3. Construction of ITS-PCR database

The ITS patterns of 1,529 bacterial isolates and 15 reference strains were analyzed using a computer software package, Gel Compar II version 4.5 (Applied Maths BVBA, Belgium). The similarity between all pairs was expressed by Dice coefficient correlation, and Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering for the construction of the dendrogram. To prove the potential of ITS-PCR for use in differentiation of major bacterial species involved in Nham fermentation, fifteen reference strains were used to establish the ITS-PCR database. The coefficient of similarity was used as a discriminating factor in the cluster analysis of the bacterial isolates in this study.

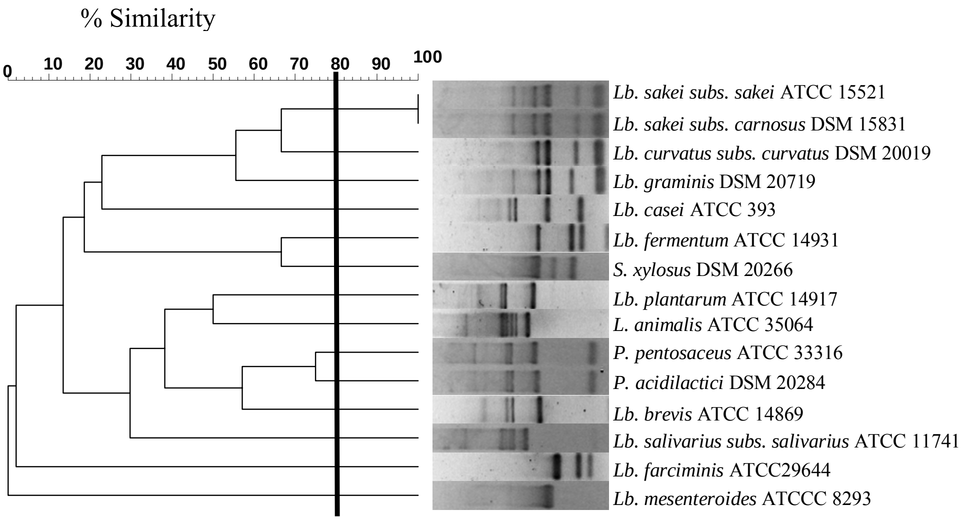


Figure 13 ITS-PCR pattern and dendrogram of reference strains.

Figure 13 shows the ITS-PCR patterns and the cluster analysis for 15 reference strains. A coefficient of similarity of 80% was selected to distinguish the clusters in order to differentiate all species tested (shown with a line in Figure 14). In this way, ITS-PCR patterns revealed that each species was represented by different patterns. However, in some cases related species have close banding patterns but this issue can be overcome by using pattern analysis software. Thus, the ITS-PCR technique use in this experiment has been proved to be able to differentiate among bacterial species, especially LAB species.

4. Identification of bacterial isolates

Among the 1,529 isolates analyzed in this study, 108 were selected as representative on the basis of different ITS-PCR patterns. The amplified PCR products were approximately 300 bp (Figure 14).

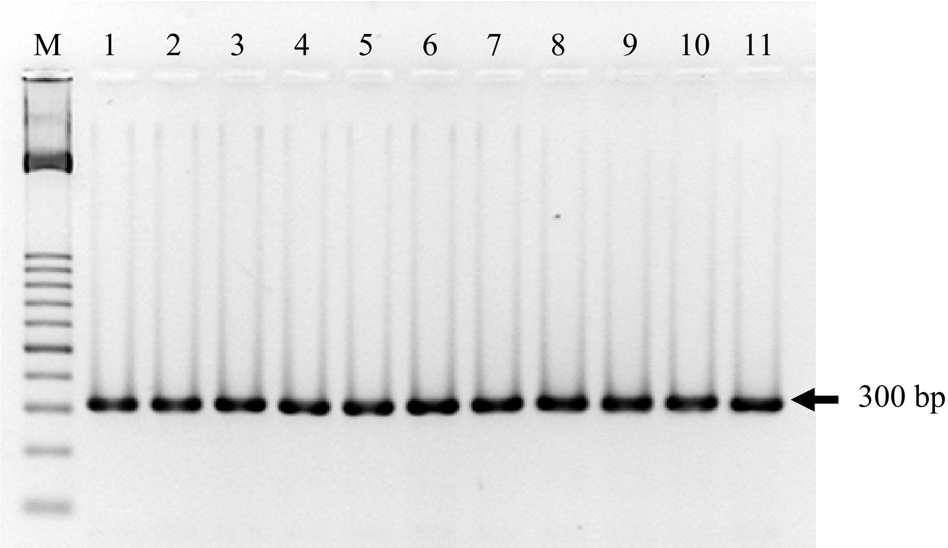


Figure 14 Agarose gel electrophoresis of partial amplified 16S rDNA of bacterial isolates (Lane 1-11). Lane M: 100 bp DNA ladder (Bioexcellence).

The partial sequence of 16S rDNA demonstrated the highest identity for various bacterial species with 16S rDNA sequence from GenBank (Table 2). Of the 108 isolates, 23 species were detected. Sixteen of 23 species showed ≥ 97% identity with LAB species. The species belonged to *Enterococus faecium*, *Enterococcus* sp, *Lactobacillus brevis, Lactobacillus curvatus, Lactococcus lactis, Lactobacillus farciminis, Lactobacillus plantarum, Lactococcus garvieve, Leuconostoc citreum, Leuconostoc fallax, Leuconostoc pseudomesenteroides, Pediocoocus pentosaceus, Vagococcus carniphilus, Weissella cibaria, Weissella* sp., and *Weissella paramesenteroides*. Seven species showed ≥ 96% identity with *Bacillus cereus*, *Klebsiella oxytoca, Klebsiella pneumoniae, Klebsiella* sp., *Macrococcus caseolyticus, Staphylococcus gallinarum* and *Staphylococcus hominis*. Several isolates have been identified as Bacterium KA55 and unidentified bacterium.

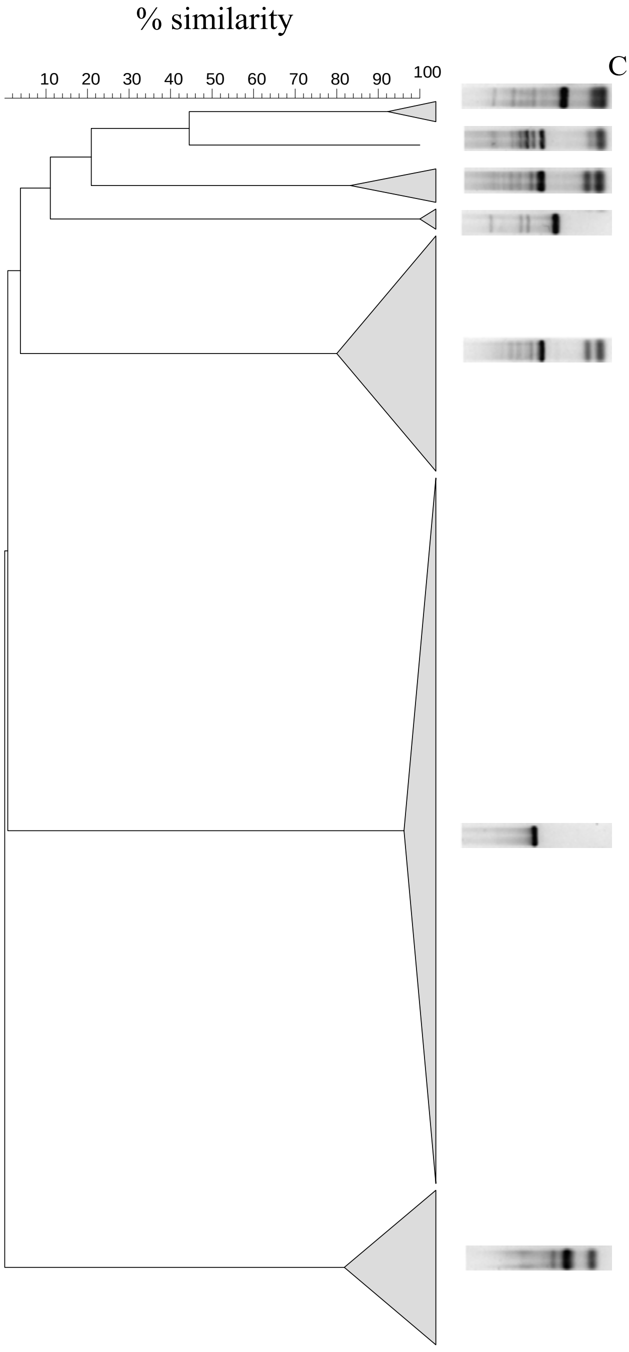
Table 2 Identification of a representative isolates

Representative strains	**Closet relative**	**Identity (%)**	**Accession no.**
CT 0/ 76	*Bacillus cereus*	99	AM 419184.1
LP 0/40, 61	Bacterium KA55	98	AY 345447.1
CT 36/38	*Enterococcus faecium*	99	EF 591817.1
LP 0/58	*Enterococcus sp.*	100	AB 079371.1
CT 0/65, LP 0/54, 55, CT 12/99	*Klebsiella oxytoca*	96-98	DQ 444288.1
CT 12/64	*Klebsiella pneumoniae*	99	DQ 831003.1
CT 36/83, CT 48/82, 86	*Lactobacillus brevis*	99-100	EF 412983.1
CT 24/37, CT 36/48, CT 48/73	*Lactobacillus curvatus*	100	EF 375891.1
LP 0/39, CT 06/19, 21, LP 6/95, CT 12/3, 7, 31, 34, LP12/27, 85, 87, CT 24/29, 39, 42, 82, CT 36/32, LP 36/36, CT 48/40, LP48/30, 67	*Lactobacillus lactis*	98-100	DQ 523490.1
CT 60/35	*Lactobacillus farciminis*	99	AJ 417499.1
LP 12/36, 49, 71, 76, 82, 83, 94, 98, 100, CT 24/4, LP 24/35, 42, 57, 75, 101, CT 36/13, 36, 77, LP 36/42, LP 48/98, CT 60/24, 31, 40	*Lactobacillus plantarum*	98-100	EF 422373.1
CT 06/1, LP 06/55, 64, CT 24/98, LP 24/88	*Lactococcus garvieve*	98-100	AB 267905.1
LP 06/96, LP 12/70, 90, CT 24/ 1, 101, CT 36/62, CT 48/38	*Leuconostoc citreum*	99-100	DQ 682946.1
CT 36/15, CT 48/100	*Leuconostoc fallax*	100	DQ 682945.1
CT 24/90, CT 36/30	*Leuconostoc pseudomesenteroides*	100	AM 491818.1
CT 0/95, 96, LP 0/83, 91	*Macrococcus caseolyticus*	98-100	EF 032686.1
LP 12/61, CT 24/7, 58, 64, 68, CT 36/18, 58, 69, CT 48/48	*Pedicoccus pentosaceus*	100	AB 300220.1
LP 0/88	*Staphylococcus gallinarum*	100	AY 647303.1
LP 06/91	*Staphylococcus hominis*	100	AY 688064.1
CT 0/71	Unidentified	96	-
LP 0/38	Unidentified	97	-
LP 0/81	*Vagococcus carniphilus*	100	AY 179329.1
CT 48/, 24, 81	*Weissella cibaria*	100	AB 300217.1
LP 0/47	*Weissella paramesenteroides*	97	AB 242999.1
CT 06/29, 98, LP 12/62, 63, 73, 79, 81, 91, LP 24/6, 100	*Weissella* sp.	99-100	DQ 682951.1
LP 0 h no. 99	*Klebsiella.* sp.	97	EF 522822.1

5. Genotypic identification of the bacterial isolates

In this study, PCR was used to amplify the ITS regions of the 1,529 isolates, which were isolated from natural and starter cultured Nham during the course of fermentation. The ITS-PCR of each isolate was subjected to restriction fragment length polymorphism (RFLP) analysis using the enzyme *Rsa*I of each sample from different fermentation times (0 h, 6, 12, 24, 36 48, 60 and 72 h). PCR fingerprinting of fermentation isolates were clustered using Dice coefficient correlation and UPGMA (unweighted pair group method using arithmetic average) and dendrograms were constructed. Clusters were discriminated at a correlation coefficient level of 80%. The identical or closely resemble band were classified in the same genetic group as indicated using the same Roman letter (Figure 15-30). The species identification obtained by 16S rDNA sequencing and the *Rsa*I restriction pattern of ITS. All identification results of the bacterial species within 1,529 isolates are presented in Table 3, where the number of isolates, for each fermentation times, belonging to specific species and percentage are reported. From these results, it showed that the microbial diversity of cultivable microorganisms in the natural and starter cultured Nham during fermentation were difference in both of bacterial diversity and bacterial composition as described below.

At 0 h of fermentation: the results of ITS-PCR profile are presented in Figure 15 and Figure 16. In natural fermentation, a total 90 isolates were grouped into six clusters and single isolate (CT 0/61) shown in figure 15. The most numerous generate group was clusters V with 53 isolates, followed by cluster IV (18 isolates), and cluster VI (12 isolates). All other clusters (I, II, and III) were small, including 3 or 2 isolates. By 16S rDNA sequencing, *Lc. garvieae, M. caseolyticus*, and *Lc. lactis* were the main bacterial species founded accounted for 58% (cluster V), 20% (cluster IV), and 13% (cluster VI), respectively. Other bacterial species which were founded in low number were *K. oxytoca* (cluster II), *B. cereus* (cluster III), and *K. pneumoniae* (CT 0/61) with 3%, 2% and 1% of total isolates recovered, respectively. In addition, two isolates has been unidentified (2% of total isolates, cluster I).



Clusters	Species	Isolates
I	Unidentified	CT 0/71, 78
	K. pneumoniae	CT 0/61
II	*K. oxytoca*	CT 0/12, 65, 77
III	*B. cereus*	CT 0/76, 84
IV	*M. caseolyticus*	CT 0/80, 81, 82, 83, 85, 86, 87, 88, 89, 90, 91, 92, 93, 95, 96, 97, 98, 99
V	*Lc. garvieae*	CT 0/ 14, 74, 16, 18, 19, 50, 03, 04, 05, 06, 07, 09, 11, 13, 15, 20, 22, 23, 24, 25, 27, 30, 31, 32, 33, 34, 36, 37, 38, 39, 40, 41, 42, 43, 45, 46, 47, 49, 51, 52, 54, 55, 56, 57, 58, 59, 60, 62, 63, 66, 69, 72, 75
VI	*Lc. lactis*	CT 0/01, 02, 08, 21, 26, 28, 29, 35, 44, 48, 53, 64

Figure 15 Abridged dendrogram and representing restriction pattern of ITS with of bacterial isolates obtained from natural Nham at 0 h of fermentation. Identified clusters are indicated with roman letters.

A total of 93 isolates from starter cultured Nham were group into seven different clusters and seven single isolates as shown in Figure 16. Cluster VI was the biggest with 49 isolates followed by cluster V (10 isolates), cluster III (9 isolates), I (8 isolates) and cluster VII (6 isolates). The remaining clusters (II and IV) were contained only 2 isolates. A representative isolate of each cluster was subjected to 16S rRNA sequence analysis which revealed that 53% of total isolates were *Lc. garvieae* (cluster VI), followed by *Lc. lactis* (cluster I, LP0/43 and LP0/39) and *M. caseolyticus* (cluster V). Each species were accounted for 11% of total isolates. It is noteworthy that 10% of recovered isolates were identified as Bacterium KA55 (cluster III). In addition, six isolates has been unidentified (6% of total isolates, cluster VII). A low number of other bacterial species including various LAB namely, *Vc. carniphilus*, *S. gallinarum*, *Enterococcus* sp., *W. paramesenteroides, Weissella* sp., *P. pentosaceus*, *K. oxytoca* and *Klebsiella* sp. Furthermore, it should be noted more bacterial diversity in starter cultured Nham than natural Nham at the start of fermentation.

At 6 h of fermentation: Cluster analysis of 86 isolates from natural fermentation is reported in Figure 17. Four genetic clusters and three single isolates, CT 6/98, CT 6/04 and CT 6/97 have been identified. Clusters I and IV were the major clusters with 66 and 12 isolates, respectively, whereas the remaining two clusters were composed of 3 and 2 isolates. According to 16S rDNA sequence, the presence of 5 LAB species was detected. *Lc. garvieae* and *Lc. lactis* remained the major species representing 77% (cluster I) and 15% (cluster IV and CT6/04), respectively while *M. caseolyticus* disappeared. The proportion of other LAB species, *Weissella* sp., *Ln. pseudomesenteroides* and *Ln. citreum* were significantly lower at 3.0% (cluster III and CT6/98), 3.0% (cluster II) and 1% (CT6/97), respectively.

Cluster analysis of the 95 isolates obtained from starter cultured Nham is reported in Figure 18. Four major clusters and two of single isolate clusters were obtained. Cluster III was the biggest cluster comprised of 78 isolates, followed by cluster IV (10 isolates), cluster I (4 isolates) and cluster II (2 isolates), respectively.

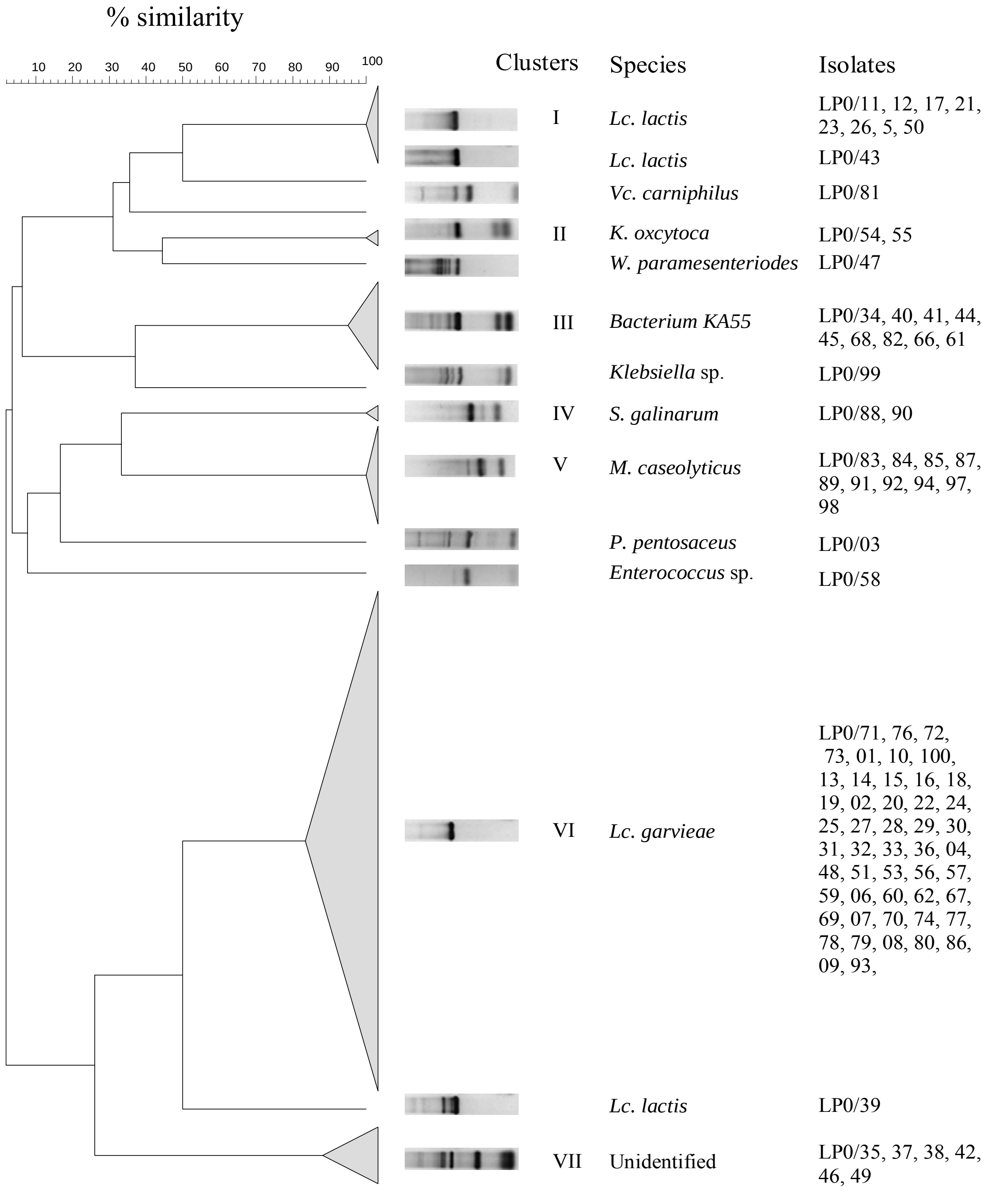


Figure 16 Abridged dendrogram and representing restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from starter cultured Nham at 0 h of fermentation. Identified clusters are indicated with roman letters.

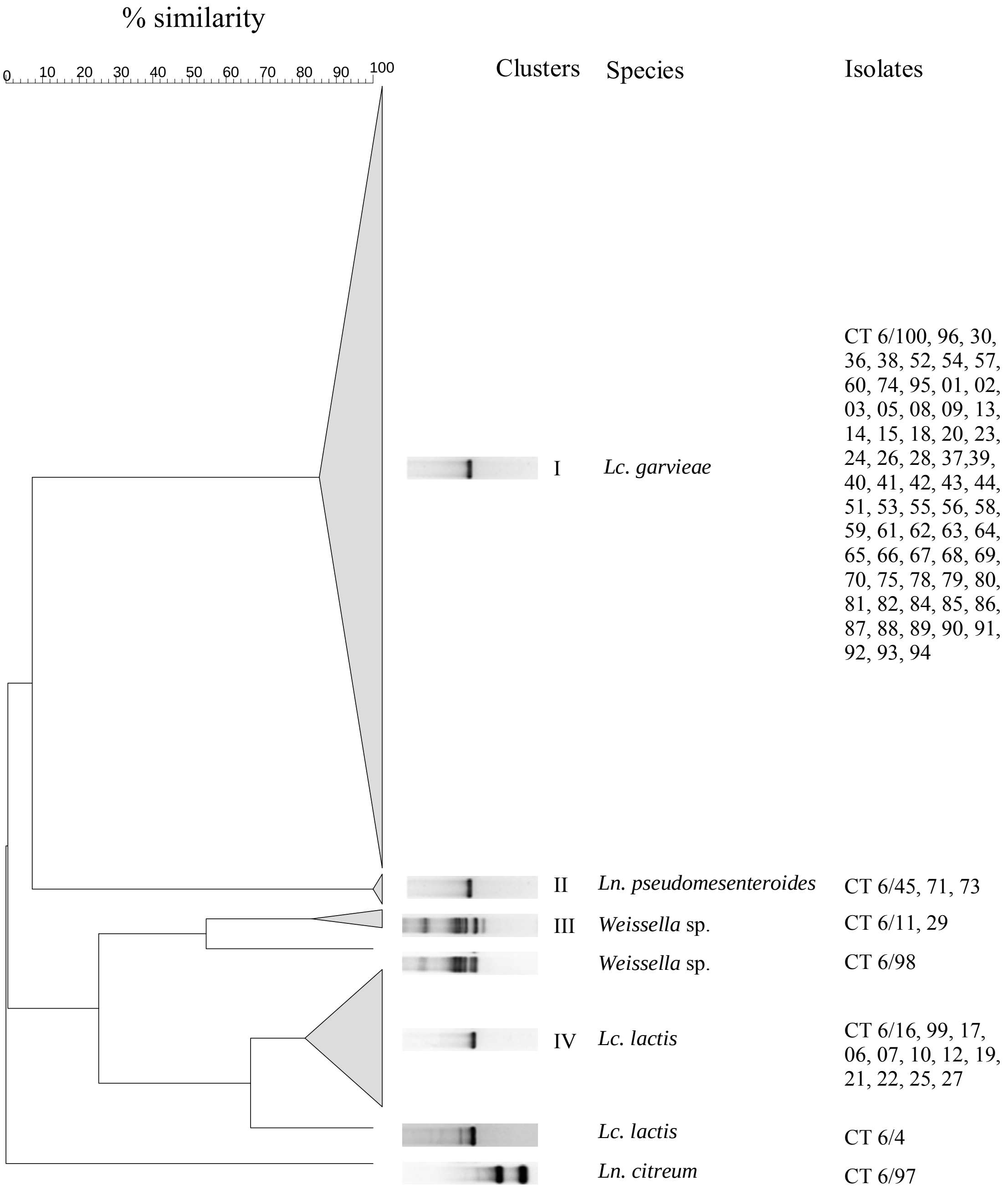
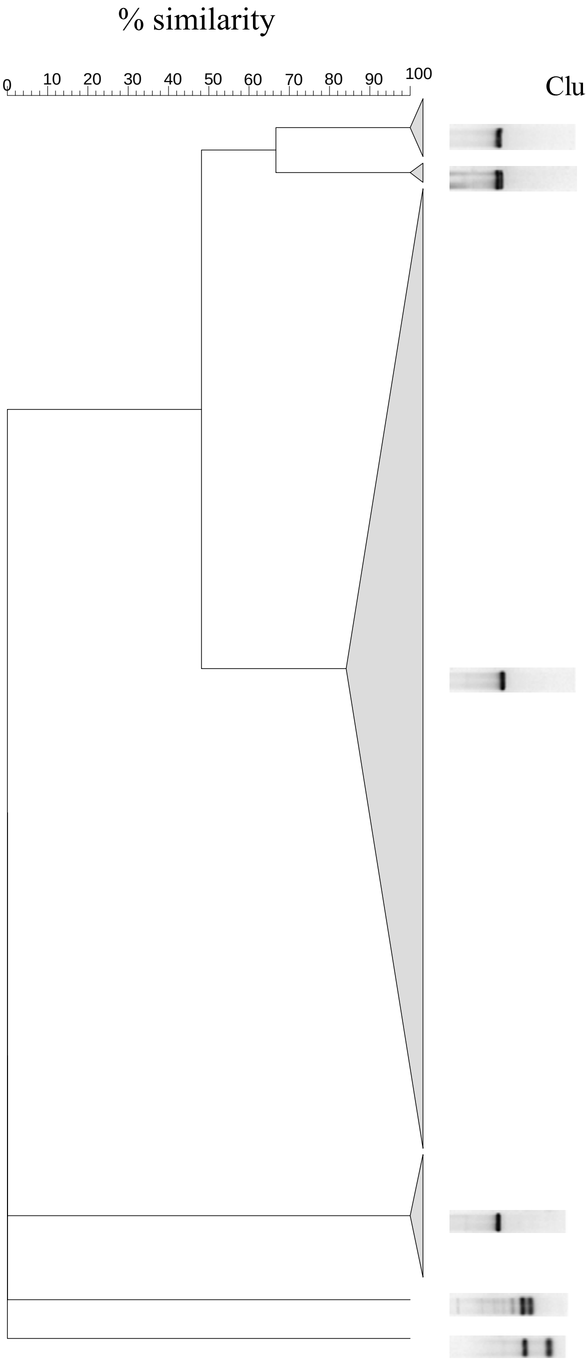


Figure 17 Abridged dendrogram and representing restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from natural Nham at 6 h of fermentation. Identified clusters are indicated with roman letters.



Clusters	Species	Isolates
I	*Ln. pseudomesenteriodes*	LP6/22, 62, 88, 92
II	*Lc. garvieae*	LP6/01, 25
III	*Lc. garvieae*	LP6/13, 68, 36, 37, 38, 41, 42, 44, 45, 59, 60, 63, 67, 03, 04, 05, 06, 07, 08, 10, 100, 11, 12, 14, 15, 16, 17, 18, 20, 21, 23, 24, 26, 28, 29, 30, 31, 32, 33, 34, 35, 39, 40, 43, 46, 47, 48, 49, 50, 51, 52, 53, 55, 56, 57, 58, 61, 64, 65, 66, 69, 71, 72, 73, 75, 76, 77, 78, 81, 84, 86, 87, 89, 09, 90, 93
IV	*Lc. lactis*	LP6/02, 19, 27, 54, 82, 83, 95, 97, 99
	S. hominis	LP6/91
	Ln. citreum	LP6/96

Figure 18 Abridged dendrogram and representing restriction pattern of ITS with *Rsa*I of bacteria strain isolated from starter cultured Nham at 6 h of fermentation. Identified clusters are indicated with roman letters.

The 16S rDNA sequencing of representative isolate from each cluster was performed. *Lc. garvieae* and *Lc. lactis* were the dominant species, accounted for 82% (clusters II and III) and 12% (cluster IV) of total isolates, respectively. In additional, no isolate belonging to *M. caseolticus* has been founded. Less dominant species were *Ln. pseudomesenteroides* at 4% (cluster I), *Ln. citreum* (LP6/96) and *S. hominis* (LP 6/91) at 1% each. It is interesting to notice that, *M. caseolyticus* was only founded at the 0 h of fermentation and absence from of both types of Nham fermentation at 6 h of fermentation

At 12 h of fermentation: The total of 98 isolates obtained from natural fermentation was grouped into three major clusters and six single isolate clusters, as shown in Figure 19. The largest group was cluster II which contained 51 isolates, followed by cluster III (29 isolates) and cluster I (13 isolates). According to 16S rDNA sequence, *Lc. lactis* and *Lb. garvieae* were the major bacterial species associated with natural fermentation represent with 66% (cluster I and cluster II) and 30% (cluster III) of total isolates. Other species identified at lower percentage were *Weissella* sp., *K. pneumoniae* and *K. oxytoca*. It should be noted that the species *K. pneumoniae* and *K. oxytoca* remain founded in natural fermentation.

On the contrary, the starter cultured Nham showed a different microbial diversity from natural Nham (Figure 20). A total of 89 isolates were subjected to analysis and seven major clusters and five single isolate clusters were obtained. Clusters VI, I, and VII were the largest grouped comprised of 33, 25 and 17 isolates, respectively. In additional, four smaller clusters (II and III-V) which contained between 5 and 2 isolates have been identified. However, by 16S rDNA sequencing, *Lc. lactis* has been founded to be the dominant LAB constituting 37% of total isolates (cluster VI and LP 12/27), followed by *Lb. plantarum*, 34% (clusters I and II), *Lc. garvieae* 19% (cluster VII), *Weissella* sp. 7 % (clusters III-V), *Ln. citreum* 2% (LP12/70 and LP12/90) and *P. pentosaceus* 1% (LP 12/61). Strikingly, it is interesting to notice that the isolates identified as *Lb. plantarum*, which was the dominant species in starter cultured Nham.

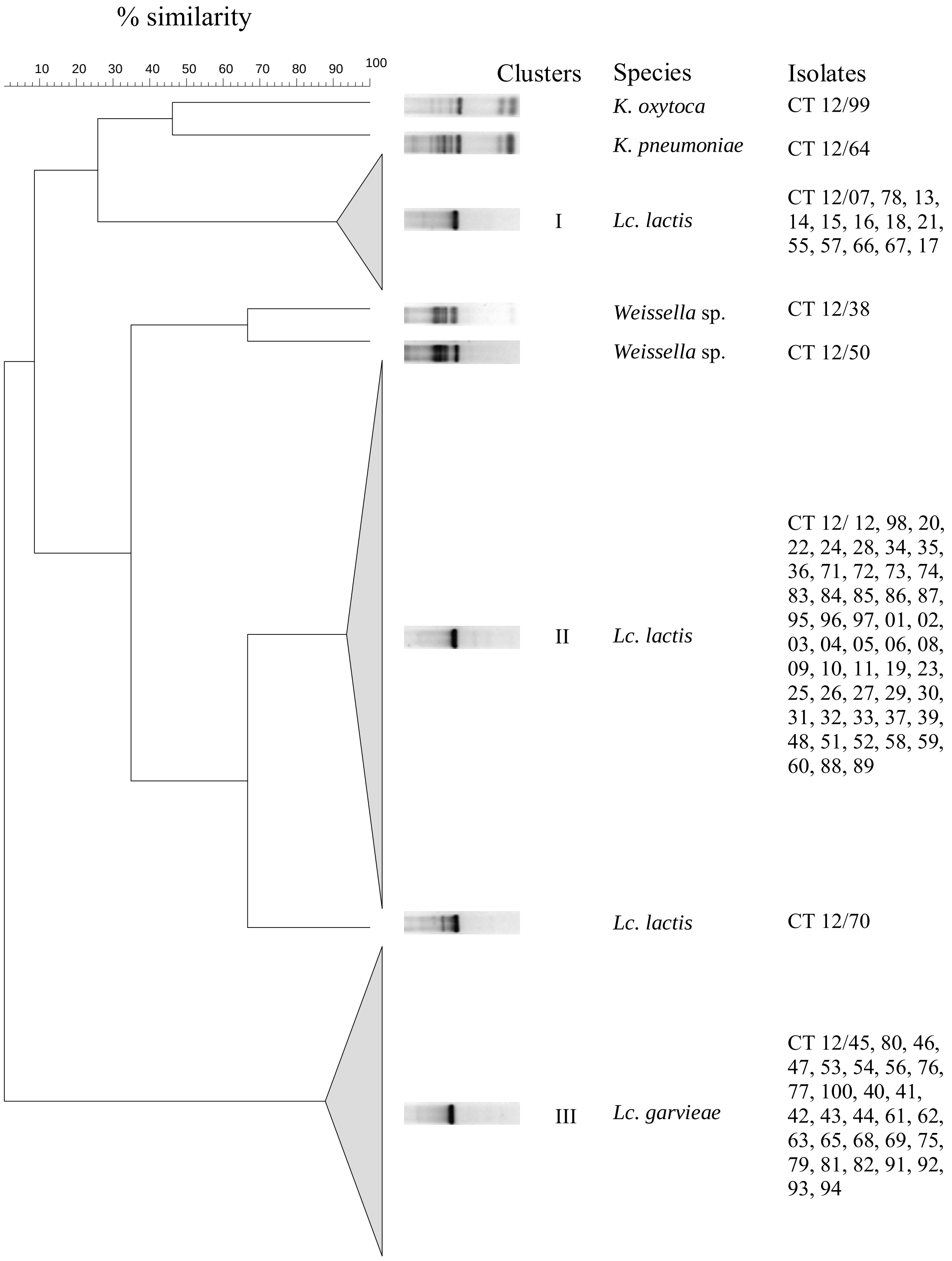


Figure 19 Abridged dendrogram and representing restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from natural Nham at 12 h of fermentation. Identified clusters are indicated with roman letters.

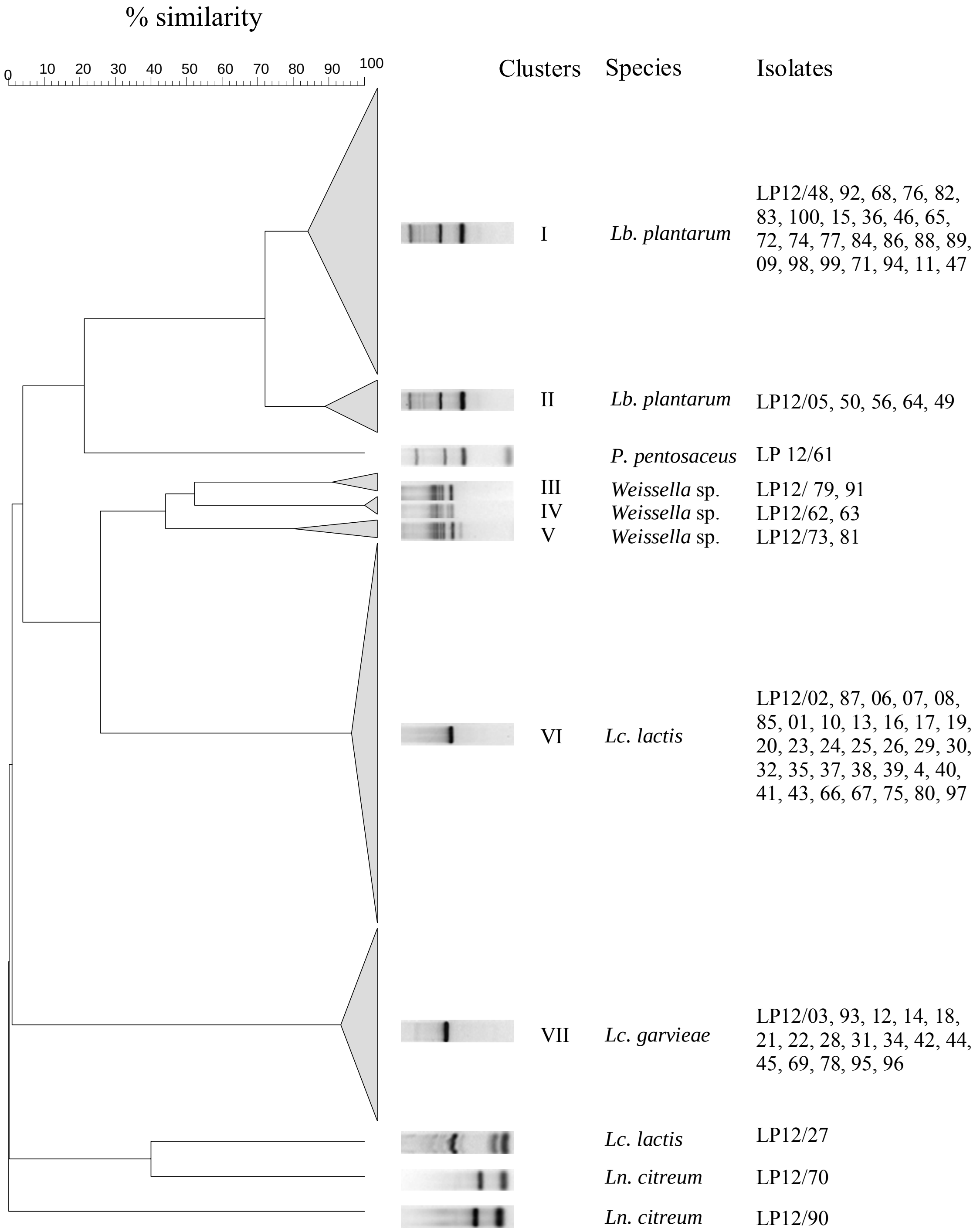
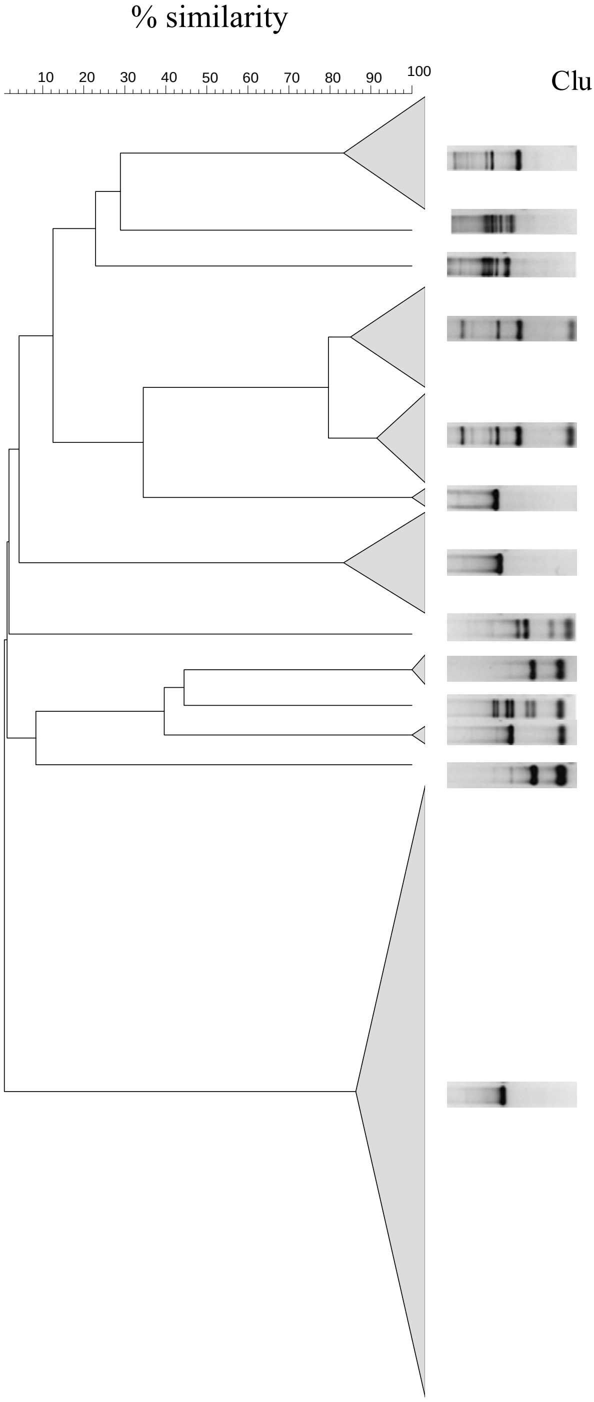


Figure 20 Abridged dendrogram and representing restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from starter cultured Nham at 12 h of fermentation. Identified clusters are indicated with roman letters.

At 24 h of fermentation: The results obtained by the cluster analysis of 100 isolates from natural fermentation are shown in Figure. 21. Eight distinct clusters and five single isolate clusters were obtained. Cluster VIII was the largest cluster comprised of 52 isolates. Cluster I was formed by 10 isolates. Clusters II and V were comprised of 9 isolates each. Cluster III was composed of 8 isolates. The remaining clusters (cluster IV, VI and VII) were constituted by 3 or 2 isolates. For further identification, nine different species of LAB were detected by using 16S rDNA sequencing, *Lc. lactis* remained a dominant species but with decreased proportion, comprising of 52% of total isolates (cluster VIII), followed by *P. pentosaceus* at 17% (clusters II and III) and *Lb. plantarum* at 10% (cluster I), *Lc. garvieae* comprised of 9% (cluster V) and *Ln. citreum* represented with 5% (cluster VI, CT24/101 and CT24/74) of total isolates. Other species of LAB that were founded at 2 isolates (2%) each, were identified as *Ln. pseudomesenteroides* (cluster IV) and *Ln. fallax* (cluster VII). In addition, single isolates each has been identified as *W. cibaria* and *Lb. curvatus*.

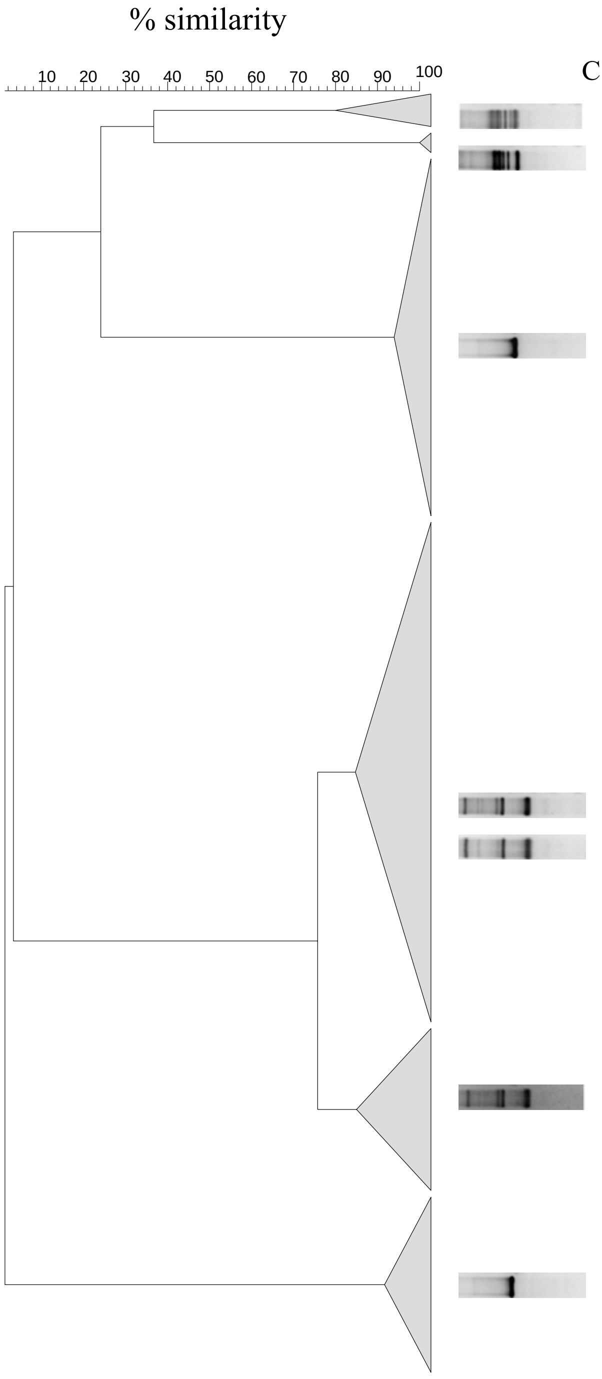
In Figure 22, the dendrogram resulting from the analysis of 99 isolates obtained from starter cultured Nham are shown. Six clusters have been determined. Clusters IV was the biggest cluster with 39 isolates, followed by cluster III (28 isolates), cluster VI (14 isolates), cluster V (13 isolates), cluster I (3 isolates) and cluster II (2 isolates). According to 16S rDNA sequencing, *Lb. plantarum* was the predominant species representing 53% of total isolates (clusters IV and V), followed by *Lc. lactis* (cluster III, 28%), *Lc. garvieae* (cluster VI, 14%), *Weissella* sp. (cluster I, 3%) and *W. cibaria* (cluster II, 2%). It is interesting to notice that *Lb. plantarum* became the predominant species in starter cultured Nham at this sampling period, while *P. pentosaceus* were the dominant species founded in natural fermentation.

At 36 h of fermentation: One hundred isolates from natural fermentation were grouped into eight clusters and two single isolates (CT 36/58 and CT 36/38) (Figure. 23).



Clusters	Species	Isolates
I	*Lb. plantarum*	CT 24/ 30, 63, 68, 73, 104, 106, 04, 06, 08, 10,
	W. cibaria	CT 24/24
	W. cibaria	CT 24/80
II	*P. pentosaceus*	CT 24/14, 19, 22, 25, 02, 05, 07, 11, 26
III	*P. pentosaceus*	CT 24/34, 44, 58, 59, 64, 72, 79, 67
IV	*Ln. paramesenteroides*	CT 24/47, 90
V	*Lg. garvieae*	CT 24/95, 98, 96, 43, 65, 91, 92, 93, 105
	Lb. curvatus	CT 24/37
VI	*Ln. citreum*	CT 24/1, 21, 69
	Ln. citreum	CT 24/101
VII	*Ln. fallax*	CT 24/94, 97
	Ln. citreum	CT 24/74
VIII	*Lc. lactis*	CT 24/86, 88, 87, 03, 12, 13, 15, 16, 17, 18, 20, 23, 27, 28, 29, 31, 32, 33, 35, 36, 38, 39, 40, 41, 42, 45, 46, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 60, 62, 66, 71, 75, 81, 82, 83, 84, 85, 99, 100, 102, 103, 61

Figure 21 Abridged dendrogram and representing restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from natural Nham at 24 h of fermentation. Identified clusters are indicated with roman letters.



Clusters	Species	Isolates
I	*Weissella* sp.	LP 24/05, 06, 100
II	*W. cibaria*	LP 24/80, 90
III	*Lc. lactis*	LP 24/28, 79, 44, 54, 59, 01, 04, 08, 09, 14, 21, 24, 25, 27, 36, 38, 39, 40, 41, 45, 47, 55, 56, 72, 82, 89, 97, 98
IV	*Lb. plantarum*	LP 24/46, 68, 64, 65, 66, 67, 02, 07, 10, 11, 22, 23, 34, 43, 60, 61, 62, 69, 70, 74, 76, 77, 81, 83, 85, 86, 91, 92, 99, 17, 87, 71, 73, 75, 84, 51, 53, 57, 95
V	*Lb. plantarum*	LP 24/18, 50, 19, 29, 30, 31, 32, 33, 35, 49, 15, 37, 48
VI	*Lc. garvieae*	LP 24/63, 93, 03, 12, 13, 16, 20, 26, 42, 52, 78, 88, 94, 96

Figure 22 Abridged dendrogram and representing restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from starter cultured Nham at 24 h of fermentation. Identified clusters are indicated with roman letters.

The most numerous genetic group were clusters III with 49 isolates, followed by cluster I (25 isolates) and cluster VI (13 isolates). Five small clusters (II, IV, V, VII and VIII) were contained only 3 or 2 isolates. By sequence analysis of 16S rDNA, the three major clusters were identified as *Lb. plantarum* (49%, cluster III), *P. pentosaceus* (25%, cluster I and CT 36/58) and *Lc. lactis* (13%, cluster VI). Other LAB species identified at lower numbers were *Ln. citreum*, *Lc. curvatus*, *Ln. pseudomesenteroides*, *Lb. brevis* and *Ln. fallax* accounted for 3% and 2% of total isolates. One isolates (CT 36/38) could identified as *E. faecium*. In additional, *Lc. garvieae* was only founded during 24 h of fermentation and absence in both types of Nham fermentation at 36 h of fermentation Moreover, the LAB species was founded to be less heterogeneous in natural fermentation.

Cluster analysis of the 99 isolates obtained from starter cultured Nham is reported in Figure 24. There were four clusters and two single isolates, LP 36/42 and LP 36/94. Cluster I was the largest genetic group with 82 isolates, followed by cluster III (10 isolates), cluster IV (4 isolates) and cluster II (2 isolates). Further identification by 16S rDNA sequencing, there were 5 different species of LAB being detected, *Lb. plantarum* remained a predominant species with an even larger proportion, comprising 83% of total isolates, followed by *Lc. lactis* (cluster III, 10%), *Ln. citreum* (IV, 4%), *Weissella* sp. (cluster II, 2%) and *Ln. pseudomesenteroides* (LP36/94, 1%). It should be noted that the isolates identified as *Lb. plantarum* were the predominant species present in both types of Nham fermentation, while *P. pentosaceus* was founded only in natural fermentation.

At 48 h of fermentation: The cluster analysis of 100 isolates obtained from natural fermentation is shown in Figure 25. A total of five clusters were determined. Five isolates did not share any similarity with other analyzed, forming single-isolate clusters, namely CT 48/24, CT 48/81, CT 48/13, CT 48/38 and CT 48/73. Clusters I represented the most numerous genetic group including 52 isolates, followed by clusters II (24 isolates) and cluster IV (15 isolates), clusters III and V with 2 isolates each.

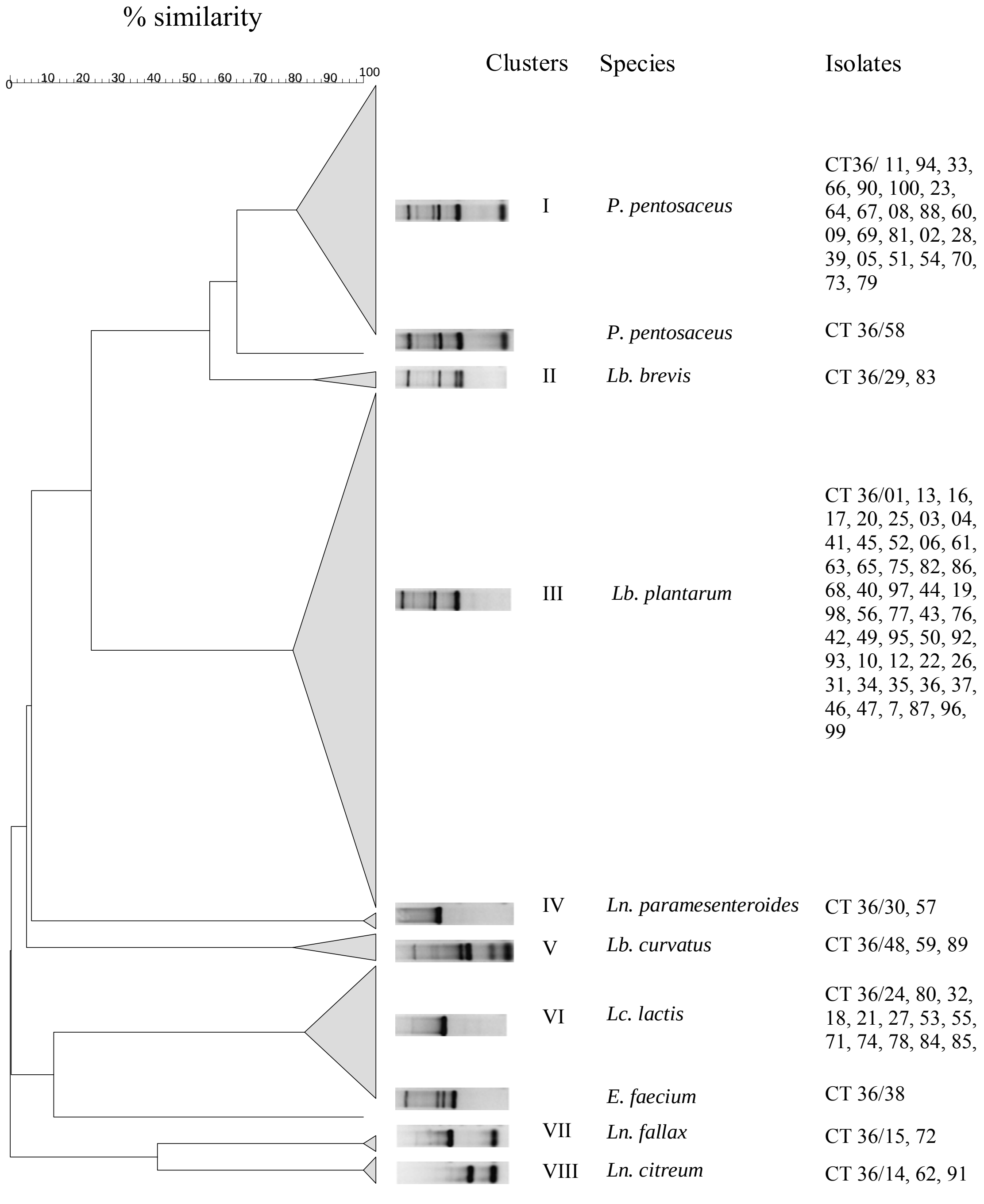


Figure 23 Abridged dendrogram and representing restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from natural Nham at 36 h of fermentation. Identified clusters are indicated with roman letters.

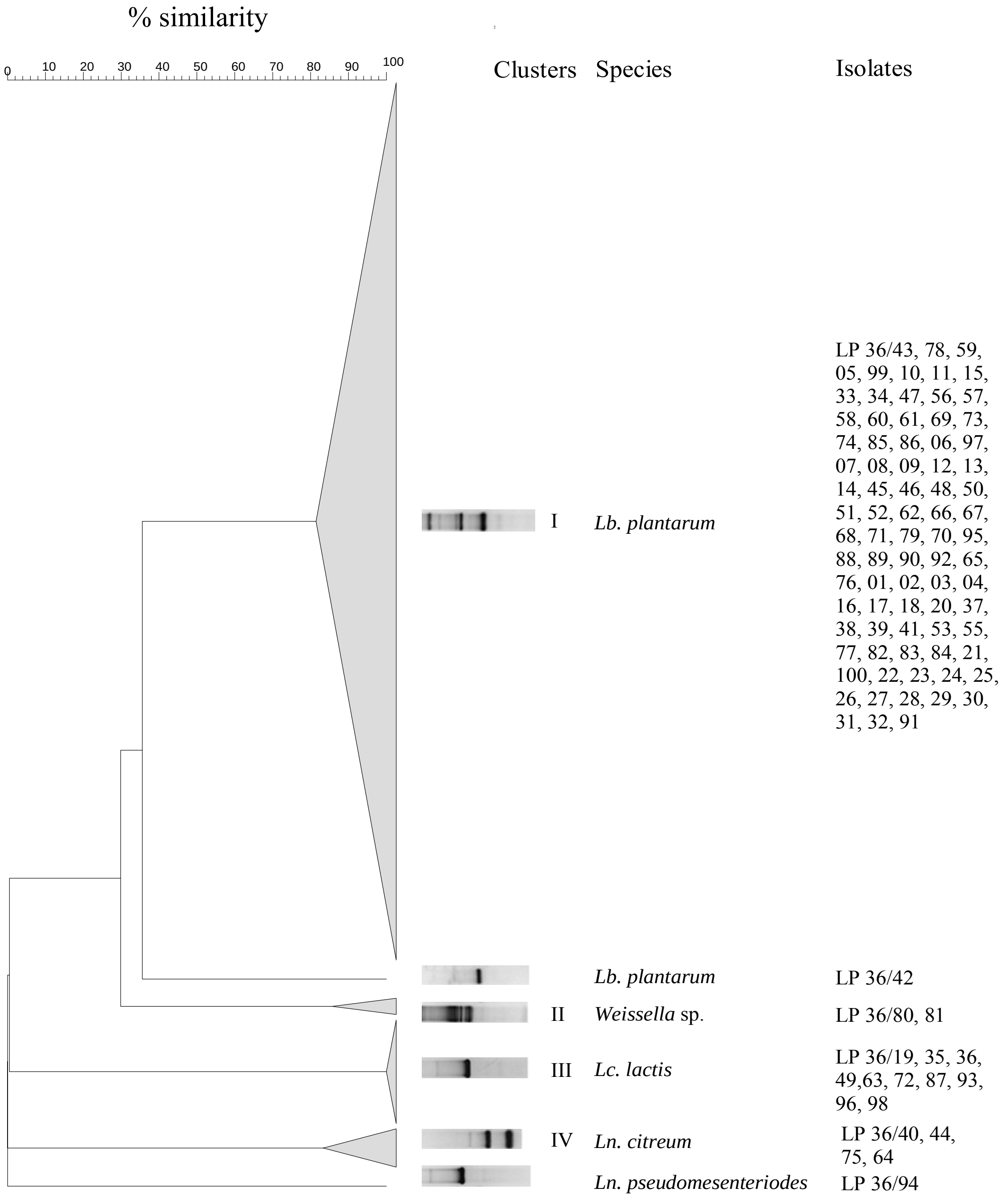


Figure 24 Abridged dendrogram and representing restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from starter cultured Nham at 36 h of fermentation. Identified clusters are indicated with roman letters.

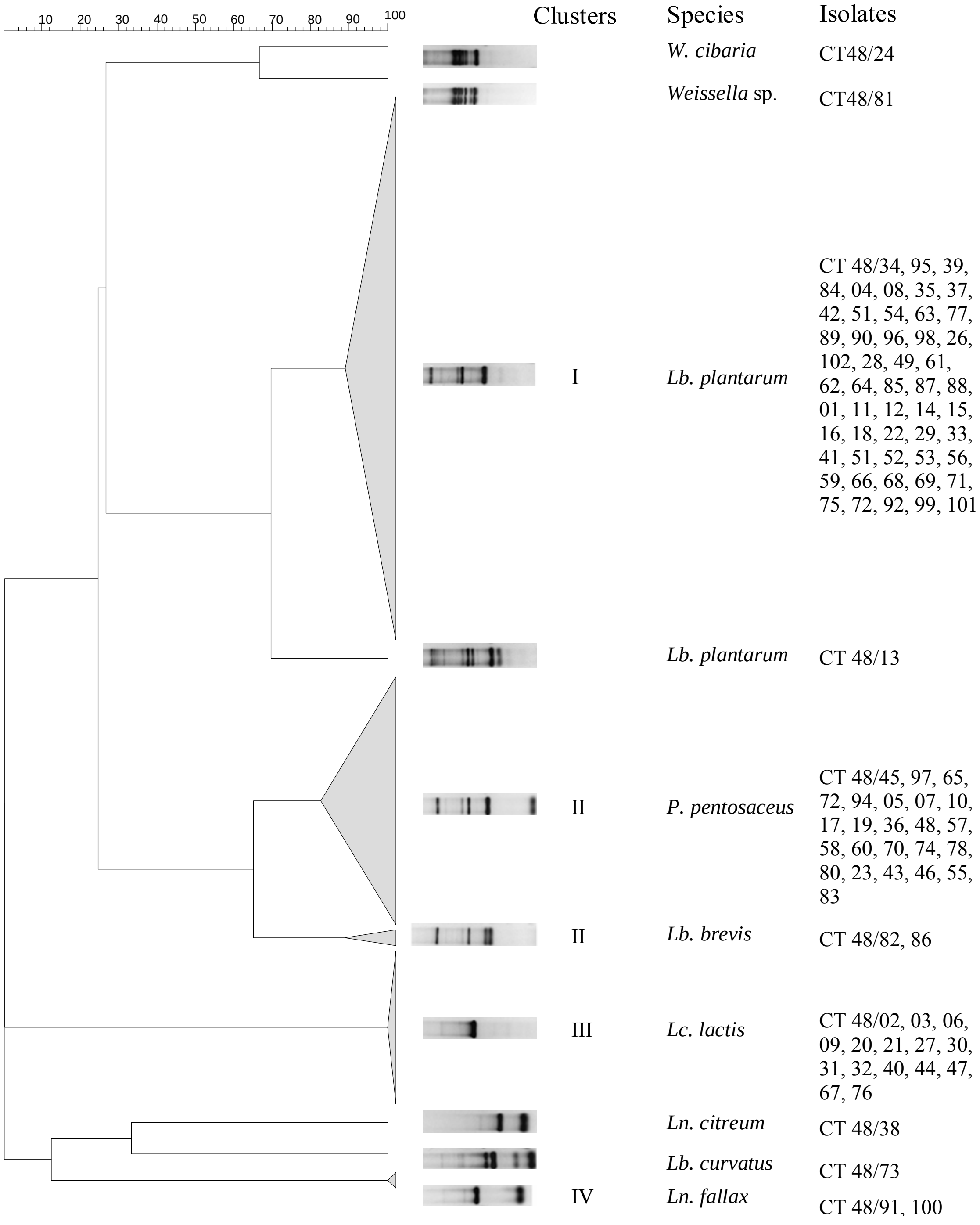


Figure 25 Abridged dendrogram and representing restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from natural Nham at 48 h of fermentation. Identified clusters are indicated with roman letters.

Representative of the ITS-PCR clusters were subjected to 16S rDNA analysis. *Lb. plantarum* which was the predominant species coming from cluster I and CT48/13 (53 from 100 isolates), followed by, *P. pentosaceus* (24%) coming from cluster II and *Lc. lactis* (15%) coming from cluster III. Other species of LAB were comprised of 2 isolates, namely *Lb. brevis* (cluster II) and *Ln. fallax* (cluster IV). *Ln. citreum* (CT 48/38), *Lb. curvatus* (CT48/73), *W. cibaria* (CT48/24) and *Weissella* sp. (CT48/81) were also identified comprised of 1% of total isolates.

In Figure 26, the dendrograms resulting from the analysis of 99 isolates from starter cultured Nham are shown. Two clusters and four single-isolate clusters were identified. Clusters I was the largest genetic grouped with 78 isolates followed by cluster II which comprised of 17 isolates. By 16S rDNA sequencing, the two major clusters were assigned to *Lb. plantarum* (80%, cluster I) and *Lc. lactis* (17%, cluster II). The proportion of other LAB species, *Ln. pseudomesenteroides* (LP 48/61) and *Ln. fallax* (LP48/100) were founded at 1%.

At 60 h of fermentation: Clusters shown in Figure 27 were obtained for LAB species isolated from natural Nham. A total of 100 isolates was grouped into analysis and 8 clusters and 4 single-isolate clusters. Clusters I was the largest genetic group with 46 isolates, followed by cluster V (28 isolates) and cluster VII (7 isolates). Five small clusters (II, III, IV, VI and VIII) were comprised of 4, 3 or 2 isolates, respectively. By sequence analysis of 16S rDNA, *Lb. plantarum* was present in high number in cluster I, II and III (55 isolates representing the 55%), followed by *P. pentosaceus* (28%) coming from cluster V and *Lc. lactis* (7%) coming from cluster VII. The proportion of other bacterial species, *W. cibaria* and *P. pentosaceus* were clearly lower at 4.0% (cluster IV) and 2.0% (cluster VIII), respectively. Other species of LAB were identified as *Ln. pseudomesenteroides* (CT 60/95), *Lb. brevis* (CT 60/95), *Ln. fallax* (CT 60/63) and *Lb. farciminis* (CT 60/35) at 1% of total isolates.

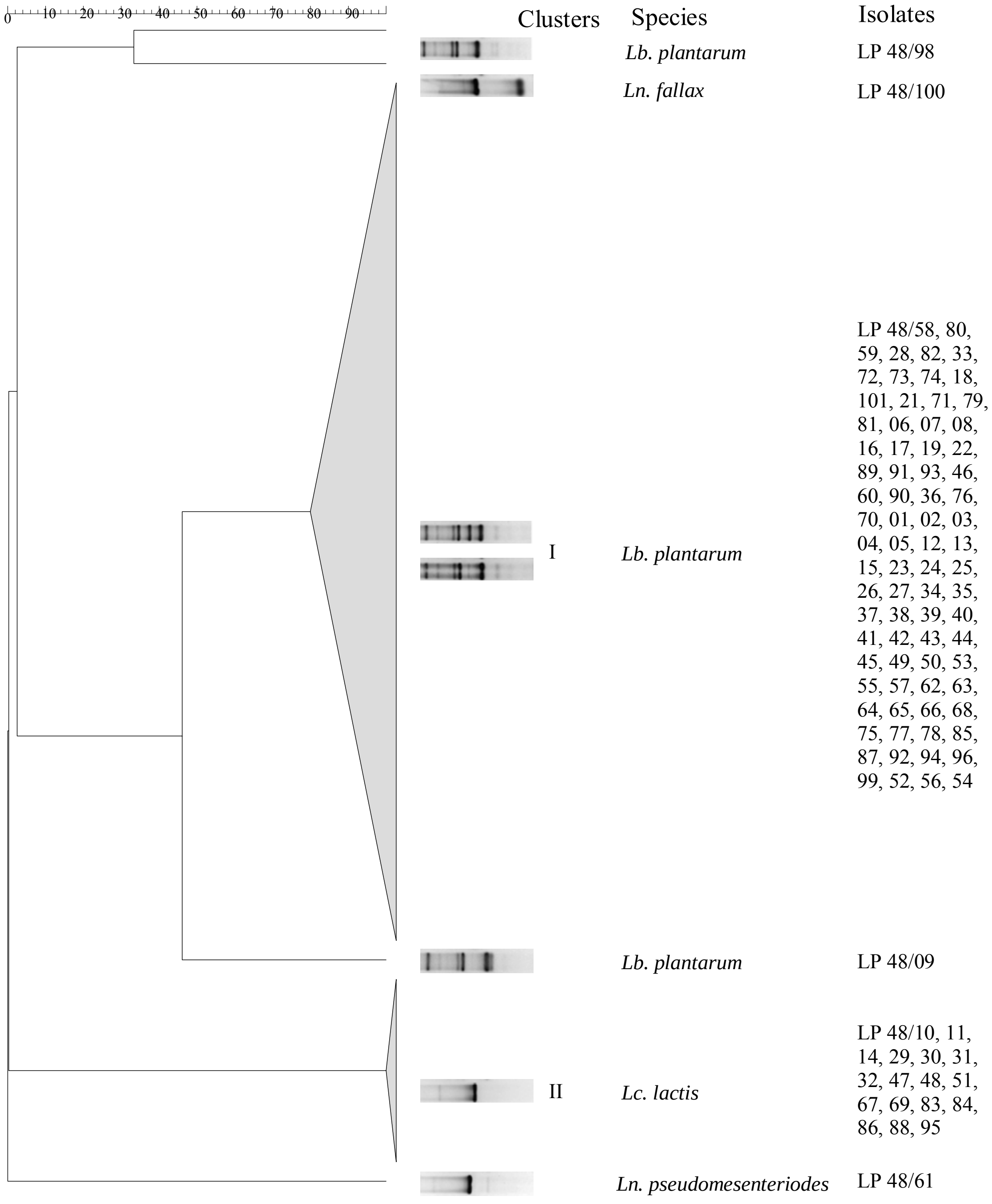


Figure 26 Abridged dendrogram and representing restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from starter cultured Nham at 48 h of fermentation. Identified clusters are indicated with roman letters.

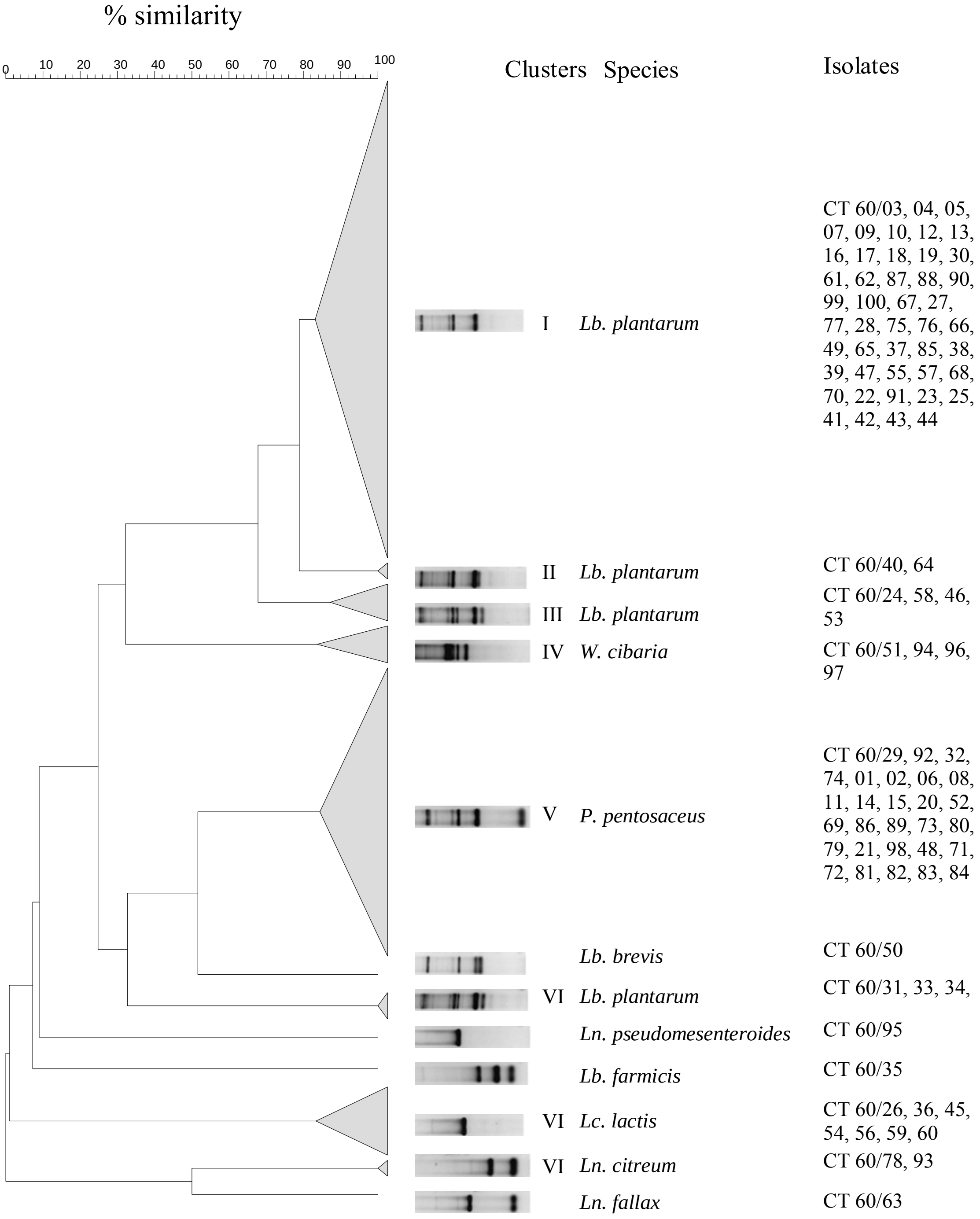


Figure 27 Abridged dendrogram and representing restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from natural Nham at 60 h of fermentation. Identified clusters are indicated with roman letters.

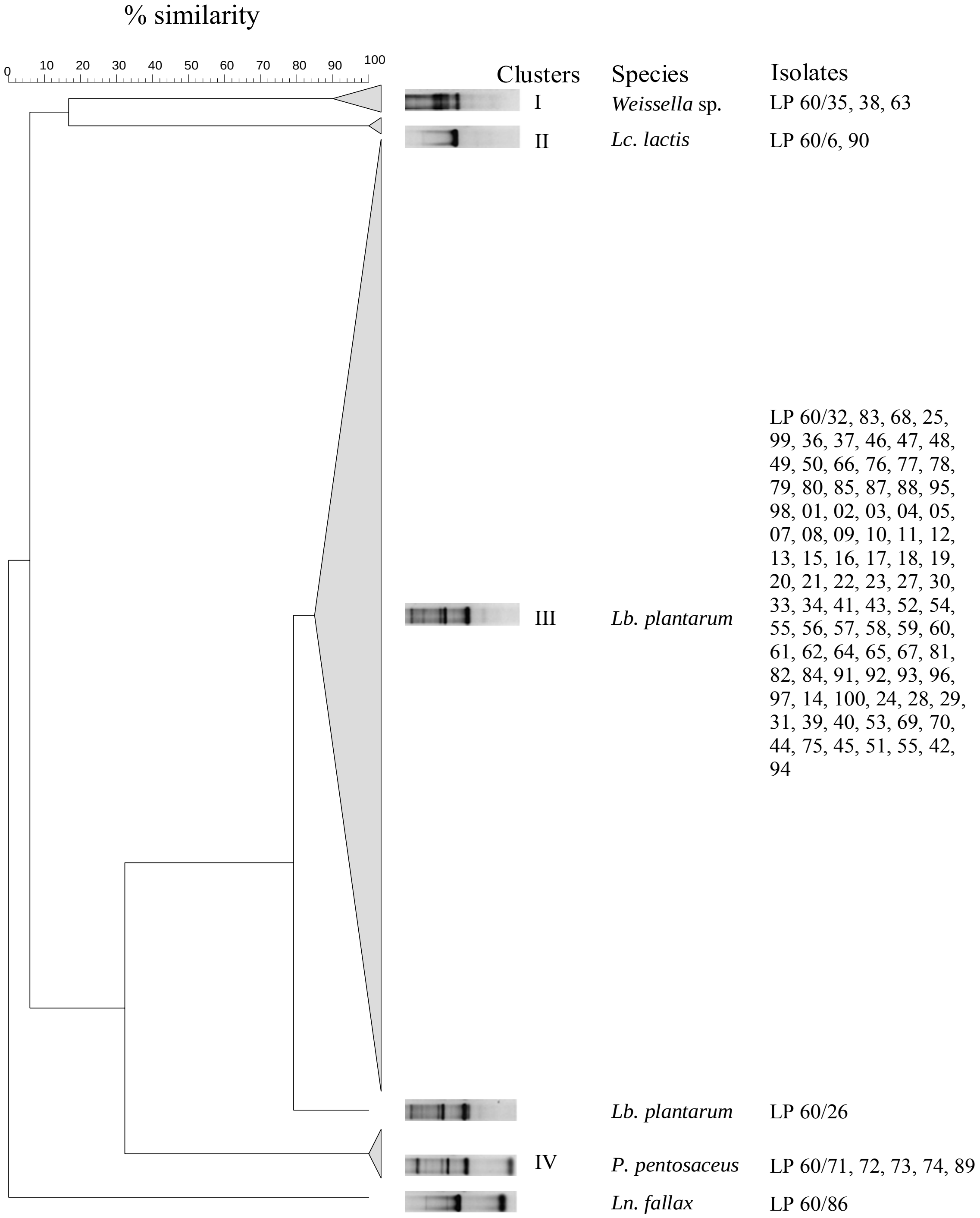


Figure 28 Abridged dendrogram and representing restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from starter cultured Nham at 60 h of fermentation. Identified clusters are indicated with roman letters.

Cluster analysis of the 100 isolates obtained from starter cultured Nham is reported in Figure 28. These were four clusters and 2 single isolate clusters, LP 60/26 and LP 60/86. Cluster III was the biggest cluster with 88 isolates. All other clusters (IV, I and II) were small, including 5, 3 and 2 isolates, respectively. According to 16S rDNA sequencing, *Lb. plantarum* was presented in the highest numbers (89 isolates representing the 89%) coming from cluster III and LP 60/26. A low number of other LAB species namely *Weissella.* sp.*, Lc. lactis* and *Ln. fallax* were also founded at 5% (cluster IV), 3% (cluster VII), 2% (cluster II) and 1% (LP 60/86) of total isolates, respectively.

At 72 h of fermentation: Figure 29 shows the cluster analysis of 100 isolates obtained from natural fermentation at the end of fermentation. Five clusters and one single isolate cluster were determined. Cluster III was the largest cluster with 68 isolates followed by cluster I which comprised of 21 isolates. In additional, three small clusters (II, IV and V) which have 4 or 2 isolates have been identified. Based on ITS-PCR database and 16S rDNA sequencing, *Lb. plantarum* and *P. pentosaceus* remain predominated in the fermentation but at an even larger proportion, comprising 68% (cluster III) and 21% (cluster I), respectively. Both cluster II and V had 4 isolates which were identified as *Lb. brevis* (4%) and *Lc. lactis* (4%), respectively. *Ln. fallax* (cluster IV) and *Ln. citreum* (CT72/81) were also detected, which accounted for 2% and 1% of total isolates, respectively.

A total of 80 isolates from starter cultured Nham were subjected to analysis and only one cluster was obtained as shown in Figure 30. Cluster I was form by homogeneous composition with 80 isolates. For further identification by using ITS-PCR database and 16S rDNA sequencing, *Lb. plantarum* was the only predominant specie founded at the end of fermentation.

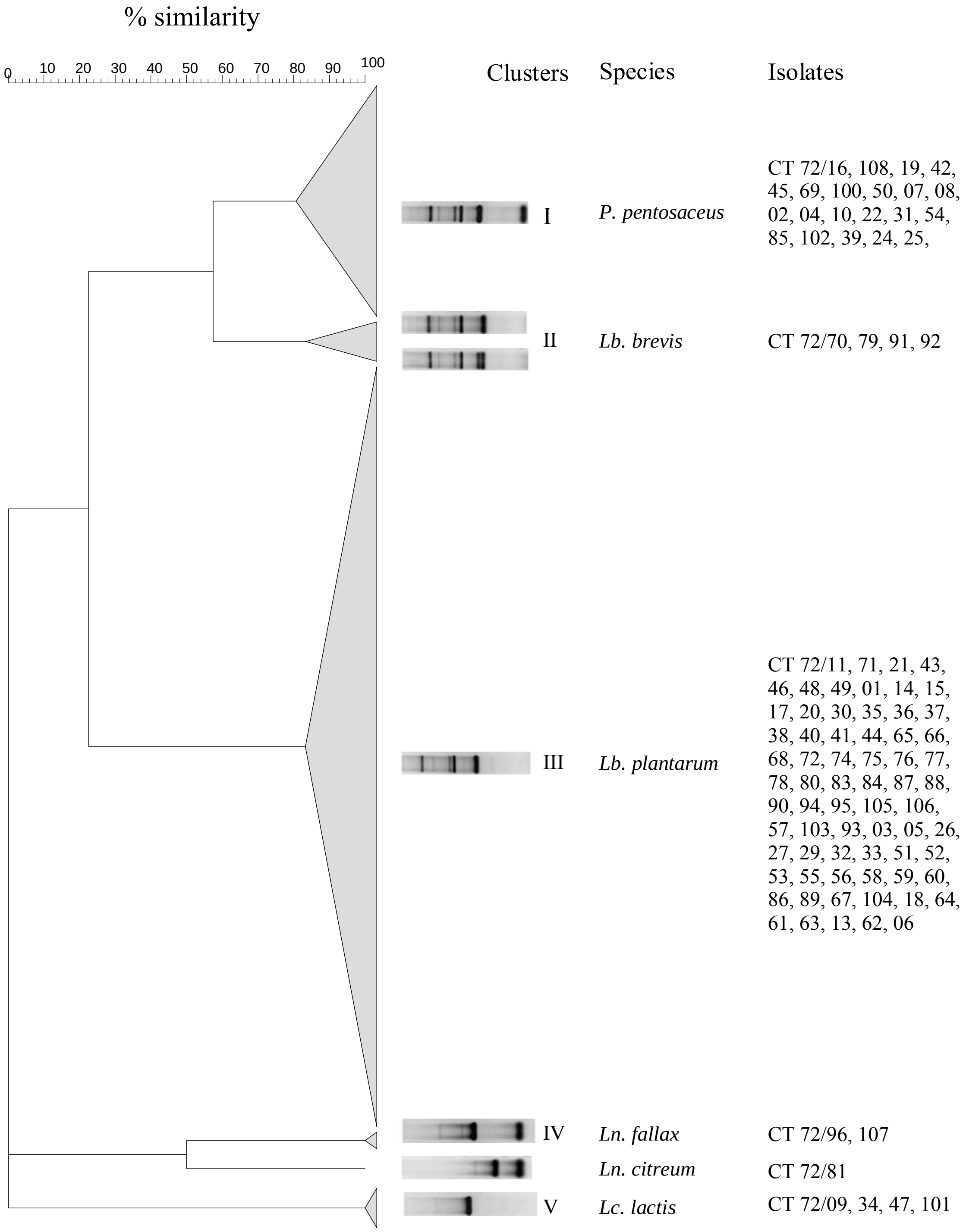


Figure 29 Abridged dendrogram and representing restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from natural Nham at 72 h of fermentation. Identified clusters are indicated with roman letters.

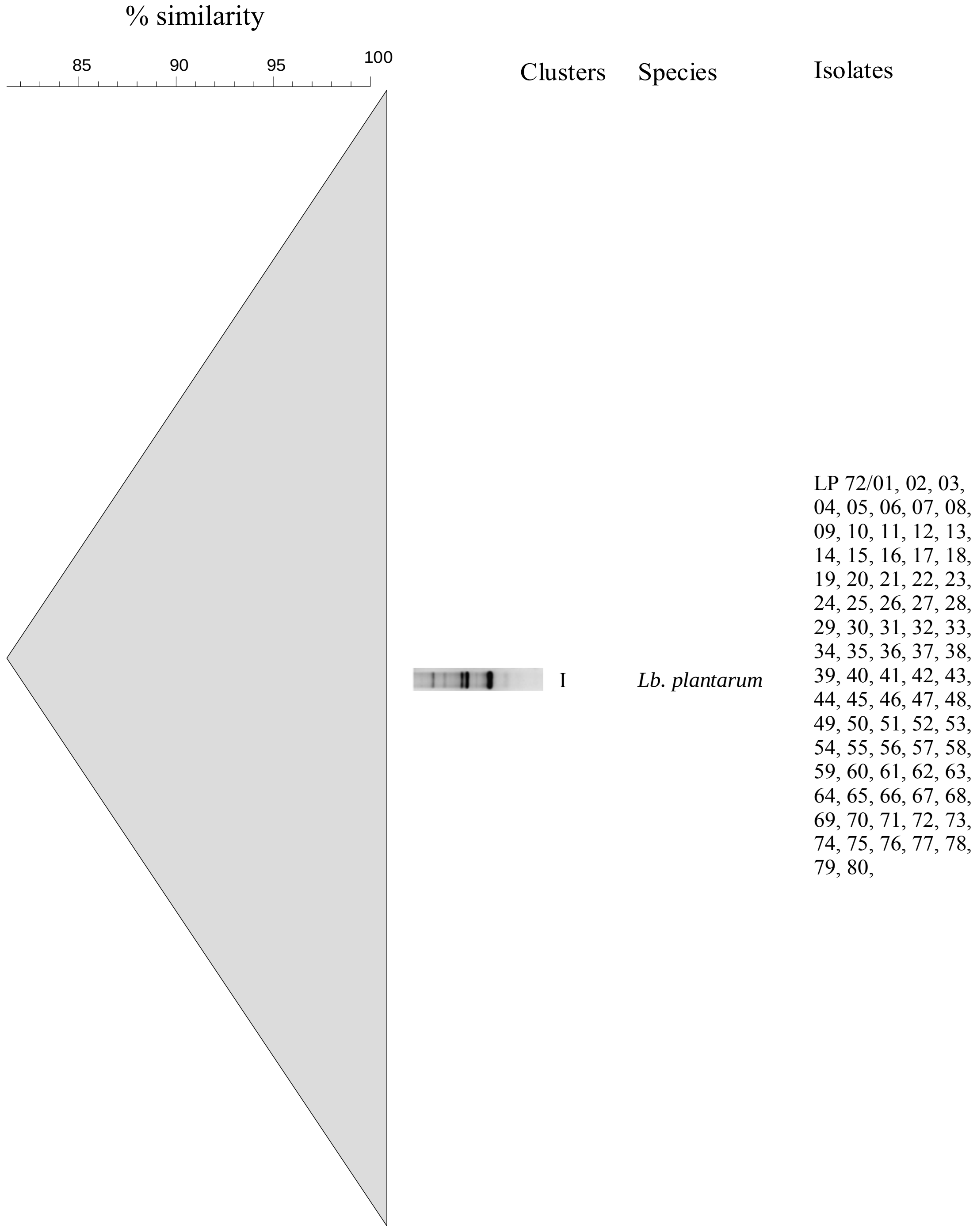


Figure 30 Abridged dendrogram and representing restriction pattern of ITS with *Rsa*I of bacterial isolates from starter cultured Nham at 72 h of fermentation. Identified clusters are indicated with roman letters.

Table 3 Numbers and proportions of bacterial isolates distributed between different species identify by ITS-PCR database

Time	Sample	Species	Total isolates	No. of isolates	%
0 hr	Control	*Lactococus garvieae*	91	53	58
		Macrococcus caseolyticus		18	20
		Lactococcus lactis		12	13
		Klebsiella oxytoca		3	3
		Unidentified		2	2
		Bacillus cereus		2	2
		Klebsiella pneumoniae		1	1
	Starter	*Lactococus garvieae*	93	49	53
		Macrococcus caseolyticus		10	11
		Lactococcus lactis		10	11
		Bacterium KA55		9	10
		Unidentified		6	6
		Klebsiella oxytoca		2	2
		Staphylococus gallinarum		2	2
		Vagococcus carniphilus		1	1
		Enterococus sp.		1	1
		Weissella paramesenteroides		1	1
		Pediococcus pentosaceus		1	1
		Klebsiella sp.		1	1
6 hr	Control	*Lactococus garvieae*	86	66	77
		Lactococcus lactis		13	15
		Weissella sp.		3	3
		Leuconostoc pseudomesenteroides		3	3
		Leuconostoc citreum		1	1
	Starter	*Lactococus garvieae*	95	78	82
		Lactococcus lactis		11	12
		Leuconostoc pseudomesenteroides		4	4
		Staphylococcus hominis		1	1
		Leuconostoc citreum		1	1
12 hr	Control	*Lactococus lactis*	98	65	66
		Lactococcus garvieae		29	30
		Weissella sp.		2	2
		Klebsiella oxytoca		1	1
		Klebsiella pneumoniae		1	1
	Starter	*Lactococcus lactis*	89	33	37
		Lactobacillus plantarum		30	34
		Lactococus garvieae		17	19
		Weissella sp.		6	7
		Leuconostoc citreum		2	2
		Pediococcus pentosaceus		1	1
24 hr	Control	*Lactococcus lactis*	100	52	52
		Pediococcus pentosaceus		17	17
		Lactobacillus plantarum		10	10
		Lactococcus garvieae		9	9
		Leuconostoc citreum		5	5
		Leuconostoc pseudomesenteroides		2	2
		Leuconostoc fallax		2	2
		Weissella cibaria		2	2
		Lactobacillus curvatus		1	1

Table 3 (Continued)

Time	Sample	Species	Total isolates	No. of isolates	%
24 hr	Starter	*Lactobacillus plantarum*	99	52	53
		Lactococcus lactis		28	28
		Lactococcus garvieae		14	14
		Weissella cibaria		3	3
		Weissella sp.		2	2
36 hr	Control	*Lactobacillus plantarum*	100	49	49
		Pediococcus pentosaceus		25	25
		Lactococcus lactis		13	13
		Leuconostoc citreum		3	3
		Lactobacillus curvatus		3	3
		Leuconostoc pseudomesenteroides		2	2
		Lactobacillus brevis		2	2
		Leuconostoc fallax		2	2
		Enterococcus faecium		1	1
	Starter	*Lactobacillus plantarum*	99	82	83
		Lactococcus lactis		10	10
		Leuconostoc citreum		4	4
		Weissella sp.		2	2
		Leuconostoc pseudomesenteroides		1	1
48 hr	Control	*Lactobacillus plantarum*	100	53	53
		Pediococcus pentosaceus		24	24
		Lactococcus lactis		15	15
		Lactobacillus brevis		2	2
		Leuconostoc fallax		2	2
		Weissella cibaria		1	1
		Leuconostoc citreum		1	1
		Lactobacillus curvatus		1	1
		Weissella sp.		1	1
	Starter	*Lactobacillus plantarum*	99	80	81
		Lactococcus lactis		17	17
		Leuconostoc fallax		1	1
		Leuconostoc pseudomesenteroides		1	1
60 hr	Control	*Lactobacillus plantarum*	100	55	55
		Pediococcus pentosaceus		28	28
		Lactococcus lactis		7	7
		Weissella cibaria		4	4
		Leuconostoc citreum		2	2
		Lactobacillus brevis		1	1
		Leuconostoc fallax		1	1
		Leuconostoc pseudomesenteroides		1	1
		Lactobacillus farciminis		1	1
	Starter	*Lactobacillus plantarum*	100	89	89
		Pediococcus pentosaceus		5	5
		Weissella sp.		3	3
		Lactococcus lactis		2	2
		Leuconostoc fallax		1	1
72 hr	Control	*Lactobacillus plantarum*	100	68	68
		Pediococcus pentosaceus		21	21
		Lactococcus lactis		4	4
		Lactobacillus brevis		4	4
		Leuconostoc fallax		2	2
		Leuconostoc citreum		1	1
	Starter	*Lactobacillus plantarum*	80	80	100

6. Succession of LAB species during Nham fermentation

As reported in Figure 31 and Figure 32, dynamics changes in the ecology of the major bacterial species during fermentation were observed. From our results, it demonstrated that both types of Nham showed a dramatic succession of LAB species during fermentation. The predominant species of LAB from natural Nham displayed the higher diversity than in starter cultured Nham. We identified 4 main different LAB species (*Lc. garvieae*, *Lc. lactis*, *Lb. plantarum* and *P. pentosaceus*) while only 3 main LAB species (*Lc. garvieae*, *Lc. lactis* and *Lb. plantarum*) were identified in starter cultured Nham. However, the distribution of major LAB species fairly similar in both type of Nham fermentation during the early phase (0-6 h) of fermentation but clearly different when the fermentation proceeded to 12 h and continue to differ until the end of fermentation as described below.

At the start (0 h) of fermentation, *Lc. garvieae* was the predominant species accounted for more than 50% of total isolates in both types of Nham. Other predominant species were *M. caseolyticus* and *Lc. lactis*. After 6 h of fermentation, *Lc. garvieae* remained dominant species with increasing in proportion to 77% and 82% in natural and starter cultured Nham, respectively. *Lc. lactis* remained constant while *M. caseolyticus* disappeared in both types of Nham fermentation.

After 12 h of fermentation, the significant difference in species composition was observed between natural and starter cultured Nham. In starter cultured Nham, the microbial diversity dramatically changed from 82% of *Lc. garvieae* and 12 % of *Lc. lactis* to 37% of *Lc. lactis*, 34% of *Lb. plantarum* and 19% of *Lc. garvieae*. In contrast to the natural fermentation, the microbial diversity dramatically changed from 77% of *Lc. garvieae* and 15 % of *Lc. lactis* to 65% of *Lc. lactis* and 30% of *Lc. garvieae*.

After 24 h of fermentation, the LAB species from natural Nham displayed the higher diversity, with 9 species identified while the species composition of starter cultured Nham were comprised of 5 species. *Lb. plantarum* became the predominant species (53%) followed by *Lc. lactis* (28%) and *Lc. garvieae* (14%) in starter cultured Nham. On the other hand, natural Nham *Lc. lactis* remained a dominant species but gradually declined to 52% of the isolates followed by *P. pentosaceus* (17%), *Lb. plantarum* (10%) and *Lc. garvieae* (9%). It is a noteworthy that *Lb. plantarum* became the predominant species in starter cultured Nham, while these species were only found at a small percentage in natural Nham at this sampling time.

After 36 h of fermentation, the natural fermentation remained higher heterogeneous microbial diversity than those in starter cultured Nham. The LAB species dramatically changed from 52% of *Lc. lactis*, 17 % of *P. pentosaceus* and 9 % of *Lc. garvieae* to 49 % of *Lb. plantarum*, 25 % of *P. pentosaceus* and 13% of *Lc. lactis* in natural Nham. In comparison to starter cultured Nham, *Lb. plantarum* remained the dominant species with an even larger proportion comprised of 83 % of the isolates while *Lc. lactis* was strongly reduced to only 10 % of total isolates. Moreover, it is interesting to notice that *Lc. garvieae* disappeared in both types of Nham fermentation at this period.

After 48 h of fermentation, *Lb. plantarum* remained a dominant species accounted for 53 % of isolates followed by *P. pentosaceus* (24% of total isolates) and *Lc. lactis* (15% of total isolates) in natural Nham. On the contrary, *Lb. plantarum* and *Lc. lactis* remained dominant species comprised of 81 % and 18 % of isolates, respectively in starter cultured Nham.

From 60 h through the end of fermentation, in natural Nham, *Lb. plantarum* species were continually increased from 55% to 68% of total isolates while *P. pentosaceus* species were not dramatically reduced from 28% to 21% of total isolates. In contrast to starter cultured Nham, *Lb. plantarum* was the only dominant species continually increased from 89% of total isolates at 60 h to 100% of total isolates at the end of fermentation.

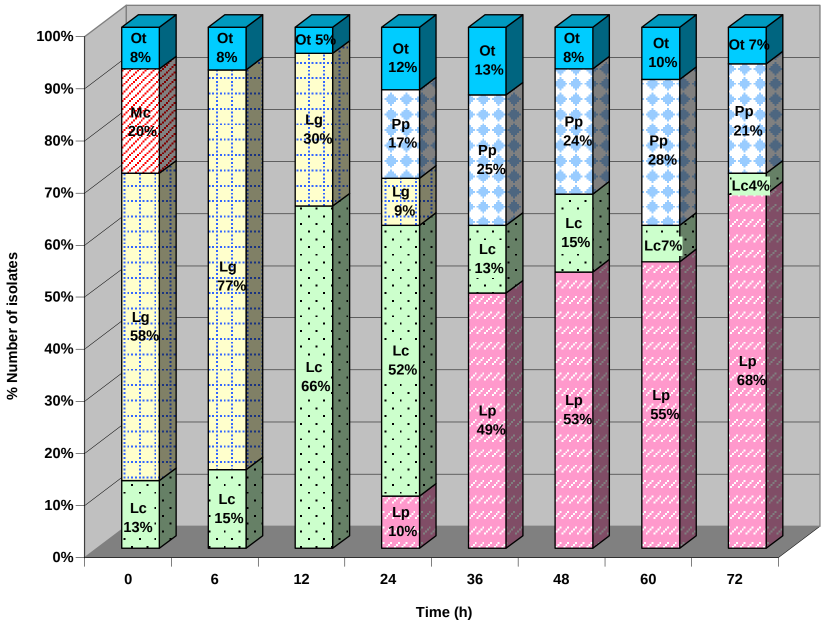


Figure 31 Distribution of the predominant species from natural Nham fermentation ; Lc: *Lc. lactis,* Lg: *Lc. garvieae,* Mc: *M. caseolyticus,* Lp*: Lb. plantarum*, Pp*: P. pentosaceus* and Ot : Other

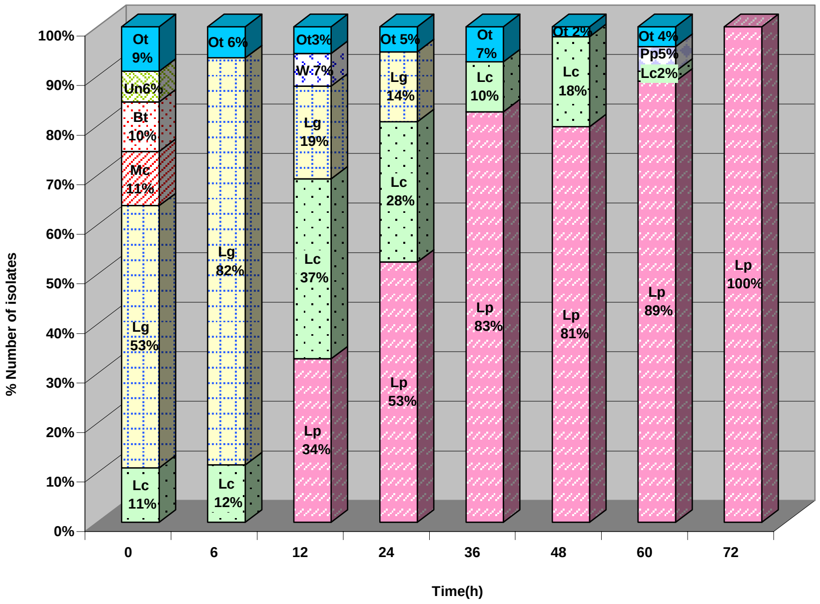


Figure 32 Distribution of the predominant species from starter cultured Nham fermentation; Bt : *Bacterium KA55,* Lc*: Lc. lactis,* Lg*: Lc. garvieae*, Mc: *M. caseolyticus*, Lp: *Lb. plantarum*, Pp: *P. pentosaceus*, Un: Unidentified,Ws**:** *Weissella* sp. and Ot : Other