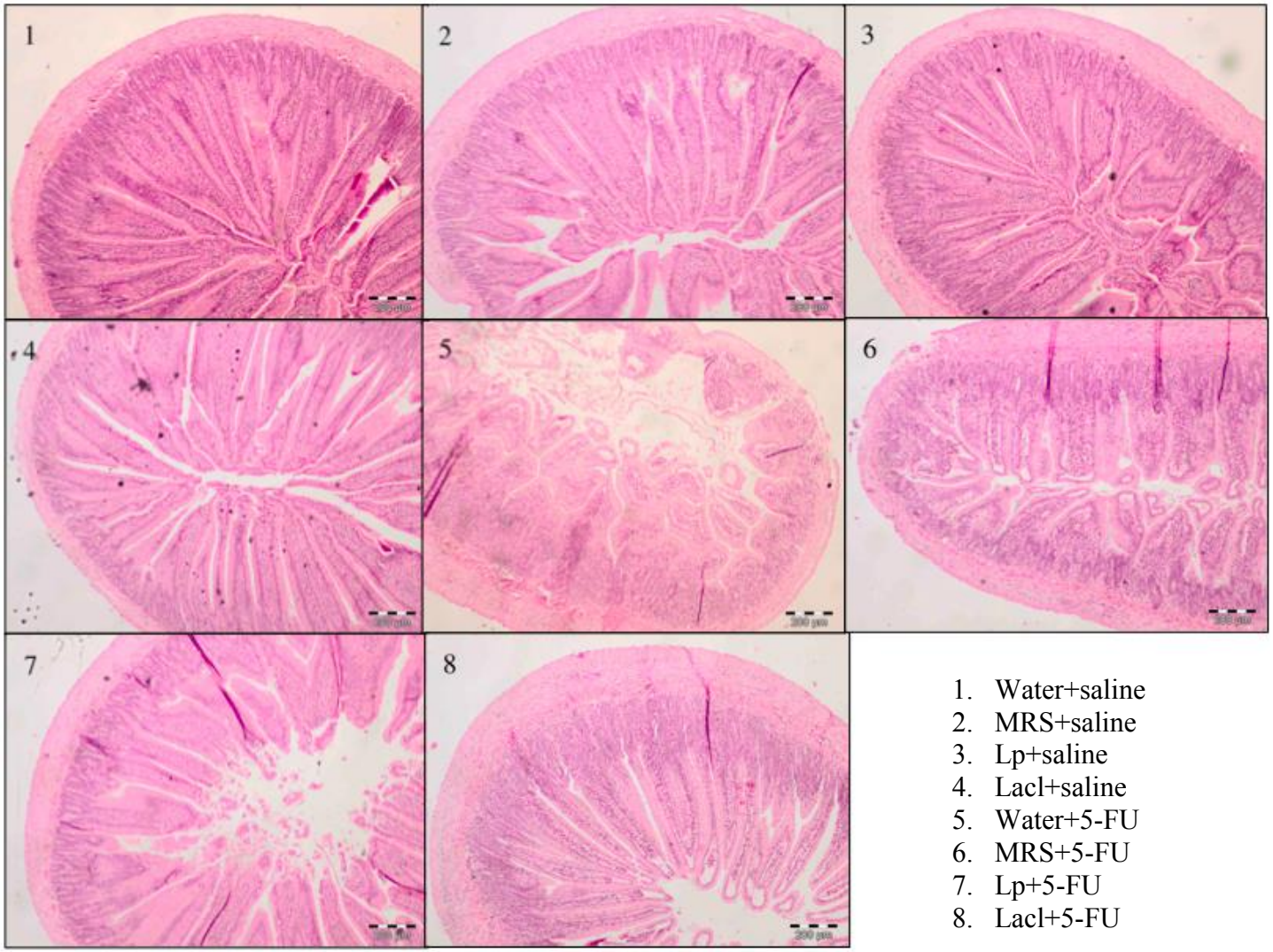
Appendix 7 Diameter of inhibition zone (mm) of *Lactobacillus plantarum* strains against pathogenic bacteria

No	Strains	*Bacillus cereus*	*Enterococcus faecalis* ATCC 29212	*Listeria monocytogenes*	*Staphylococcus aureus* ATCC 25923	*Streptococcus pyogenes* ATCC 10389	*Escherichia coli* 99364-1	*Escherichia coli* 99386-3	*Salmonella typhimurium* P135	*Salmonella sofia*
1	S1.30	0	10.5	10	0	11	8.5	8.5	0	0
2	*L. plantarum* ATCC 14941	0	10.5	10	0	11	8	8	0	0
3	SL0.17	0	9.5	9	0	11	8	8.5	0	0
4	SL2.2	0	9.5	9	0	11	8	8.5	0	0
5	SL2.4	0	0	0	0	0	8	8	0	8
6	SL2.5	0	9	9	0	10	8	8	0	0
7	SL2.6	0	9	0	0	0	0	0	0	0
8	SL2.7	0	8.5	8	0	9.5	8	8	0	0
9	SL2.9	0	9	9	0	10.5	8	8	0	0
10	SL2.10	0	9	9	0	10.5	8	0	0	0
11	SL3.1	0	0	8	0	10.5	8	8	0	8
12	SL3.2	0	9	8.5	0	10.5	8	8	0	8
13	SL3.3	0	9	8.5	0	10.5	8	0	8	0
14	SL3.4	0	8.5	9	0	10.5	8	0	8	0
15	SL3.5	0	9	9	0	10	8	0	8	8
16	SL3.6	0	9	9	0	9.5	8	0	8	0

17	SL3.7	0	9	9	0	9.5	8	0	8	8
18	SL3.8	0	9	9	0	10	9	9	8	8
19	GD.1	0	9.5	8	0	9.5	8	8.5	0	8
20	GD.2	0	9.5	8	0	9.5	8	8	0	0
21	GD.3	0	9.5	8	0	10	8	8	0	0
22	GD.4	0	9	8	0	10	8	8	0	0
23	GD.5	0	9.5	7.5	0	10	0	8	0	0
24	GD.6	0	9	8	0	9	0	8	0	0

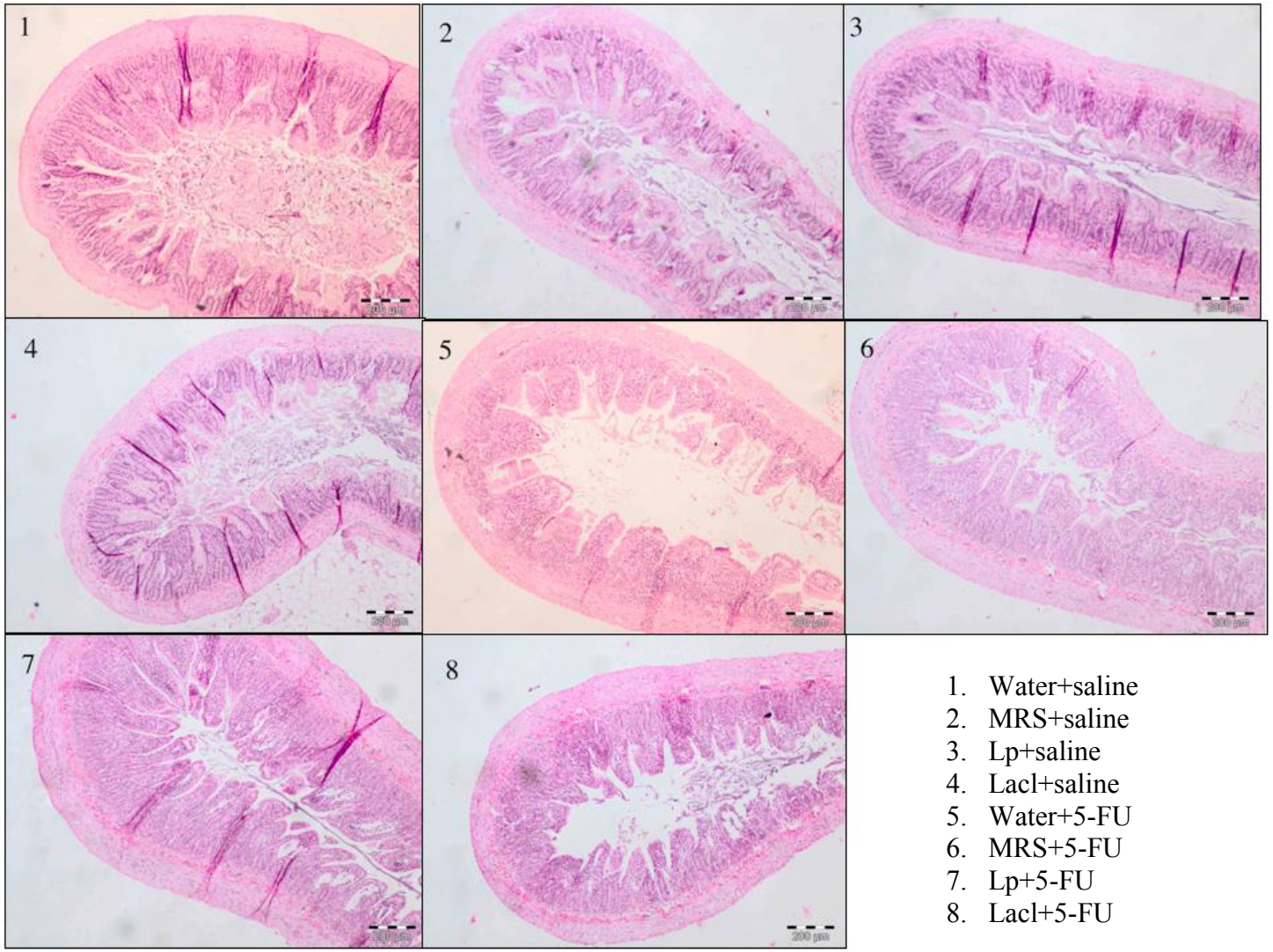
*The diameter was including the well diameter (6 mm)

Appendix 8 Photomicrograph of jejunum post-injection with saline and 5-fluorouracil (5-FU) (magnification 40X)



1. Water+saline
2. MRS+saline
3. Lp+saline
4. Lacl+saline
5. Water+5-FU
6. MRS+5-FU
7. Lp+5-FU
8. Lacl+5-FU

Appendix 9 Photomicrograph of ileum post-injection with saline and 5-fluorouracil (5-FU) (magnification 40X)



1. Water+saline
2. MRS+saline
3. Lp+saline
4. Lacl+saline
5. Water+5-FU
6. MRS+5-FU
7. Lp+5-FU
8. Lacl+5-FU

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