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Contact Info

Editor
International Food Research Journal
Faculty of Food Science and Technology
Universiti Putra Malaysia
43400 UPM Serdang
Selangor
Malaysia

Phone: +603-89468361
Fax: +603-89423552
Email: ifrj@upm.edu.my

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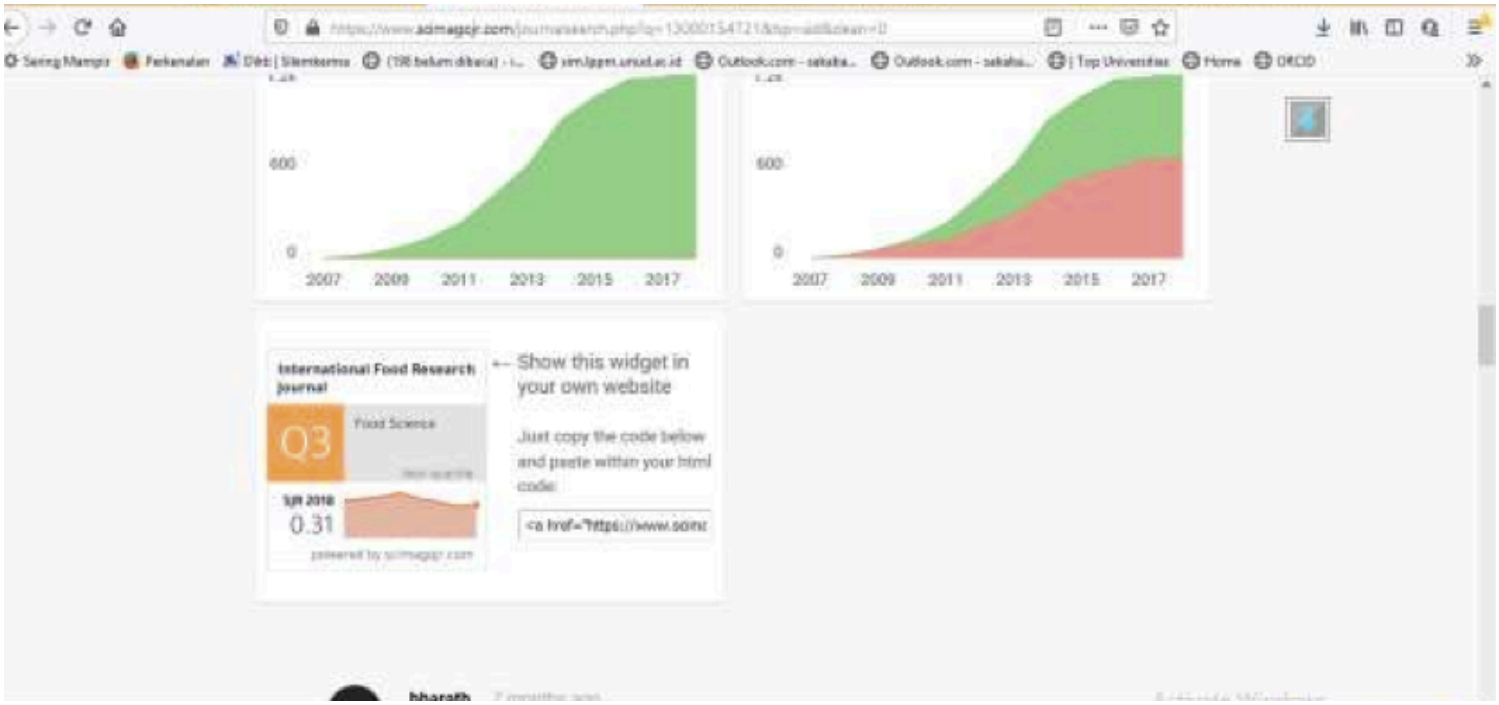
International Food Research Journal

Volume 17, No. 2, 2010

Gadekar, Y.P; Kokane, R.D; Suradkar, U.S; Thomas, R; Das, A. K; Anjaneyulu, A.S.R.
Review article : shelf stable meat pickles- a review
View abstract
View references
1
Obodai, M; Amoa-Awua, W; Odamtten, G.T.
Physical, chemical and fungal phenology associated with the composting of 'wawa' sawdust (Triplochiton scleroxylon) used in the cultivation of oyster mushrooms in Ghana
View abstract
View references
2
Sujaya, I.N; Nocianitri, K.A; Asano, K.
Diversity of bacterial flora of Indonesian ragi tape and their dynamics during the tape fermentation as determined by PCR-DGGE
View abstract
View references
3
Usha, M.R; Fauziah, M; Tunung, R; Chai, L.C; Cheah, Y.K; Farinazleen, M.G; Son, R.
Occurrence and antibiotic resistance of Campylobacter jejuni and Campylobacter coli in retail broiler chicken
View abstract
View references
4
Omemu, A.M; Omeike, S.O.
Microbiological hazard and critical control points identification during household preparation of cooked ogi used as weaning food
View abstract



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Diversity of bacterial flora of Indonesian *ragi tape* and their dynamics during the *tape* fermentation as determined by PCR-DGGE

[a,]*Sujaya, I. N., [b]Nocianitri, K. A. and [c]Asano, K.

[a]*Integrated Laboratory for Bioscience and Biotechnology, Udayana University, Bukit Jimbaran Campus, Badung, Bali 80 362, Indonesia*
[b]*Laboratory of Biochemistry, Faculty of Agricultural Technology, Udayana University, Bukit Jimbaran Campus, Badung, Bali 80 362, Indonesia*
[c]*Laboratory of Applied Microbiology, Department of Molecular Bioscience, Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, North 9, West 9, Sapporo, Hokkaido 060 8589, Japan*

Abstract: The diversity of bacterial flora of *ragi tape*, an Indonesian traditional dry starter, was analyzed using culture independent methods, the Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE). The result revealed the lactic acid bacteria were the predominant bacterial flora of *ragi tape*. *Weissella* spp., *Pediococcus pentosaceus, Lactobacillus* spp. and *Enterococcus* spp., were detected in all 9 *ragi tape* samples, *Bacillus cereus* detected in 3 out of 9 samples, swine manure bacterial species related to *Clostridium perfringent* was detected in 3 out of 9 samples. Remaining uncultured bacteria of either *Eubacterium moniliforme, Clostridium sardiniensis*, or *Clostridium baratii* was detected in one out of 9 samples. *Pediococcus, Weissella* and *Enterococcus* consistently developed during the *tape* fermentation; while the *Lactobcillus* sp. started to grow after 24 h fermentation then its population likely decrease afterwards. The active growth of LAB during the *tape* fermentation implies that these bacteria might play significant contribution on the flavor of *tape* and *brem*.

Keywords: *Ragi tape*, lactic acid bacteria, *brem*, DGGE

Introduction

The traditional starchy starter such as the Chinese yeast, Indonesian *ragi*, Nepal's *muncha,* Vietnamese *banh men,* and other similar dry starters are the common starter cultures found in the Orient (Steinkraus 1996; Hesseltine and Ray 1989; Thanh *et al.,* 2008). The starters appear in several forms such as pellet (tablet like) and round and grain-like form of *mucha*, which mainly contain raw starch and use in the production of various starch-based fermented foods. The products are mainly found in two types, namely; the grainy-rice paste, Indonesian and Malaysian *tape* (Djien 1974; Kato *et al.,* 1976; Cronk *et al.,* 1977, 1979; Merican and Yeoh 1989) and alcoholic beverages, Balinese *brem*, Chinese *lau cho* and Malaysian *samsu* (Steinkraus 1996; Sujaya *et al.,* 2002).

Numerous studies have been conducted to describe the microorganisms that are responsible for the fermentation. They are mostly fungi and yeasts (Dwidjosepoetro and Wolf 1972; Djien 1974; Cronk *et al.,* 1977, 1979; Hesseltine 1983; Kuriyama *et al.,* 1991; Sujaya *et al.,* 2003; Abe *et al.,* 2004). Only few works however, were done to determine the bacterial flora in these traditional starters. The study on bacterial flora of *ragi tape* has gained interest since they contribute to the overall quality of the fermented product (Ardana and Fleet, 1989).

In order to understand the role of bacterial flora of *ragi tape* in the Balinese rice wine fermentation, it is of primary importance to show reliable description of the bacterial community. The previous studies showed that lactic acid bacteria (LAB) were the predominant bacterial flora of *ragi tape*, which comprised at least four different genera such as *weissella, lactobacilli, enterococci* and *pediococci* (Sujaya *et al.,* 2001, 2003).

The application of culture independent approaches (DNA based methods) is very important since in recovering the whole microbial diversities; only limited number of strains could be identified using the culture-based method. Recently, numbers of culture independent approaches have been successfully

*Corresponding author.
Email: *inengah_sujaya@yahoo.com*
Tel. +62 361 744 8773 ; Fax. +62 361 744 8773

applied in describing structure of a very complex microbial community such as gastrointestinal tract (Zoetendal *et al.,* 1998; Suau *et al.,* 1999; Hayashi *et al.* 2002), soil (el Fantroussi *et al.,* 1999; Duineveld *et al.,* 2001) as well as traditional fermented foods such as *pozol* in mexico (Ampe *et al.,* 1999; ben Omar *et al.,* 2000). The molecular methods provided realistic view of microbial community by which the uncultivable microbes could be described. The objective of this study was to verify the diversity of bacterial community of Indonesian *ragi tape* and to elucidate the possible role of the LAB in fermentation of Balinese rice wine (*brem*).

Material and Methods

Collection of ragi tape samples

Ragi tape were obtained from different parts of Indonesia, where the *tape* making is assumed to be the most popular such as at Sumatera Island (3 samples), Java Island (4 samples) and two samples collected in Bali Island where the starter is applied both for making *tape* and rice wine (*brem*).

DNA extraction from ragi tape

About 2.5 g powdered *ragi tape* was dispensed in 25 ml of PBS buffer using 50 ml of falcon tubes. The suspension was sonicated for 60 sec; briefly vortexed for 15 min, following centrifugation at 100*g* for 2 min. The supernatant containing bacterial cells was collected. These steps were repeated twice then supernatant pool was centrifuged at 2,500*g* for 20 min. The resulting pellet was washed twice in PBS buffer then dissolved in 1 ml PBS buffer, adding with 15 µl of 0.225 g/ml labiase (Seikagaku Corp., Tokyo, Japan), 50 µl of 2.5 g/ml lysozyme (Wako, Osaka, Japan), and 20 µl of 2 mg/ml of N-acetylmuramidase (Seikagaku Corp., Tokyo, Japan). The suspension was incubated at 37°C for 30 min and DNA was isolated using UltraClean™ Soil DNA Kit (MO BIO Lab. Inc, Solana Beach, CA) as instructed by the manufacturer.

Determination of LAB during the fermentation of glutinous rice (tape)

A mixture of black and white glutinous rice (10 g and 20 g, respectively) representing the row material for making *brem* (Balinese rice wine) was washed and soaked using tap water for 4 h in 200 ml beaker. The rice was rinsed and added with 30 ml distilled water and then autoclaved at 121°C for 15 min. After cooling, it was inoculated with 0.3% (w/w) powdered *ragi tape* and incubated at 30°C at 37°C. The progress of fermentation was followed through destructive sampling in every specified time intervals (12 hours). DNA was isolated from 5 g of fermented rice as those described above.

PCR Denaturing Gradient Gel Electrophoresis (DGGE) analysis

Amplification was performed in GeneAmp PCR Systems 9700 (PE Applied Biosystems) using GC-388F: 5'-CGCCCGCCGCGCGCG GCGGGCGGGGCGGGGGCACGGGGGG -ACTCCTACGGGAGGCAGCAG-3' and GC-518R: 5'-ATTACCGCGGCTGCTGG-3' (Muyzer *et al.,* 1993). The PCR was carried out in 50 µl of reaction mixture containing, about 100 ng of DNA, 50 pmol each primer, 1X PCR buffer, 10 mM each DNTPs, 75 mM $MgCl_2$, 2.5U *AmpliTaq* gold. All reagents used in PCR amplification were provided by Applied Biosystems, Japan. DNA was denatured 95°C for 5 min, following two cycles at 80°C for 1 min, 65°C for 1 min, 72°C for 3 mins; 18 cycles at 94°C for 1 min, 64°C for 1 min, 72°C for 3 mins; 9 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 3 mins; two cycles at 95°C for 1 min, 55°C for a min, 72°C for 3 min. The last cycle was extended at 72°C for 5 mins. DNA amplification was checked by electrophoresis of 2 µl of amplicons through 1.5% agarose gel in 1X TAE buffer, stained with 5µg/ml EtBr then viewed using UV transluminator.

Amplicons from 100 µl PCR reaction mixtures were concentrated by ethanol precipitation then dissolved in 10 µl autoclaved water. Prior to run, sample was heated at 95°C for 5 mins, and then 60°C for 60 mins and was left at 25°C for overnight. The samples were applied on 10% acrylamide containing urea-formamide as denaturant in concentration 30-60% (100% denaturant contained per 100 ml; acrylamide/Bis 40%, 25 ml; 50 x TBE buffer, 2 ml; formamide (deionized), 40 ml; urea, 42 g). All reagents were provided by BIO-RAD, Hercules, CA. Electrophoreses (DGGE) was performed on The DCode™ Universal Mutation Detection System (BIO-RAD, Hercules, CA) at 60°C in 1 x TAE, 65 volts, 500 mA for about 14 h. Gel was stained using SYBR Green (BioWhittaker Molecular Application, Rockland, ME USA).

For sequencing of respective DGGE bands, PCR amplification was performed in 50 µl reaction mixture containing small pieces of gels as DNA template (gel volume approx. 2 µl), 50 p mole of each primer (388F without incorporation of GC-clamp and 517R); 1 x PCR buffer; 10 mM of each dNTP; 75 mM of $MgCl_2$; 2.5U of Ampli*Taq* Gold™. PCR conditions were; pre-denaturation for 2 mins at 94°C, followed by 30 cycles; denaturation for 30 secs at 95°C, annealing

for 30 secs at 50°C, extension for 2 mins at 72°C. A final extension for 5 mins at 72°C was added. PCR products were purified using SUPREC™ PCR (Takara Biomedicals, Otsu, Japan) then were sequenced using Big Dye Primer Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems). Sequences were automatically analyzed on a 3100 Genetic Analyzer (PE Applied Biosystems). The sequences were subjected to the GenBank for sequence homology.

Results

Culture independent analysis of LAB in ragi tape

The traditional dry-starter (*ragi tape*) found in four islands in Indonesia (Sumatera, Jawa, and Bali Islands) appeared almost in similar forms, round and tablet-like in varied sizes (inner diameter ranged from 1.5 to 2.5 cm) (Figure 1). All starters contain raw starch as the carrier of microorganisms. The starters were produced in home industry with or without trade mark and sold in a 10-20 tablets in plastic bag container.

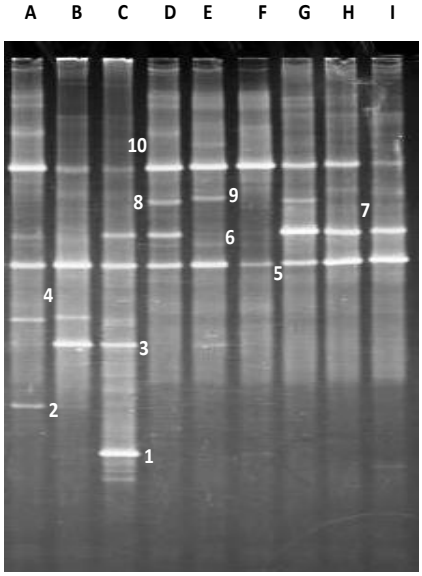

Figure 1. Profile of bacterial flora of *ragi tape* etermined using PCR-DGGE method. Bands were excised and sequenced as described in Materials and Methods section, and its respective identities are given in Tabel 1.

The culture independent analysis of the bacterial flora of *ragi tape* are given in Figure 2 dan Table 1. It was shown that *Weissella kimchii, Weissella confusa, Lactobacillus fermentum* group (further designated as *Weissella* spp. - *Lb. fermentum* group), *Enterococcus* spp. (*Enteroccoccus villorum* and *Enteroccoccus gallinarum*), *Pediococcus pentosaceus* were LAB detected in all *ragi tape* from different sources. *Lactobacillus* spp., *Bacillus* cereus, and species related to swine manure or *Clostridium perfringens* were detected in three samples. *Eubacterium moniliforme*, *Clostridium sardiniensis, Clostridium baratii* were detected in one sample. It was found that the *ragi tape* found in Sumatera Island (Figure 1, panels A, B, C, and D) were more diverse than those found in Jawa and Bali Island.

Culture independent analysis of LAB during the glutinous rice tape fermentation

The Balinese *rice wine* was prepared in laboratory scale using black and white glutinous rice as those practices in home inbdustry, inoculated with *ragi tape*. Diversity of bacterial flora was observed progressively from 12 to 24 h of fermentation. *Lactobacillus* spp. (Figure 2, band number 4) started to grow after 12 h and then its corresponding band became less distinct; indicating decreased the population in rice mash. Meanwhile, bacterium corresponded to band number 1 was started to proliferate after 24 h fermentation and rapidly disappeared after 36 h fermentation. All these showed that the succession of LAB took place during the *tape* fermentation. The three main LAB found in the starter, *Weissella* spp. - *Lb. fermentum, Enterococcus* spp. and *P. pentosaceus* were consistently detected throughout the fermentation (Figure 2 and Table 2)

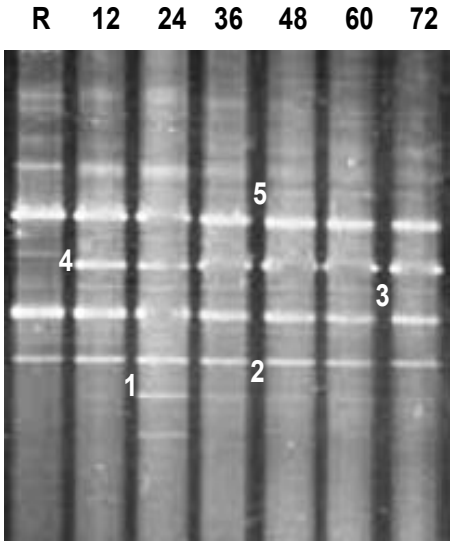

Figure 2. Profile of dynamic growth of LAB during the glutinous rice fermentation. The *ragi tape* used to inoculate glutinous rice (R). The bands were excised and sequenced as described in Materials and Methods section, and its respective identities are shown in Tabel 2.

Discussion

The raw starch containing traditional dry starters are the common way practiced by the people in the Orients in order to develop fermented foods. The starter is used for the production of soybean paste (Indonesian *kecap* and *tempe*), alcoholic beverages (Balinese rice wine (*brem*) and Vietnamese rice wine), *tape* (Indonesian) fermented cassava or rice) and *ruou nep* (Vietnamese traditional snack). Raw starch in dry starters serves as the carrier of microorganisms for making alcoholic beverages. The starter preparations are believed to vary in different countries, though it is thought that the traditional preparation is always by incorporation of spices or herbs, which selectively promotes the growth of desirable microorganisms for the fermentation.

W. confusa, W. paramesenteroides, P. pentrosaceus, E. faecium and *L. curvatus* were the major LAB identified in *ragi tape* using common cultivation methods (Hadisepoetro *et al.,* 1979; Hesseltine and Ray, 1988; Ardana and Fleet, 1989; Sujaya *et al.,* 2001). The culture independent analysis showed that a more diversified bacterial flora detected in *ragi tape* (Figure 1). Bacterial flora of *ragi tape* was dominated by the group of certain species from *weissella, enterococci, lactobacilli* and *pediococci*. Similar findings were also reported found in Vietnamese *banh men* (Thanh *et al.,* 2008). It was shown that at least 4 LAB species were involved in the *tape* fermentation (Figure 2). *Lactobacillus* sp. were detected after 12 h fermentation which might have gradually decreased in numbers as fermentation progressed. The presence of *Bacillus cereus*, *Clostridium perfringent* and *Eu. moniliforme*/*C.*

Tabel 1. List of DGGE bands of *ragi tape* and identifications by DNA sequencing

Band	Putative species	Related GenBank sequences	Homology (%)
1	Uncultured bact.	AF371840	97
	Eubact. moniliforme	L34622	97
	C. sardiniensis	X73446	97
	C. baratii	X68174	97
2	Sewine manure bact.	AY167966	97
	Sewine manure bact. 5	AY16796	97
	C. perfringent	Y12699	97
3	*B. cereus*	AE016999	98
	B. cereus	AE016999	98
	B. cereus	AY279196	98
4	*Lactobacillus* sp.	AF157037	93
	Lactobacillus sp.	AF157034	93
	Lactobacillus sp.	AF157031	93
5	Uncultured bact.	AJ487025	100
	P. pentosaceus	AJ30531	100
	P. pentosaceus	AJ018215	100
6-7	*Enterococcus* sp.	AF098492	98
	Uncultured bact.	AF371532	98
	Uncultured bact.	AF371531	98
	E. villorum	AF335596	98
8-9	*Lactobacillus* sp.	AF349935	98
	Uncultured bact.	AF349926	98
	L. fermentum	AF 477499	98
10	*W. kimchii*	AF312874	100
	L. fermentum	AF477498	100
	W. confusa	AF497497	100

Tabel 2. List of DGGE bands of fermented glutinous rice and identifications by DNA sequencing

Band	Putative species	Related GenBank sequences	Homology (%)
1	*Lactobacillus* sp.	AF157037	93
	Lactobacillus sp.	AF157034	93
	Lactobacillus sp.	AF157031	93
2	Uncultured bact.	AJ487025	100
	P. pentosaceus	AJ30531	100
	P. pentosaceus	AJ018215	100
3	*Enterococcus* sp.	AF098492	98
	Uncultured bact.	AF371532	98
	Uncultured bact.	AF371531	98
	E. villorum	AF335596	98
4	*Lactobacillus* sp.	AF349935	98
	Uncultured bact.	AF349926	98
	L. fermentum	AF 477499	98
5	*W. kimchii*	AF312874	100
	L. fermentum	AF477498	100
	W. confusa	AF497497	100

sardiniensis/ C. baratii and some bacteria related to swine manure. These bacteria were mostly isolated in *ragi tape* produced in Sumatera Island and sold in the market without trade mark (Figure 1, panel A to D). The presence of these bacteria in *rage tape* suggests that sanitary procedures must be seriously considered to ensure the safety of the products. Some results also revealed that *Bacillus* spp. might be a part microbial constituent in traditional dry starter and demonstrated happen in *banh men* (Thanh *et al.,* 2008). The present of this bacterium was not detected in tape fermentation; suggest that the fermentation environment such as low pH and ethanol content in the rice mash suppress the growth of this bacterium.

The dynamic succession of LAB during the fermentation was observed especially the present of *Lactobacillus* sp. growth after 24 h then its population decreased (Figure 2). It also demonstrated that the diversity of major LAB in samples was shown in a more accurate compare to culture based methods, and it strongly described that *Weissella* spp., *P pentosaceus* and *Enterococus* spp. were the main LAB in Indonesian *ragi tape*. The consistent growth of these LAB during the fermentation of glutinous rice (*tape*), where this condition is similar to that practiced in Balinese rice wine fermentation (*brem*), therefore these LAB may contribute in the flavor of the *tape* and *brem*.

Conclusions

Culture independent analysis PCR-DGGE of LAB in *ragi tape* revealed that the LAB especially *Weissella* spp., *Enterococus* spp., and *P. pentosaceus,* was the most dominant bacterial flora, where small variation was observed on the present of *Bacillus* sp. and likely intestinal bacteria such as *Clostridium* and or *Eubacterium*. Though the later bacteria might be regulate by the fermentation environment nevertheless the sanitation during the *ragi* preparation need to be improved. The succession growth of LAB was take places during the fermentation of glutinous rice where *Lactobacillus* sp. detected after 24h fermentation then its population decreased.

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