ISOLATION AND ANTIMICROBIAL POTENCY OF INDIGENOUS LACTIC ACID BACTERIA ISOLATED FROM DADIH, A TRADITIONAL FERMENTED BUFALLO MILK FROM INDONESIA

by

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Table of Contents

Page

Table of Contents ..... **i**

List of Figures ..... **v**

List of Tables ..... **vi**

Glossary ..... **vii**

Summary ..... **ix**

Declaration ..... **xi**

Acknowledgments ..... **xii**

CHAPTER ONE **1**

GENERAL INTRODUCTION **1**

1.1 Background information **1**

1.2 Naturally fermented milk (NFM) products **2**

- **Origin of indigenous micro-flora** **4**
- **Small-scale producers/ rural communities** **4**
- **Traditional vessels** **4**
- **Incubation temperatures** **5**
- **Poor standard sanitary conditions** **5**

1.3 Dadih **5**

1.4 General characteristics of lactic acid bacteria **7**

1.5 Identification of lactic acid bacteria **7**

1.5.1 Phenotypic methods 8

1.5.2 Genotypic methods 10

1.6 Bacteriocins of lactic acid bacteria **11**

1.7 Bacteriocin detection methods **13**

1.8 Characterization of bacteriocin activities **14**

1.9 Potency of naturally fermented milk products as habitat of bacteriocin-producing LAB **15**

1.10 Molecular identification and characterization of micro-flora to support bacteriocin detection **16**

1.11 Objectives of this study **18**

CHAPTER TWO **19**

ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF INDIGENOUS LACTIC ACID BACTERIA FROM DADIH **19**

2.1 Introduction **19**

2.1.1 Aims **20**

2.2 MATERIALS AND METHODS **20**
2.2.1 Materials **20**
2.2.1.1 Dadih samples 20
2.2.1.2 Bacterial strains 20
2.2.2.3 Media 20
2.2.2.3.a Solid media 20
2.2.2.3.b Liquid media 20
2.2.2.4 Chemicals and reagents 21
2.2.2.5 Enzymes 21
2.2.2.6 PCR reagents and purification kit 21
2.2.2.7 Buffer solutions 21
2.2.2 Methods **22**
2.2.2.1 Isolation of LAB 22
2.2.2.2 Phenotypic characterization of LAB 22
2.2.2.3 Molecular characterization of LAB 23
2.2.2.3.a DNA template preparation for PCR 23
2.2.2.3.b PCR amplification 23
2.2.2.3.c Agarose gel electrophoresis 23
2.2.2.3.d RFLP analysis 24
2.2.2.4 Molecular identification of LAB 24
2.2.2.4.a PCR amplification 24
2.2.2.4.b Purification of DNA products 24
2.2.2.4.c DNA sequencing analysis 25
2.3 Results **26**
2.3.1 Predominant microorganisms in dadih **26**
2.3.2 Biochemical and microscopic characterization of bacteria and yeasts **27**
2.3.3 Molecular characterization of LAB **28**
2.3.4 Molecular identification of LAB **29**
2.4 Discussion **33**
2.5 Conclusion **35**
CHAPTER THREE **37**
INVESTIGATION OF LACTIC ACID BACTERIA ISOLATED FROM DADIH FOR PRODUCTION OF BACTERIOCINS **37**
3.1 Introduction **37**
3.1.1 Aims **37**
3.2 Materials and Methods **38**
3.2.1 Materials **38**
3.2.1.1 Bacterial strains 38
3.2.1.2 Media 38
3.2.1.2.a Solid media 38
3.2.1.2.b Liquid media 38
3.2.1.3 Chemical and reagents 39
3.2.1.3.a Chemicals 39
3.2.1.3.b Enzymes 39

3.2.1.3.c Buffer solutions 39
3.2.1.3.d PCR reagents 39
3.2.2 Methods 39
3.2.2.1 Screening of antimicrobial activity 39
3.2.2.1.a Agar spot test 40
3.2.2.1.b Agar well-diffusion 40
3.2.2.2 Conventional detection of bacteriocins 41
3.2.2.3 Production kinetics of an antimicrobial product from a potential LAB isolate 41
3.2.2.4 Molecular detection of bacteriocins 42
3.2.2.5 Bacteriocin induction in co-culture 42
3.3 Results 44
3.3.1 Screening of antimicrobial activity 44
3.3.2 Detection of bacteriocin activity 44
3.3.3 Production kinetics of antimicrobial substances from *Lactobacillus plantarum* S1.30 46
3.3.4 Molecular detection of genes related to bacteriocin activity 47
3.3.5 Detection of bacteriocin in a co-cultivation approach 48
3.4 Discussion 48
3.5 Conclusion 50
CHAPTER FOUR 51
MOLECULAR CHARACTERIZATION AND IDENTIFICATION OF YEASTS ISOLATED FROM DADIH 51
4.1 Introduction 51
4.1.1 Aims 51
4.2 Materials and Methods 52
4.2.1 Materials 52
4.2.1.1 Isolates 52
4.2.1.2 Media 52
4.2.1.3 Chemical and Reagent 52
4.2.1.4.a Chemical 52
4.2.1.4.b Enzymes 52
4.2.1.4.c PCR reagents and purification kit 52
4.2.1.4.d Buffer solutions 53
4.2.2 Methods 53
4.2.2.1 Molecular characterization 53
4.2.2.2 Evaluation of lactate-utilizing yeasts 53
4.3 Results 54
4.3.1 Molecular characterization and identification of dadih yeasts 54
4.3.2 Utilization of lactic acid by yeasts 55

4.4 Discussion **56**
4.5 Conclusion **57**
CHAPTER FIVE **58**
GENERAL DISCUSSION AND CONCLUSION **58**
References **62**
Appendices **73**
Appendix A Phenotypic and molecular characterization of bacteria isolated from dadih **73**
Appendix B Cellular morphology of LAB, yeasts and acetic acid bacteria isolated from dadih (magnification: 1000x) **77**
Appendix C Molecular characterization of LAB isolated from dadih **78**
Appendix D Phenotypic and molecular characterization of yeasts isolated from dadih **82**
Appendix E Antimicrobial screening of LAB isolated from dadih using agar spot test **84**

List of Figures

Figure 1 Dadih manufacturing process 6
Figure 2 Proportion of LAB and yeasts in dadih from different sites (S and G) and from two and three day-fermentations 27
Figure 3 PCR amplification of 16S-23S rRNA gene ISR 29
Figure 4 Representation of PCR products that were sequenced generated by universal primers targeting 16S rRNA V1-V2 variable regions 30
Figure 5 Cladogram of partial 16S rRNA gene sequences of the selected isolates representing 30
Figure 6 Gel electrophoresis of PCR-amplified 16S-23S rRNA- ITS region digested with *RsaI* 31
Figure 7 Gel electrophoresis of PCR-amplified tRNA genes of the *Lb. paracasei* strains digested with *Hinf*I 31
Figure 8 Agarose gel electrophoresis of *Lb. paracasei* using species-specific primers 32
Figure 9 Agarose gel electrophoresis of PCR-amplified species-specific *Lb. plantarum* S1.30 (16-F and plantarum-R) 32
Figure 10 The number of dadih isolates selected randomly from the isolation medium 33
Figure 11 Effect of growth phase on antimicrobial production from *Lb. plantarum* S1.30 against *L. monocytogenes* and *Lb. sakei* ATCC 15521 47
Figure 12 Amplification product of plantaricin A (450 bp) 48
Figure 13 5.8S rRNA-ITS PCR products and their restriction analysis with the endonuclease 55
Figure 14 The utilization of D/L-lactate by dadih yeasts incubated for 48 h 56

List of Tables

Table 1 Naturally fermented milk products with identified micro-flora and bacteriocin-producing bacteria 2
Table 2 Morphological and biochemical characteristics of lactic acid bacteria 9
Table 3 Classification of bacteriocins 12
Table 4 List of primers used in this study 25
Table 5 PCR amplification condition used for the characterization and identification of LAB isolated from dadih 26
Table 6 Grouping of LAB based on ITS-PCR results 28
Table 7 List of bacterial strains used in this study as indicator bacteria and controls 43
Table 8 Antimicrobial activity of selected dadih LAB strains 45
Table 9 Characterization and identification of dadih yeasts 54

Glossary

ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BLIS	Bacteriocin-like inhibitory substances
bp	Base pair(s)
CFS	Cell-free supernatant
CFU	Colony Forming Units
CO_2	carbon dioxide
dATP	2-deoxy-adenosine-5-triphosphate
dCTP	2-deoxy-cytidine-5-triphosphate
dGTP	2-deoxy-guanosine-5-triphosphate
DNA	Deoxyribose nucleic acid
dNTPs	Deoxynucleotide triphosphates
dTTP	2-deoxy-thymin-5-triphosphate
EDTA	Ethylenediamine tetra acetic acid
g	gram(s)
g	Gravity
h	Hour(s)
ISR	Internal spacer region
ITS	Intergenic transcribed spacer
L	litre
LAB	Lactic acid bacteria
mg	milligram(s)
$MgCl_2$	Magnesium chloride
MilliQ H_2O	Milli-Q filtered deionized water
min	Minute(s)
ml	millilitre
mm	millimetre(s)
mM	millimoles L^{-1}
MRS	de Man, Rogosa and Sharpe
NaCl	Sodium chloride
NCFS	Neutralized cell-free supernatant

NFM	Naturally fermented milk
PCR	Polymerase chain reaction
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal RNA
TAE	Tris-acetic EDTA
Taq	*Thermus aquaticus* DNA polymerase
Tris	Tris (hydroxymethyl) aminomethane
TSA	Tryptone soya agar
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
µg	Microgram(s)
µl	Microlitre(s)
µM	Micromoles L^{-1}
v/v	Volume per volume
w/v	Weight per volume
YPD	Yeast peptone dextrose

Summary

Dadih is a traditional fermented fresh buffalo milk from Indonesia that is categorized as a functional food or probiotic. Identification and characterization of the indigenous micro-flora is essential for understanding how the microbial ecology impacts on the chemistry, flavor and potential health benefits of dadih. Additionally, a study on antimicrobial potency from dadih is important to understand how a product made under natural conditions does not become contaminated with food pathogens such as listeria or salmonella. A number of dadih bacteria and yeast isolates were collected from two different locations in Bukit Tinggi, West Sumatra with the aim of investigating the LAB and their bacteriocins present in dadih. Biochemical characterization identified 66 isolates of lactic acid bacteria, 51 isolates of yeast and 2 isolates of a Gram-negative, catalase-positive bacteria. A genotypic approach (PCR ITS-RFLP) and further assessment with 16S rRNA gene sequencing indicated that *Lactococcus lactis* subsp. *lactis* and *Lactobacillus plantarum* were common species found in dadih. In previous studies, the latter species exhibited probiotic and anti-pathogenic properties. However, we also found five species from three different microbial groups which have never been observed in dadih before; LAB (*Lactobacillus paracasei* and *Leuconostoc pseudomesenteroides*), acetic acid bacteria (*Acetobacter cerevisiae)* and yeast (*Candida stellimalicola* and *Pichia jadinii*). Interestingly, RFLP patterns of *Lb. paracasei* were able to distinguish two different types, consistent with differences in cell morphology. Microbial composition of the two dadih analyzed were different: *Lb. paracasei* was the dominant bacteria in one and *Lb. lactis* subs. *lactis* in the other. *P. jadinii* was the principal yeast in both dadih samples. Both yeast species isolated from the dadih were able to utilize lactic acid, indicating that they can impact on dadih by further metabolising the lactic acid produced by the LAB. The presence of LAB, yeasts and acetic acid bacteria in dadih indicate that the micro-flora is complex with the potential for synergistic interactions in dadih.

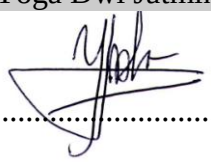
The antimicrobial potency of dadih LAB has been demonstrated in this study. Of the four dadih LAB identified, three strains of dadih LAB inhibited the pathogenic indicator bacteria, namely *Lb. plantarum*, *Lc. lactis* subsp. *lactis* and *Lb. paracasei* and these clearly had the potential to produce bacteriocins. In addition, the organic acids produced by dadih LAB played an important role in inhibiting pathogenic indicator bacteria. The greatest antimicrobial activity was demonstrated by *Lb. plantarum* S1.30 and further analysis of antimi-

crobial activity was focused on this isolate. A bacteriocin could not be identified in *Lb. plantarum* S1.30 with the conventional methods, but the application of PCR-based method has detected the genes responsible for the synthesis of bacteriocin-like peptide important in the induction of bacteriocin production. This result suggests that bacteriocin produced by *Lb. plantarum* S1.30, called plantaricin, could be regulated by a quorum sensing mechanism.

Declaration

This thesis presents work carried out by myself and does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; to the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, are clearly acknowledged.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background information

Dadih, Indonesian fermented buffalo milk, has been produced and consumed for hundreds of years, especially by local people in the province of West Sumatra. Dadih is categorized as a functional food or probiotic that provides health benefits to humans and also economic benefits to the local community (Collado et al. 2007b).

In recent times, traditional fermented milk products have been widely studied due to their beneficial effects on human health, including therapeutic, prophylactic and nutritional values. Microorganisms involved in the fermentation are the primary contributors to these beneficial attributes. In contrast to industrial fermented products which use a very large inoculum of starter cultures, in traditional techniques, fermentation occurs spontaneously without addition of any starter cultures. These types of products are usually called naturally fermented milk (NFM) products. NFM products could facilitate the growth of well-adapted micro-flora that compete with the contaminating microorganisms, and adjust in the environmental conditions and the food substrate (Holzapfel 2002). NFM contains a mixture of indigenous micro-flora with lactic acid bacteria (LAB) predominating (O'Sullivan et al. 2002). However, the role of additional metabolic products, such as antimicrobial substances which also could bring beneficial effect to humans still needs to be investigated.

As one of the sources of the human commensal microbiota, NFM products are considered a significant source of prospective commercial starter cultures for development of new fermentation products (Hansen 2002). Moreover, in terms of metabolic products, the NFM flora has untapped potential that requires further investigation. One of the prospective products is bacteriocins, which can be applied for killing or inhibiting the growth of undesirable microorganisms in the food industry (O'Sullivan et al. 2002). This thesis investigates the role of NFM products as a potential source of bacteriocin-producing indigenous LAB with particular reference to dadih.

1.2 Naturally fermented milk (NFM) products

NFM products arise from spontaneously fermented fresh milk without the addition of a starter culture and are normally manufactured on a small-scale in developing countries (Narvhus & Gadaga 2003). The origin of NFM micro-flora may come from milk itself, milking tools, workers and the fermentation environment (Holzapfel 2002). Back-slopping is commonly used in some NFM products where microorganisms from earlier fermentation products are re-introduced to initiate the new fermentation. For example, this method is used for producing raib from Morroco, dahi from India and airag from Mongolia (Holzapfel 2002; Narvhus & Gadaga 2003). There are many different types of NFM from many developing countries in Africa and Asia (Table 1).

Table 1 Naturally fermented milk products with identified micro-flora and bacteriocin-producing bacteria

No	Products	Substrates	Country	Micro-flora	Bacteriocin producers	Activity against	Ref.
1.	Airag/ Koumiss/ Chigee	Mare milk	Mongolia	*Lactococcus garvieae*, *Lc. lactis* ssp. *lactis*, *Streptococcus parauberis*, *Enterococcus faecium*, *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides*	*Enterococcus durans*	*Lactobacillus* spp., *E. coli*, *S. aureus* and *Listeria innocua*	(Ying, et al.2004; Batdorj et al. 2006)
2.	Amasi and mafi	Cow milk	South Africa, Zimbabwe	*Lc. lactis* subsp. *lactis*, *Leu. mesenteroides* subsp. *dextranicum*, *Leu. citreum*, *Leu. lactis*, *Lb. delbrueckii* subsp. *lactis* and *Lb. plantarum*	-	-	(Beukes et al. 2001; Todorov et al. 2007)
3.	Amasi/ hodzeko/ mukaka wakakora	Cow milk	Zimbabwe	LAB *(Lb. helveticus, Lb. plantarum, Lb. delbrueckii* subsp. *lactis, Lb. casei* subsp. *casei and Lb. casei* subsp. *pseudoplantarum)* Yeasts (*Saccharomyces cerevisiae, Candida lusitaniae, C. colliculosa, S. dairenensis, Dekera bruxillensis, C. lipolytica, C. tropicalis*)	*Lb. plantarum* AMA-K	*Listeria innocua* and *Enterococcus faecalis*	(Feresu & Muzondo 1990; Gadaga et al.2000; Todorov et 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*, *Lb. casei* subsp. *casei* and *Lb. casei* subsp. *rhamnosus*, *Lb. paramesenteroides*, *Lb. rhamnosus*, *Lb. plantarum*); yeasts (*Endomyces lactis*), others (*Micrococcus varians*, *Staphylococcus saprophyticus* and *Bacillus cereus* var. *mycoides*)	*Lc.lactis* subsp. *lactis* (IS-10285 & IS-16183), *Lb. brevis* IS-26958 and *Lb. casei* IS-7257	*E. coli*, *Listeria monocytogenes* and *S. aureus*	(Hasono et al.1989; Zakaria et al. 1998; Surono, 2003)
5.	Dahi (yoghurt-like)	Cow and buffalo milk or	India	*Streptococcus bovis*, *Lb. fermentum*, *Lb. delbrueckii* ssp. *bulgaricus*, *Lb.*	*Streptococcus bovis* J2 40-2, *Lc. lactis* CM1	*Listeria monocytogenes*, *Enterococcus*	(Mitra et al.2007; Rashid et

		mixture		*delbrueckii* ssp. *lactis*, *E. faecium*, *S. thermophilus*, *Leu. mesenteroides* ssp. *mesenteroides*, *Leu. mesenteroides* ssp. *dextranicum*, *Lc. lactis* ssp. *lactis*, *Lb. raffinolactis* and *Pediococcus. pentosaceus*		*faecalis* and *Pediococcus dextranicus*	al. 2007; Harun-ur-Rashid et al. 2009; Mitraet al.2010)
6.	Dangke (cheese-like)	Buffalo milk	Makasar, Indonesia	LAB (*Lactobacillus* spp.)	-	-	Abdul Razak, Univer-sity of Tadulako (Personal commu-nication, 2008)
7.	Kule naoto	Cow milk	Maasai, Kenya	*Lb. plantarum*, *Lb. fermen-tum*, *Lb. paracasei and Lb. Acidophilus*	-	-	(Mathara et al. 2004)
8.	Kurut	Yak milk	Qinghai, China	LAB and yeasts	-	-	(Zhang et al. 2008)
9.	Raïb	Cow milk	Morocco	*Lc. lactis*, *Enterococcus faecium*, *E. faecalis*	*Lactococcus lactis*, *E. faecium*, *E. faecalis*	*Listeria mono-cytogenes*	(Elotmani et al. 2002)
10.	Rob	Cow milk	Sudan	LAB (*Lb. fermentum*, *Lb. acidophilus*, *Lc. lactis* and *Streptococcus salivarius*); yeasts (*Saccharomyces cere-visiae* and *Candida kefyr*)	-	-	(Abdelga dir et al. 2001)
11.	Sethemi	Cow milk	South Africa	LAB (lactobacilli, leu-conostocs and lactococci), yeasts (*Debaryomyces han-senii*, *Saccharomyces cerevisiae*, *Cryptococcus curvatus*, *Cryptococcus humicola and Kluyveromyces marxianus*)	-	-	(Kebede et al. 2007)
12.	Shubat	Camel milk	China	LAB (*Lb. sakei*, *E. faecium*, *Lb. helveticus*, *Leu. lactis*, *E. faecalis*, *Lb. brevis* and *Weissella hellenica)*, yeasts (*Kluyveromyces marxianus*, *Kazahtan uiosporus* and *Candida ethanolica)*.	-	-	(Rahman et al. 2009)
13.	Suusac	Camel milk	Kenya	LAB (*Lb. curvatus*, *Lb. plantarum*, *Lb. salivarius*, *Lc. raffinolactis* and *Leu. mesenteroides* subsp. *mesenteroides*); yeasts (*Candida krusei*, *Geotrichum penicillatum* and *Rhodotorula mucilaginosa*)	(Watanabe et al. 2008)-	-	(Lore 2005)
14.	Tarag (yo-ghurt-like)	Milk of cow/ camel/ yak/ goat	Mongolia	LAB (*Lb. delbrueckii* subsp. *bulgaricus*, *Lb. helveticus*, and *S. thermophilus*), yeasts (*Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *Issatchenkia orientalis*, and *Kazachstania unispora*)	-	-	(Watana be et al. 2008)
15.	Gioddu	Sheep or goat milk	Sardinia	*Lb. paracasei*, *Lb. plantarum* and *Lb. reuteri*	*Lb. paracasei* Olb46As2, Olb46As3, and	*Lb. sakei* LMG 2312	(Ortu et al. 2007b)

					Olo45As2		
16.	Jben	Cow or goat milk	Morocco	*Lc. lactis*	*Lc. lactis* CCMM/IAV/BK2	*Listeria monocytogenes*	(Benkerroum et al. 2000)

Even though different regions will produce a slightly different type of fermented milk product, there are significant similarities:

- **Origin of indigenous micro-flora**

Fermentation occurs naturally with a wide variety of microorganisms involved in the process, including LAB, yeasts and other microbes. The indigenous microbes may originate from non-pasteurized milk and the environment including containers and equipment used during fermentation, the workers and the weather. The different local traditions used in the manufacturing process, such as the quality of the raw materials (milk) are also expected to result in a different microbial composition in the various products.

- **Small-scale producers/ rural communities**

The people who produce these products mainly come from rural areas and utilize a very simple technology. The skill required to manufacture the product is inherited from their ancestors and can help to complement their income. Nevertheless, the number of dairy farmers producing NFM products is decreasing with the introduction of modern foods, so that the existence of these traditional foods is in danger of vanishing. Special attention from governments and dairy scientists is imperative to protect and further develop these products to increase the benefits to the local communities.

- **Traditional vessels**

The utilization of traditional and natural containers also contributes to the uniqueness of NFM. Dadih, for example, uses bamboo tubes as the vessel and banana leaves as a lid (Hasono et al.1989). On the other hand, an earthenware pot is generally utilized in amasi and dahi manufacturing (Rashid et al. 2007; Todorov et al. 2007). Unfortunately, in some regions, the utilization of plastic containers to substitute for the traditional vessels is increasing and this could influence the quality of the final products.

- **Incubation temperatures**

Ambient temperature is chiefly utilized for incubation of the milk with fermentation normally taking 2 – 3 days in tropical climates. However, in cooler regions, prolonged incubation may be necessary. Kurut, for example, a Chinese fermented yak milk, requires 7-8 days of incubation at about 10-15^{o} C (Zhang et al. 2008).

- **Poor standard sanitary conditions**

Poor sanitation and handling during preparation of NFM products is the main concern related to food safety issues. Undesirable microorganisms are still detected although at a lower than expected level (Gran et al. 2003). On one hand, this situation could induce LAB to produce antimicrobial compounds. On the other hand, the survival of food spoilage and pathogenic bacteria may increase the incidence of food-borne illness in the consumers. A good manufacturing process and a hazard analysis (HACCP) concept need to be applied for production of NFM products.

1.3 Dadih

Dadih is a traditional Indonesian fermented milk product prepared from non-pasteurized buffalo milk and is proven to have a beneficial effect on the health of consumers (Pato 2003). This product is manufactured traditionally and naturally by rural communities in the Province of West Sumatra. The process of preparation of dadih includes 3 simple steps (Figure 1): fresh buffalo milk is filtered and transferred into fresh bamboo tubes, the bamboo tubes containing buffalo milk are capped with banana leaves previously wilted by heating, and finally the containers are incubated at room temperature for at least 24 hours (Suryono 2003).



Figure 1 Dadih manufacturing process
1. Milking from buffalo; 2. Filtration; 3. Pouring; 4. Capping; 5. Incubation (at least 24 h); 6. Final products

Previous studies have reported on the diversity of micro-flora in dadih (Yodoamijoyo et al. 1983; Hasono et al. 1989; Zakaria et al. 1998). The microorganism composition reported by these researchers varies depending on scientific methodology and the many factors that influence the production of dadih (Purnomo 1996). The studies, however, have consistently shown the predominance of LAB species including *Leu. paramesenteroides*, *Lb. rhamnosus* and *Lb. lactis* ssp. *lactis* isolated using a selective medium (Yodoamijoyo et al. 1983; Hasono et al. 1989; Zakaria et al. 1998).

The probiotic effect in dadih that enhances human health has been reviewed and investigated (Pato 2003; Collado et al. 2007b, a). Probiotic properties have been found using an indigenous dadih LAB strain, *Lb. plantarum* IS-10506. This strain showed the highest potential to

compete with pathogenic bacteria and decreasing their adherence to intestinal cells (Collado et al. 2007b). Moreover, results indicate that LAB isolates derived from dadih, particularly *Lb. casei* subsp. *casei* R-52, exhibit anti-mutagenic activity against the mutagenic properties of terasi (Surono & Hosono 1996). This has led Pato (2003) to conclude that dadih consumption has the potential to reduce the cancer risk, in particular colon cancer, that exists from consuming mutagenic compounds contained in some food products. The diverse microflora is best explained by the fact that production of dadih is the result of natural fermentation without the addition of starter cultures. The ability of specific LAB isolates to adapt to highly competitive ecological niches formed in natural fermentation may depend on the production of potent antimicrobial substances (Piard & Desmazeaud 1992), especially bacteriocins. Interestingly, bacteriocin-like substances of some LAB dadih have been detected that are active against *Escherichia coli* and *Listeria monocytogenes*. Nonetheless, further bacteriocin confirmation tests, such as proteolytic sensitivity and temperature stability, are still needed to be conducted to characterize its proteinaceous properties. As a consequence, indigenous LAB strains which may originate from buffalo milk, bamboo tubes or banana leaves are of great interest as sources of improved bacteriocins.

1.4 General characteristics of lactic acid bacteria

Lactic acid bacteria have recently acquired a prominent status in food fermentation as some of the strains have been shown to possess a beneficial effect to humans. These bacteria are characterised by being Gram-positive, non-spore-forming, coccoid- or rod-shaped, catalase-negative, aerobic to facultative anaerobic. The trait of most importance to the food industry is their ability to form lactic acid as the major product (homofermenters) or to lactic acid and other products, such as acetate, ethanol, carbon dioxide, formate or succinate (heterofermenters) during glucose fermentation (Schleifer et al. 1995; Schleifer & Ludwig 1995). Even though around 20 genera have been identified to be members of LAB, basically only 12 genera are the most important genera from the perspective of food scientists; *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Axelsson 2004). The morphological and biochemical characteristics of each genus are presented in Table 2.

1.5 Identification of lactic acid bacteria

A range of methods have been developed for the identification of microorganisms including

LAB. The methods can be divided into two main groups, phenotypic and genotypic methods. Since precise identification of LAB is a prerequisite of their role as commercial starter cultures in the fermentation industry or as probiotic candidates with their health promoting properties, a comprehensive study of LAB is essential (Mckay & Baldwin 1990; Botina, Tsygankov & Sukhodolets 2006). Application of a polyphasic approach that combine phenotypic, genotypic and phylogenetic methods for identifying potential novel LAB strains has become the current trend, (Vamdamme et al. 1996).

1.5.1 Phenotypic methods

Phenotypic methods are mainly based on morphological, biochemical, and physiological tests (Botina et al. 2006). Classification of LAB to genus level can be based on the characteristics shown in Table 2. To obtain more accurate results in the classification at species level, additional biochemical tests are required, such as carbohydrate fermentation profiles, haemolysis test, extracellular polysaccharide formation, utilization of growth factors, enzymatic activities, growth response in milk media, and serological assays (Axelsson 2004). This traditional approach of identifying LAB is time consuming and rather tedious, and therefore the use of a commercial identification system such as API 50 CHL (Bio Meriéux S.A., Marcy I'Etoile, France) based on biochemical characteristics is currently more popular (Botina et al. 2006).

Although phenotype characterization can yield an ambiguous result and is quite unreliable (Botina et al. 2006), this technique is still the standard in bacterial systematics. As part of a biological system, phenotypic information of microorganisms is very valuable for some purposes, such as understanding microbial behaviour and predicting their activities in certain conditions; gaining a better understanding of microbial physiology especially related to technological/ functional important properties; and supporting the genotypic characterization results (Bochner 2009).

Table 2 Morphological and biochemical characteristics of lactic acid bacteria (Axelsson 2004)

Character	*Aeroc.*	*Carnob.*	*Enteroc.*	*Lactob.*	*Lactoc.*	*Leucon.*	*Oenoc.*	*Pedioc.*	*Streptoc.*	*Tetragenoc.*	*Vagoc.*	*Weissella*
Cellular morphology	coccoid	rods	coccoid	rods	coccoid	coccoid	coccoid	coccoid	coccoid	coccoid	coccoid	coccoid / rods
Tetrad formation	+	-	-	-	-	-	-	+	-	+	-	-
Mode of glucose fermentation[a]	-	-[b]	-	±	-	+	+	-	-	-	-	+
Growth at 10° C	+	+	+	±	+	+	+	±	-	+	+	+
Growth at 45 ° C	-	-	+	±	-	-	-	±	±	-	-	-
Growth at 6.5% NaCl	+	ND[c]	+	±	-	±	±	±	-	+	-	±
Growth at 18% NaCl	-	-	-	-	-	-	-	-	-	+	-	-
Growth at pH 4.4	-	ND	+	±	±	±	±	+	-	-	±	±
Growth at pH 9.6	+	-	+	-	-	-	-	-	-	+	-	-
Isomeric forms of lactic acid during glucose fermentation	L	L	L	D, L, DL[d]	L	L	D	L, DL[d]	L	L	L	D, DL[d]

+ positive; - negative; ± varies between species; ND not determined
[a]Test for glucose fermentation under standard condition: - homofermentative; +heterofermentative
[b]Small amount of CO_2 can be produced, depending on media
[c]No growth in 8% NaCl has been reported
[d]Production of D-, L-, or DL-lactic acid varies between species

1.5.2 Genotypic methods

Genotypic identification of LAB which is based on a molecular approach is widely used due to more accurate, rapid and reproducible results in comparison with the classical phenotypic approach (Singh et al. 2009). Molecular approaches for LAB systematic studies (with special attention to organisms isolated from traditional fermented milk products) include pulse field gel electrophoresis (PFGE) (Ventura & Zink 2002; Lei & Jakobsen 2004), random amplified polymorphic DNA (RAPD) analysis (Ortu et al. 2007b; Chao et al. 2009; Franciosi et al. 2009), PCR-denaturing gradient gel electrophoresis/ PCR-DGGE (Ercolini et al. 2001; Temmerman et al. 2004; Ongol & Asano 2009), PCR-RFLP (Yavuz et al. 2004; Yu et al. 2009) and DNA sequencing (An et al. 2004; Ortu et al. 2007a; Rashid et al. 2007; Rahman et al. 2009). The genotypic methods are easy and inexpensive and have a high discriminatory power (Temmerman et al. 2004). In their review, a range of molecular methods that have frequently been utilized for identification of LAB have been successfully analysed, taking into account the advantages and disadvantages of each method. The methods categorized with a high level of reproducibility and of low labour-intensity are PCR using species-specific primers and rep-PCR. PFGE was considered as a "gold standard" for LAB classification by Axelsson (2004) due to its high discrimination at the strain level; this technique, however, is dependent on expensive tools and requires a complicated procedure and analysis (Temmerman et al. 2004; Singh et al. 2009). PCR-RFLP, on the other hand, gives a highly consistent result and requires an intermediate workload (Temmerman et al. 2004).

PCR – RFLP seems to be more valuable in bacterial characterization rather than in bacterial identification. This can be seen from many LAB studies that utilize this method as a preliminary investigation for species identification (Lei & Jakobsen 2004; Ferchichi et al. 2008; Belgacem et al. 2009; Yu et al. 2009). This genetic fingerprinting method is based on observation of banding patterns following digestion of amplicons with restriction enzymes (Temmerman et al. 2004). While useful for grouping, to identify the unknown strains further molecular assessment, such as DNA sequencing or PCR with species-specific primers, is essential. In contrast, RFLP analysis can be applied for identification of yeasts without any further confirmation as database of restriction patterns are available (Guillamón et al. 1998; Esteve-Zarzoso et al. 1999; Villa-Carvajal et al. 2006). However, DNA sequencing is still required if new species are obtained (Heras-Vazquez et al. 2003) due to the small number of restriction profiles of LAB currently available, which are mostly limited to lactobacilli

(Yavuz et al. 2004; Moreira et al. 2005), RFLP analysis is most important in the characterization of strains of LAB.

In order to obtain consistent restriction patterns, the PCR amplification target that yields a unique profile for each species should be selected. Many LAB studies use the 16S-23S rRNA spacer region as the target of PCR amplification (Berthier & Ehrlich 1998; Nour 1998; Lee et al. 2008). More discussion on this will be presented in section 1.10. For comprehensive identification of potential LAB, the utilization of a polyphasic approach is still recommended through the combination of phenotypic, chemotaxonomic and genotypic methods (Axelsson 2004).

1.6 Bacteriocins of lactic acid bacteria

Bacteriocins produced by LAB genera have attracted the attention of the food industry for their potential use as additives with GRAS (generally recognized as safe) status in improving food safety and quality (Oppegård et al. 2007). Numerous studies have been conducted to identify and characterize various bacteriocins produced by LAB from different sources (Jamuna & Jeevaratnam 2004; Batdorj et al. 2006; Chen et al. 2007; Ghrairi et al. 2008). Bacteriocins are defined as proteinaceous substances produced by bacteria that exhibit an antagonistic effect toward other bacteria competing for the same ecological niche (Klaenhammer 1993). In this case, the inhibitory ability showed by bacteriocins is potentially used against food spoilage and pathogenic bacteria. These target organisms can be either closely-related bacteria or bacteria from diverse genera (Vandenbergh 1993; De Martinis et al. 2002; Deegan et al. 2006). Many publications divide bacteriocins into three major classes. This classification is based mainly on molecular weight, amino acid sequence and modes of action as can be seen in Table 3 (Nes et al. 1996; Héchard & Sahl 2002; Ross et al. 2002; Papagianni 2003).

Bacteriocins are ribosomally synthesized proteins with a narrow-spectrum of inhibition (Cleveland et al. 2001). The bacteriocins do not kill the bacteriocin producers as these bacteria express protection genes on the same operon using a mechanism that is not fully understood (Jack et al. 1995; McAuliffe et al. 2001). In the case of mode action, the inhibitory effect of bacteriocins toward target-cells is directed toward the cell membrane of the bacterial target (Héchard & Sahl 2002).

Table 3 Classification of bacteriocins

Class	Sub-class	Features	Bacteriocins	Producer strains
	Lantibiotics, ribosomally synthesized peptides that undergo posttranslational modifications. Molecular weight 2– 5 kDa. Contain lanthionine and h-methyl lanthionine			
I	Ia	Flexible, elongated molecules	Nisin A Nisin Z Lacticin 481 Lactocin S Plantaricin C Plantaricin W Cytolysin	*Lactococcus lactis* NIZOR5, 6F3 *Lactococcus lactis* N8, NIZO22186 *Lactococcus lactis* CNRZ481 *Lactobacillus sake* L45 *Lactobacillus plantarum* *Lactocbacillus plantarum* LMG 2379 *Enterococcus faecalis* FA22
	Ib	Globular peptides with no net charge or net negative charge	Mersacidin	*Bacillus* sp. Strain HIL Y-85,54728
	Heat stable peptides formed exclusively by unmodified amino acids. Ribosomally synthesized as inactive prepeptides that become activated by posttranslational cleavage of the N-terminal leader peptide. Molecular weight < 10 kDa.			
II	IIa	Pediocin-like bacteriocins. Single peptides that contain a YGNGVN-terminus. Also known as Listeria-active peptides.	Pediocin PA-1 Bavaricin MN Divercin V41 Mesentericin Y105 Leucocin A Enterocin P	*Pediococcus acidilactici* *Lactobacillus sake* *Carnobacterium divergens* *Leuconostoc mesenteroides* *Leuconostoc gelidum* *Enterococcus faecium*
	IIb	Two peptide bacteriocins	Lactacin F Plantaricin EF & JK Lactococcin G	*Lactobacillus johnsonii* *Lactobacillus plantarum* C11 *Lactococcus lactis* LMG 2081
	IIc	Other bacteriocins. Bacteriocins produced by the cell's general secretory pathway	Plantaricin A Lactococcin A Lactococcin 972	*Lactobacillus plantarum* C11 *Lactococcus lactis* *Lactococcus lactis* IPLA 972
III	Large heat labile proteins (>30 kDa)		Helvetin J Enterolysin	*Lactobacillus helveticus* *Enterococcus faecium*

Sources: (Klaenhammer 1993; Nes et al. 1996; Héchard & Sahl 2002; Ross et al. 2002; Papagianni 2003)

Nisin, a good example of a bacteriocin, actively disrupts cell wall synthesis, leading to pore formation in the bacterial cell membrane, disturbance of the proton motive force (PMF) and resulting in cell death (McAuliffe et al. 2001). Many factors affect bacteriocin activity including bacteriocin quantity and composition, the cell membrane structure of target cells, interaction between bacteriocin and immune factors and extracellular conditions (Héchard & Sahl 2002).

Evaluation of the use of bacteriocins as food preservatives requires consideration of the food grade status of the bacteriocin-producing strains, the target spectrum, stability in a range of environmental conditions, human toxicity and the contributions to food safety and quality (O'Sullivan et al. 2002). Based on these requirements, LAB isolated from traditional fermented milk have a huge potential as a source of bacteriocin candidates.

1.7 Bacteriocin detection methods

Screening of bacteriocin-producing LAB from various potential sources, either living materials (humans, animals and plants) or food products, is an important preliminary step for obtaining prospective bacteriocins. The conventional techniques for screening bacteriocin producers *in vitro* are based on culture dependent methods or agar plate diffusion assays, namely simultaneous and deferred antagonism method (Heng et al. 2007). In the simultaneous antagonism method, colonies of the test bacteria on agar medium are overlaid with soft agar containing indicator bacteria. After incubation in optimum conditions for 48 – 72 hr, antagonistic activity can be detected by the inhibition zone surrounding the test cultures (De Martinis et al. 2002). Subsequently, the bacteriocin activities and properties need to be confirmed using the deferred antagonism method. For this method, agar well-diffusion, agar spot test, paper disc assay and flip-streak are popular analytical techniques used to screen bacteriocin producers (De Martinis et al. 2002). The putative bacteriocin producer cultures can be used directly in each technique; however, cell-free supernatants of the strains can be only applied in agar well-diffusion and paper disc assay.

Besides bacteriocins, LAB also produce other antimicrobial compounds, such as lactic acid, hydrogen peroxide and diacetyl. Therefore the use of traditional assessment techniques is dependent on growth conditions that eliminate non-bacteriocin activities. Furthermore, because bacteriocins are proteinaceous substances, their activities can be inactivated by proteolytic enzymes. Considering these facts, Heng et al (2007) suggested to use the term of BLIS (bacteriocin-like inhibitory substance) for referring to antagonistic substances which have not been characterized as proteins. The simplest way to determine if the substance is a protein is by adding protease enzymes to the wells (van Belkum et al. 1989) or by spotting the proteolytic enzymes, next to the test bacteria (De Martinis et al. 2002).

One of the disadvantages of culture-based methods for detecting bacteriocins is that they are not expressed in all conditions or are not easily detected (Tagg 1992). The reason is the complexity of bacteriocin structures and their sometimes unstable characteristics which result from complex regulatory mechanisms controlling the release of these antimicrobial peptides. Another limitation of the conventional methods is the influence of environmental factors (physical, chemical and nutritional) towards bacteriocin production (Nes & Johnsborg 2004; Heng et al. 2007). Some bacteriocins will be produced optimally in depleted condition rather than in optimum growth conditions for the producers (Heng et al. 2007). Therefore, a molecular approach is important either for confirmation purpose or for the detection of bacterio-

cins not expressed in conventional assays.

The molecular approach is considered as a rapid method for detection of bacteriocins. Based on knowledge of LAB genome sequences and "conserved amino acid sequences" (YGNGVXCXXXXCXV and LDNAIE, in the N-terminal region and the histidine kinase, respectively), primers for PCR amplification can be designed for detecting genes responsible for bacteriocin biosynthesis, especially in class II bacteriocin (Heng et al. 2007; Yi et al. 2010). A range of primers have been generated and used in several studies (Odenyo et al. 1994; Remiger et al. 1996; Rojo-Bezares et al. 2007; Suwanjinda et al. 2007; Knoll et al. 2008). While the PCR-based method is reliable and rapid for bacteriocin detection it requires identification of the bacteria in order to determine the appropriate primers.

1.8 Characterization of bacteriocin activities

Characterization of a potential bacteriocin includes assessing its tolerance to extreme environmental conditions (e.g heating and pH) and its activity in the presence of enzymes (Piard & Desmazeaud 1992). The biochemical information is necessary for preliminary assessment of the stability of the bacteriocin in a food system, assessment of conditions for optimum bacteriocin activity and also contributes to the classification of the bacteriocin (Cintas et al. 2001; Parada et al. 2007). This physical-chemical evaluation utilizes the neutralized cell-free supernatants (CFS) of the bacteriocin producers as the test material. The treated supernatant fluids are dispensed into the well (4-8 in diameter) in the agar lawn (agar well-diffusion) or spotted on the surface of the agar lawn (agar spot test), seeded with indicator bacteria to evaluate the antimicrobial activities.

The thermal stability is assessed at pasteurization temperatures (63 ^{o}C for 30 min or 72 ^{o}C for 15 min), 100 ^{o}C for 60 min and sterilization temperature (121 ^{o}C for 15 min). If bacteriocin activities are categorized as heat stable/ heat tolerant, it indicates that the molecular size of the bacteriocin is small and that it has relatively a simple protein structure (without a tertiary structure) (Piard & Desmazeaud 1992). Next, a wide pH range from 2.0 to 12.0 is used to test stability at different pH. The pH of the extracellular environment of bacteriocin producers is generally acid or neutral pH, so acid tolerant bacteriocins are highly preferred used for food biopreservation. For enzymatic sensitivity, a range of enzymes is used, such as proteolytic enzymes (proteinase-K, papain, trypsin, pronase, pepsin and α-chymotrypsin), hydrolytic enzymes, lipolytic enzymes (lipase and α-amylase) and catalase. Apart from characterization of the proteinaceous nature of the bacteriocin, the sensitivity to proteolytic enzymes is impor-

tant for ensuring that bacteriocins are safe if they are ingested (Piard & Desmazeaud 1992; Cintas et al. 2001; Parada et al. 2007).

1.9 Potency of naturally fermented milk products as habitat of bacteriocin-producing LAB

In recent years, the search for bacteriocins from traditional fermented foods has increased significantly. The properties of bacteriocins derived from NFM, however, have not been investigated fully (Table 1). Ecologically, the level of competitiveness in NFM products is relatively high, which provides ideal conditions for LAB to produce their potent growth-inhibiting substances, including bacteriocins. Wouters et al (2002) observed that the isolation rate of bacteriocin-producing wild-lactococci from fermented milk products from non-dairy environments is quite high in comparison with lactococci isolated from milk itself. The presence of bacteriocins has been detected in a number of NFM samples namely airag, amasi, dahi and dadih earthenware (Beukes et al. 2001; Surono 2003; Ying et al. 2004; Rashid et al. 2007).These indigenous products utilize non-pasteurized milk fermented in natural containers, which are made from animal materials (bull skin) and earthenware (Beukes et al. 2001; Surono 2003; Ying et al. 2004; Rashid et al. 2007). In the case of dadih, the antagonistic activity resulting from neutralized cell-free supernatant was still categorized as putative bacteriocins, as the evidence of the sensitivity of the antimicrobial substances to proteolytic enzymes was not obtained (Surono 2003).

A complex interaction among indigenous micro-flora in NFM facilitates a quorum sensing mechanism as a part of the adaptation strategy of the bacteria (Gobbetti et al. 2007). Quorum sensing is the intercellular communication between bacteria and is an important component of the physiological response to changing environmental conditions and competition with other microbes (Kleerebezem & Quadri 2001). In the case of Gram-positive bacteria, this behaviour is known as cell-density-dependent regulation, which means it only occurs when the number of bacterial cells is high (Dunny 1997; Risoen et al. 2000; Bassler 2002). Signalling molecules released by bacterial cells are generally used as the means of communication (Quadri 2002). One response to quorum sensing is bacteriocin biosynthesis mediated by extracellular molecule signals/ signalling pheromones, referred to as inducing peptides (IPs). After the concentration of IPs in the environment achieves a certain thresh-hold as a result of the increasing cell numbers, antimicrobial peptides/ bacteriocins will be produced in response to signal transduction activation in the bacterial cells (Kleerebezem & Quadri 2001; Quadri

2002). The regulation system for bacteriocin produced by Class I (lantibiotics) is different from that of Class II (non-lantibiotics). In lantibiotics, bacteriocin production is categorized as a constitutive phenotype, in which bacteriocins have a dual activity either as active anti microbial substances or inducing peptides (Kleerebezem 2004). Well-studied bacteriocins in this category are nisin and subtilin. However, bacteriocin synthesis in non-lantibiotics has been shown to be inducible and involves the autoinducing peptides (Quadri 2002).

Although quorum sensing mechanisms can be found in a wide range of environments, the uniqueness of the NFM environment might contribute to the finding of novel and potent natural antimicrobial peptides. The involvement of other microbial groups apart from LAB and yeasts in dadih fermentation (Hasono et al. 1989) could select for dadih LAB that possess a competitive advantage by producing antimicrobial peptides. Investigation of bacteriocin-producing LAB in such products is challenging but important for increasing the understanding of NFMs such as dadih. Since accurate and reliable information on the composition of the micro-flora is vital for supporting bacteriocin investigations, application of molecular approaches is required for detection and identification of bacteriocins.

1.10 Molecular identification and characterization of micro-flora to support bacteriocin detection

Two main purposes for the utilization of molecular methods in studying micro-flora composition in a complex environment are firstly to identify and characterize the bacterial diversity in the samples; and secondly to monitor or investigate the existence of targeted bacteria in the environments (McCartney 2002). In case of lactobacilli, the phenotypic and physiological characteristics of LAB genera are relatively similar. Singh et al. (2009) identified that the identical ecological niche for their growth seems to be the main reason for these similarities. Consequently, classical microbiological techniques have been supplemented with molecular technique as the former approach is not sufficient to differentiate *Lactobacillus* strains and species (Axelsson 2004; Singh et al. 2009). One such molecular identification method, a combination of non-PCR and PCR-based method (i.e. amplified rDNA restriction analysis/ ARDRA and amplified fragment length polymorphism/ AFLP) has been used recently in studying microorganisms from both dairy and non-dairy environments.

The 16S rRNA genes and the 16S-23S rRNA intergenic spacer region (ISR) are commonly used as targets for amplification. Difficulties arise, however, when an attempt is made to implement 16S rRNA gene amplification as the sole method. One of the reasons is that the genes in this region are too conserved; as a consequence closely related species cannot be distinguished confidently (Pot & Tsakalidou 2009). Phenotypic and physiological characterizations are still required to support the result of 16S rRNA analysis. However, the use of these traditional methods as a complement to molecular studies is time and labour consuming. Alternatively, molecular characterization using 16S-23S rRNA intergenic transcribed spacer (ITS) as the target is much more efficient and reliable, especially when closely related species are under consideration. A higher heterogeneity in the spacer region compared to the 16S rRNA structural genes in both sequence and length is the advantage in using this region for PCR target to identify microbial strains (Barry et al. 1991; Berthier & Ehrlich 1998). This variability in this spacer region is mainly associated with the existence and arrangement of tRNA genes, namely $tRNA^{Ile}$, $tRNA^{Ala}$ and $tRNA^{Glu}$ (Nour 1998), and other components such as enzyme recognition sites (RNase III), a *boxA* unit and special sequences in which insertion and deletion frequently occur (Berthier & Ehrlich 1998; Nour 1998; Clementino et al. 2001).

In recent times, PCR amplification of 16S-23S rRNA ISR has been successfully applied for typing indigenous LAB derived from traditional fermented products (Abdelgadir et al. 2001; Paludan-Muller et al. 2002; Lei & Jakobsen 2004; Sawadogo-Lingani et al. 2007; Vieira-Dalode et al. 2007; Belgacem et al. 2008; Dolci et al. 2008; Belgacem et al. 2009). A study conducted by Abdelgadir et al. (2001) is the first investigation implementing ITS-PCR of indigenous LAB isolated from Sudanese rob, a NFM product. Based on sequence analysis in ITS region, many species-specific primers for the LAB group have been developed for rapid detection and evaluation of LAB in a variety of traditional fermented milk products (Tilsala-Timisjarvi & Alatossava 1997; Song et al. 2000; Tilsala-Timisjarvi & Alatossava 2001; Kabadjova et al. 2002; Rachman et al. 2003; Kwon et al. 2004). Restriction fragment length polymorphism (RFLP) is also widely utilized to complement ITS-PCR for characterization and identification of both bacteria and yeasts.

Besides determining the microbial composition of a sample, the results of molecular identification will play an important part in detection of bacteriocins. Species information will be important as the basis for molecular detection or confirmation of bacteriocin production, especially when conventional methods to detect bacteriocins are unsuccessful.

1.11 Objectives of this study

Based on all the evidence of the potential of dadih as source of bacteriocin-producing LAB, the specific aims of this study were:

a) To isolate, characterize and identify indigenous microorganisms from dadih.
b) To examine antimicrobial activity of LAB isolates against indicator bacteria.
c) To investigate the presence of bacteriocins from LAB with antimicrobial activity against indicator bacteria.

CHAPTER TWO

ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF INDIGENOUS LACTIC ACID BACTERIA FROM DADIH

2.1 Introduction

Prior to gaining a full understanding of the health benefits of dadih, it is important to establish the microorganisms that are present in dadih and that account for this benefit. While several studies have analysed the LAB present in dadih the methods used have not been able to differentiate closely related species. Biochemical and physiological characteristics were used as a basis of the identification of dadih LAB (Yodoamijoyo et al. 1983; Hasono et al. 1989; Zakaria et al. 1998). Some recent studies conducted by Surono (2003) and Collado et al (2007a) investigated probiotic properties among dadih LAB that were identified using API 50 test kit (BioMerieux, France). Subsequently Collado et al (2007b) have sequenced the 16S rRNA genes to confirm the identity of the potential probiotic candidates.

Although molecular methods have been applied to identify the dadih LAB strains, an accurate and reliable characterization technique is required to assist in identification. In the present study, the intergenic/ internal transcribed spacer (ITS) regions were used as a basis of the characterization of LAB isolated from dadih before molecular identification was performed. Due to greater variation in the ITS region compared to that of the 16S rRNA structural gene, the PCR targeting of this region has proven to be an accurate method for characterization of bacteria at species level (Tilsala-Timisjarvi & Alatossava 1997; Rachman et al. 2003; Yavuz et al. 2004). In particular, PCR-amplified 16S-23S rRNA ITS region combined with the restriction fragment length polymorphism (RFLP) has permitted rapid differentiation of closely related LAB species (Song et al. 2000; Ferchichi et al. 2008; Lee et al. 2008). If isolates are analysed that are difficult to differentiate based on the restriction profiles of the 16S-23S rRNA ITS region, as reported by Rachman et al (2003) and Ferchichi et al (2008), a pair of primers (23S/p10-R and tAla-F) amplifying the tRNA genes inside the ITS region can be used. These primers are recommended, because they can produce a single DNA fragment suitable for RFLP analysis (Kabadjova et al. 2002). To our knowledge, this is the first study on the application of PCR ITS-RFLP of micro-flora isolated from dadih.

2.1.1 Aims

To isolate, characterize and identify indigenous lactic acid bacteria from dadih

2.2 MATERIALS AND METHODS

2.2.1 Materials

2.2.1.1 Dadih samples

Dadih was collected from two different regions/ villages in Bukit Tinggi, West Sumatra, Indonesia, namely the Sianok and Gadut regions. Two different products were obtained from Sianok identified as S1 (fermented for 2 days) and S2 (fermented for 3 days). Meanwhile, dadih from Gadut (G) was obtained after three days of fermentation.

2.2.1.2 Bacterial strains

The following strains were used as control organisms (Table 7): *Lactobacillus sakei* ATCC 15521, *Lactococcus lactis* subsp. *lactis* ATCC 11454, *Lactobacillus casei*, *Lactobacillus hilgardii*, *Lactobacillus fermentum*. The last three lactobacilli strains were provided by the Institute of Medical and Veterinary Science (IMVS), Adelaide, South Australia.

All bacteria stocks were maintained in snap freeze medium at -80 ^{o}C. Fresh cultures were prepared by taking a small portion of the frozen stocks using sterile wooden sticks and then inoculating them onto appropriate solid media.

2.2.2.3 Media

2.2.2.3.a Solid media

The media used for isolation and cultivation of lactic acid bacteria was de Man, Rogosa and Sharpe (MRS) Agar base manufactured by Oxoid Ltd, Basingstoke, UK and was prepared according to the manufacturer's instructions.

Yeast peptone dextrose (YPD) agar and broth supplemented with 2% of D-glucose was used for cultivation of yeast isolates. Dehydrated base (Oxoid Ltd, Basingstoke, UK) was prepared according to manufacturer's instructions.

2.2.2.3.b Liquid media

The following dehydrated media manufactured by Oxoid Ltd, Basingstoke, UK were utilized for bacterial cultivation and were prepared according to manufacturer's instructions:

- de Man, Rogosa and Sharpe (MRS) broth was used for cultivating of LAB.

- MRS broth with glycerol (snap freeze medium) was used for the maintenance of LAB stocks at -80 ^{o}C.

2.2.2.4 Chemicals and reagents

The chemicals and reagents used in this study were:

- Hydrogen peroxide (H_2O_2) 3% (v/v), sodium chloride (NaCl) and bromo-cresol purple.
- Saline solution (0.75%, w/v of NaCl) was used for dilution solution during isolation of LAB
- Gram staining reagents: crystal violet, Gram's iodine, acetone-ethanol (95%) and dilute carbol-fuchsin.

2.2.2.5 Enzymes

Restriction endonucleases (*Rsa*I, *Hae*III, *Hinf*I, *EcoR*I, *Hind*III and *Sph*I) were purchased from New England BioLabs and used according to manufacturer instructions.

2.2.2.6 PCR reagents and purification kit

The oligonucleotide primers used in this study are listed in Table 4. The purified synthetic oligonucleotide primers were purchased from GeneWorks Pty Ltd, Adelaide, and were re-suspended in sterile MilliQ water (100 µM) and then stored at -20 ^{o}C as stock solutions.
Taq DNA polymerase, 10x buffer, 25 mM magnesium chloride ($MgCl_2$) and dNTPs were purchased from Promega (Madison, Wis. USA).
DNA ladder (100 bp) was purchased from either New England BioLabs Inc. (molecular weight: 100 – 1,517 bp) or Bioline (Aust) Pty Ltd., Alexandria, NSW, Australia (molecular weight: 100-1000 bp).
Agarose for electrophoresis was purchased from Promega (Madison, Wis. USA).
Loading dye (15% w/v Ficoll, 0.1% bromothymol blue, 0.1 mg/ml RNAse) was purchased from Bioline (Aust) Pty Ltd., Alexandria, NSW, Australia.
The DNA purification kit used was Ultra Clean™ PCR Clean-up™ Kit purchased from Mo-Bio Laboratories, Inc., Solena Beach, CA, USA.

2.2.2.7 Buffer solutions

TAE buffer (40 mM Tris, 1.14 ml of glacial acetic acid, 1 mM EDTA) was used for agarose gel electrophoresis.

2.2.2 Methods

2.2.2.1 Isolation of LAB

Isolation of LAB was conducted in the Laboratory of Microbiology, Indonesia Institute of Technology, Indonesia.

100 g dadih samples were diluted in 900 ml of saline solution (0.75% w/v NaCl) and then homogenized by using a vortex mixer. One ml of homogenized solution was inoculated into 9 ml of saline solution to make a serial dilution series from 10^{-1} to 10^{-7}. By using a pour plate method, 1 ml from each dilution was dispensed into sterile Petri dishes in duplicate (dilutions of 10^{-5}, 10^{-6} and 10^{-7}). For differentiation between LAB and yeast colonies, a modified MRS agar (Oxoid) supplemented with 10% (w/v) of bromo-cresol purple was used. Using a pour plate technique, 15 ml of the medium was poured into Petri dishes containing 100 µl of samples. After the agar had solidified, the plates were incubated at 37 ^{o}C for 48 hours. After incubation, the plates were examined for colour changes, with yellow indicating production of acid from glucose by the colonies. Colonies producing yellow discoloration were identified as LAB, while yeast colonies did not change the colour of the medium (it is not expected that yeast would produce significant acid so there is no change in the colour of the medium).

Single colonies representing bacteria and yeasts were randomly picked, and then streaked onto MRS agar using a quadrant technique. After incubation for 48 hours, a single colony was transferred onto a fresh slant MRS agar, and then forwarded to the University of South Australia (UniSA), Adelaide. After the samples arrived in UniSA, the samples were subcultured and then stored at -80 ^{o}C in snap freeze medium.

2.2.2.2 Phenotypic characterization of LAB

Phenotypic characterization conducted in this study was by Gram reaction, cell morphology and the catalase test. The catalase test was performed as follows. A small amount of the culture was smeared on a clean dry slide. Then, one drop of hydrogen peroxide (3%, v/v) was added in 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*.

2.2.2.3 Molecular characterization of LAB

Molecular characterization of the isolates was conducted by PCR and then analysed with RFLP. The target genes were in the region between the 16S and 23S rRNA, called the intergenic transcribed spacer (ITS). The oligonucleotide primers used were R139 F (position 1388-1406 of 16S rRNA gene) and 23S/p7 R (position 207-189 of 23S rRNA gene) (Table 4). The primers were designed from the complete DNA sequence of *Escherichia coli* (Gene Bank Accession number V00331). The other oligonucleotide primers used in this study that also amplify the spacer region were 23S/p10 (position 456-474 of 23S rRNA) and tAla (position tRNA gene) (Rachman et al. 2003).

2.2.2.3.a DNA template preparation for PCR

Total DNA was extracted by a simple lysis method (Bell et al. 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). 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.

2.2.2.3.b PCR amplification

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 5.

2.2.2.3.c Agarose gel electrophoresis

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. Seven µl of PCR products were dispensed into the wells. The result of electrophoresis 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 (size range: 100-1,517 bp, New England BioLabs Inc.)

2.2.2.3.d RFLP analysis

A total volume of 10 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. The solution was incubated at the optimum temperature for the enzyme activity for 24 hours. Following this incubation, 5 µl of the RFLP product plus 2 µl of loading dye were dispensed into the wells of the agarose gel and then electrophoresed as mentioned in the previous section.

2.2.2.4 Molecular identification of LAB

In order to confirm the results given by PCR ITS-RFLP, other genotypic techniques were applied on the basis of 16S rRNA sequencing and species-specific PCR. Identification of LAB was conducted using PCR amplification with universal oligonucleotide primers, namely plb16-F and mlb16-R (Table 4). The primer design was based on a conserved region between the V1 and V2 variable regions in 16S rRNA and a product of around 500 bp in size (Kullen et al. 2000). A PCR amplification using species-specific primers (Table 4) was also performed when the 16S rRNA gene sequencing results produced a comparable similarity value with the other closely related species.

2.2.2.4.a PCR amplification

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 5. The result of PCR amplification was visualized using agarose gel electrophoresis as mentioned in the previous section (2.2.2.3.c). A similar master mix composition was used for species-specific PCR.

2.2.2.4.b Purification of DNA products

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.

2.2.2.4.c 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, Adelaide. The nucleotide sequences were edited by using BioEdit software (version 7.0.9.0). 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/).

Table 4 List of primers used in this study

Primer*	Target	Oligonucleotide sequence (5'-3')	Annealing T (^{o}C)	Amplicon size (bp)	References
R139-F 23S/p7-R	ITS region	TTG TAC ACA CCG CCC GTC GGT ACT TAG ATG TTT CAG TTC	49	-	(Rachman et al. 2003)
23S/p10-R tAla-F	ITS region	CCT TTC CCT CAC GGT ACT G TAG CTC AGC TGG GAG AGC	56	-	(Rachman et al. 2003)
plb16-F mlb16-R	16S rRNA (V1 and V2 regions)	AGA GTT TGA TCC TGG CTC AG GGC TGC TGG CAC GTA GTT AG	52	~500	(Kullen et al. 2000)
Casei-F Y2-R	*Lactobacillus casei*	TGC ACT GAG ATT CGA CTT AA CCC ACT GCT GCC TCC CGT AGG AGT	50	290	(Ward & Timmins 1999)
Para-F Y2-R	*Lactobacillus paracasei*	CAC CGA GAT TCA ACA TGG CCC ACT GCT GCC TCC CGT AGG AGT	50	290	(Ward & Timmins 1999)
16-F Pentosus-R	*Lactobacillus pentosus*	GCT GGA TCA CCT CCT TTC GTA TTC AAC TTA TTA GAA CG	53	220	(Berthier & Ehrlich 1998)
16-F Plantarum-R	*Lactobacillus plantarum*	GCT GGA TCA CCT CCT TTC ATG AGG TAT TCA ACT TAT G	53	220	(Berthier & Ehrlich 1998)
plnA-F plnA-R	Plantaricin A	GTA CAG TAC TAA TGG GAG CTT ACG CCA TCT ATA CG	53	450	(Remiger et al. 1996)
ITS 1-F ITS 2-R	ITS region (yeasts)	TCC GTA GGT GAA CCT GCG G TCC TCC GCT TAT TGA TAT GC	53	-	(Granchi et al. 1999)

*F, forward; R, reverse

Table 5 PCR amplification condition used for the characterization and identification of LAB isolated from dadih

Primer*	PCR amplification condition**	References
R139-F 23S/p7-R	94 °C- 1m, 49 °C- 1m, 72 °C- 1m (35 cycles)	(Rachman et al. 2003)
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)
Casei-F Y2-R	94 °C- 45s, 50 °C- 45s, 72 °C- 1m (30 cycles)	(Ward & Timmins 1999)
Para-F Y2-R	94 °C- 45s, 50 °C- 45s, 72 °C- 1m (30 cycles)	(Ward & Timmins 1999)
16-F Pentosus-R	94 °C- 1m, 53 °C- 1m, 72 °C- 1m (30 cycles)	(Berthier & Ehrlich 1998)
16-F Plantarum-R	94 °C- 1m, 53 °C- 1m, 72 °C- 1m (30 cycles)	(Berthier & Ehrlich 1998)
plnA-F plnA-R	94 °C- 1m, 53 °C- 1m, 72 °C- 1m (35 cycles)	(Remiger et al. 1996)
ITS 1-F ITS 2-R	94 °C- 1m, 56 °C- 1m, 72 °C- 1m (35 cycles)	(Granchi et al. 1999)

*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 Predominant microorganisms in dadih

This study was focused on the isolation of LAB from dadih rather than on exploration of the total microbial flora. However, the use of a selective medium of MRS agar supplemented with 10% (w/v) of bromo-cresol purple allowed differentiation of two distinct groups of microorganisms. One group was LAB, characterized by acid production and resulting in yellow discoloration of the medium around the colonies; the other group consisted of yeasts which did not produce acid, so the medium remained purple in color. In addition, the yeast colonies were larger than the LAB colonies. The number of viable microorganisms isolated from dadih using this selective medium differed from the two production sites studies. In Sianok (S1: and S2:), the total number of microorganisms was 1.48 x 10^7 CFU/g after two days fermentation (S1) and 6.75x10^7 CFU/g after three days fermentation (S2). Whereas the total number of microorganisms isolated from Gadut (three days of fermentation) was 9.40 x 10^7 CFU/g.

A total of 68 isolates from the two production sites were tentatively identified as LAB, and 56 isolates as yeasts. As we can see from Figure 2 and Appendix A, the composition of microorganisms of dadih from the 2 and 3 day fermentation products from Sianok differed in microbial composition. The number of yeasts was almost three times higher in S2 compared with S1. Conversely, the LAB in S2 declined to nearly half the number in S1. The microflora in the dadih samples obtained from Gadut (G) was dominated by yeasts. A number of the yeasts from Sianok failed to survive subculturing after transport, reducing the number of yeasts available for study.

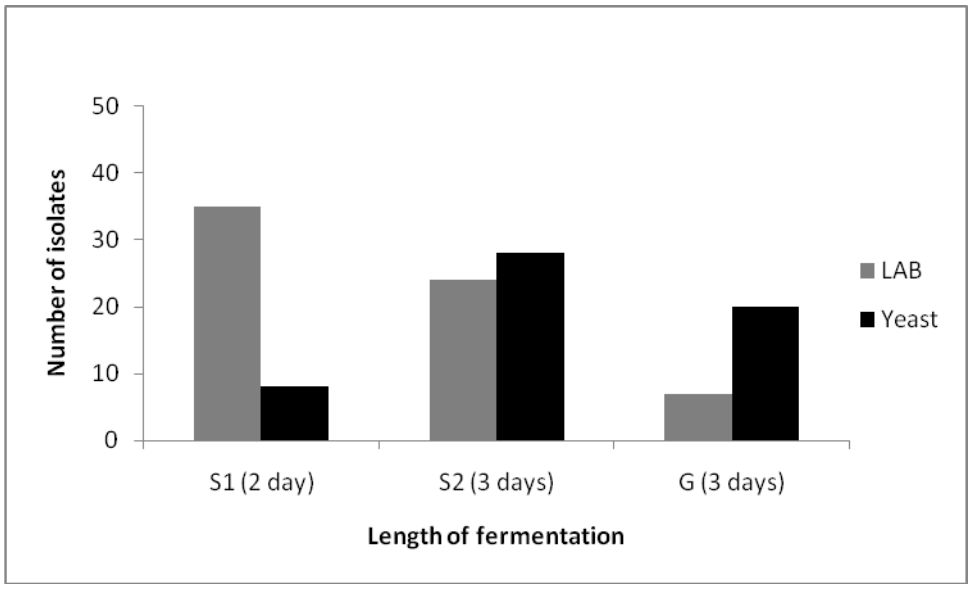


Figure 2 Proportion of LAB and yeasts in dadih from different sites (S and G) and from two and three day-fermentations

2.3.2 Biochemical and microscopic characterization of bacteria and yeasts

A total of 68 putative LAB isolates and 51 yeast isolates were further analyzed. Gram staining and the catalase assay were the general biochemical parameters used for preliminary characterization of LAB. Based on these parameters, a total of 66 isolates were characterized as potential LAB (Gram-positive and catalase-negative). Interestingly, of these 66 isolates three different cellular morphologies were observed; namely rod-shaped (37 isolates), rod-shaped with granules (20 isolates), and coccoid-shaped (nine isolates). The other two rod-shaped isolates were identified as Gram-negative and catalase-positive bacteria. Based on microscopic and colony morphology, two distinct groups of yeasts were observed. Eight isolates were characterized as being small and ovoid shaped. These isolates produced small creamy-

colored colonies. In contrast, 43 isolates were larger ovoid cells and produced larger sized colonies. Examples of the microscopic morphology of both LAB and yeasts can be seen in Appendix B. To confirm the differentiation of both LAB and yeast isolates, molecular characterization was also conducted.

2.3.3 Molecular characterization of LAB

Polymorphisms in the 16S-23S rRNA intergenic spacer region (ISR) were used as the basis of genetic differentiation of dadih LAB. The molecular characterization of yeasts will be described in chapter IV. Based on ITS-PCR results, four groups of LAB were differentiated from the amplification pattern (Table 6). The PCR products of group I revealed three ISRs of different sizes. In group II, two ISRs were produced, while only one major class was obtained for group III, IV and V (Group V isolates represent the Gram negative/catalase positive non-LAB strains). Different patterns of ITS-PCR products corresponding to group I and II can be seen in Figure 3. The ITS-PCR products of LAB are divided into three ISR categories, namely L-ISR, M-ISR and S-ISR associated with the presence and arrangement of tRNA genes ($tRNA^{Ile}$ and $tRNA^{Ala}$) within this region (Belgacem et al. 2009).

Table 6 Grouping of LAB based on ITS-PCR results

Group	Sub-groups	Cell-morphology	Number of isolates	ITS-PCR (bp)	tRNA (bp)	BLAST analysis (%)	Identification (16S rRNA gene sequencing)**
I	A	Rod	42	600, 750, 800	780	99	*Lactobacillus paracasei* S1A
	B	Rod with granules	13				*Lactobacillus paracasei* S1B
II		Rod	1	590, 800	780	98	*Lactobacillus plantarum*
III		coccoid	9	700	740	98	*Lactococcus lactis* subsp *lactis*
IV		Rod	1	800	820	99	*Leuconostoc pseudomesenteroides*
V*		Rod	2	1200	ND	99	*Acetobacter cerevisiae*

* non-LAB
**Species name as a result of 16S rRNA gene sequencing and then confirmed with species-specific primers.
ND, not determined

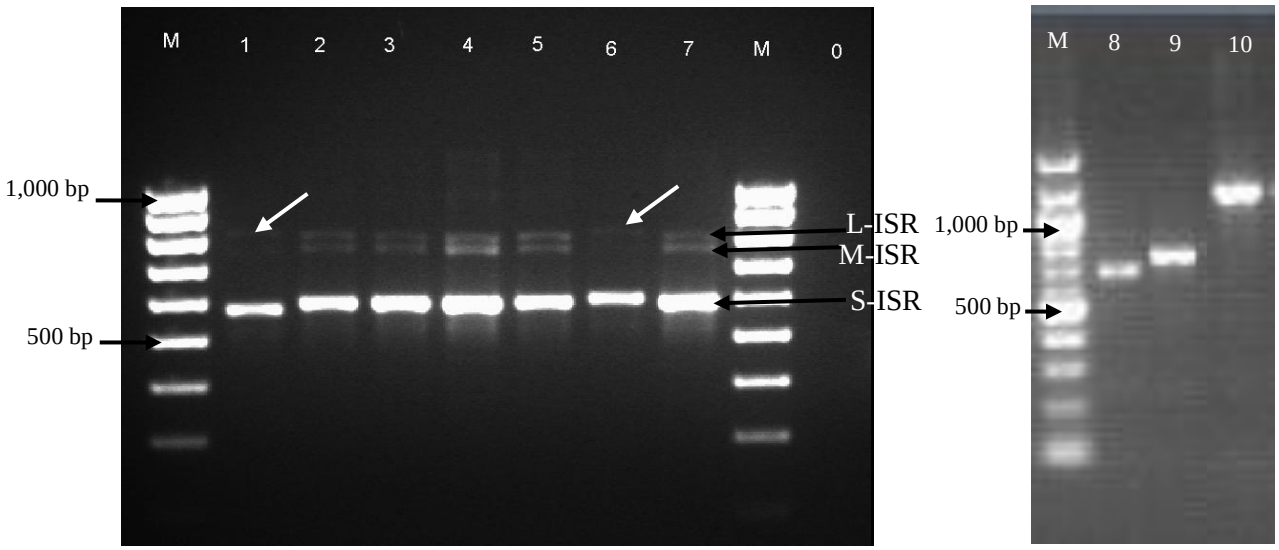


Figure 3 PCR amplification of 16S-23S rRNA gene ISR

Lane M: 100 bp DNA marker; lane 0: sterile milliQ H_2O; lane 1: group II (isolate S1.30); lane 2-5: group I (isolate S1.22, S2.05, S2.26 and S1.34), lane 6: *Lb. sakei* ATCC 15521; lane 7: *Lb. casei*; lane 8: group III (isolate S1.06); lane 9: group IV (isolate G5); lane 10: group V (isolate S2.14). The white arrow indicated the L-ISR of isolate S1.30 and *Lb. sakei* ATCC 15521

Due to the pattern of RFLP analysis of the 16S-23S rRNA-ITS region was quite difficult to interpret and did not able to distinguish between *Lb. plantarum* and *Lb. paracasei* digested with *RsaI* (Figure 6), another set of primers (23S/p10-R and tAla-F) were applied for further molecular characterization of the LAB isolates. A single band was produced from this PCR amplification (Table 6). RFLP analysis was performed to further differentiate either between the groups or within the group. A range of restriction enzymes were used for this analysis, namely *Hinf*I, *Hind*III and *Rsa*I (Appendix C). The restriction fragments that resulted were much clearer than the digestion results of 16S-23S amplification products. Moreover, when the tRNA gene region was cut with *Hinf*I, group I isolates could be differentiated into two types on the basis of different restriction patterns which confirmed the differentiation observed with the Gram-stain cell-morphology characteristics (Figure 7, Appendix C).

2.3.4 Molecular identification of LAB

To identify the isolates, representative strains were selected on the basis of phenotypic and molecular characterization, and 16S rRNA (V1 and V2 regions) sequence analysis was performed. The results show that group I was identified as *Lactobacillus paracasei*, group II as *Lactobacillus plantarum*, group III as *Lactococcus lactis* subsp. *lactis*, group IV as *Leuconostoc pseudomesenteroides*, and group V (non-LAB) as *Acetobacter cerevisiae* (Table 6).

The growth of *A. cerevisiae* on MRS medium is an unexpected finding. The results of partial 16S rRNA gene sequencing were analysed using Clustal W version 2 (http://ebi.ac.uk/tools/clustalw2/) to obtain a neighbour-joining tree, as shown in the cladogram (Figure 5). The five clusters resulting from the cladogram is consistent with the ITS-PCR results. The isolates in group I were further classified into two types according to their restriction analysis and cellular morphology (the presence of granules); designated as *Lb. paracasei* strain S1A and S1B (Figure 7 and Appendix C).

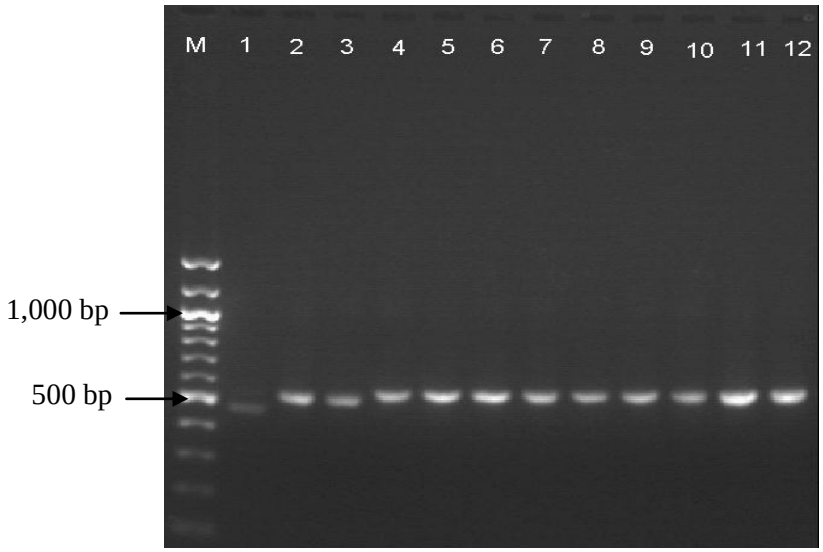


Figure 4 Representation of PCR products that were sequenced generated by universal primers targeting 16S rRNA V1-V2 variable regions

Lane M: 100 bp DNA marker; lane 1: group V (isolate S2.44); lane 2: group IV (isolate G.05); lane 3: group III (isolate G10); lane 4: group II (isolate S1.30); lane 5-10: group Ia (isolates S134-S1A, S103-S1A, S133-S1A, S226-S1A, S236-S1A and S204-S1A); lane 11-12: group Ib (isolate S205-S1B and S111-S1B)

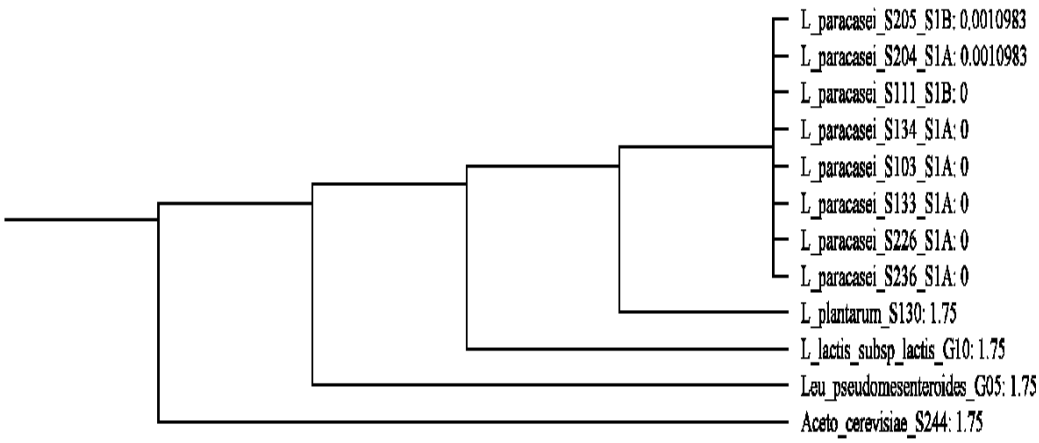


Figure 5 Cladogram of partial 16S rRNA gene sequences of the selected isolates representing the different groups

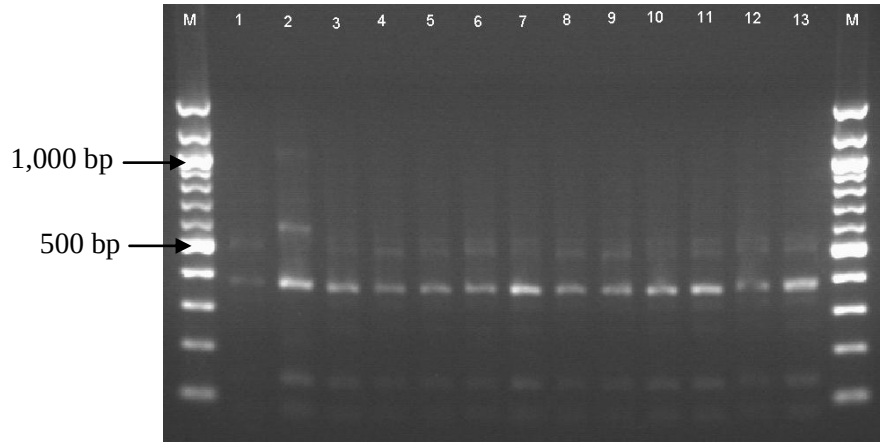


Figure 6 Gel electrophoresis of PCR-amplified 16S-23S rRNA- ITS region digested with *RsaI*
Lane M: 100 bp DNA marker; lane 1: *Lb. sakei* ATCC 15521; lane 2: *Lb. plantarum* S1.30, lane 3-13: *Lb. paracasei* strains S134-S1A, S103-S1A, S133-S1A, S226-S1A, S236-S1A, S204-S1A, S102-S1A, S119-S1A S205-S1B, S111-S1B and S126-S1B.

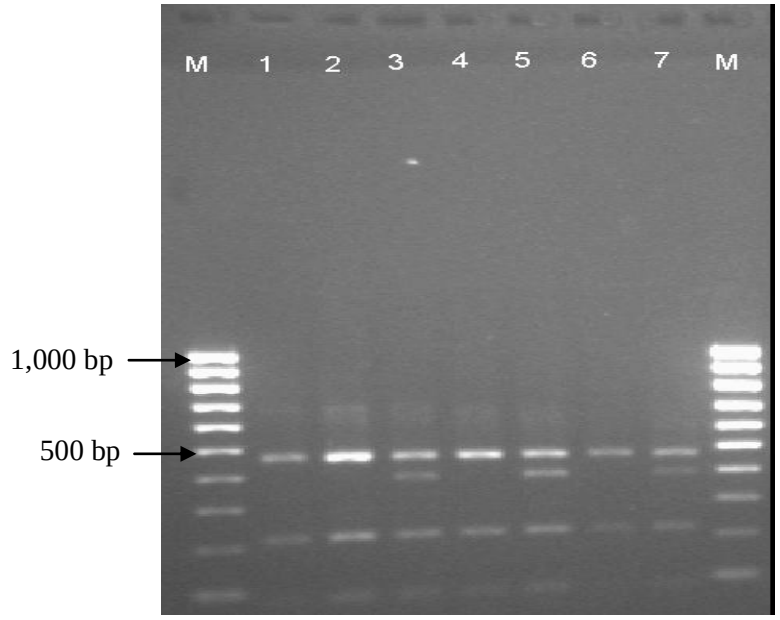


Figure 7 Gel electrophoresis of PCR-amplified tRNA genes of the *Lb. paracasei* strains digested with *Hinf*I
Lane M: 100 bp DNA marker; lane 1: *Lb. plantarum* S1.30; lane 2: *Lb. casei*, lane 3-7: *Lb. paracasei* strains S102-S1A, S111-S1B, S119-S1A, S126-S1B and S135-S1A (S1A: 3 bands; S1B: 2 bands)

Due to a high degree of similarity of *Lb. paracasei* with other closely related species, and *Lb. plantarum* with other closely related species; species-specific primers were used to confirm the results given by 16S rRNA gene sequence analysis. *Lb. paracasei*, which is a member of the *Lb. casei* group, could be distinguished from the other sub-groups (*Lb. casei, Lb. rhamnosus* and *Lb. zeae*) using species-specific oligonucleotide primers on the basis of their 16S rRNA gene sequences (Desai et al. 2006). In this case, *Lb. paracasei* can be differentiated

from *Lb. casei*, which shared a 16S rRNA sequence identity of 99% (Figure 8). The same case also occurred in *Lb. plantarum* S1.30 (Figure 9). This strain showed a 98% sequence identity with *Lb. pentosus*, but the initial designation was shown to be correct using a pair of primers designed specifically to the variation present in the intergenic spacer region (Berthier & Ehrlich 1998).

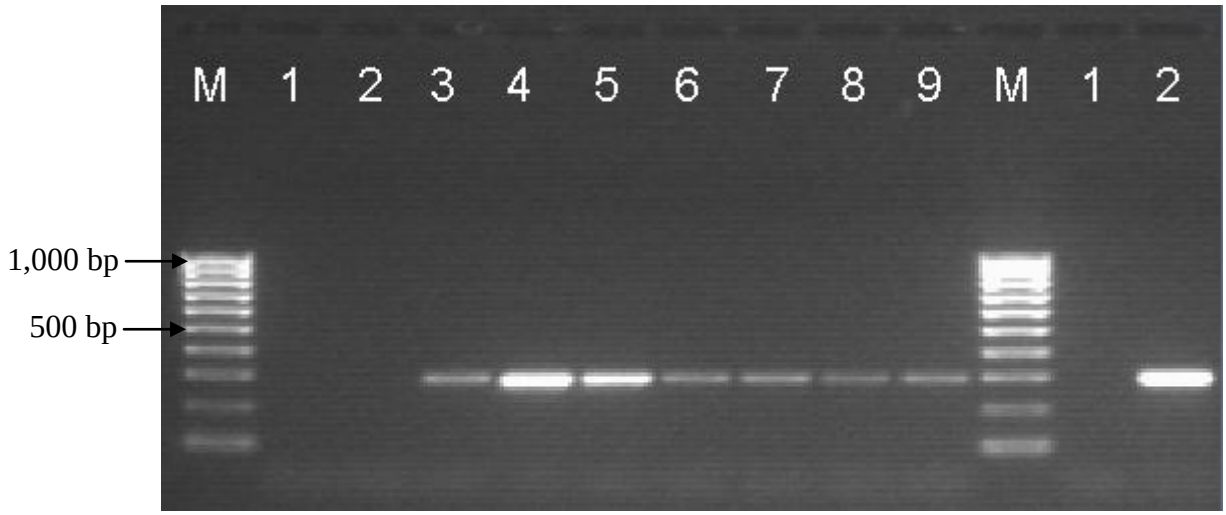


Figure 8 Agarose gel electrophoresis of *Lb. paracasei* using species-specific primers
Lane M: 100 bp DNA marker; lane 1: sterile milliQ H2O; lane 2: *Lb. casei*; lane 3-9*: Lb. paracasei* strains S2.04, S2.52, S1.35, S1.14, S1.17, S1.27 and S1.44 (290 bp). Primers: para-F and Y2-R (lane 1-9); casei-F and Y2-R (lane 1-2)

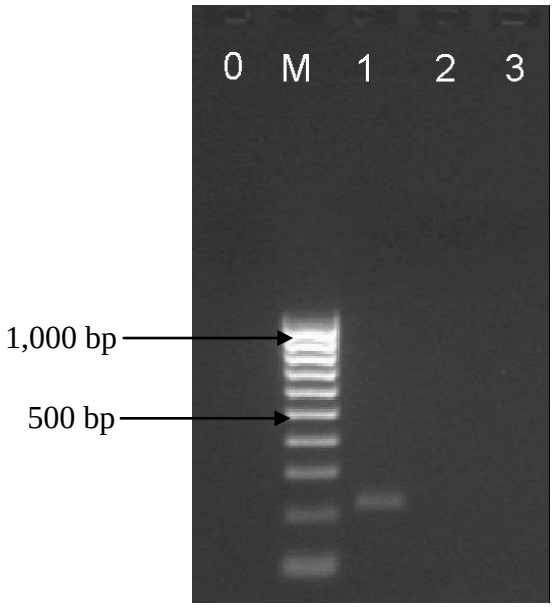


Figure 9 Agarose gel electrophoresis of PCR-amplified species-specific *Lb. plantarum* S1.30 (16-F and plantarum-R)
Lane M: 100 bp DNA marker; lane 0: sterile milliQ H2O, lane 1: *Lb. plantarum* S1.30 (220 bp); lane 2: *Lb. casei*; lane 3: *Lb. paracasei* S205-S1B

The LAB most frequent isolated from dadih was *Lb. paracasei* S1A (37 isolates or 54%), followed by *Lb. paracasei* S1B (20 isolates or 29%) and *Lc. lactis* subsp. *lactis* (9 isolates or 13%) (Figure 10). While only one strain of *Lb. plantarum* and *Leu. pseudomesenteroides* was isolated from Sianok and Gadut, respectively. Dadih from Sianok was dominated by *Lb. paracasei* S1A, whereas *Lc. lactis* subsp. *lactis* was prominent in Gadut. The presence of *A. cerevisiae* in dadih was only detected in dadih from Sianok after three days of fermentation. Conversely, *Lb. plantarum* was only detected at two days of fermentation.

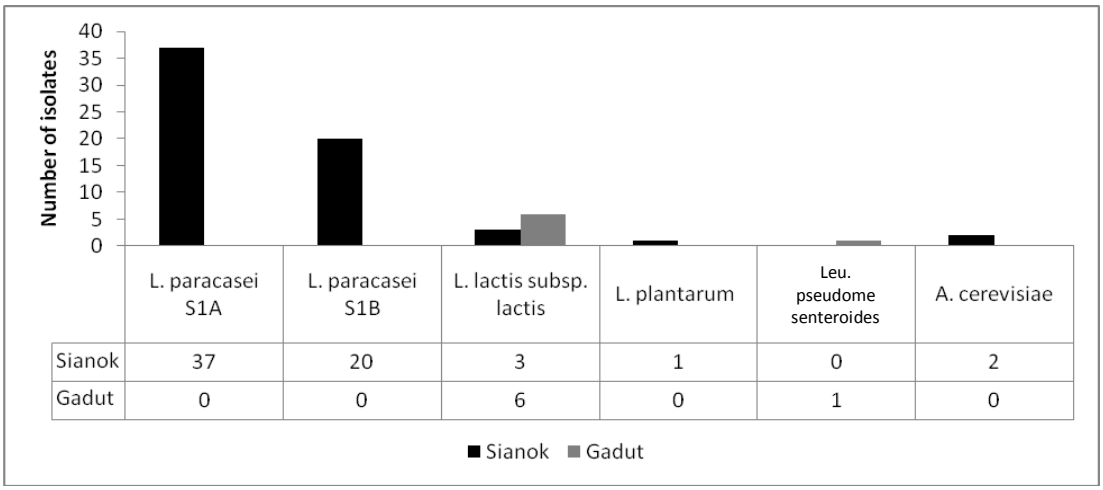


Figure 10 The number of dadih isolates selected randomly from the isolation medium

2.4 Discussion

In this study, the dadih LAB strains can be easily differentiated by their 16S-23S ITS profiles. According to Belgacem et al (2009), LAB can be grouped into three types on the basis of ITS-PCR results. This is consistent with the results of the dadih LAB isolated in this study. The first pattern was produced by *Lb. paracasei* and is characterised by the amplification of three DNA fragments, namely S-ISR (600 bp), M-ISR (750 bp) and L-ISR (800 bp). The M-ISR and L-ISR contain one tRNA (tRNAAla) and two tRNA genes (tRNAIle and tRNAAla), respectively, while the S-ISR contains no tRNA genes. *Lb. plantarum* S1.30 amplified two PCR products. tRNA genes (tRNAIle and tRNAAla) were only present in the L-ISR (800 bp) fragment in this amplification (Figure 3*). Lc. lactis* subsp. *lactis* and *Leu. pseudomesenteroides* only amplified a single M-ISR fragment (700 bp and 800 bp, respectively) that contained the tRNAAla gene. The tRNA genes in the ISR of dadih LAB were confirmed by the amplification of a single band using specific tRNA primers (23S/p10-R and tAla-F). As

shown in Appendix C, analysis with HinfI often yielded fragments whose sizes did not add up to 780 bp. One explanation could be that there was incomplete digestion of the product but this can be discounted by the results in Figure 7. However, the intensity of the bands in Figure 7 shows differences in the amounts of the 480 and 400 bp fragments and this could indicate that there are different numbers of copies of this tRNA gene in the genome where multiple copies of one with less HinfI sites gives a 480 bp band and fewer copies of one that has more HinfI sites the gives both 480 and 400 bp bands. This also could explain why these fragments do not total 780 bp. In addition, close examination of Figure 7 shows a faint band at 80 bp. For Group 1B strains inclusion of this band would equate to a full size fragment (220+480+80=780 bp). A study conducted by Han et al. (2005) reported that the restriction fragments which have closely similar in size appeared as a single band on the agarose gel. Therefore, these authors suggested utilizing polyacrylamide gels to separate these bands. Clearly these issues warrant further investigation.

This study has found that *Lb. paracasei* was the dominant strain of LAB in dadih, followed by *Lc. lactis* subsp. *lactis*. The other LAB strains obtained were *Lb. plantarum* and *Leu. pseudomesenteroides*. This finding differs with previous research that identified *Leu. mesenteroides* as the major strain found in dadih (Hasono et al. 1989; Zakaria et al. 1998). The most current investigation conducted by Surono and Nurani (2001) used biochemical identification methods to show that *Lactobacillus* sp., *Lactococcus* sp., and *Leuconostoc* sp. were the main species of LAB in the dadih. Our study has also identified two species of LAB, namely *Lb. paracasei* and *Leu. pseudomesenteroides* that have never been reported in dadih before. Even though *Lb. paracasei* has never been reported in dadih, the species is commonly isolated from other milk products. According to Ward & Timmins (1999), and supported by Desai et al. (2006), *Lb. paracasei* is the most frequently isolated species in dairy products. Dadih micro-flora appears to be highly variable, which is no doubt related to the use of traditional techniques and the spontaneous fermentation in the production process. This was also observed in microbial ecology studies of African fermented milk. The variation of microbial composition could lead to unstable product quality and a difficulty in predicting the microorganisms involved in the fermentation (Narvhus & Gadaga 2003).

This study has successfully differentiated *Lb. paracasei* into two types which differed in both their cellular morphologies and tRNA gene sequences. From a taxonomical point of view, *Lb. paracasei* is officially divided into two sub-species, namely *Lb. paracasei* subsp. *paracasei* and *Lb. paracasei* subsp. *tolerans* (Hammes & Vogel 1995). The cellular morphologies of

these two sub-species are described as rod-shaped, 0.8-1.0 by 2.0-4.0 µm in size, often with square ends and with a cell configuration that is usually either singly or in chains (Hammes & Vogel 1995). The appearance of granules in the cytoplasm of *Lb. paracasei* strains isolated from dadih has, to our knowledge, not been previously reported (Appendix B). A more detailed investigation is required to characterize the nature and role of these granules. Based on different cellular morphologies and RFLP of tRNA gene, *Lactobacillus paracasei* S1B could be categorized as a novel sub-species. However, more biochemical and physiological studies are required to confirm these findings.

Besides dadih LAB, *A. cerevisiae* was also identified molecularly using universal primers that targeted V1 and V2 variable regions of 16S rRNA genes. The 16S rRNA sequence of *A. cerevisiae* was 99% identical to the *A. cerevisiae* type strain LMG 1625^{T}. Cell (Gram-negative, rod-shaped, singly and short-chains) and colony (pale brown in colour, circular-shaped, entire in edge, raised and smooth in elevation) morphology of the dadih isolated bacteria are in agreement with the description of the *A. cerevisiae* type strain. This species was first isolated from stored beer (ale) in Toronto, Canada by Kozulis and Parsons in 1958 (Cleenwerck et al. 2002), and has since been found in vinegar, fruits/plants, and fermenting cocoa bean (Altieri et al. 2008). The occurrence of this strain in dadih is not too surprising as this species can grow in acidic condition (pH 2.5-3.5) and oxidize acetate and lactate to CO_2 and H_2O (Cleenwerck et al. 2002; Altieri et al. 2008).

However, with the exception *Lc. lactis* subsp *lactis*, the different species of LAB were found in only one of the dadih samples. On one hand, these results indicate that the micro-flora composition varied between dadih samples, but on the other hand, insufficient information was obtained to reflect the full diversity of dadih micro-flora due to limited samples and use of LAB-specific culture media. Further study is required on more dadih samples from several regions.

Yeasts were also identified in the dadih ferments. The yeasts were still able to grow in MRS medium as has been reported by Rose (1985). The complete discussion on dadih yeasts is presented in Chapter Four.

2.5 Conclusion

The micro-flora in dadih appears to be quite variable, although only three dadih samples were examined. This is likely to be the result of spontaneous fermentation of the product and

the natural materials (bamboo tubes, banana leaves and buffalo milk) used in its production. Two previously identified LAB species were found in this study, namely *Lb. plantarum* and *Lc. lactis* subsp. *lactis*. Two LAB species, *Lb. paracasei* and *Leu. pseudomesenteroides*, as well as the non-LAB bacteria *A. cerevisiae*, and two yeast species were also shown to be present in dadih. The application of PCR - RFLP analysis of the 16S-23S ISR provided a useful method to describe the indigenous microbial community in dadih. The restriction profiles of dadih LAB can be used to provide a more detailed understanding of dadih microbial ecology in future studies.

As the purpose of this study was to focus on LAB a selective medium was used. This medium is highly selective for LAB and it has been shown that several yeasts are inhibited. As a selective medium was used, it is important to realize that the microbial diversity in dadih is greater than observed, both in terms of non-LAB and yeast. A more comprehensive ecological study of dadih using generalized media is likely to highlight the true diversity of microorganisms present in this product.

CHAPTER THREE

INVESTIGATION OF LACTIC ACID BACTERIA ISOLATED FROM DADIH FOR PRODUCTION OF BACTERIOCINS

3.1 Introduction

Dadih is considered an important traditional fermented milk product with potential health benefits to local Indonesian communities. LAB isolated from dadih have been shown to have probiotic properties such as anti-mutagenic and cholesterol binding activities that are stable in bile and acid conditions (Surono 2003; Collado et al. 2007a, b). In addition dadih LAB have antimicrobial activity. The consumption of dadih is safe, even though the sanitary conditions during manufacturing are poor. The capability of the LAB to produce bacteriocins is believed to be important in increasing the safety and extending the shelf-life of the fermentation products (Talon et al. 2007). Bacteriocin-producing LAB have been isolated from a variety of NFM products, such as airag, amasi, dahi, raib and gioddu (Table 1).

A study by Collado et al (2007b) demonstrated that *Lb. plantarum* IS-10506 was the LAB in dadih that demonstrated the most active inhibitory activity to intestinal adhesion of pathogenic bacteria. In a previous study, bacteriocin-like inhibitory substances (BLIS) active against indicator bacteria were detected in dadih LAB (Surono 2003). However, whether the antimicrobial activity was mediated by antimicrobial peptides has not been confirmed. Bacteriocins are characterized as proteinaceous compounds which can be inactivated by protease enzymes (Heng et al. 2007). In this current investigation, some dadih LAB were assessed for their potential to produce antimicrobial substances, with a particular focus on bacteriocins.

3.1.1 Aims

To detect the presence of bacteriocins from indigenous LAB using both conventional methods and a PCR-based method.

3.2 Materials and Methods

3.2.1 Materials

3.2.1.1 Bacterial strains

Bacterial strains used in this study either as bacteriocin producers or indicator bacteria are listed in Table 7.

All bacterial stocks were maintained in snap freeze medium at -80 ^{o}C. Fresh cultures were prepared by taking a small portion of the frozen stocks using sterile wooden sticks and then inoculating them onto appropriate solid media.

3.2.1.2 Media

3.2.1.2.a Solid media

The media used for isolation and cultivation of lactic acid bacteria was de Man, Rogosa and Sharpe (MRS) Agar (Oxoid Ltd, Basingstoke, UK) and was prepared according to the manufacturer's instructions:

- Tryptone Soya Agar (Oxoid, CM131) was used for cultivation of non-LAB (pathogenic bacteria).
- M17 agar prepared from M17 broth (Oxoid, CM0817) supplemented with 1.5% (w/v) of Technical Agar No.3 (Oxoid, LP0013) was used for cultivation of lactococci strains.

3.2.1.2.b Liquid media

The following media manufactured by Oxoid Ltd, Basingstoke, UK were utilized for bacterial cultivation and were prepared according to manufacturer's instructions:

- de Man, Rogosa and Sharpe (MRS) broth was used for cultivating of LAB.
- MRS broth supplemented with 0.75% (w/v) of Technical Agar No.3 (LP0013) was used for cultivating bacteria in semi solid media.
- Tryptone Soya Broth (TSB) (CM129) was used for culturing of non-LAB (pathogenic bacteria). With supplementing of 0.75% (w/v) of Technical Agar No.3 (LP0013) was used as soft agar for cultivating indicator bacteria (non-LAB).
- M17 broth (CM0817) was used for cultivating of lactococci strains.

- MRS broth and TSB with glycerol (snap freeze medium) was used for the maintenance of LAB and non-LAB, respectively, at -80 ^{o}C as stocks.

3.2.1.3 Chemical and reagents

3.2.1.3.a Chemicals

Nisaplin (nisin from *Streptococcus lactis*, 2.5%) (Sigma-Aldrich: CAS number 1414-45-5): Ten grams of nisin in 50 ml of sterile milliQ water to make a concentration 0.2 mg/ml.
N-Lauroylsarcosine sodium salt (0.02 M) in sterile milliQ water was purchased from Sigma-Aldrich (CAS: 137-16-6).

3.2.1.3.b Enzymes

Proteinase-K (Merck) in Tris HCl buffer (pH 7.5) at a concentration of 1 mg/ml was used for assessment of bacteriocin activity.

3.2.1.3.c Buffer solutions

TAE buffer (40 mM Tris, 1.14 ml of glacial acetic acid, 1 mM EDTA) was used for agarose gel electrophoresis.
Tris HCl buffer (pH 7.5) was used for diluting Proteinase-K enzyme.

3.2.1.3.d PCR reagents

The oligonucleotide primers used in this study are listed in Table 4. The purified synthetic oligonucleotide primers were purchased from GeneWorks Pty Ltd, Adelaide, and they were resuspended in sterile MilliQ water (100 µM) and then stored at -20 ^{o}C for stocks.
Taq DNA polymerase, 10x buffer, 25 mM magnesium chloride ($MgCl_2$) and dNTPs were purchased from Promega (Madison, Wis. USA).
DNA Ladder (100 bp, range: 100-1,517 bp) was purchased from New England BioLabs Inc.
Agarose gel for the electrophoresis was purchased from Promega (Madison, Wis. USA).

3.2.2 Methods

3.2.2.1 Screening of antimicrobial activity

For detection of the antimicrobial activity of the LAB isolates, an agar spot test and an agar well-diffusion test were used.

3.2.2.1.a Agar spot test

The agar spot-on-lawn or agar spot assay was conducted to screen antimicrobial producers. This method followed Fleming et al (1990) with a little modification. The LAB isolates as the test bacteria were prepared as follows. The LAB isolates from the frozen stocks were revived on MRS agar and the plates were incubated at 37 ^{o}C in 5% CO_2. After an overnight incubation, a single colony was removed and then streaked onto the surface of fresh MRS agar. The incubation conditions and time were as above. Meanwhile, the LAB indicator bacteria (*Lb. casei*, *Lb. fermentum* and *Lc. lactis* subsp. *lactis* LM0230) were cultured using MRS broth. The cultures were revived from frozen stocks, and then subcultured twice in 15 ml of fresh MRS broth at 37 ^{o}C in 5% CO_2. These overnight cultures were inoculated into 5 ml of soft MRS agar (0.75 %, w/v), and mixed gently. The mixtures were then poured over the plates where the colonies of the test bacteria were growing. After an overnight incubation at 37 ^{o}C in 5% CO_2, the plates were checked for the presence of the inhibition zones around the producer colonies. The inhibition zones were measured including the diameter of the colony. This experiment was carried out in duplicate.

3.2.2.1.b Agar well-diffusion

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 in 5% CO_2. 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 hours.

At the same time, the indicator bacteria (Gram-positive and Gram-negative pathogenic bacteria, Table 7) were also prepared in the same way, except for the incubation conditions, which was under aerobic condition (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 containing 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.

3.2.2.2 Conventional detection of bacteriocins

The detection of bacteriocins was performed using the well-diffusion assay as described previously. Instead of using the culture suspensions directly, as in the bacteriocin detection method described above, a cell-free supernatant (CFS) of putative bacteriocin-producing LAB was used. The CFS was obtained by centrifuging (5,444 x g for 20 minutes at 4 ^{o}C, Sigma 3-16K) the overnight cultures (15 ml) of candidate LAB, followed by adjusting of the supernatant to pH 6.5 to exclude the organic acid activity. Next, the neutralized-CFS (NCFS) was filtrated through a 0.2 µm membrane filter (PES membrane, Millex® GP, Millipore, Carrigtwohill Co, Cork, Ireland). This sterile neutralized supernatant (80 µl) was dispensed into the wells which had been prepared as described previously. The presence of a clear zone of inhibition indicated the result of bacteriocin-like inhibitory substance (BLIS) activity.

Two modified procedures were used during either the preparation of the bacterial cultures or sterilization of NCFS in order to detect the BLIS activity when the standard method described above was unsuccessful. In the first modification, 5% (v/v) of Tris HCl 0.05M (pH 7.5) and N-lauroylsarcosine sodium salt (pH 6.5) was added to the bacterial suspensions immediately before they were centrifuged. This treatment aimed to release bacteriocin that might be attached to the cell membrane of the bacteriocin producers (Stern, personal communication, 2008). The second modification was to heat the NCFS at 80 ^{o}C for 15 minutes using a dry block heater (Thermoline Australia) to sterilize the NCFS solution (instead of using filter sterilization). This technique was used in case the bacteriocin compounds were being trapped in the membrane filter (Tagg, personal communication, 2008).

In addition to these two modifications, a protease test was used in an attempt to confirm the proteinaceous nature of the antimicrobial product from the dadih LAB. The method used in this study was described by van Belkum et al (1989) with a minor modification. A 4-mm well was made next to the well (6 mm in diameter) containing the putative-bacteriocin producer cultures. Subsequently, 50 µl of proteinase-K (1 mg/ml) was dispensed into the small wells. After incubation at 37 ^{o}C in 5% CO_2 for 24 hours, the inhibition of antimicrobial activity of the producers was observed. Nisin (0.2 mg/ml) was used as a positive control. In this test LAB strains were used as the indicator bacteria.

3.2.2.3 Production kinetics of an antimicrobial product from a potential LAB isolate

The growth and antimicrobial activity of a putative-bacteriocin producer strain was assessed *in vitro*. An overnight broth culture (10 %, v/v) was inoculated into fresh MRS

broth (200 ml). The culture was incubated at 37 ^{o}C in 5% CO_2 for 24 hours, and every two hours 15 ml of culture was removed to determine the cell count, pH, and antimicrobial activity. After 12 hours incubation, the sample was removed in every three hours. The total number of bacterial cells was determined using the spread plate method by serial dilution (10^{-1} – 10^{-7}). The acidity level of sample was examined using digital pH meter (Hanna Instruments). The inhibitory activity in both the cell suspensions and the NCFS was evaluated using the well-diffusion assay as described previously with *Listeria monocytogenes* and *Lactobacillus sakei* ATCC 15521 as the indicator bacteria. The antimicrobial activity was indicated by the diameter of clear zones of inhibition growth surrounding the producer colonies. The evaluation of each parameter was conducted in duplicate.

3.2.2.4 Molecular detection of bacteriocins

A PCR-based method was used to detect *pln*A, a gene associated with bacteriocin activity using specific primers selected from the published literature (Remiger et al. 1996). The LAB strains used were those showing the greatest activity in inhibiting the growth of both Gram-positive and Gram-negative bacteria in the agar spot test and agar well-diffusion test even though they were negative for bacteriocin production in the cell-free supernatant tests. The PCR amplification and the electrophoresis methods were performed as described in the previous chapter (2.2.2.3.b and 2.2.2.3.c).

3.2.2.5 Bacteriocin induction in co-culture

Induction of bacteriocin production through co-culture was attempted according to Maldonado et al. (2004). The antimicrobial producer used in this study was the LAB strains which were not inhibitory in the agar spot test or agar well-diffusion test. The method of the bacteriocin induction is as follows: The overnight broth culture (1 %, v/v) was inoculated into 100 ml of fresh MRS broth and then 0.5% of overnight culture of inducer bacteria (*L. monocytogenes* and *Lb. sakei* ATCC 15521) was immediately added. Each co-cultivation was incubated at 37 ^{o}C in 5% CO_2 for 24 hours. Both antimicrobial activity of the viable cells and bacteriocin activity of NCFS were evaluated using the agar well-diffusion method as described previously with *L. monocytogenes* and *Lb. sakei* ATCC 15521 as the indicator bacteria. Non-neutralized CFS and heating treatment (80 ^{o}C) for 15 min) instead of membrane filtration were also utilized. The diameter of the inhibition zones was measured including the diameter of the well itself. These experiments were conducted in duplicate.

Table 7 List of bacterial strains used in this study as indicator bacteria and controls

Strains	Collection code	Characteristic	Source
Lactobacillus sakei* ATCC 15521	-	Bacteriocin sensitive indicator (non-bacteriocin producer)	American Type Culture Collection
Lactococcus lactis* subsp. *lactis* ATCC 11454*	-	Nisin producer	
Lactococcus lactis* subsp. *lactis* LM0230	-	Non-nisin producer (plasmid free)	Dairy Research Institute, Melbourne
Lactococcus* lactis subsp. *lactis* DL11*	-	Nisin A producer	Dairy Research Institute, Melbourne
Lactobacillus casei*	-	LAB	IMVS, South Australia
Lactobacillus hilgardii*	-	LAB	IMVS, South Australia
Lactobacillus fermentum*	-	LAB	IMVS, South Australia
Salmonella sofia*	1-31 (99-5503892)	Gram negative - multiple antibiotic resistance	University of South Australia
Salmonella kiambu*	5-4 (00-5500436)	Gram negative - multiple antibiotic resistance	University of South Australia
Salmonella typhimurium* P135	3-1 (99-5503917)	Gram negative - multiple antibiotic resistance	University of South Australia
Salmonella* subsp. *sofia*	1-32 (99-5503848)	Gram negative - multiple antibiotic resistance	University of South Australia
Salmonella agona*	2-17 (00-5500390)	Gram negative - multiple antibiotic resistance	University of South Australia
Escherichia coli*	99364-1	Gram negative - multiple antibiotic resistance	University of South Australia
Escherichia coli*	99386-3	Gram negative - multiple antibiotic resistance	University of South Australia
Escherichia coli*	99385-5	Gram negative - multiple antibiotic resistance	University of South Australia
Escherichia coli*	99416-1	Gram negative - multiple antibiotic resistance	University of South Australia
Escherichia coli*	99363-1	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

* indicator bacteria

** positive control (bacteriocin producers)

3.3 Results

3.3.1 Screening of antimicrobial activity

The antimicrobial activity of four LAB species isolated from dadih, namely *Lb. paracasei*, *Lb. plantarum*, *Lc. lactis* subsp *lactis* and *Leu. pseudomesenteroides* were screened using the agar spot test. Only strains of *Lb. paracasei, Lb. plantarum* and *Lc. lactis* subsp *lactis* exhibited an inhibition zone against LAB indicator bacteria (*Lb. casei*, *Lb. fermentum* and *Lc. lactis* subsp. *lactis* LM0230) (Appendix E). Interestingly, the only *Lc. lactis* subsp *lactis* strain that produced antimicrobial activity was isolated from dadih from Sianok. Representative strains of these three dadih LAB was further tested against Gram-negative and Gram-positive pathogenic bacteria by the agar well-diffusion assay (Table 8).

Lb. plantarum S1.30 demonstrated greater antimicrobial activity than other strains, with inhibitory activity against all bacterial indicator strains tested. In contrast, the antimicrobial activity of *Lb. paracasei* strains was variable. Two representatives of *Lc. lactis* subsp *lactis* isolated from dadih from Sianok showed an ability to inhibit all of the indicators strains, except *B. cereus*. The dadih LAB strains showed the greatest activity against *S. pyogenes* ATCC 10389, followed by *L. monocytogenes*.

3.3.2 Detection of bacteriocin activity

The antimicrobial activity of dadih LAB (*Lb. plantarum* S1.30, *Lb. paracasei* and *Lb. lactis* subsp *lactis*) was assessed using the agar well-diffusion method. The neutralized-cell free supernatant of these antimicrobial producers was tested against *Lb. sakei* ATCC 15521 and *L. monocytogenes*, but no bacteriocin activity was detected.

To try to identify antimicrobial peptide activity, modified procedures were investigated. The first modification, the addition of N-lauroylsarcosine sodium salt to the growth media of the producers failed to detect any bacteriocin activity. The second modification, the sterilization of N-CFS using heating (80 ^{o}C for 15 minutes) also failed to detect any bacteriocin activity. In short, no antimicrobial activity was detected by either method.

A further test was applied to investigate the nature of the inhibitory activity of the producer strains. The effect of proteinase-K on the inhibition zones produced by *Lb. plantarum* S1.30 and *Lc. lactis* subsp *lactis* S2.24 was assessed but there was no effect. This suggested that the antimicrobial activity of the producers was not mediated by proteins, raising the possibility that bacteriocins were not the active agents in the antimicrobial activity.

Table 8 Antimicrobial activity of selected dadih LAB strains

Indicator bacteria	Ll 11454	Ll DL11	Lm	Lc	Lp	*Lc. lactis*				*Lb. paracasei*																
						G 6	G 2	S1 06	S2 24	S1 19	S2 26	S1 36	S1 03	S1 02	S1 25	S2 21	S2 52	S2 18	S2 42	S2 04	S14 0	S2 51	S1 11	S2. 05	S1 33	S1 31	S1 39
Streptococcus pyogenes ATCC 10389	++	++	+++	++	+++ +	-	-	++ +	++ +	++ +	++ +	++ +	++ +	++	++	++ +	++	++ +	++	++ +	++ +	++ +	+++	++ +	++ +	++ +	+++
Listeria monocyto-genes	+	+	++	++	++	-	-	+	++	++	+	++	++	++	++	++	++	++	++	++	+	++	++	+	++	+	+
Staphylococcus aureus ATCC 25923	-	-	+	+	++	-	-	+	+	-	-	-	+	-	-	-	-	-	-	+	++	+	+	++	+	+	+
Bacillus cereus	-	-	+	+	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Enterococcus faecalis ATCC 29212	-	-	++	++	++	-	-	+	++	+	+	+	+	++	+	++	++	++	++	++	+	+	+	+	+	+	+
Salmonella subsp. *sofia*	-	-	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salmonella kiambu	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	
Salmonella typhi-murium P135	-	-	-	-	+	-	-	+	+	-	-	+	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+
Salmonella sofia	-	-	-	-	+	-	-	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salmonella agona	-	-	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-
Escherichia coli 99364-1	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Escherichia coli 99386-3	-	-	-	-	++	-	-	+	++	-	+	+	-	+	+	+	+	+	+	++	++	+	+	++	++	+	+
Escherichia coli 99385-5	-	-	+	+	+	-	-	+	+	+	-	+	+	+	+	++	+	++	+	+	+	+	+	+	+	+	+
Escherichia coli 99416-1	-	-	+	+	+	-	-	+	++	+	+	+	-	+	+	+	+	+	+	++	+	+	+	+	+	+	+
Escherichia coli 99363-1	-	-	+	+	++	-	-	-	+	-	+	+	+	++	+	++	++	++	+	+	+	+	+	+	+	+	+

The diameter was including the well diameter (6 mm)
-, <6 mm (no antimicrobial activity)
+, 6-9 mm; ++, 10-14 mm; +++, 15-20 mm; ++++, >20 mm
Ll 11454: *Lc. lactis* subsp. *lactis* ATCC 11454
Lm: *Leu. mesenteroides;* Lc: *Lb. casei*
Ll DL11: *Lc. lactis* subsp. *lactis* DL11
Lp: *Lb. plantarum* S1.30
Sources of dadih samples: G: Gadut; S1: Sianok (two days of fermentation); and S2: Sianok (three days of fermentation)

3.3.3 Production kinetics of antimicrobial substances from *Lactobacillus plantarum* S1.30

Lactobacillus plantarum S1.30 was utilized for further study, as this strain exhibited the greatest antimicrobial activity, especially against *Lb. sakei* ATCC 15521 and *L. monocytogenes*. A study of the inhibitory activity of this potential antimicrobial producer was performed in a static broth culture with unregulated pH. As can be seen in Figure 11, the pH of the culture decreased from 6 to 3.54 during an overnight incubation at 37 ^{o}C in 5% CO_2 as the bacterial cell count increased. Exponential growth of *Lb. plantarum* S1.30 occurred between two and 18 hours of incubation, and early stationary phase started at 18 hours of incubation. The production of antimicrobial substances from *Lb. plantarum* S1.30 against *Lb. sakei* ATCC 15521 began after 2 hours of incubation and gradually increased, reaching maximum activity (20 mm in diameter) after 15-24 hours of incubation. When *Lb. plantarum* S1.30 was tested against *L. monocytogenes*, the antagonistic activity was detected after 8 hours of incubation and steadily increased until the end of incubation. The maximum activity (12 mm in diameter) occurred at 21-24 hours of incubation. The increase in antimicrobial activity was positively correlated with the increasing acidity level. The antimicrobial activity of *Lb. plantarum* S1.30 commenced during the exponential phase and the maximum activity was reached in early stationary phase. However, longer incubation times should be assessed to further understand the dynamics of antimicrobial activity demonstrated by the dadih LAB. The culture supernatants were also tested (with and without neutralization to assess the effect of acid conditions on the target organisms) but no inhibition zones were observed (results not shown).

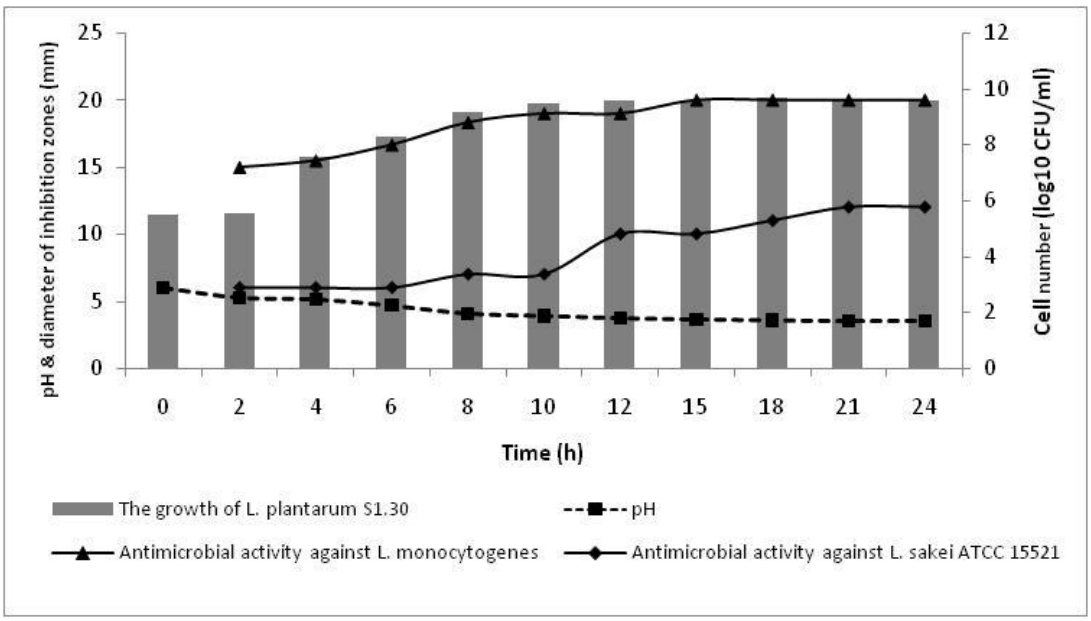


Figure 11 Effect of growth phase on antimicrobial production from *Lb. plantarum* S1.30 against *L. monocytogenes* and *Lb. sakei* ATCC 15521

3.3.4 Molecular detection of genes related to bacteriocin activity

Due to the failure to detect bacteriocins through an agar diffusion based method, a PCR-based method was carried out by using specific primers targeting a bacteriocin regulation gene, *pln*A. *Lb. plantarum* S1.30 was used as the source of DNA template as this strain demonstrated the best antimicrobial activity. A pair of *pln*A primers targeting the plantaricin A gene was selected for this study, because plantaricin A has frequently been found in a variety of *Lb. plantarum* isolates from a wide range of environments. *Pln*A is an induction factor that can induce the production of bacteriocin in *Lb. plantarum* (Diep et al. 1995; Diep et al. 2009). The PCR amplification resulted a-450 bp-DNA fragment (Figure 12), which indicated that plantaricin A was present in *Lb. plantarum* S1.30.

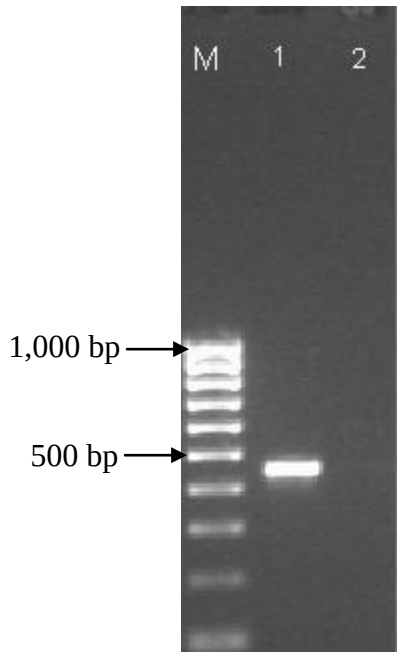


Figure 12 Amplification product of plantaricin A (450 bp)
Lane M: 100 bp DNA marker; lane 1: *Lb. plantarum* S1.30 (plantaricin A); lane 2: sterile milliQ H_2O

3.3.5 Detection of bacteriocin in a co-cultivation approach

Since plantaricin biosynthesis is known to be regulated by quorum sensing mechanisms, *Lb. plantarum* S1.30 was co-cultivated with either *L. monocytogenes* or *Lb. sakei* ATCC 15521. Both the neutralized and non-neutralized CFS of this co-culture, which was sterilized using both membrane filtration and heating were used in the agar well-diffusion technique to investigate the antimicrobial activity. No antimicrobial activity as a result of bacteriocin activity or the activity of other antimicrobial compounds was detected.

3.4 Discussion

In this study, three strains of dadih LAB, namely *Lb. plantarum* S1.30, and *Lc. lactis* subsp *lactis* S1.06 and S2.24, demonstrated antimicrobial activity against all Gram-negative and Gram-positive pathogenic bacteria tested (Table 8), with the exception of *B. cereus* for *Lc. lactis* subsp. *lactis* strains. However, only *Lb. plantarum* s1.30 was selected for further tests as this strain showed significantly greater antimicrobial activity in comparison with the other strains. Moreover, it also consistently inhibited all the indicator bacteria. Unfortunately, all the conventional methods used in this study, including a co-cultivation approach were unsuccessful in detecting any specific bacteriocin activity by *Lb. plantarum* S1.30.

Many factors can influence the production of bacteriocins. Belgacem et al (2008) pointed out that the likelihood of obtaining bacteriocin-producing LAB from fermented products was va-

riable and affected by many aspects. These include intrinsic factors associated with the products, variation in nutrients in the growth medium, variation in methodology used, and the selection of indicator bacteria. These factors may have contributed to the detection of only one potential antimicrobial producer from the dadih samples in this study. Moreover, the failure to detect bacteriocins using conventional methods may have been caused by the growth condition (pH and temperature), the composition of the media used (Rojo-Bezares et al. 2007), or the complex regulatory system of bacteriocin biosynthesis (Heng et al. 2007).

Investigation of the regulatory mechanism for bacteriocin biosynthesis is a plausible avenue to explore as regulation is triggered by a quorum sensing mechanism. In support of this, plantaricin was detected in *Lb. plantarum* S1.30 using PCR. However, the *pln*A gene is not directly responsible for plantaricin biosynthesis. Diep et al. (1995) argued that plantaricin A is a bacteriocin-like peptide which triggers bacteriocin biosynthesis when it is present in the growth media. Some *Lb. plantarum* strains containing *pln*A in their genome exhibited bacteriocin activity, such as strains C11, WCFS1 (type strain), J51 and V90 (Diep et al. 2009). In case of *Lb. plantarum* S1.30 and other dadih LAB more investigations are required to evaluate *pln*A or related genes in inducing bacteriocin production.

The failure to detect bacteriocin production by *Lb. plantarum* S1.30 might be related to the composition of the growth medium, especially the lack of milk protein fragments, as recently proposed by Georrgalaki et al (2010). This group reported that the bacteriocin (macedocin) could not be detected if either MRS or M17 media were use, leading them to cultivate the macedocin producers (*Streptococcus macedonicus* ACA-DC 198) in milk media. They concluded that the production of macedocin was not directly affected by the presence of lactose or galactose, but that the organisms produced a heat-stable protein when cultured on milk media and that this protein induced macedocin production. These findings suggest an approach that could be investigated for detection of bacteriocin production by dadih bacteria.

The results obtained from kinetic studies of antimicrobial production showed that the organic acids produced by *Lb. plantarum* S1.30 played an essential role in inhibiting the growth of target bacteria. The beneficial activities of *Lb. plantarum* isolated from dadih have been demonstrated in the previous research (Surono et al. 2008). A cell suspension of this bacterium actively removed the toxin, microcystin-LR, in the presence of 1% glucose (Surono et al. 2008). Furthermore, in an *in vitro* study, this highly adhesive bacterium was able to com-

pete with the pathogen which was trying to colonize the mucus layer of the intestinal epithelium (Collado et al. 2007b).

3.5 Conclusion

Even though antimicrobial activity was not detected in all dadih LAB, there was one potential bacteriocin producer, which was *Lb. plantarum* S1.30. The failure to detect antimicrobial activity in a wider range of dadih LAB with the conventional methods warrants further investigation to see if milk media would be more suitable for culture of the LAB. In addition, a study of quorum sensing by dadih LAB and investigations of *pln*A and similar genes could help determine why antimicrobial activity was demonstrated by cultures of *Lb. plantarum* S1.30 but no bacteriocin could be detected.

CHAPTER FOUR

MOLECULAR CHARACTERIZATION AND IDENTIFICATION OF YEASTS ISOLATED FROM DADIH

4.1 Introduction

The existence of yeasts in a range of spontaneously fermented milk products has been investigated by several researchers (Gadaga et al. 2000; Abdelgadir et al. 2001; Lore et al. 2005; Kebede et al. 2007; Zhang et al. 2008; Rahman et al. 2009). As well as being potential spoilage microorganisms, the presence of indigenous yeasts in NFM products may contribute to the development of characteristic fermentation metabolites, providing important texture and flavor (Narvhus & Gadaga 2003).

The occurrence of indigenous yeasts in dadih has been noted but not well studied (Hasono et al. (1989). Although not a focus of this current study, yeasts were found to make up a significant proportion of the isolates that formed colonies on MRS selective medium, a medium that has been shown to be only slightly selective for yeasts Rose (1985). The addition of acid production indicator (bromo-cresol purple) in the medium permits the differentiation of yeast and bacteria.

PCR amplification of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers (ITS) region followed by restriction fragment length polymorphism (RFLP) has been used to identify the species present in dadih. To study the potential effect of these yeast on dadih their ability to utilize lactic acid, the major metabolite of LAB, was evaluated.

4.1.1 Aims

- To characterize and identify yeasts isolated from dadih using RFLP analysis of the intergenic transcribed spacer (ITS) region
- To evaluate the capability of the yeast in utilizing lactate *in vitro*

4.2 Materials and Methods

4.2.1 Materials

4.2.1.1 Isolates

Fifty one of yeasts isolated from dadih were included in the study. A phenotypic characterization was carried out among these yeast isolates to cluster them into two groups.

4.2.1.2 Media

Yeast peptone dextrose (YPD) agar and broth supplemented with 2% of D-glucose was used for cultivation of yeast isolates. Dehydrated base (Oxoid Ltd, Basingstoke, UK) was prepared according to manufacturer's instructions.

Minimal media containing yeast nitrogen base without amino acids (Difco, BD Diagnostics, USA) and 2% of lactic acid (Sigma-Aldrich) was used for evaluation of lactate utilizing by yeasts.

4.2.1.3 Chemical and Reagent

4.2.1.4.a Chemical

Lactic acid solution (85%) with CAS number 50-12-5 was ACS reagent purchased from Sigma-Aldrich Pty Ltd., Sydney, Australia.

K-DLATE kits manufactured by Megazyme International Ireland Ltd., Bray Business Park, Bray, Co. Wicklow, Ireland. This kit was used for measuring the concentration of D-lactic acid (D-lactate) and L-lactic acid (L-lactate)

4.2.1.4.b Enzymes

Restriction endonucleases were purchased from New England BioLabs and used with buffer supplied (*Hae*III, *Hinf*I and *Eco*RI).

4.2.1.4.c PCR reagents and purification kit

The oligonucleotide primers used in this study are listed in Table 4. The oligonucleotide primers were synthesized by GeneWorks Pty Ltd, Adelaide, and they were resuspended in sterile MilliQ water to a concentration of 100 µM, and then stored at -20 ^{o}C for stocks.

Taq DNA polymerase, 10x buffer, 25 mM magnesium chloride ($MgCl_2$) and dNTPs were purchased from Promega (Madison, Wis. USA).

DNA ladder (100 bp, range: 100-1,517 bp) was purchased from New England BioLabs Inc.

Agarose gel for the electrophoresis was purchased from Promega (Madison, Wis. USA).

DNA purification kit used was Ultra Clean™ PCR Clean-up™ Kit purchased from MoBio Laboratories, Inc., Solena Beach, CA, USA.

4.2.1.4.d Buffer solutions

TAE buffer (40 mM Tris, 1.14 ml of glacial acetic acid, 1 mM EDTA) was used for agarose gel electrophoresis.

4.2.2 Methods

4.2.2.1 Molecular characterization

PCR-RFLP analysis of the internal transcribed spacer (ITS) region was used for differentiation of 51 yeast isolates. The oligonucleotide primers used in this experiment were ITS 1 and ITS 4 (Table 4). Colony-PCR was performed in this study where a small amount of yeast colony used directly as DNA templates for the PCR amplification. A small amount of a single colony was taken from the solid media, and placed in the bottom of sterile PCR tubes using a sterile loop. Subsequently, the colony in the PCR tubes was heated in a Microwave oven (Panasonic) for one minute. After this, the master mix reagents were dispensed into the tubes. The DNA amplification was carried out Mycycler thermal cycle (Bio-Rad Laboratories, Hercules, CA, USA) with 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) and 1.5 µl of 5 µM each primer. To obtain a final volume of 25 µl, a quantity of sterile milliQ water was added. The PCR reaction was run with amplification condition as shown in Table 5. The amplified products were detected using agarose gel electrophoresis as described in the previous chapter (2.2.2.3.c). After obtaining a good yield of product following electrophoresis, RFLP analysis was performed using the protocol described previously (section 2.2.2.3.d).

Since species identification of yeast can be determined by comparing the result of RFLP patterns with the existing databases, sequencing of the ITS region product was also performed when restriction profiles of the reference strains are not yet available. The method of DNA purification and sequencing has been described previously (2.2.2.4.b and 2.2.2.4.c).

4.2.2.2 Evaluation of lactate-utilizing yeasts

A number of yeast isolates were selected to represent the different strains obtained from the dadih samples. Each selected isolate was revived from frozen stocks and subcultured twice in YPD broth at 37 ^{o}C in 5% CO_2. Following this incubation, 1% (v/v) of cultures was inocu-

lated into minimal medium containing 2% of lactic acid, and then incubated at 37 ^{o}C in 5% CO_2 for 48 hours. The cell count and pH were measured before and after incubation in duplicate. A spread plate method was used to determine the total yeast counts. The utilization of lactic acid was shown by subtracting between initial and final concentration of D/L-lactate measured using K-DLATE kit (Megazyme, Ireland). The reagent preparation and calculation of the lactate were performed according to the manufacturer's instructions. Minimal medium without cultures was used as a control. The isolates were also grown in the minimal medium without lactic acid.

4.3 Results

4.3.1 Molecular characterization and identification of dadih yeasts

A total of 51 yeast isolates were characterized and identified using PCR amplification of the 5.8S rRNA - ITS region combined with RFLP and gene sequencing. The restriction analysis results were consistent with cellular and colony appearance, which differentiated the yeast isolates into two groups (Table 9, Figure 13, Appendix B and D). The restriction enzymes of *Hae*III and *Hinf*I successfully digested the amplicons and confirmed the presence of two 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, the two species were identified as *Pichia jadinii* and *Candida stellimalicola*. (The restriction profile of *P. jadinii* was also in agreement with the study conducted by (Villa-Carvajal et al. 2006)). Interestingly, *P. jadinii* (20 isolates) was only found in dadih from Gadut. In dadih collected from Sianok, *C. stellimalicola* was the only identified yeast species in the two day ferments while the three day ferments yielded seven isolates of *C. stellimalicola*. At the same time, *P. jadinii* was dominant in both two and three day ferments (six and 17 isolates, respectively).

Table 9 Characterization and identification of dadih yeasts

Group	Cell-morphology	Number of isolates	5.8S rRNA-ITS-PCR (bp)	RFLP (bp)			BLAST analysis (%)	Identification
				*Hae*III	*Hinf*I	*EcoR*I		
I	Large ovoid	43	575	390, 145	300, 275	-	97	*Pichia jadinii*
II	Small ovoid	8	520	-	300, 220	-	98	*Candida stellimalicola*

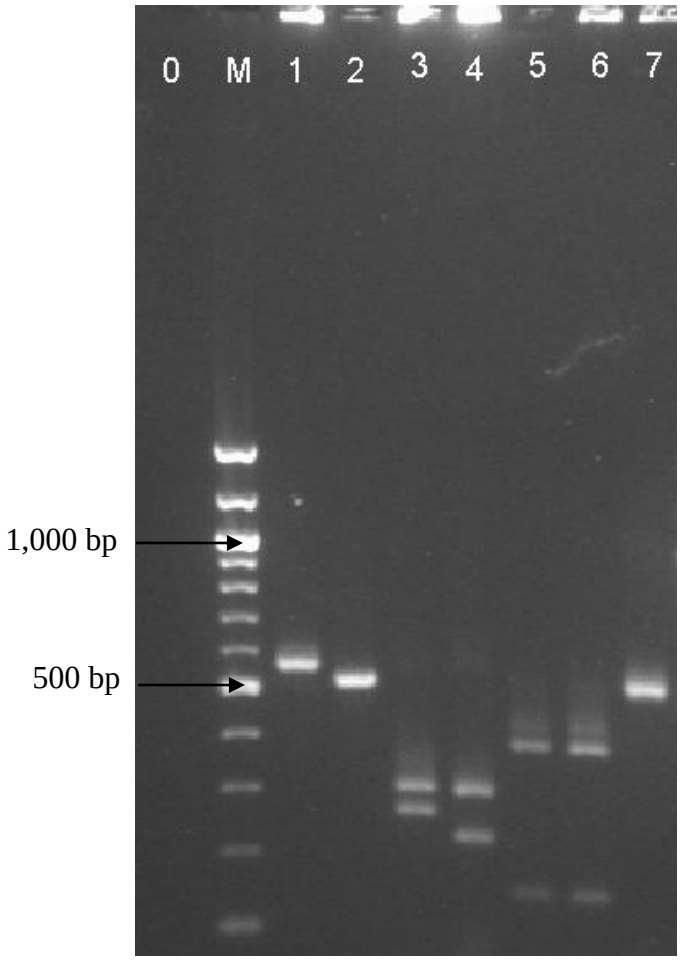


Figure 13 5.8S rRNA-ITS PCR products and their restriction analysis with the endonuclease *Hinf*I and *Hae*III

Lane M: 100 bp DNA marker; lane 0: sterile milliQ H_2O; lane 1: group I *(*isolate S1.04), lane 2: group II (isolate S2.31), lane 3: group I (isolate G.20), lane 4: group II (isolate S2.29), lane 5-6: group I (isolate G.19 and S2.03), lane 7: group II (isolate S2.33) (*Hinf*I: lane 3-4; *Hae*III: lane 5-7).

4.3.2 Utilization of lactic acid by yeasts

The isolation of significant numbers of yeasts from the dadih samples suggested that they may influence the product quality. Although *P. jadinii* and *C. stellimalicola* are unable to utilize lactose for their growth (Lodder 1970; Suzuki et al. 1994), it was important to determine whether they were able to utilize lactic acid, the main product of LAB and the main carbon source in dadih. During incubation in lactate medium, the concentration of total lactate declined slightly with both yeasts and was correlated with the reduction in acidity level of the medium. All of the test yeast strains were more active in utilizing D-lactate rather than L-lactate. Interestingly, co-cultivation of the two yeasts did not increase use of lactate significantly (Figure 14). However, the changes detected were small.

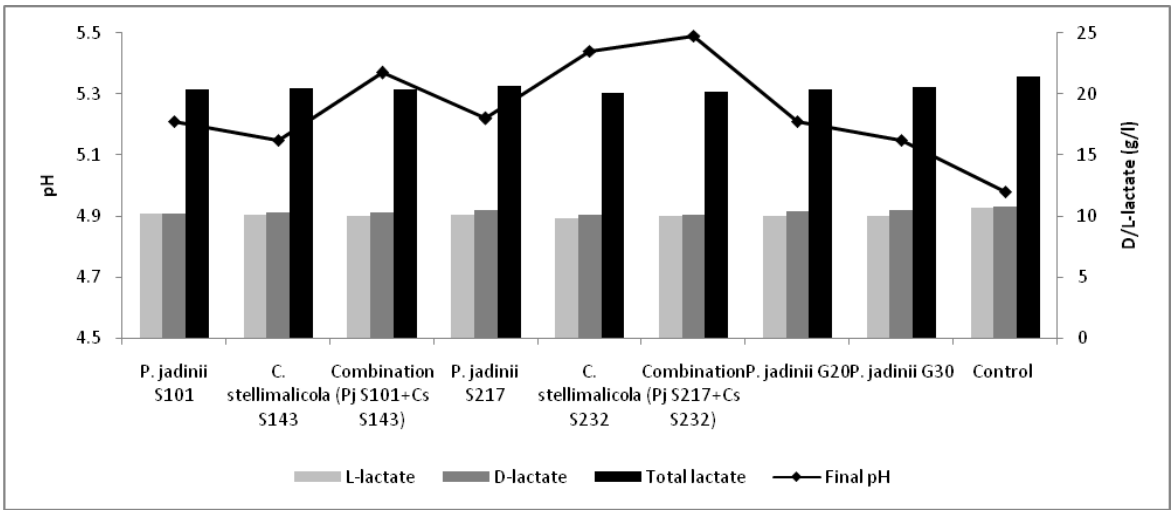


Figure 14 The utilization of D/L-lactate by dadih yeasts incubated for 48 h
Control: without cultures. P: *Pichia*; C: *Candida*

4.4 Discussion

In this study two yeast species, namely *P. jadinii and C. stellimalicola* were shown to be present in significant numbers in dadih. This can be seen from the proportion of dadih LAB and yeasts isolated from the dadih samples (Figure 2). To our knowledge, this is the first report on the isolation of these two yeast species from dadih which means that the role of yeast in determining the characteristics and properties of dadih may have been overlooked. The morphological study of *P. jadinii* showed that the cellular characters were consistent with the literature, in that they were ovoid in shaped and large in size (3.5-4.5) x (7-13) µm (Lodder 1970). *C. stellimalicola* was first isolated from star apple (Ma-Fueng) in Thailand (Suzuki et al.1994). In comparison with *P. jadinii, C. stellimalicola* is smaller. The cells are (1.8-4.0) x (1.8-4.2) µm, and ovoid in shape (Suzuki et al. 1994). *P. jadinii* (anamorph of *C. utilis*) and *C. stellimalicola* was classified in a similar group on the basis of 18S rRNA gene sequence divergence (Suzuki & Nakase 2002). Hence, based on the phylogenetic studies these yeast species are considered closely related.

The occurrence of these two yeasts in dadih indicates that these species could adjust the acidic properties of dadih as, although unable to utilize lactose, they can use lactate and this may explain their presence in dadih (Figure 14). The lactate utilization is also a known trait of these species (Lodder 1970; Suzuki et al. 1994). It should be noted that in our study the cul-

tures were incubated without shaking to mimic the static conditions used in traditional production of dadih; as the two are obligate aerobes, it is predicted that if oxygenation was increased, growth and utilization of D/L lactate would be improved. Their role in dadih requires further study. It is expected that these yeasts could live in the semi-aerobic condition found in the dadih fermentation process and may have adapted to the dadih environment by assimilating the lactic acid. The source of these yeasts also remains unknown and a further ecological study looking at the bamboo containers and the banana leaf covers, as well as the milk requires more detailed analysis.

4.5 Conclusion

P. jadinii and *C. stellimalicola* were detected as non-LAB cultures of dadih. Their ability to utilize lactic acid was suggested by small changes in lactate content in the growth medium, indicating that they could modify the sensory characteristics of dadih. However, this requires more investigation. A more detailed analysis using an appropriate yeast medium in place of MRS would be useful to further establish the dadih yeast ecology. In addition, the restriction profiles of the dadih yeasts can be used as a basis of the rapid identification of yeasts isolated from dadih or other products in the future.

CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSION

In recent years there has been increased interest in NFM products in the food biotechnology area. Two important reasons identified for this phenomenon are the improvements in NFM product quality and the discovery of new types of starter cultures. Dadih is one of the NFM products that has been reported to contain micro-flora with potentially beneficial properties, such as probiotic activity and the ability to remove toxic products. Conventionally the probiotic properties have been attributed to the presence of LAB genera in dadih. However, the microbial composition in every spontaneous fermentation milk product is variable dependent on the production process and the locality. This study was not designed to be an ecological study of the micro-flora of dadih but rather was focussed on developing a better understanding of LAB in dadih. However, the isolation of significant numbers of yeasts encouraged the inclusion of these organisms in the study. Based on this background, the purposes of this study were determined as: 1) isolation, characterization and identification of indigenous microorganisms from dadih, 2) examination of antimicrobial activity of LAB isolates against indicator bacteria, and 3) investigation of bacteriocins from LAB with antimicrobial activity against indicator bacteria.

Using phenotypic and molecular methods, a total of 66 LAB isolates were identified as *Lb. plantarum* (1 isolate), *Lc. lactis* subsp. *lactis* (9 isolates), *Lb. paracasei* S1A (42 isolates), *Lb. paracasei* S1B (13 isolates) and *Leu. pseudomesenteroides* (1 isolate). The latter three LAB species have not been isolated from dadih before. It should be recognized that if a wider range of dadih samples were cultured or samples covering more fermentation periods, more species could well have been found. Of the 51 isolates of yeast, 43 were identified as *P. jadinii* and eight were identified as *C. stellimalicola*. As indicated previously, the study did not set out to isolate yeasts as only MRS medium was used. The role of yeasts in determining the characteristics and properties of dadih has been overlooked previously and if media more appropriate for yeast isolation were used it may be that additional yeast species would be found. In addition, two acetic acid bacteria isolates were also identified as *A. cerevisiae*. Again, the study was not directed at this species. This is the first report of non-LAB microorganisms from dadih. A properly developed ecological study may well reveal even more non-LAB in this NFM. The most frequently isolated LAB from dadih was *Lb. paracasei,* while *P. jadinii*

was the principal yeast found in dadih. However, this study is limited by the lack of dadih samples. Three dadih samples from two regions (Sianok and Gadut) and two time-points in fermentation are probably inadequate to truly indicate the diversity of the micro-flora of dadih.

The RFLP analysis successfully identified two different types of *Lb. paracasei.* A unique cellular morphology was shown in *Lb. paracasei* S1B which had granule-like structures within the cell bodies. The two recognized type strains, *Lb. paracasei* subsp. *paracasei* and *Lb. paracasei* subsp. *tolerans* do not have granule-like structure inside their cells (Hammes & Vogel 1995). A thorough investigation is needed to further characterize this unusual strain as it may represent a third sub-species. The DNA sequencing of 16S rRNA genes and the use of species-specific primers in the PCR ITS-RFLP analysis could be useful strategies for further studies on the microbial composition of dadih. However, researchers must bear in mind the weakness in using PCR ITS-RFLP alone as there is limited information in public databases on PCR ITS-RFLP profiles, especially for the LAB genera.

This present study showed that the micro-flora in dadih consists of a combination of LAB, yeasts and acetic acid bacteria. A complete understanding of the interactions of LAB, yeasts and acetic acid bacteria in spontaneously fermented products has not been elucidated. However, interaction between LAB and yeasts has previously been reported in naturally fermented products (Gadaga et al. 2000; Abdelgadir et al. 2001; Lore et al.2005; Kebede et al. 2007; Zhang et al. 2008; Rahman et al. 2009). A synergistic interaction between LAB and yeasts contributes to the quality of African NFM products (Narvhus & Gadaga 2003). The authors reported that the growth of yeasts in NFM products was dependent on their ability to metabolize milk components or the metabolic products produced by LAB. In this study both yeasts isolated from dadih showed a tendency to utilize the lactic acid produced by dadih LAB, rather than milk lactose (Lodder 1970; Suzuki et al. 1994) although only small changes in lactate concentration in the growth medium was observed. Meanwhile, the concept that sequential growth of different microorganisms occurs in dadih was strengthened by the isolation of acetic acid bacteria, *A. cerevisiae*. The principal characteristic of this bacterium is the production of acetic acid from ethanol (Stasiak & Blazejak 2009). The *Lactobacillus* spp. and *Leuconostoc* sp. isolated from this study are categorized as facultatively heterofermentative, as they are able to ferment lactose into lactic acid, ethanol, and CO_2 (Hammes & Vogel 1995). The ethanol produced by both dadih LAB and yeasts is highly probably utilized by *A. cerevisiae.* Additionally, this acetic acid bacteria was also reported to be able to oxidize lac-

tate to CO_2 and H_2O (Cleenwerck et al. 2002). However, a specific interaction of LAB, yeast and acetic acid bacteria in dadih requires further investigation, especially their role in developing dadih quality (flavours and textures).

Antimicrobial activity is important to assure the safety level of dadih. Our study showed that antimicrobial activity was detected from dadih LAB against a range of indicator bacteria. However, one strain, *Lb. plantarum* S1.30, had far greater antimicrobial activity in comparison with the other isolated LAB. Bacteriocin/plantaricin activity was not detected through the conventional methods, but the application of molecular methods has detected the genes responsible for the synthesis of a bacteriocin-like peptide. The role of this peptide has been recognized as an induction factor for the production of plantaricin (Diep et al. 1995). This indicates that the bacteriocin production of *Lb. plantarum* S1.30 is likely to be regulated by quorum sensing, and that an inducer is involved. The other possible reasons for inability to detect bacteriocins were that the medium and the incubation conditions used for cultivating the LAB producers were unsuitable for the synthesis of bacteriocins.

From a public health perspective the critical step in dadih fermentation is at the start of the fermentation when buffalo or humans involved in the preparation of dadih are likely to contaminate the buffalo milk. The occurrence of pathogenic bacteria, such as *Escherichia coli* and *Staphylococcus aureus* in Zimbabwean NFM products has been reported by Gran et al (2003). The bacteriocin-producing LAB from dadih are present in the early stages of fermentation, producing highly competitive conditions which may not favour the survival of the pathogens. Whereas, the dadih samples obtained in our study were at the final stage of fermentation when any pathogens present are likely to have been killed by antimicrobial factors (probably bacteriocins) produced by LAB.

In conclusion, this study has updated information on the micro-flora found in dadih and their potency as antimicrobial producers. The traditional knowledge used in making dadih should be preserved and maintained as part of the conservation of indigenous culture with potential benefits to the wider community. This project has just started the process of improving our understanding of the microbial complexity of dadih and much more research is needed if dadih is to be developed commercially as a NFM.

FUTURE RESEARCH NEEDS

- An ecological assessment of the total indigenous micro-flora in dadih at different stages in the fermentation process is important to assist in understanding the interactions in the indigenous micro-flora. This should include wide-ranging isolation and characterization techniques to ensure that yeasts and other non-LAB organisms can be isolated and characterized, as well as molecular techniques such as PCR-DGGE and molecular profiling and sequencing.

- Establishing where each of the species of microorganisms in dadih originates: what role does the unpasteurized buffalo milk, or the bamboo tube and banana leaf play?

- Improving the understanding of the production of bacteriocins by LAB in dadih by modification of media components and incubation conditions.

- Further characterization of the technologically important characteristics of dadih LAB through a series of physiological and biochemical tests.

- To further characterize strains of *Lb. paracasei* and *A. cerevisiae* found in dadih using a polyphasic approach involving phenotypic and molecular techniques.

- To further characterize *Lb. paracasei* strain S1B to see if it represents a new sub-species of *Lb. paracasei*.

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APPENDICES

Appendix A Phenotypic and molecular characterization of bacteria isolated from dadih

No	Original code	Gram Staining	Catalase	Cell Shape	ITS PCR		RFLP					Identification (16S rRNA)
					Band size (bp)	Σ	RsaI	HaeIII	HinfI	EcoRI	SphI	
1	S1.02	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
2	S1.03	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
3	S1.05	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
4	S1.06	+	-	Coccoid	700	1	-	270, 450	-	-	ND	*Lactococcus lactis* subsp. *lactis*
5	S1.08	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
6	S1.09	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
7	S1.10	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
8	S1.11	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
9	S1.12	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
10	S1.14	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
11	S1.15	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
12	S1.17	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
13	S1.19	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
14	S1.20	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
15	S1.21	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
16	S1.22	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
17	S1.23	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
18	S1.24	+	-	Coccoid	700	1	-	270, 450	-	-	ND	*Lactococcus lactis* subsp. *lactis*

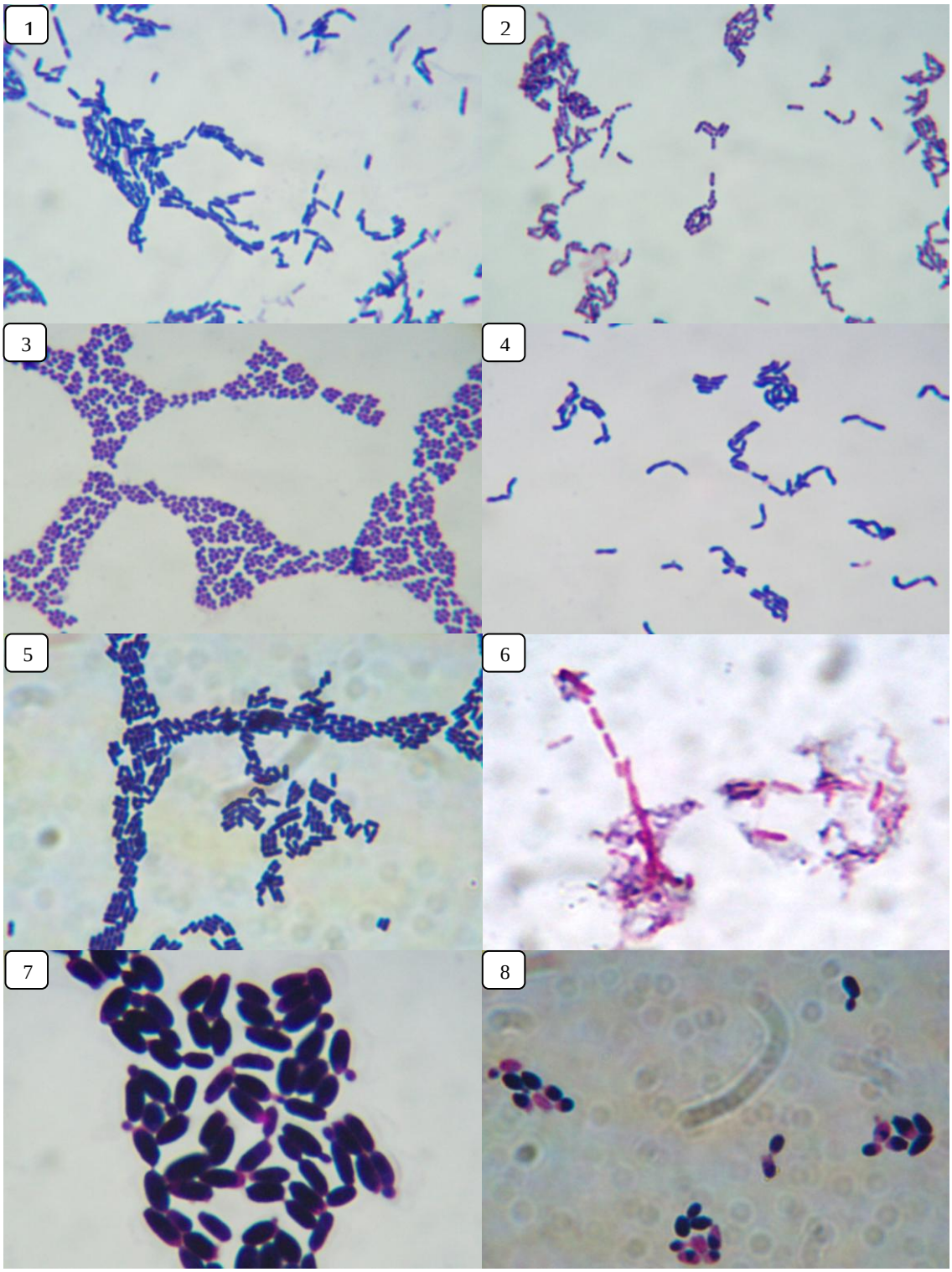
19	S1.25	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
20	S1.26	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
21	S1.27	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
22	S1.30	+	-	Rod	590, 800	3	140, 450, 700	600, 500	ND	-	-	*Lactobacillus plantarum*
23	S1.31	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
24	S1.33	+	-	Long-Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
25	S1.34	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
26	S1.35	+	-	Long-Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
27	S1.36	+	-	Long-Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
28	S1.37	+	-	Long-Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
29	S1.39	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
30	S1.40	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
31	S1.42	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
32	S1.44	+	-	Long-Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
33	S1.45	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
34	S1.46	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
35	S1.47	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
36	S2.4	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
37	S2.5	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND		*Lactobacillus paracasei*
38	S2.8	+	-	Rod + granule	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
39	S2.10	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
40	S2.11	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
41	S2.14	-	+	Rod	1200	1	-	120. 210,	400, 700	-	-	*Acetobacter cerevisiae*

								320, 490				
42	S2.18	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
43	S2.20	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
44	S2.21	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
45	S2.22	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
46	S2.23	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
47	S2.24	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
48	S2.25	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
49	S2.26	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	-	-	*Lactobacillus paracasei*
50	S2.36	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
51	S2.38	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
52	S2.39	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
53	S2.40	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
54	S2.41	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
55	S2.42	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
56	S2.44	-	+	rod	1200	1	-	120. 210, 320, 490	410, 710	-	-	*Acetobacter cerevisiae*
57	S2.45	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
58	S2.49	+	-	Rod + granules	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
59	S2.50	+	-	Coccoid	700	1	-	270, 450	-	-	ND	*Lactococcus lactis* subsp. *lactis*
60	S2.51	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	-	ND	-	*Lactobacillus paracasei*
61	S2.52	+	-	Rod	600, 750, 800	3	150, 390, 590	580, 340	ND	ND	-	*Lactobacillus paracasei*
62	G2	+	-	Coccoid	700	1	-	270, 450	-	-	ND	*Lactococcus lactis* subsp. *lactis*
63	G3	+	-	Coccoid	700	1	-	270, 450	-	-	ND	*Lactococcus lactis* subsp.

												lactis
64	G5	+	-	Rod	800	1	600	-	-	-	-	*Leuconostoc pseudomesente-roides*
65	G6	+	-	Coccoid	700	1	-	270, 450	-	-	ND	*Lactococcus lactis* subsp. *lactis*
66	G8	+	-	Coccoid	700	1	-	270, 450	-	-	ND	*Lactococcus lactis* subsp. *lactis*
67	G10	+	-	Coccoid	700	1	-	270, 450	-	-	ND	*Lactococcus lactis* subsp. *lactis*
68	G28	+	-	Coccoid	700	1	-	270, 450	-	-	ND	*Lactococcus lactis* subsp. *lactis*

ND, not determined

Appendix B Cellular morphology of LAB, yeasts and acetic acid bacteria isolated from dadih (magnification: 1000x)



1. *Lactobacillus paracasei* S1.19 (rod-shaped)
2. *Lactobacillus paracasei* S2.38 (rod-shaped with granules)
3. *Lactococcus lactis* subsp. *lactis* G.08 (ovoid-cocci-shaped)
4. *Leuconostoc pseudomesenteroides* G.05 (rod-shaped)
5. *Lactobacillus plantarum* S1.30 (rod-shaped)
6. *Acetobacter cerevisiae* S2.14 (rod-shaped, Gram-negative and catalase-positive)
7. *Pichia jadinii* S1.38 (large-ovoid-shaped)
8. *Candida stellimalicola* S1.43 (small-ovoid-shaped)

Appendix C Molecular characterization of LAB isolated from dadih

No	Original code	Cell Shape	tRNA (bp)	RFLP			Identification (16S rRNA)
				***Hinf*I**	***Hind*III**	***Rsa*I**	
1	S1.02	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
2	S1.03	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
3	S1.05	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
4	S1.06	Coccoid	740	680, 60	510, 230	300, 120	*Lactococcus lactis* subsp. *lactis*
5	S1.08	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
6	S1.09	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
7	S1.10	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
8	S1.11	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
9	S1.12	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
10	S1.14	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
11	S1.15	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
12	S1.17	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
13	S1.19	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
14	S1.20	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
15	S1.21	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
16	S1.22	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
17	S1.23	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
18	S1.24	Coccoid	740	680, 60	510, 230	300, 120	*Lactococcus lactis* subsp. *lactis*
19	S1.25	Rod + granules	780	220, 480	200, 230,	100, 120,	*Lactobacillus paracasei*

					350	220	
20	S1.26	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
21	S1.27	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
22	S1.30	Rod	780	235, 360	780	340, 100	*Lactobacillus plantarum*
23	S1.31	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
24	S1.33	Rod	780				*Lactobacillus paracasei*
25	S1.34	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
26	S1.35	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
27	S1.36	Rod	780				*Lactobacillus paracasei*
28	S1.37	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
29	S1.39	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
30	S1.40	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
31	S1.42	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
32	S1.44	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
33	S1.45	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
34	S1.46	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
35	S1.47	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
36	S2.4	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
37	S2.5	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
38	S2.8	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
39	S2.10	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
40	S2.11	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
41	S2.14	Rod	ND	ND	ND	ND	*Acetobacter cerevisiae*

42	S2.18	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
43	S2.20	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
44	S2.21	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
45	S2.22	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
46	S2.23	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
47	S2.24	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
48	S2.25	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
49	S2.26	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
50	S2.36	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
51	S2.38	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
52	S2.39	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
53	S2.40	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
54	S2.41	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
55	S2.42	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
56	S2.44	rod	ND	ND	ND	ND	*Acetobacter cerevisiae*
57	S2.45	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
58	S2.49	Rod + granules	780	220, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
59	S2.50	Coccoid	740	680, 60	510, 230	300, 120	*Lactococcus lactis* subsp. *lactis*
60	S2.51	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
61	S2.52	Rod	780	220, 400, 480	200, 230, 350	100, 120, 220	*Lactobacillus paracasei*
62	G2	Coccoid	740	680, 60	510, 230	300, 120	*Lactococcus lactis* subsp. *lactis*
63	G3	Coccoid	740	680,	510,	300,	*Lactococcus lactis* subsp. *lactis*

				60	230	120	
64	G5	Rod	820	500, 260, 60	350, 300, 170	350, 100	*Leuconostoc pseudomesenteroides*
65	G6	Coccoid	740	680, 60	510, 230	300, 120	*Lactococcus lactis* subsp. *lactis*
66	G8	Coccoid	740	680, 60	510, 230	300, 120	*Lactococcus lactis* subsp. *lactis*
67	G10	Coccoid	740	680, 60	510, 230	300, 120	*Lactococcus lactis* subsp. *lactis*
68	G28	Coccoid	740	680, 60	510, 230	300, 120	*Lactococcus lactis* subsp. *lactis*

ND, not determined
Rod: rod-haped without granules inside the cells.
Rod+granules: rod-shaped with granules inside the cells.

Appendix D Phenotypic and molecular characterization of yeasts isolated from dadih

No	Original code	Cell-Morphology	Colony Morphology	PCR-ITS size (bp)	Cata-lase test	RFLP			Identification (5.8S rRNA - ITS)
						*Hinf*I	*Hae*III	*Eco*RI	
1	S1.01	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
2	S1.04	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
3	S1.28	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
4	S1.29	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
5	S1.32	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
6	S1.38	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
7	S1.43	Small ovoid	Small creamy	520	+	300, 220	-	-	*Candida stellimalicola*
8	S2.1	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
9	S2.2	Small ovoid	Small creamy	520	+	300, 220	-	-	*Candida stellimalicola*
10	S2.3	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
11	S2.6	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
12	S2.7	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
13	S2.12	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
14	S2.13	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
15	S2.15	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
16	S2.16	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
17	S2.17	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
18	S2.19	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
19	S2.27	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
20	S2.28	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
21	S2.29	Small ovoid	Small creamy	520	+	300, 220	-	-	*Candida stellimalicola*
22	S2.30	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
23	S2.31	Small ovoid	Small creamy	520	+	300, 220	-	-	*Candida stellimalicola*
24	S2.32	Small ovoid	Small creamy	520	+	300, 220	-	-	*Candida stellimalicola*
25	S2.33	Small ovoid	Small creamy	520	+	300, 220	-	-	*Candida stellimalicola*
26	S2.34	Small ovoid	Small creamy	520	+	300, 220	-	-	*Candida stellimalicola*
27	S2.35	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*

28	S2.37	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
29	S2.46	Small ovoid	Small creamy	520	+	300, 220	-	-	*Candida stellimalicola*
30	S2.48	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
31	S2.53	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
32	G9	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
33	G11	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
34	G12	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
35	G13	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
36	G14	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
37	G15	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
38	G16	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
39	G17	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
40	G18	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
41	G19	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
42	G20	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
43	G21	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
44	G22	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
45	G23	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
46	G24	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
47	G26	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
48	G27	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
49	G29	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
50	G30	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*
51	G31	Large ovoid	Large creamy	575	+	300, 275	390, 145	-	*Pichia jadinii*

Appendix E Antimicrobial screening of LAB isolated from dadih using agar spot test

Isolates	*Lb. casei*	*Lb. fermentum*	*Lc. lactis* subsp. *lactis* LM0230
Lc. lactis subsp. *lactis* DL11	-	-	+
Lb. hilgardii	+	+	+
Lb.paracasei:			
S1.02	+	+	+
S1.03	++	++	+
S1.05	+	+	+
S1.08	+	++	+
S1.09	+	+	+
S1.10	+	+	+
S1.11	+	+	+
S1.12	+	+	+
S1.14	+	+	+
S1.15	+	+	+
S1.17	++	+	+
S1.19	++	+	+
S1.20	++	+	+
S1.21	+	+	+
S1.22	++	+	+
S1.23	++	+	+
S1.25	++	+	+
S1.26	++	+	+
S1.27	++	+	+
S1.31	++	+	+
S1.33	+	+	+
S1.34	+	+	+
S1.35	+	+	+
S1.36	+	+	+
S1.37	+	+	+
S1.39	+	+	+
S1.40	+	+	+
S1.42	+	+	+
S1.44	+	+	+
S1.45	+	+	+
S1.46	+	+	+
S1.47	+	+	+
S2.4	+	+	+
S2.5	+	+	+
S2.8	+	+	+
S2.10	+	+	+
S2.11	+	+	+
S2.14	+	+	+
S2.18	+	+	+
S2.20	+	+	+
S2.21	+	+	+

S2.22	+	+	+
S2.23	+	+	+
S2.24	+	+	+
S2.25	+	+	+
S2.26	+	+	+
S2.36	+	+	+
S2.38	+	+	+
S2.39	+	+	+
S2.40	+	+	+
S2.41	+	+	+
S2.42	+	+	+
S2.44	+	+	+
S2.45	+	+	+
S2.49	+	+	+
S2.51	+	+	+
S2.52	+	+	+
***Lb. plantarum* S1.30**	++	++	+
***Lc. lactis* subsp. *lactis*:**			
S1.06	-	-	+
S1.24	-	-	+
S2.50	-	-	-
G2	-	-	-
G3	-	-	-
G6	-	-	-
G8	-	-	-
G10	-	-	-
G28	-	-	-
***Leu. mesenteroides* G5**	-	-	-

+/++: inhibition zone
-: no inhibition zone