7. Construction of $(GTG)_5$-PCR genomic fingerprint database

The $(GTG)_5$-PCR genomic fingerprint of 413 *Lb. plantarum* isolates and four reference strains were analyzed using a computer software package, GelCompar*II* version 4.5 (Applied Maths, BVBA, Belgium). After background subtraction and gel normalization, the fingerprint patterns were subjected to cluster analysis using unweighted pair group method with arithmetic average (UPGMA). A similarity measuring based on overall densitometric profile (curve-based) using Pearson's product moment correlation was used. To determine the potential of rep-PCR for use in monitoring of unmarked *Lb. plantarum* BCC9546 during Nham fermentation, four reference strains of *Lb. plantarum*, including *Lb. plantarum* BCC9546 were used to establish the $(GTG)_5$-PCR genomic fingerprint database.

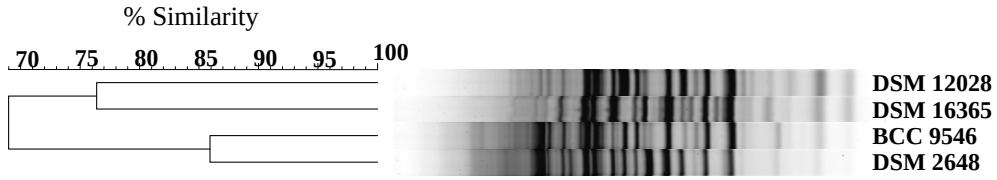


Figure 33 Dendrogram and $(GTG)_5$-PCR genomic fingerprint of reference strains.

All reference strains clearly grouped in separate clusters as shown in Figure 33. The $(GTG)_5$-PCR genomic fingerprint displayed a high heterogeneity among four reference strains of *Lb. plantarum*. It was found that the complexity of the $(GTG)_5$-PCR band pattern was not the same for all strains. Generally, the number of the bands ranged between 9 and 14, with size varying from 200 – 5,000 bp. In conclusion, the $(GTG)_5$-PCR technique use in this experiment has been proved to be able to differentiate among reference strains and can offer a tool to monitoring succession of unmarked *Lb. plantarum* BCC9546.

8. Reproducibility of rep-PCR fingerprinting

To determine the minimum percentage similarity necessary for strains discrimination. The level of similarity obtained between repeats established a discrimination threshold below which pattern were considered to be different. Each PCR reactions is controlled for reproducibility by inclusion of the reference strain *Lb. plantarum* BCC 9546. PCR amplification and electrophoresis were performed in 19 separate trials starting from same DNA preparation and using the same PCR reagents. None of the strain tested showed qualitative difference in banding pattern, i.e. presence versus absence of a band. On the other hand, minor quantitative variations in band intensity were occasionally found, but with no pronounced effect on the stability of cluster analysis. The similarity index between 19 separate of PCR amplification have obtained banding patterns of the same strain ranging between 88-99% (Figure 34).

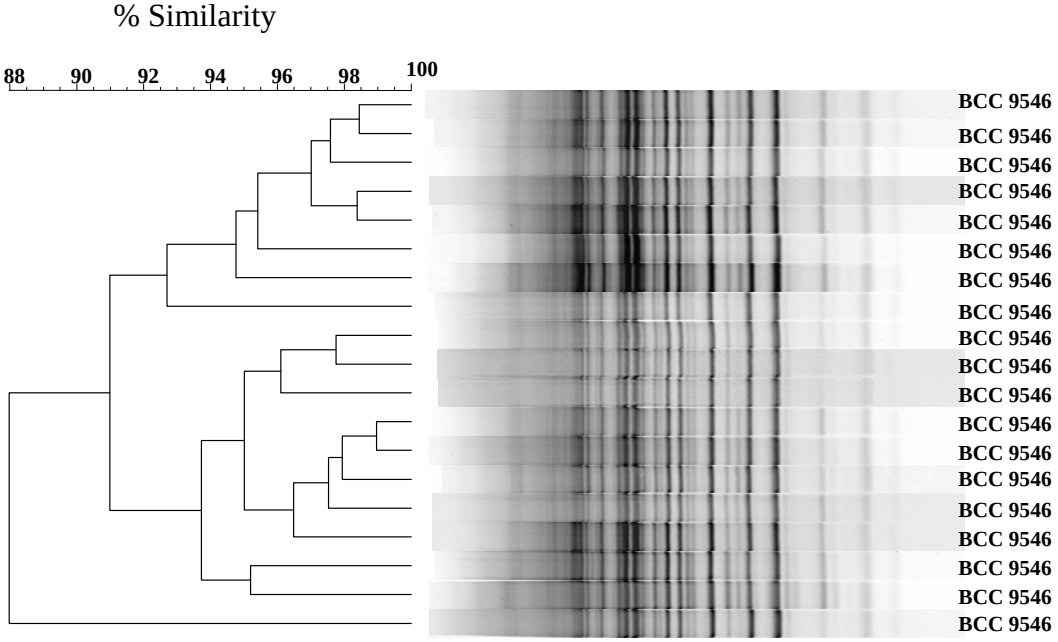
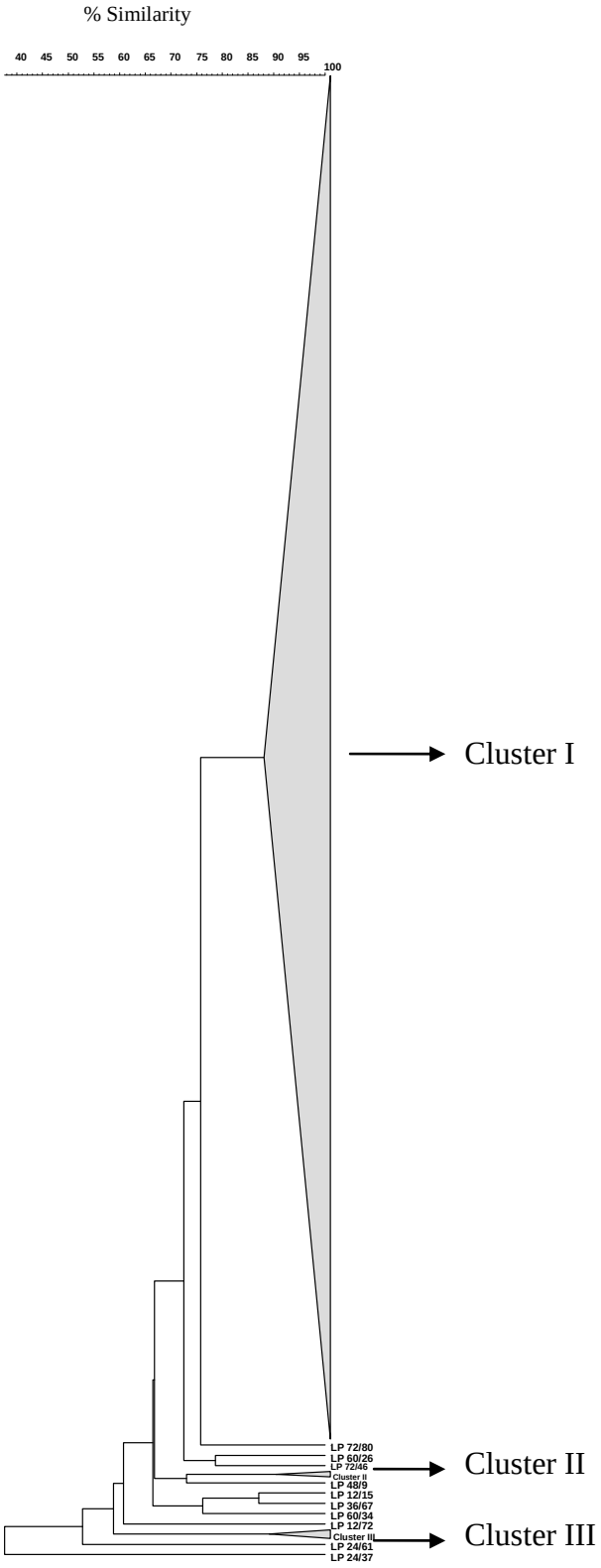


Figure 34 Dendrogram and reproducibility of (GTG)5-PCR genomic fingerprint.

9. Characterization of *Lb. plantarum* during Nham fermentation

The populations of *Lb. plantarum* isolates obtained from Nham fermentation were analyzed in order to understand the role of the starter culture in Nham fermentation. The repetitive sequence-based PCR (rep-PCR) using primer $(GTG)_5$ was used to monitoring the process of starter culture, *Lb. plantarum* BCC9546 during Nham fermentation. Cluster analysis of 413 *Lb. plantarum* strains isolated from starter cultured Nham and 19 internal reference strains (BCC 9546) were reported in Figure 35. A similarity coefficient of 88 % was selected as the discrimination cut off for strains differentiation considered in the study. There were three clusters and ten single-strain clusters, namely LP 72/80, LP 60/26, LP 72/46, LP 48/9, LP 12/15, LP 36/67, LP 60/34, LP12/72, LP 24/61 and LP 24/37, being identified. Cluster I contained the largest cluster, followed by cluster III and clusters II. Cluster I contained 19 internal reference strains (BCC 9546) and 399 isolates isolated from fermentation time at 12 h (28 isolates), 24 h (47 isolates), 36 h (81 isolates), 48 h (77 isolates), 60 h (87 isolates) and 72 h (78 isolates). Cluster III contained three isolates from fermentation time at 24 h (LP 24/18, LP 24/22 and LP 24/19), while cluster II had two isolates obtained from fermentation time at 48 h (LP 48/71 and LP 48/72). It is noteworthy that clusters I grouped together with BCC 9546 internal reference strain, indicating that this cluster can be characterized as BCC 9546 strains.

In previous studies, the $(GTG)_5$-PCR genomic fingerprint has been shown to be a very powerful method for species and strain typing (Cocolin *et al*., 2004, Gevers *et al*., 2001 and Tamang *et al*., 2005). In this study the $(GTG)_5$-PCR genomic fingerprint has proved to be a very rapid and highly reliable characterization of *Lb. plantarum* isolates at the strain level. The most common patterns found in 413 of *Lb. platarum* strains isolated from starter cultured Nham were shown in Figure 36. The $(GTG)_5$ primer set generated in total 8 - 12 fragments of about size of 200 - 6,000 bp. A total of 13 different $(GTG)_5$-PCR banding patterns (P1-P13) were obtained. The pattern 1 (P1) was the major pattern containing majority of *Lb. plantarum* strains isolated from all different time point (cluster I) and BCC 9546, therefore representing the starter culture isolate.



Cluster	No. of Strains in the cluster	Time of fermentation (hrs)					
		12	24	36	48	60	72
I	399	28	47	81	77	87	79
II	2	-	2	-	-	-	-
III	3	-	-	-	3	-	-

Figure 35 Abridged dendrogram and of the $(GTG)_5$-PCR genomic fingerprint of *Lb. plantarum* isolates obtained from starter cultured Nham.

Other patterns (P2-P13), which generated unique and different genomic fingerprints, were observed in a small number that can be considered representative for the natural strain occur in Nham fermentation.

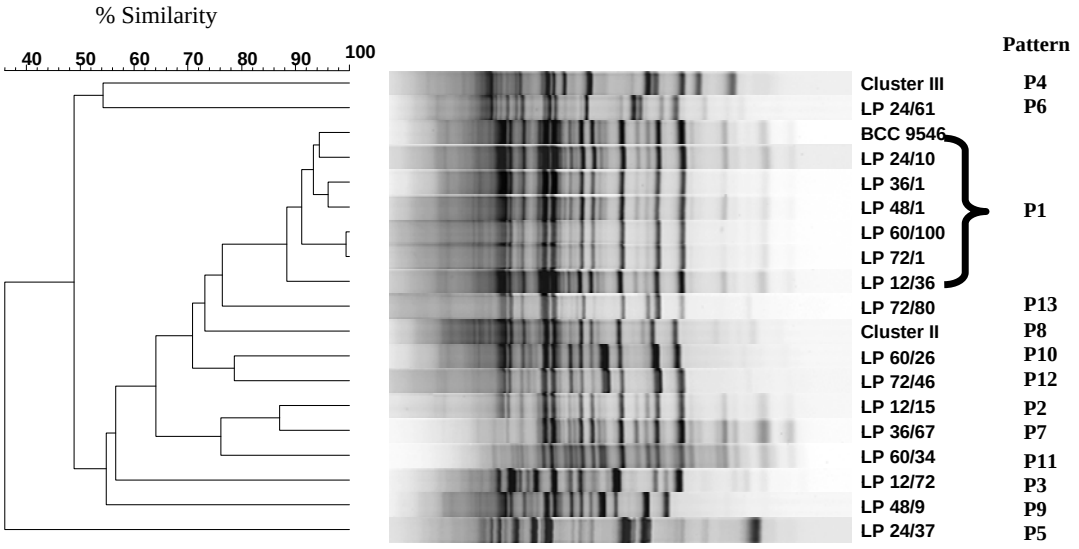


Figure 36 Dendrogram and $(GTG)_5$-PCR banding patterns of representative strains (P1-P13) found in starter cultured Nham and BCC 9546.

The percentages of each patterns were different among pattern considered, as shown in Table 4. During fermentation time at 12, 24, 36, 48, 60 and 72 h, P1 was the main representative of *Lb. plantarum* strains founded in Nham fermentation. This type of rep-PCR pattern accounted for 93.33 %, 90.38 %, 98.78 %, 96.25 %, 97.75 % and 97.50 %, respectively. Other strains appeared at low percentages were P2-P13, which accounted for 1-2 % of total isolates. Pattern 1 (P1) could be found in starter cultured Nham since fermentation reach 12 h which correlated with the initial appearance of *Lb. plantarum*. In additional, this diversity was not different in the various stages of fermentation. P1 or BCC 9546 pattern was the predominant strains throughout the fermentation, while P2-P13 was less dominant. It can be concluded that *Lb. plantarum* BCC 9546 was present in significant numbers during the fermentation.

Table 4 Distribution of $(GTG)_5$-PCR fingerprints of *Lb. plantarum* strains from starter culture Nham during fermentation.

Time (h)	Pattern	Total strains	No. of strains	%
12	P1	30	28	93.33
	P2		1	3.33
	P3		1	3.33
24	P1	52	47	90.38
	P4		3	5.88
	P5		1	1.96
	P6		1	1.96
36	P1	82	81	98.78
	P7		1	1.27
48	P1	80	77	96.25
	P8		2	2.50
	P9		1	1.25
60	P1	89	87	97.75
	P10		1	1.12
	P11		1	1.12
72	P1	80	78	97.50
	P12		1	1.30
	P13		1	1.30