APPENDICES

Appendix A

Media and chemical reagents

1. MRS Broth/Agar (1 liter)

Proteose Peptone No. 3 (Difco)	10.0	g
Glucose (Merck)	10.0	g
Meat Extract (Merck)	10.0	g
Yeast extract (Merck)	5.0	g
Tween 80 (Merck)	1.0	g
K_2HPO_4(Merck)	2.0	g
Sodium Acetate (Merck)	5.0	g
Diammonium Citrate (Merck)	2.0	g
$MgSO_4.7H_2O$(Carlo)	0.2	g
$MnSO_4.H_2O$(Carlo)	0.05	g
(Agar)	15.0	g

2. Nutrient broth (NB)/Nutrient agar (NA)

Bacto Peptone (Difco)	10.0	g
Meat Extract (Merck)	3.0	g
(Agar)	15.0	g

3. Plate Count Agar (Merck) (1 liter)

Bacto Peptone (Difco)	10.0	g
Meat Extract (Merck)	5.0	g
Sodium chloride	5.0	g
Agar	15.0	g

4. Selective Medium

Violet red bile agar (VRBG) supplemented with 1% glucose

5. Yeast Malt Extract Broth/Agar (1 liter)

Bacto Peptone (Difco)	5.0	g
Meat Extract (Merck)	3.0	g
Glucose (Merck)	10.0	g
Agar	15.0	g

6 TE pH 8.0

10 mM Tris·Cl (pH 8.0)
1 mM EDTA (pH 8.0)

Appendix B
Methods of chemical analysis

1. Organic Acids Analysis

Organic acids in Nham were extracted according to the method of Visessanguan *et al*. (2004). Coarsely ground samples (5g) were extracted with 45 ml of nonpure water using an Ultra Turrax homogenizer at 8000 rpm for 30 sec. The homogenate was centrifuged at 3000 *x*g for 10 min and to the resulting supernatant (0.9 ml) 0.1 ml of 10 mg/ml formic acid was added as an internal standard. The mixture was deproteinised with 2 ml of 0.5N perchloric acid. The mixture was left for 5 min at room temperature (~25°C) and centrifuged at 12000 x g for 10 min to remove the precipitated proteins. The supernatant was collected and filtered through a 0.45 μm membrane filter prior to analysis by high performance liquid chomatography (HPLC). The HPLC system included an Aminex HPX-87H ion exclusion column (300mm × 7.8mm i.d.). The column was heated to 65°C. The Waters Separation Module 2690 was operated to give a flow rate of 0.6 ml/min of the solvent, 0.02N H_2SO_4. A sample, 20μl, was injected and a photo diode array (Model Waters 996), set at the wavelength of 210 nm, was used as the detector. Data was processed and analyzed using Millennium 32 software. The organic acid concentration was expressed as a percentage of each organic acid in (w/w).

2. Total sugar analysis

2.1 Total sugar hydrolyzation

5 g of Nham was hydrolyzed in 1 M hydrochloric acid at 80^{o}C for 6 hours. At the end of the reaction, the hydrolyzate was immediately cooled down in ice bath, and neutralized to pH 7. The neutral hydrolyzate was centrifuged twice; each supernatant was collected in the same 50 ml volumetric flask, and the total amount of supernatant was adjusted to the volume of 50 ml. Protein and pigment was eliminated from the solution by precipitating with saturated lead acetate. The clear solution was added with 20 μl of 50 mg/ml methyl-α-glycopyranoside (an internal standard) and freeze-dried. Oxime TMS derivative of sugars was prepared and analyzed using GC method.

2.2 Preparation of oxime-trimethylsilyl (TMS) derivatives of sugar

Sugar oxime TMS derivatives were prepared by adding 1 ml of solution of hydroxylamine hydrochloride in anhydrous pyridine (2.5 g/100 ml) into an anhydrous sugar extract. The mixture was heated at 75^{o}C for 30 min. Then, the mixture was transferred to a 1.5 ml micro-tube and centrifuged at 12,000 rpm for 10 min. 0.7 ml of supernatant was transferred to 8 ml vial and then heated at 55^{o}C under a nitrogen stream to evaporate pyridine. Dried sample was reconstituted in 0.5 ml of anhydrous pyridine. The solution was added with 0.2 ml of chlorotrimethylsilane (TMSCl) and immediately added with 0.3 ml of hexamethyldisilazane (HMDS). After standing for 1 hour and centrifuging at 12,000 rpm for 10 min, 0.7 ml of supernatant was heated at 55^{o}C under a nitrogen stream to dryness. Then, 0.7 ml of isooctane was added to reconstitute dried sugar derivatives.

2.3 GC-FID analysis of sugar derivatives

This study used GC-17A (Shimadzu, Japan) equipped with a DB-1 fused-silica capillary column (30 m x 0.25 mm i.d., 0.25 mm film thickness, J&W Scientific, Folsom, CA, USA) and a flame ionization detector (FID). The column temperature was initially set at 170^{o}C for 35 min, then increased to 260^{o}C at the rate of 10^{o}C/min and maintained for 14 min, and risen to 280^{o}C at the same heating rate and continued on this temperature for 10 min. Injector and detector temperature were set at 310 and 320°C, respectively. Helium was used as carrier gas at flow rate of 1.5 ml/min. Injection volume was limited to 1 μl at split ratio 10:1. Each chomatographic peak was identified as a free sugar by relating its retention time to that of standard sugars. Linear equations formulated from calibration curves plotted between peak-area ratios (sugar/internal standard) and sugar amounts were used for quantification of free sugars.

Appendix C
Methods of nucleic acid extraction

1. Cell Lysis

1.5 ml of overnight culture was transferred to 2.0 ml micro-tube and centrifuged at 11, 000 rpm for 1 min. to remove the supernatant. Pellet cells were suspended in 480 µl of 50 mM EDTA. The solution was added with 60 µl of 10 mg/ml lysozyne enzymes and 5 µl of 2,500 U/ml mutanolysin. The mixture was incubated at 37^{o}C overnight in circulating bath or thermomixer at 400 rpm. Then, the mixture was centrifuged at 13,000 rpm, 4^{o}C for 2 min to remove the supernatant. Pellet cells were added with 600 µl of Nuclei Lysis Solution and gently pipetted up and down. After, the mixture was incubated at 80^{o}C for 10 min. in block heater and then cooled to room temp. The solution was added with 10 µl of RNase solution (10mg/ml) and then pipette gently to mix. Incubate at 37^{o}C for 1 h. and then cool to room temperature.

2. Protein precipitation

The solution was added with 200 µl of Protein Precipitation Solution and mixed by vortex for 20 sec. After that the solution was sit on ice for 5 min. The solution was centrifuged at 13,000 rpm, 4 ^{o}C for 5 min. The supernatant was transferred to a clean micro-tube and centrifuged at 13,000 rpm, 4 ^{o}C for 5 min.

3. DNA precipitation

The supernatant was transfer to a clean micro-tube contained with 600 µl of isopropanol. The solution was gently mixed by inversion until the threadlike strands of DNA form a visible mass. The solution was centrifuged. at 13,000 rpm, 4 ^{o}C for 2 min. The supernatant was removed and pellet DNA was added with 600 µl of room temperature 70% EtOH. The solution was gently mixed by inversion in several times.

4. Pure DNA

Aspire the EtOH and air–dry the pellet for 10-15 min. Rehydrate the DNA pellet in 50 µl of TE buffer at 50^{o}C overnight. Stored the DNA solution at -20 ^{o}C**.**

Appendix D

Results of microbiological and chemical analysis

Appendix Table D1. Changes in pH and lactic acid during Nham fermentations

Sampling Time (h)	pH		Lactic acid concentration (g/100 g Nham)	
	Control	Starter	Control	Starter
0	6.04	5.96	0.36	0.45
6	6.01	6.01	n.d.	n.d.
12	5.75	5.70	0.85	0.88
24	4.97	4.98	1.17	1.05
36	4.66	4.57	1.49	1.69
48	4.62	4.39	1.67	2.04
60	4.41	4.28	n.d.	n.d.
72	4.39	4.24	2.08	2.38

Note: n.d.: not determined

Appendix Table D2. Changes in total glucose during Nham fermentations

Sampling Time (h)	Total glucose concentration (mg/100g nham)	
	Control	Starter
0	1397.49	1590.40
12	1264.04	1251.15
24	1144.55	816.73
36	872.39	1.69
48	723.14	699.25
72	498.56	524.83

Appendix Table D3. Changes in total fructose during Nham fermentations

Sampling Time (h)	Total fructose concentration (mg/100g nham)	
	Control	Starter
0	559.50	584.52
12	556.57	534.75
24	560.69	448.23
36	478.70	344.28
48	295.91	249.14
72	133.91	0.00

Appendix Table D4. Changes in free fructose during Nham fermentations

Sampling Time (h)	Free fructose concentration (mg/100g nham)	
	Control	Starter
0	4.78	4.43
12	0.00	0.00
24	20.86	330.33
36	103.08	370.11
48	118.18	293.29
72	68.45	35.95

Appendix Table D5. Changes in free sucrose during Nham fermentations

Sampling Time (h)	Free sucrose concentration (mg/100g nham)	
	Control	Starter
0	514.26	529.58
12	482.75	480.68
24	361.79	215.25
36	146.95	11.84
48	72.02	4.76
72	9.59	0.00

Appendix Table D6. Changes in LAB populations during Nham fermentations

Sampling Time (h)	Lactic Acid Bacteria population (log CFU/g)	
	Control	Starter
0	6.92	6.91
6	7.84	8.18
12	8.40	8.38
24	8.52	8.53
36	8.82	8.62
48	8.88	8.74
60	8.92	8.74
72	8.97	8.62

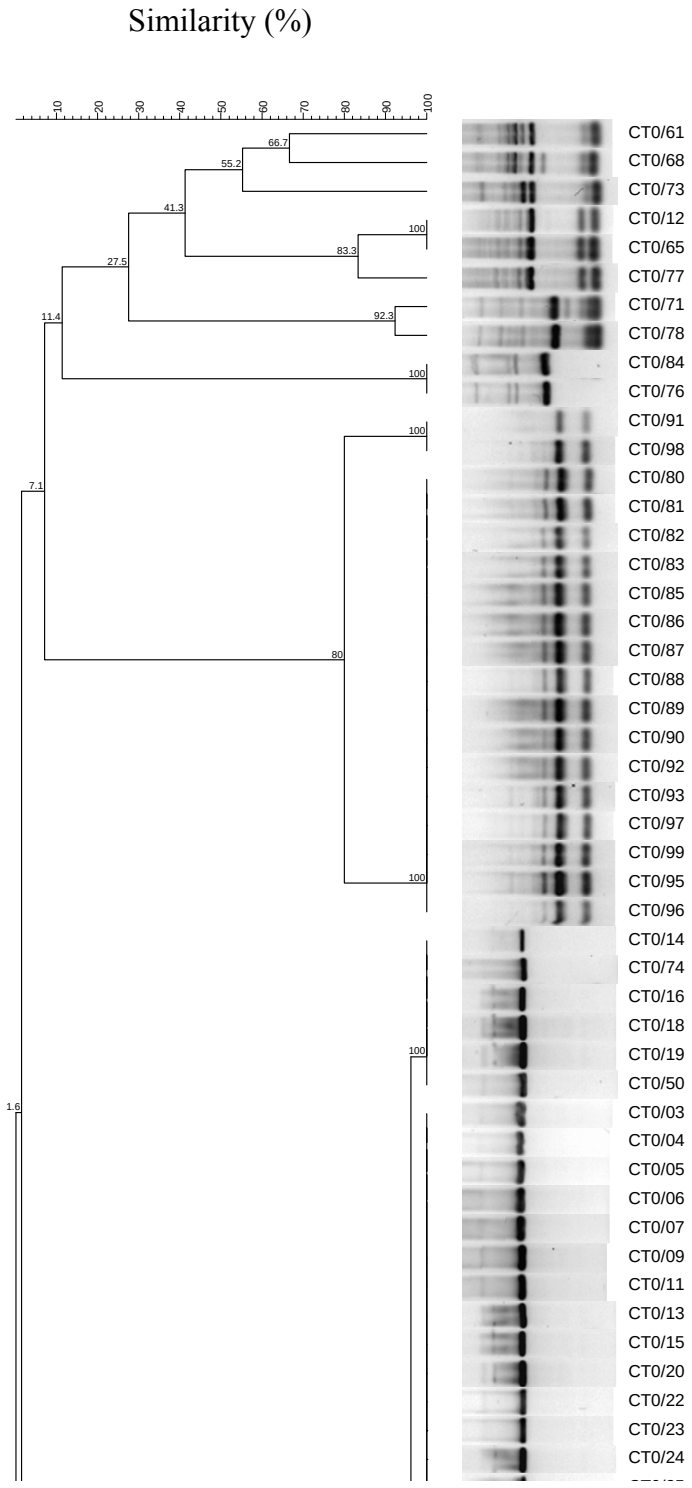
Appendix Table D7. Changes in aerobic bacteria counts during Nham fermentations

Sampling Time (h)	Aerobic Bacteria population (log CFU/g)	
	Control	Starter
0	6.88	7.01
6	8.10	8.02
12	8.29	8.54
24	8.59	8.52
36	8.11	7.96
48	8.13	7.81
60	7.89	6.80
72	7.41	6.58

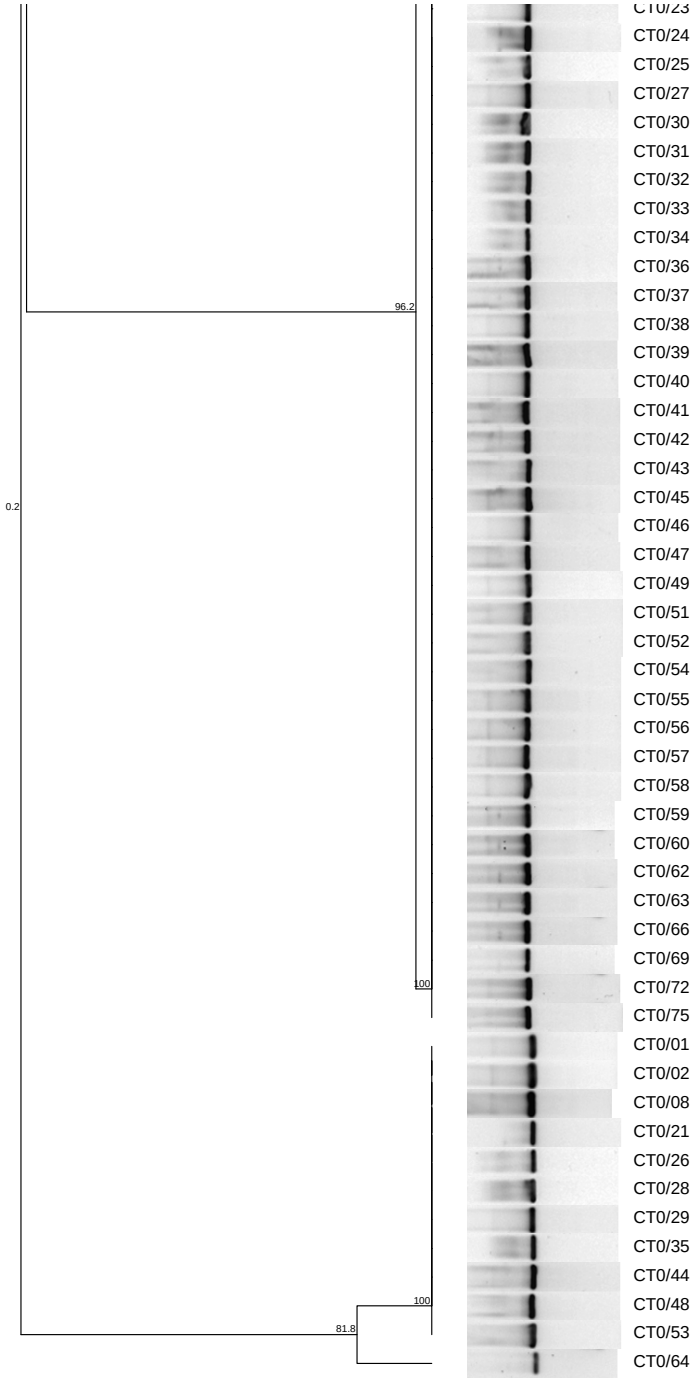
Appendix Table D8. Changes in *Enterobactericeae* counts during Nham fermentations

Sampling Time (h)	*Enterobactericeae* population (log CFU/g)	
	Control	Starter
0	5.87	6.40
6	6.03	6.23
12	6.98	6.14
24	6.40	6.10
36	6.14	5.42
48	5.66	5.11
60	5.21	3.71
72	4.57	3.57

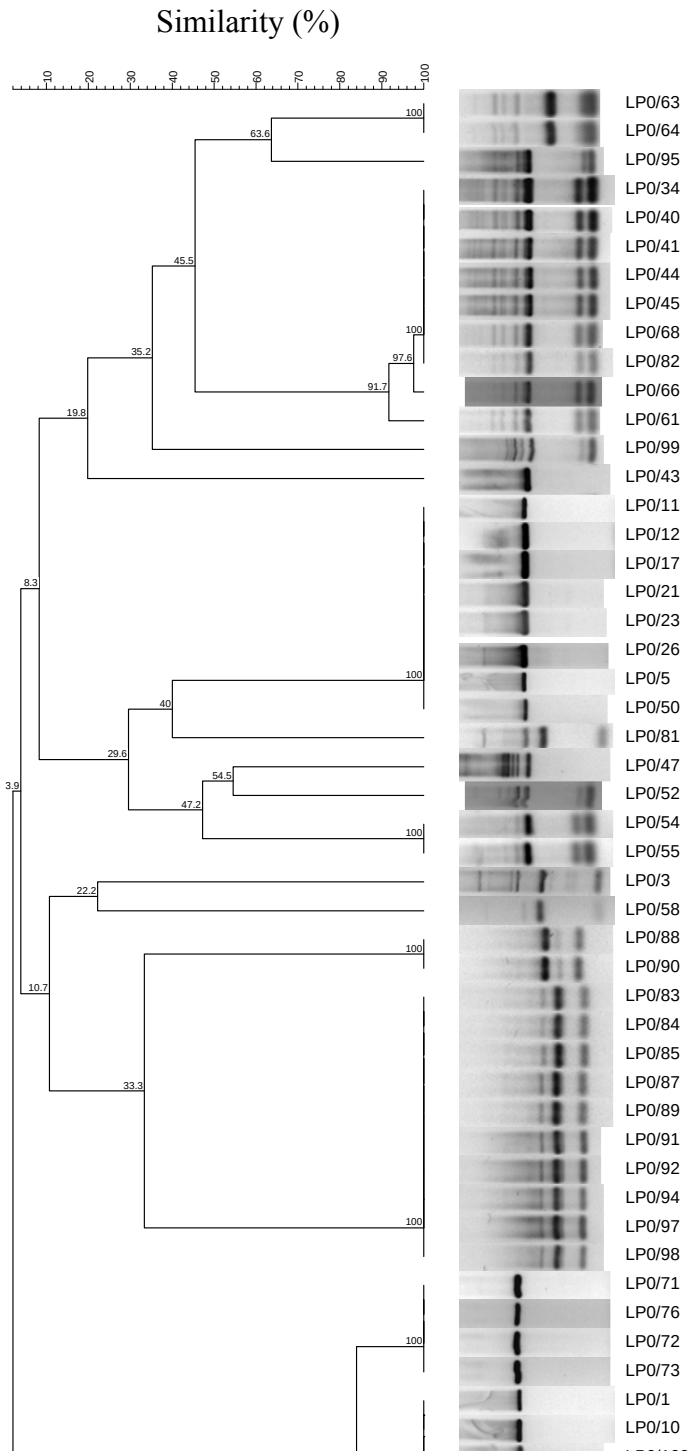
Appendix E
Dendrogram of ITS profiles



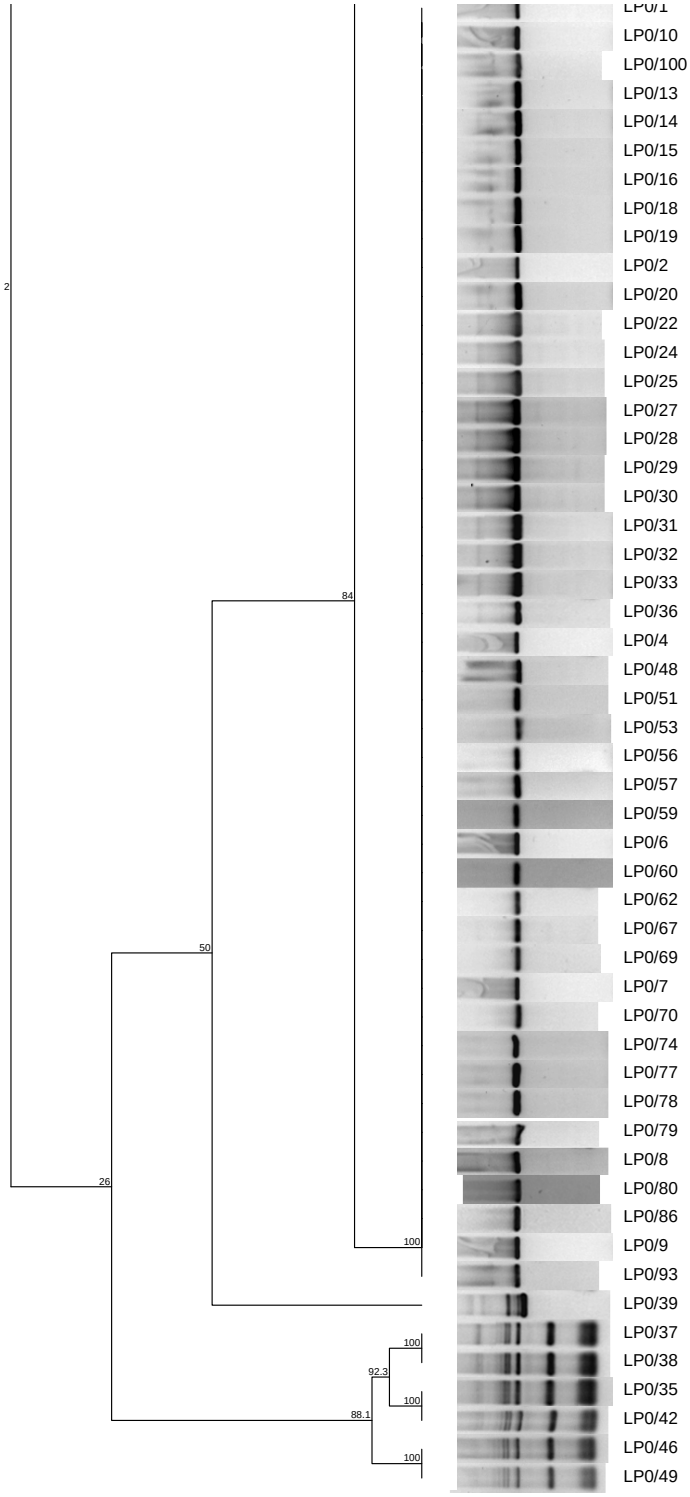
Appendix Figure E1 Dendrogram and corresponding restriction pattern of ITS-PCR with *Rsa*I of bacterial isolates obtained from natural Nham at 0 h of fermentation



Appendix Figure E1 (Continued)



Appendix Figure E2 Dendrogram and corresponding restriction pattern of ITS-PCR with *Rsa*I of bacterial isolates obtained from starter cultured Nham at 0 h of fermentation.



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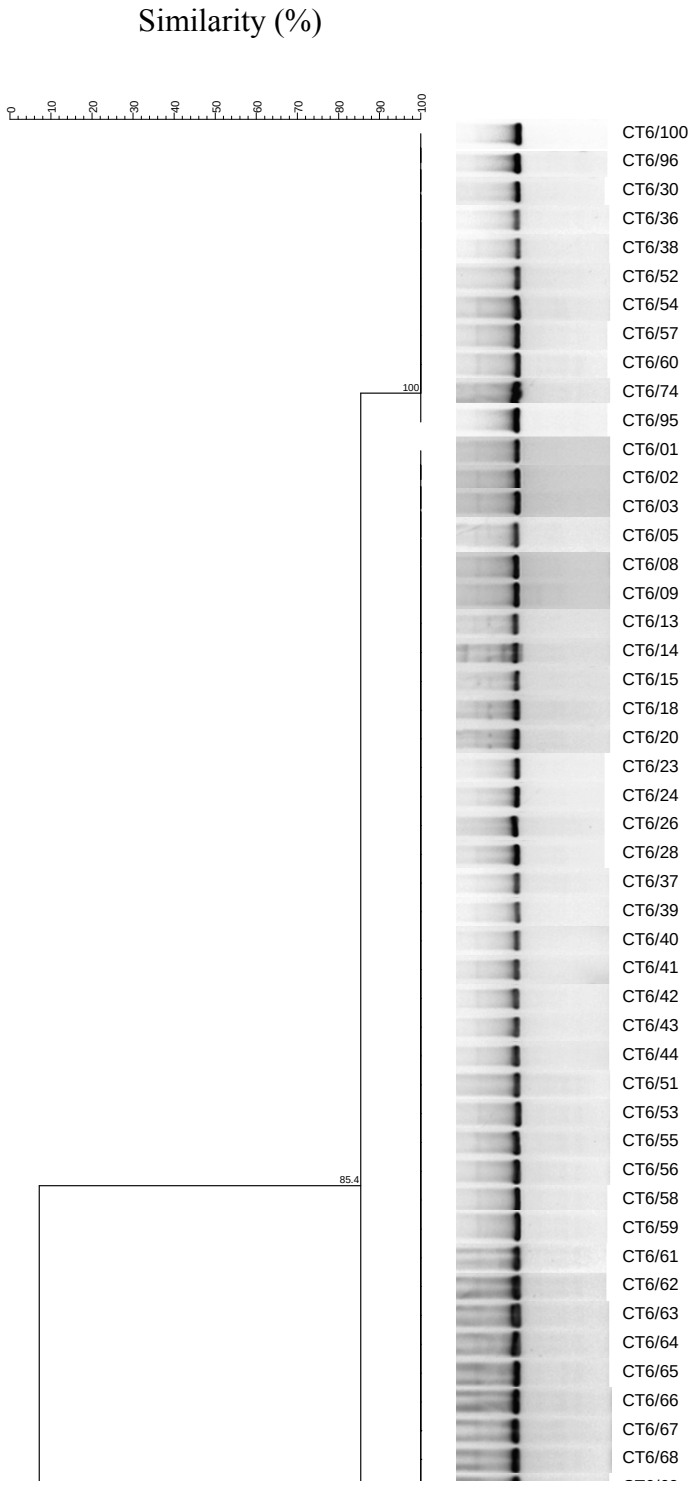
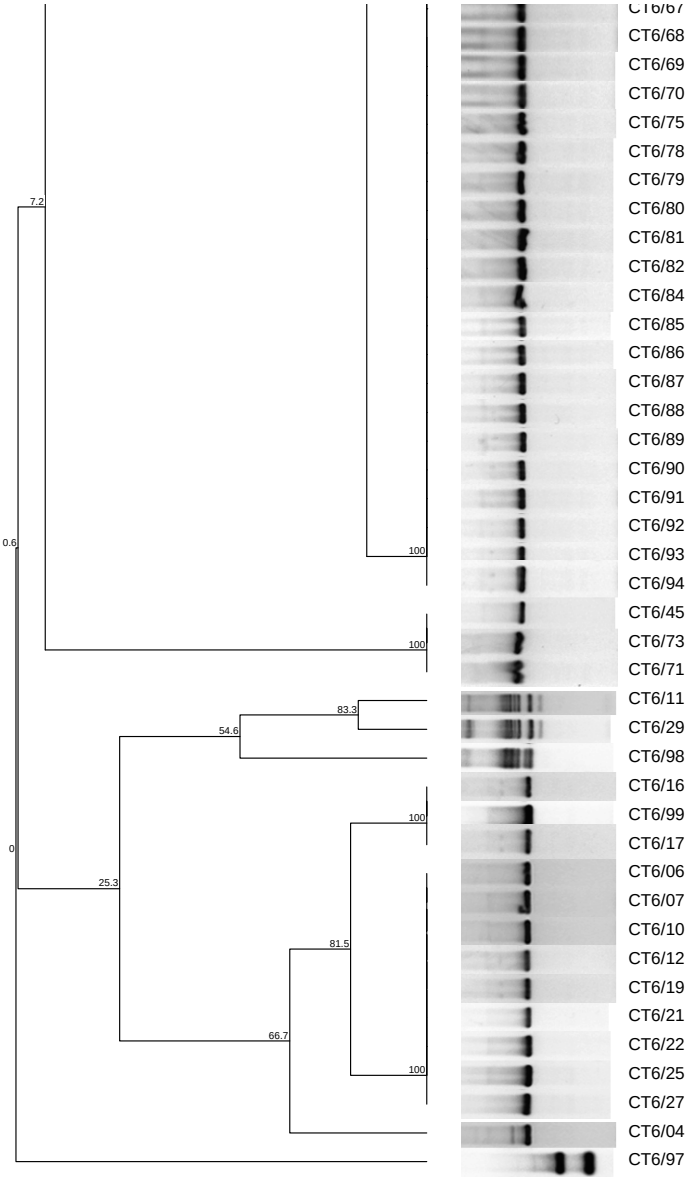


Appendix Figure E3 Dendrogram and corresponding restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from natural Nham at 6 h of fermentation.



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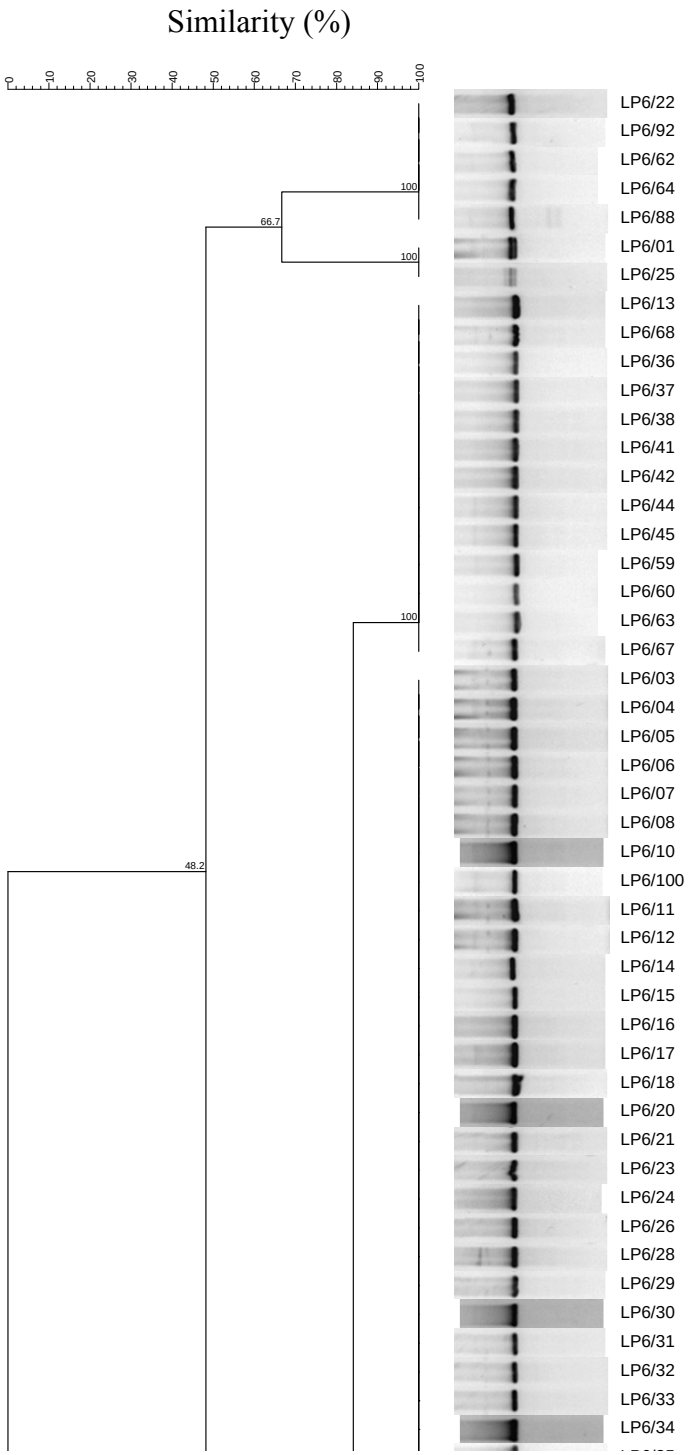
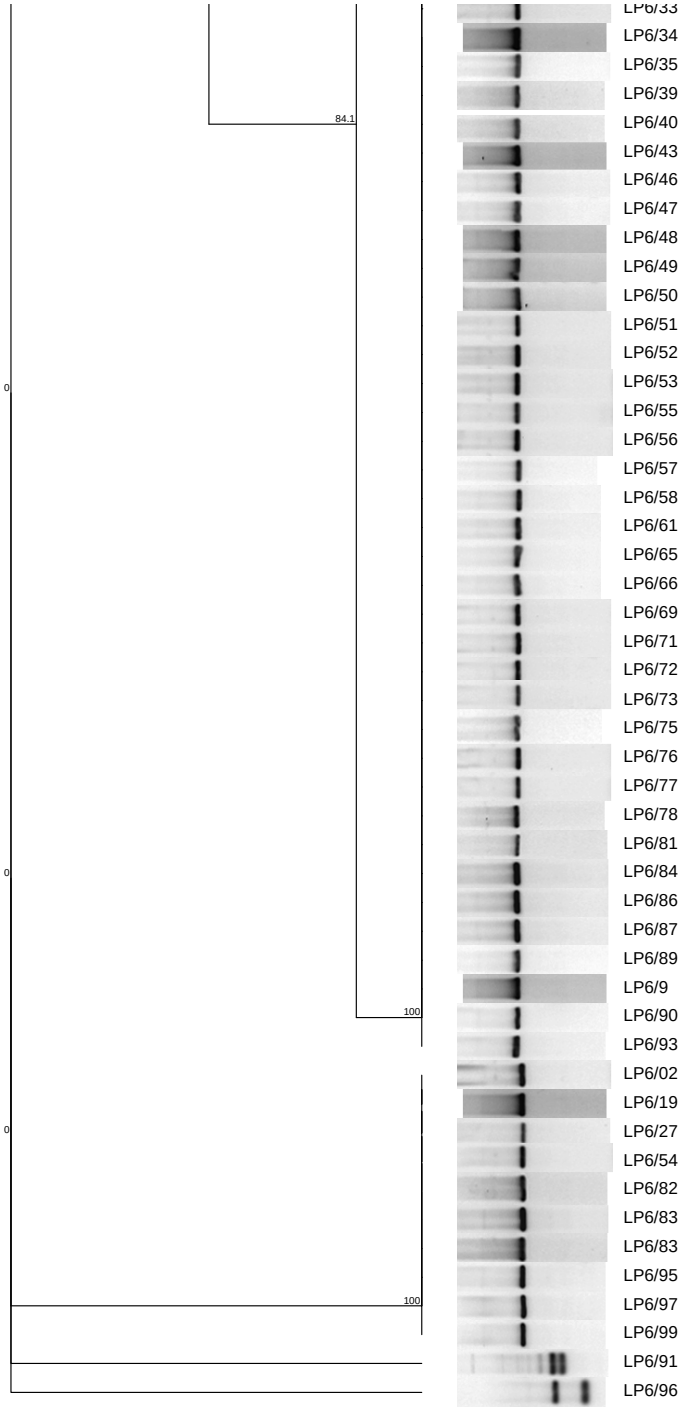


Appendix Figure E4 Dendrogram and corresponding restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from starter cultured Nham at 6 h of fermentation.



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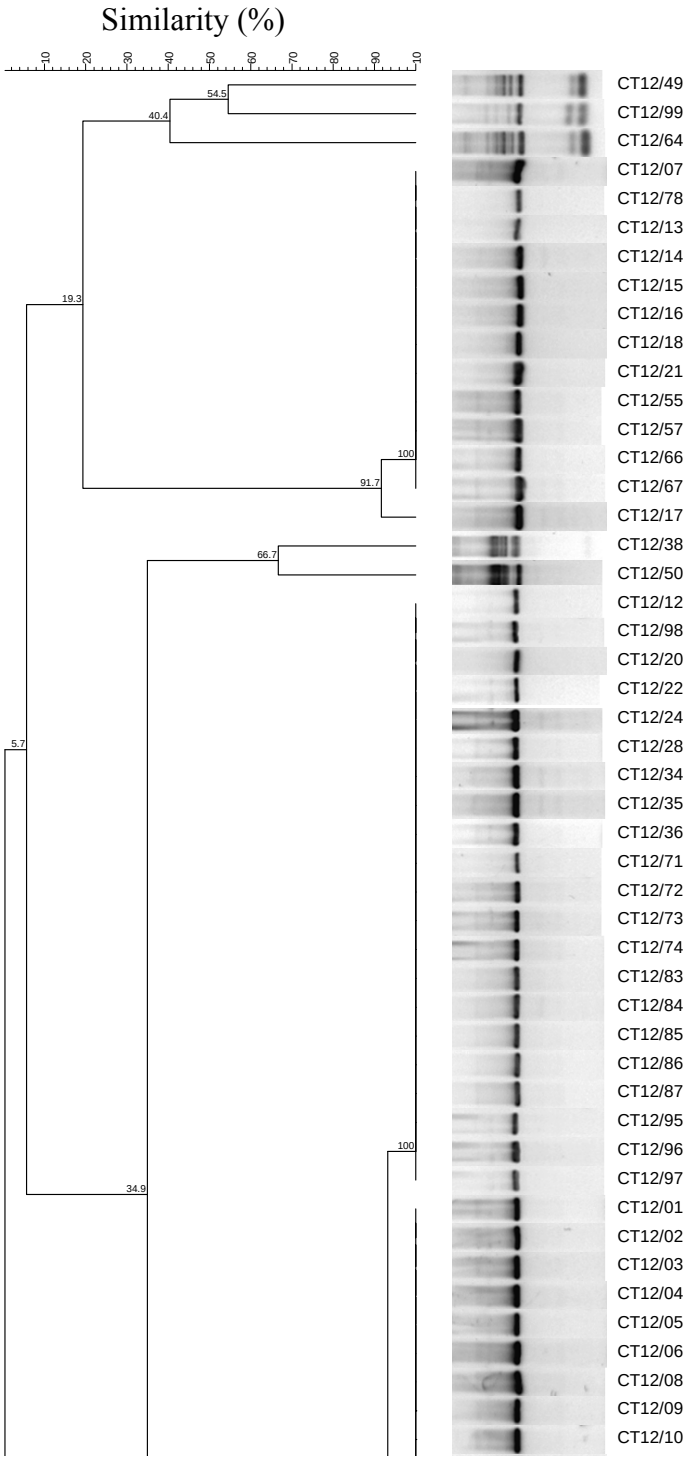
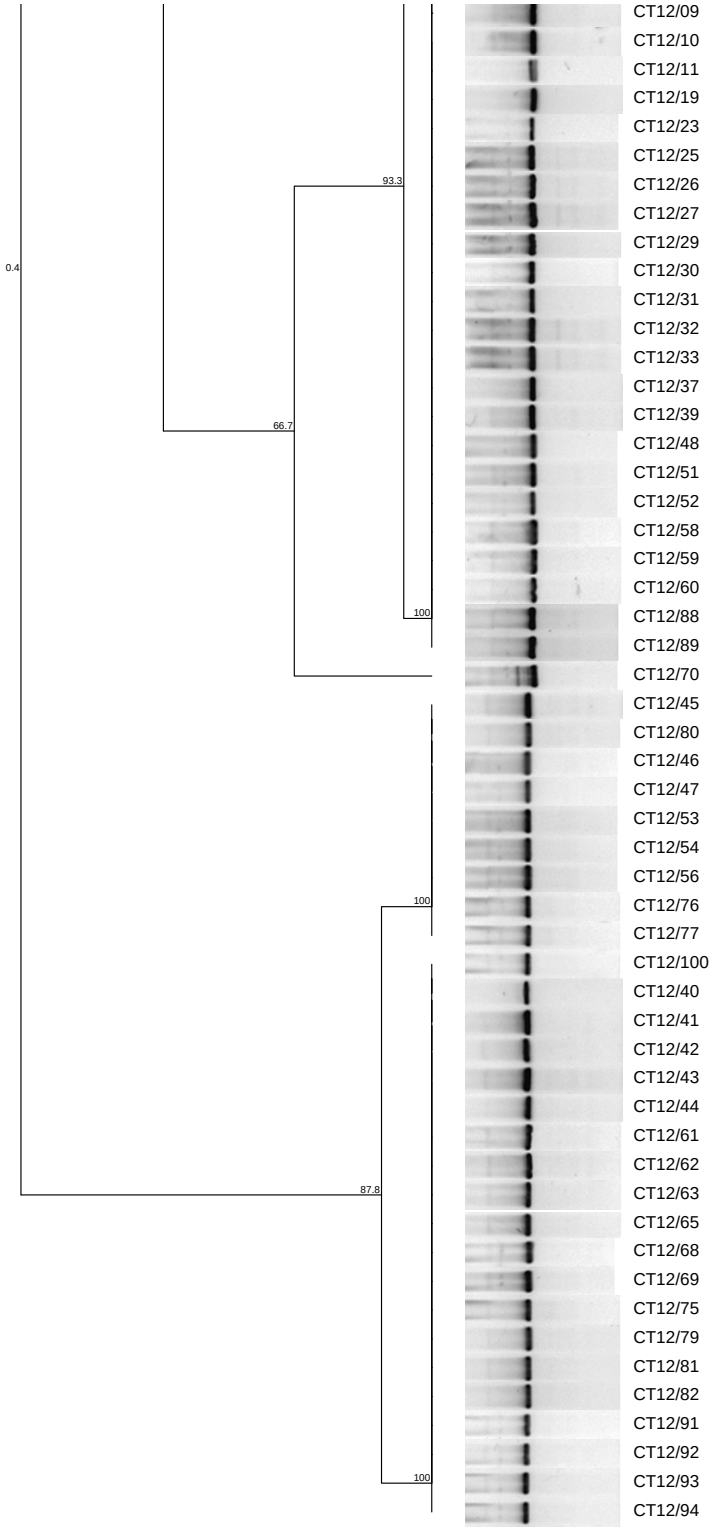


Appendix Figure E5 Dendrogram and corresponding restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from natural Nham at 12 h of fermentation.



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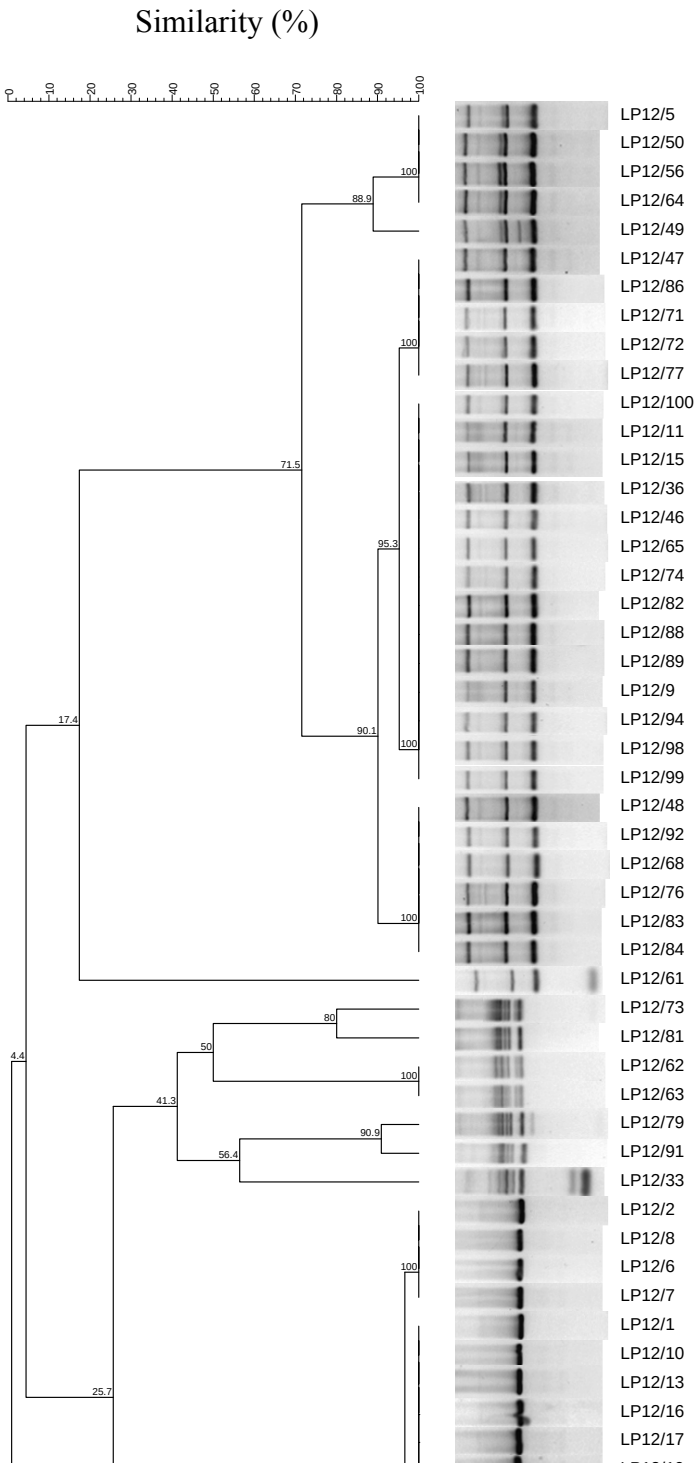
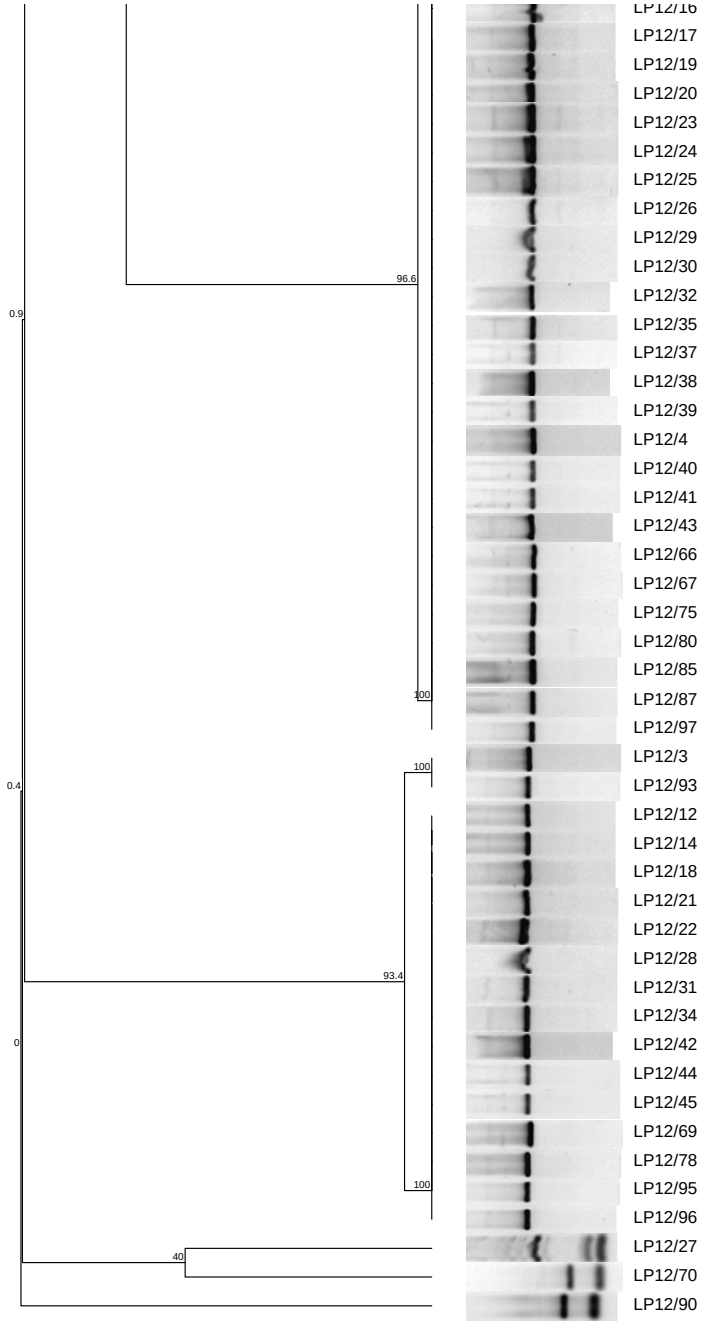


Appendix Figure E6 Dendrogram and corresponding restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from starter cultured Nham at 12 h of fermentation.



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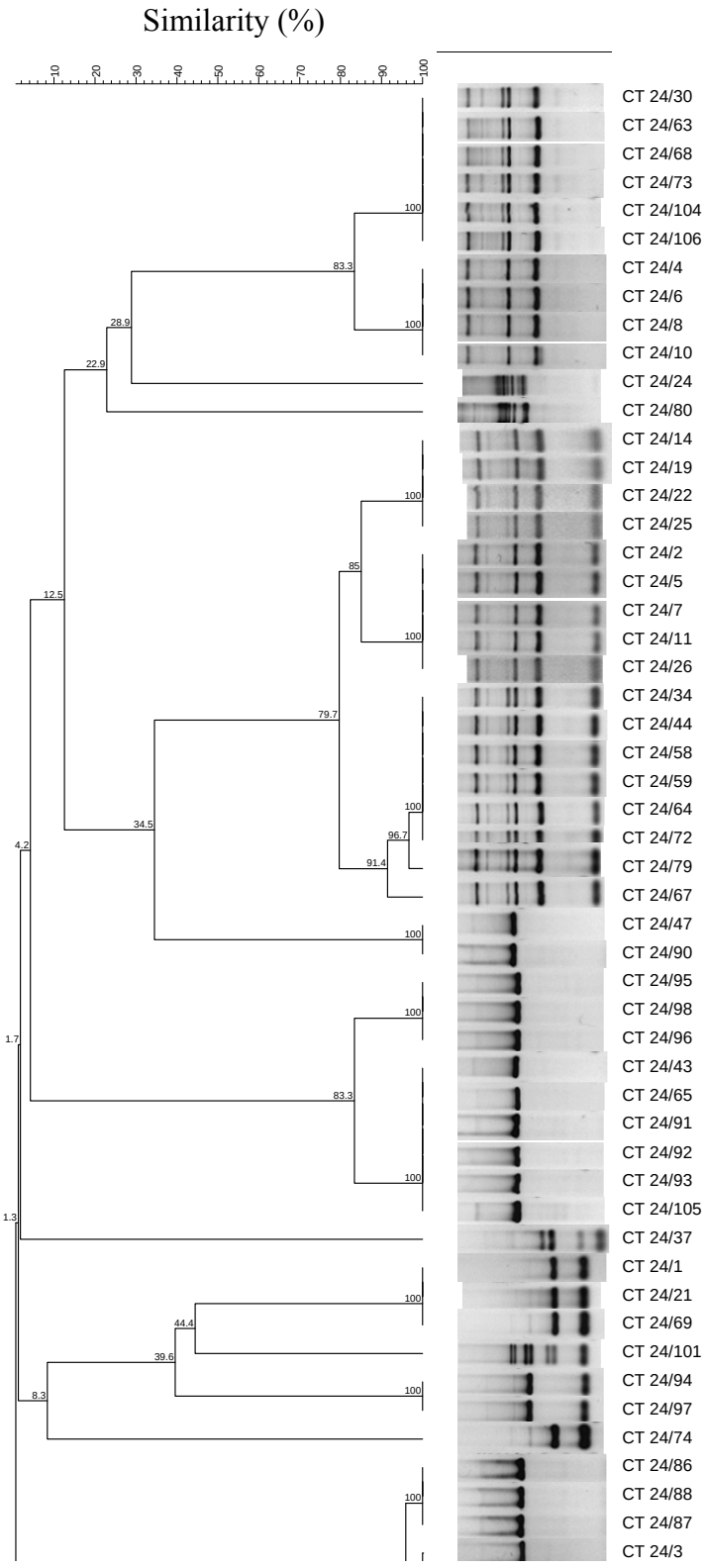
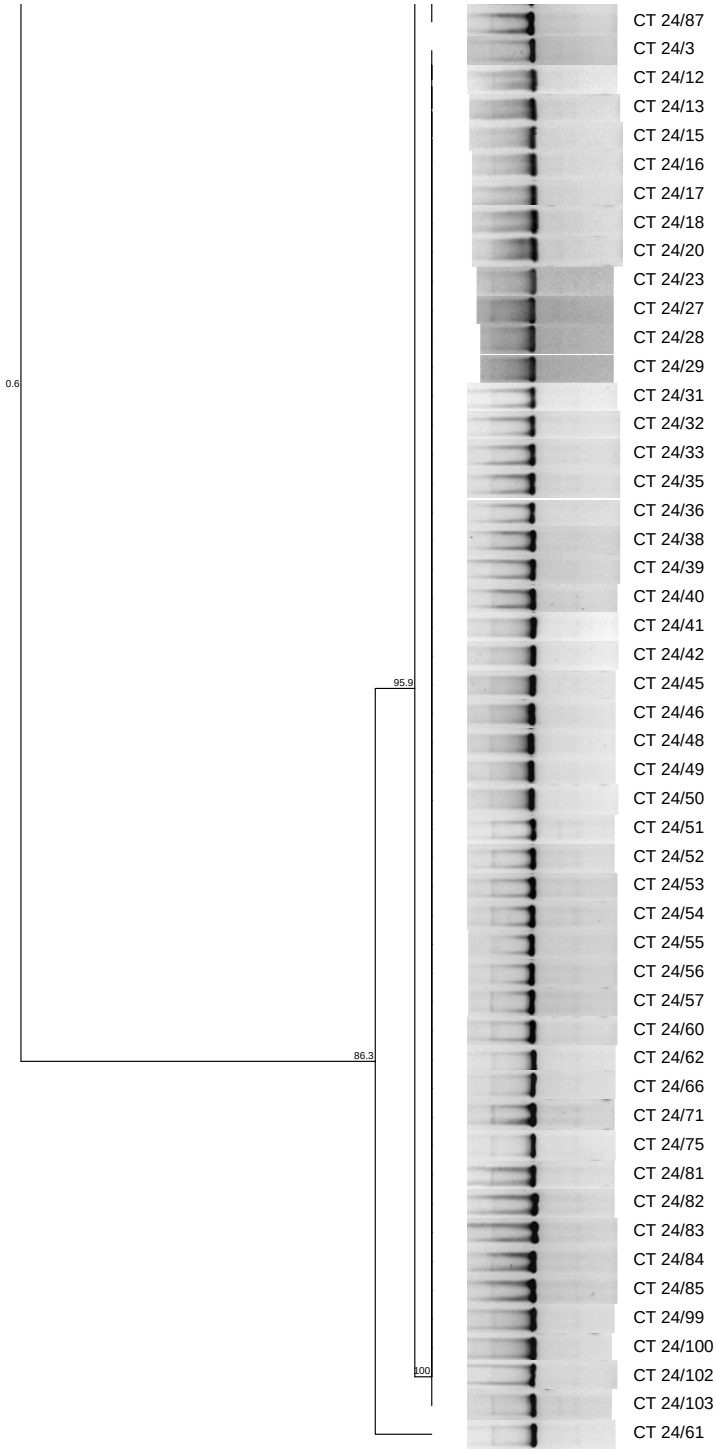


Appendix Figure E7 Dendrogram and corresponding restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from natural Nham at 24 h of fermentation.



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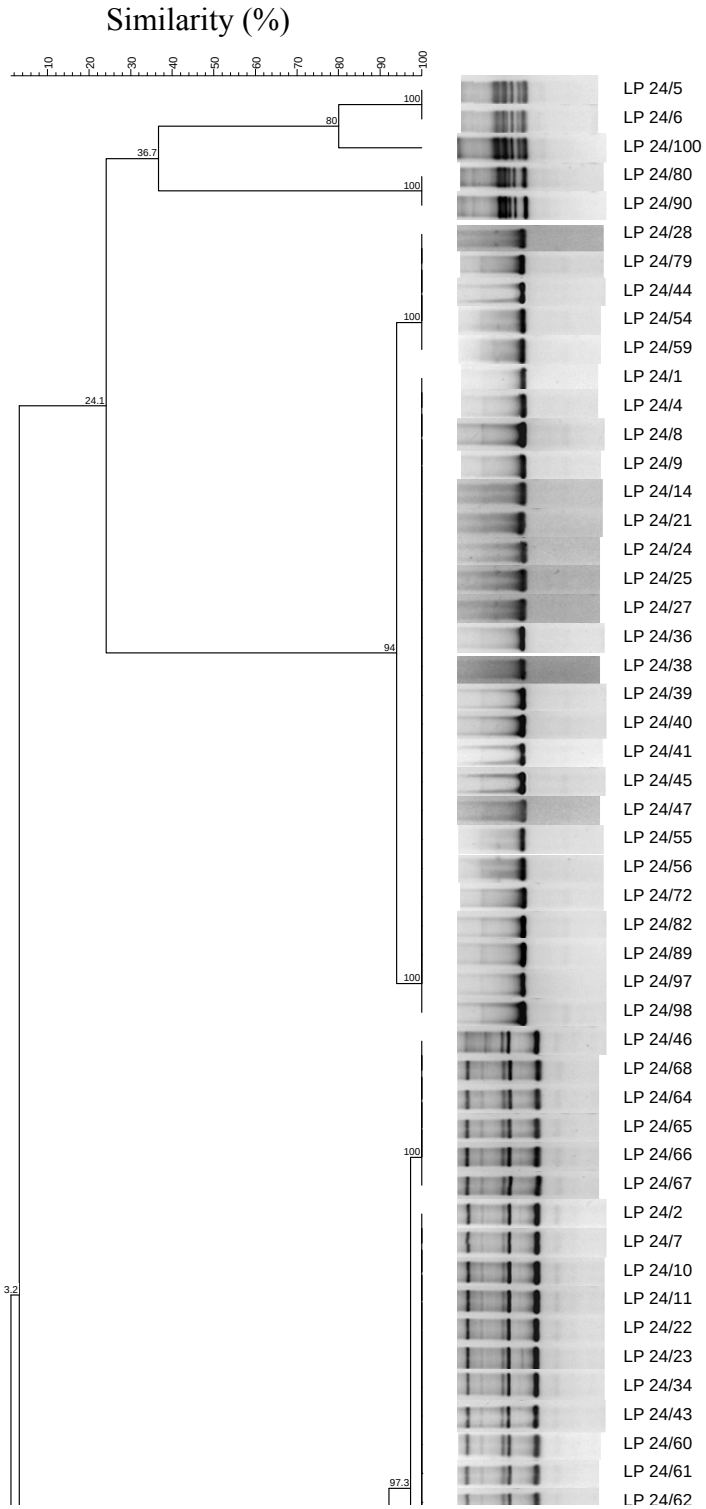
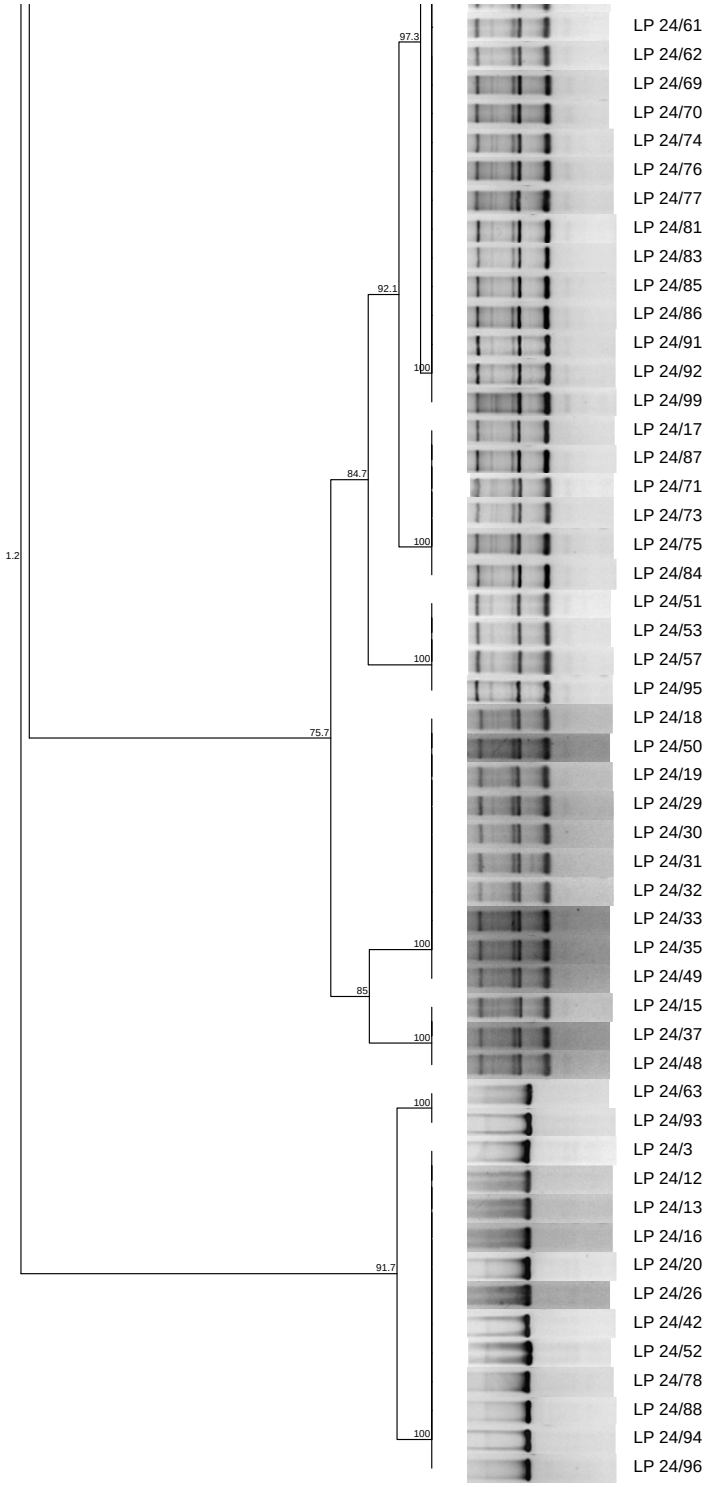


Appendix Figure E8 Dendrogram and corresponding restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from starter cultured Nham at 24 h of fermentation.



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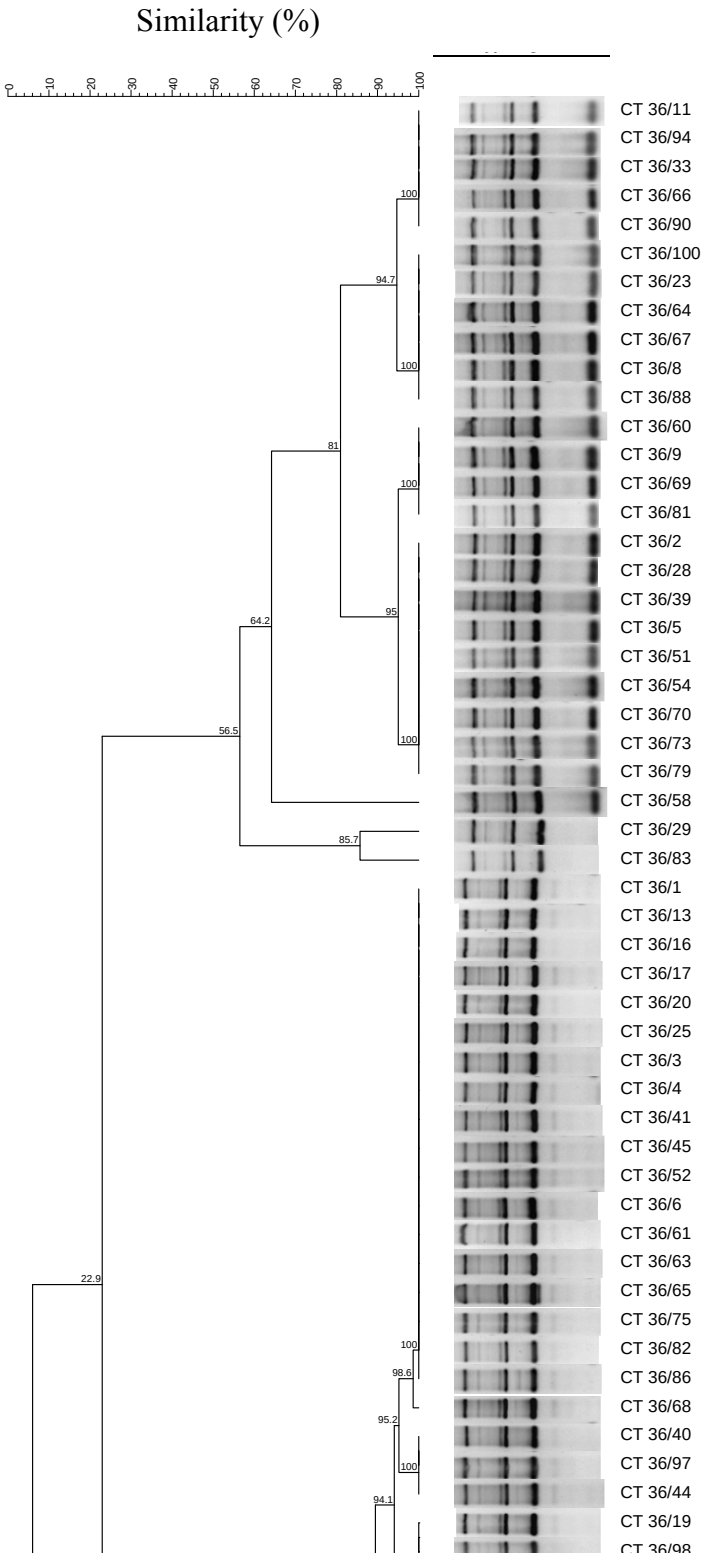
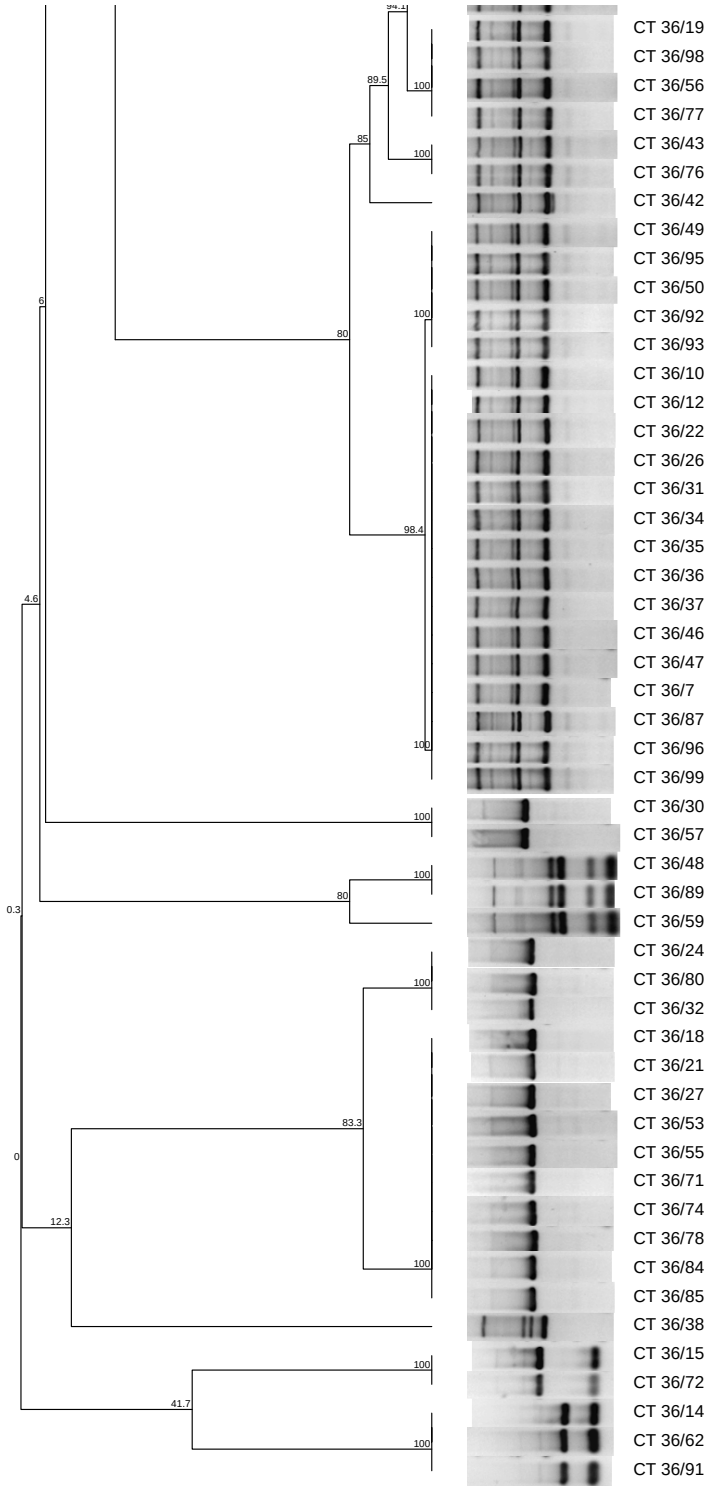
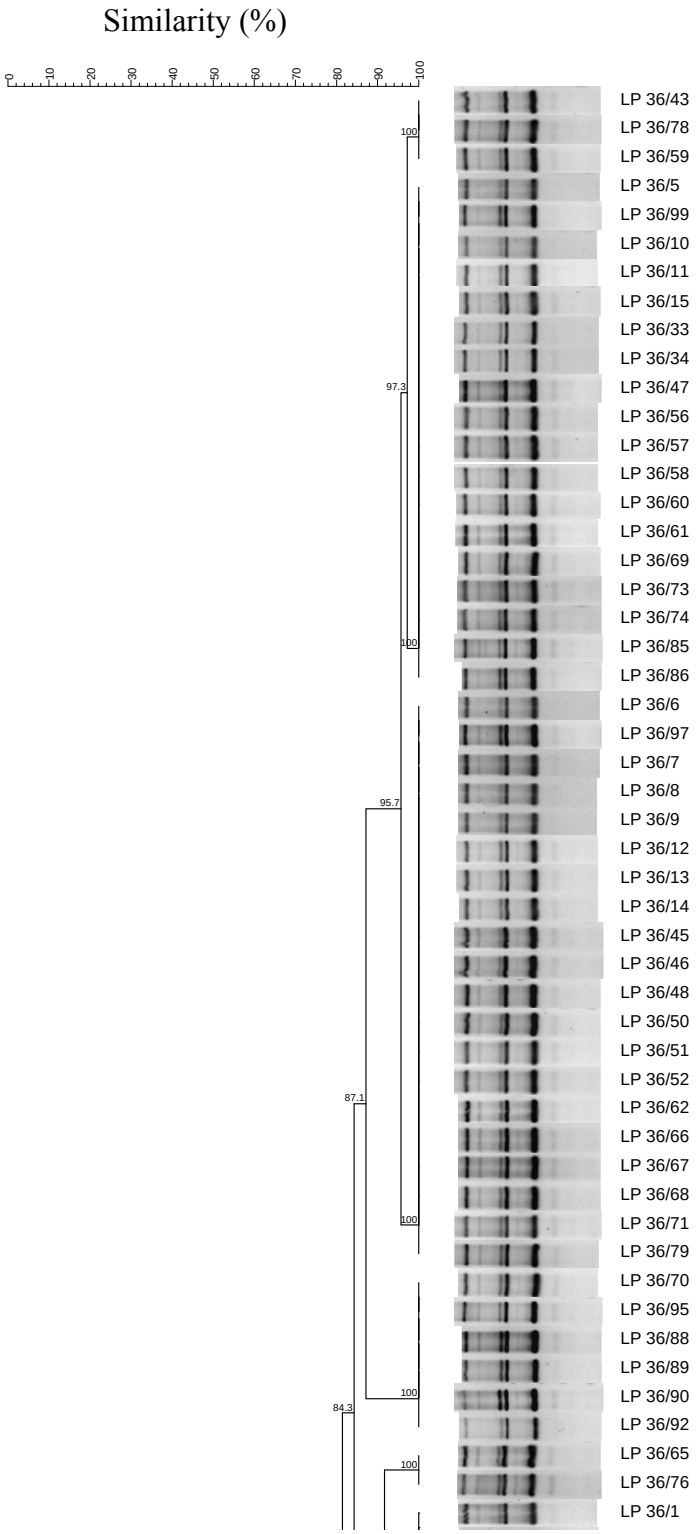


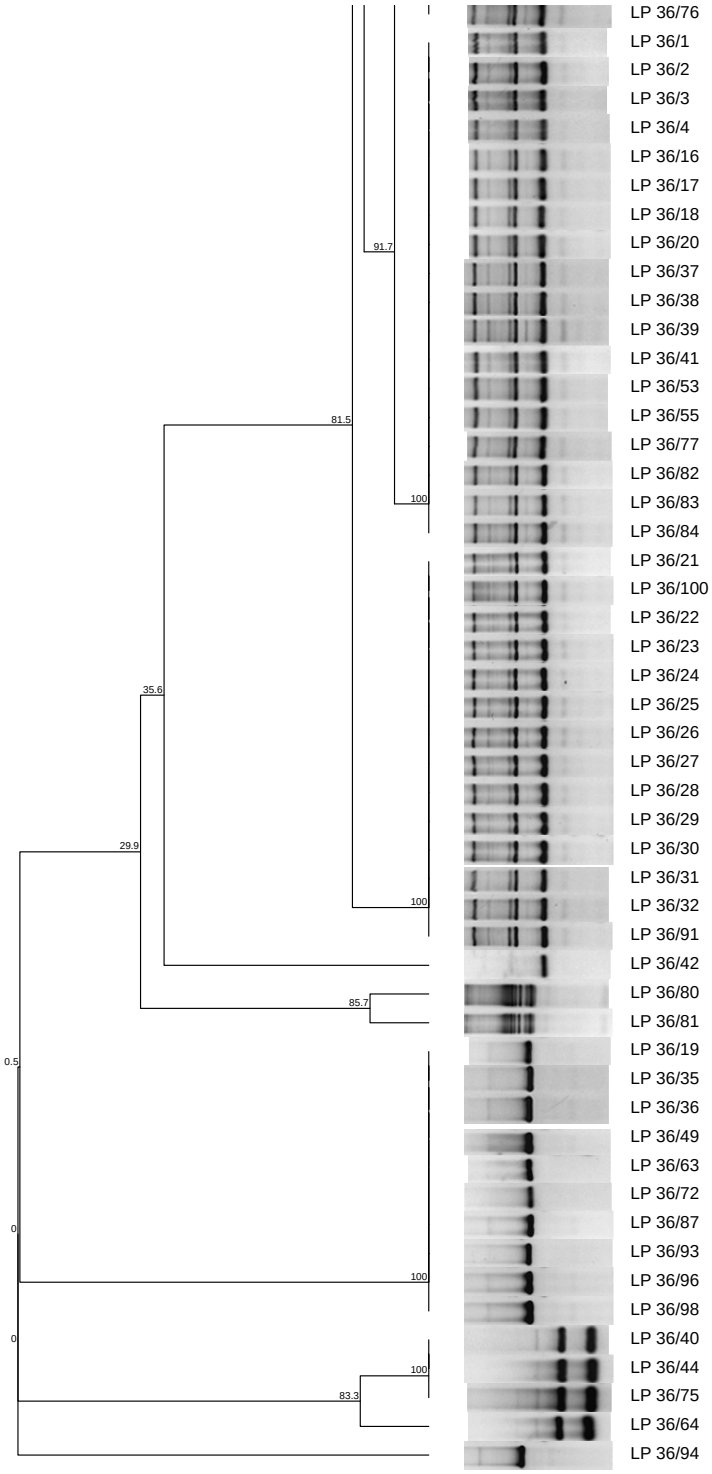
Appendix Figure E9 Dendrogram and corresponding restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from natural Nham at 36 h of fermentation.



Appendix Figure E9 (Continued)



Appendix Figure E10 Dendrogram and corresponding restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from starter cultured Nham at 36 h of fermentation.



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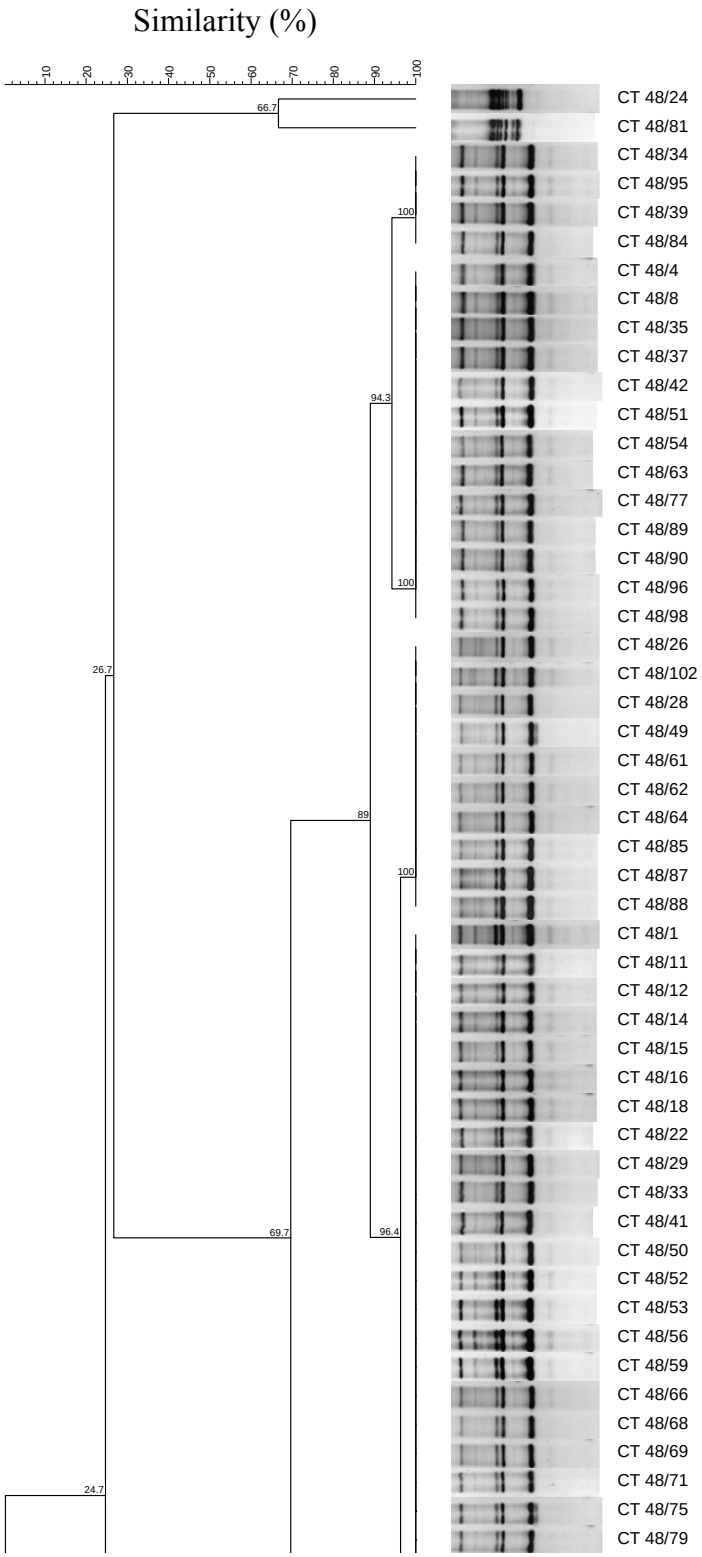
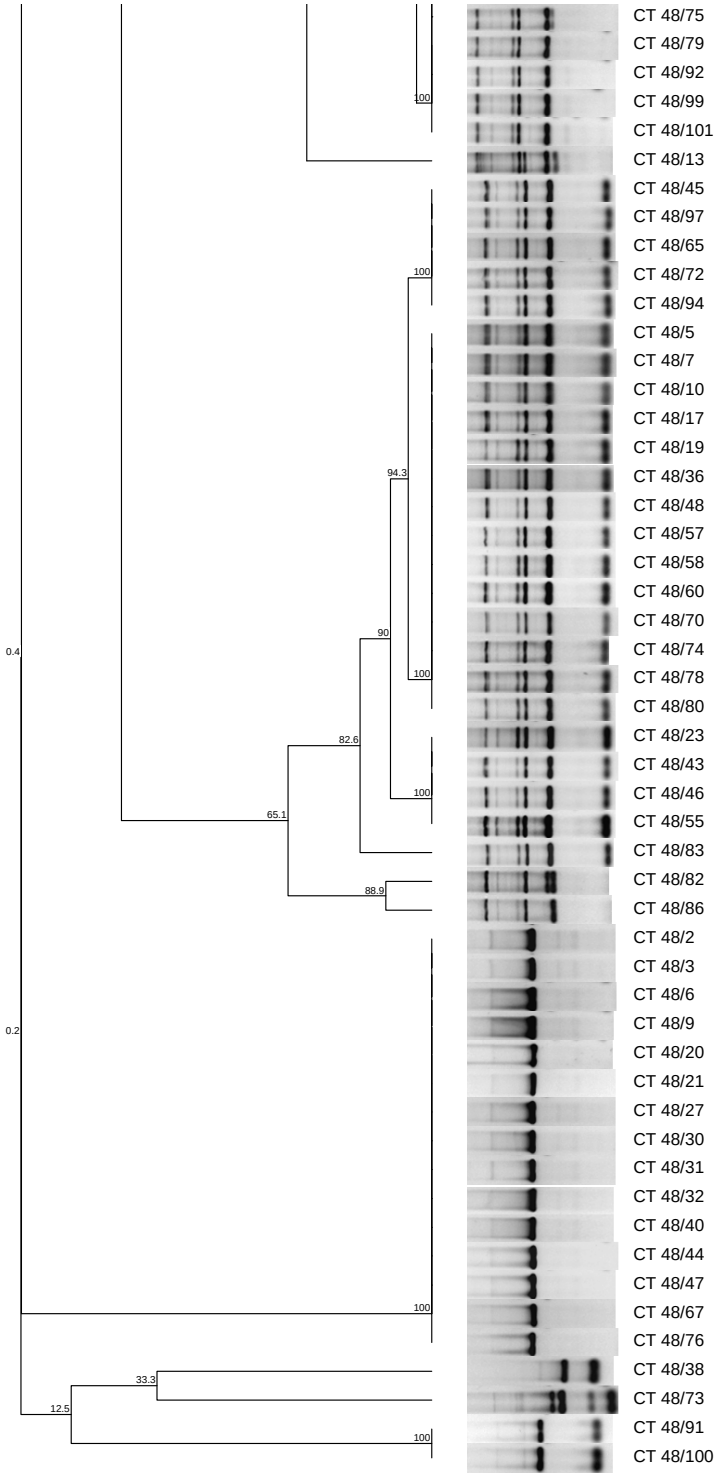


Appendix Figure E11 Dendrogram and corresponding restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from natural Nham at 48 h of fermentation.



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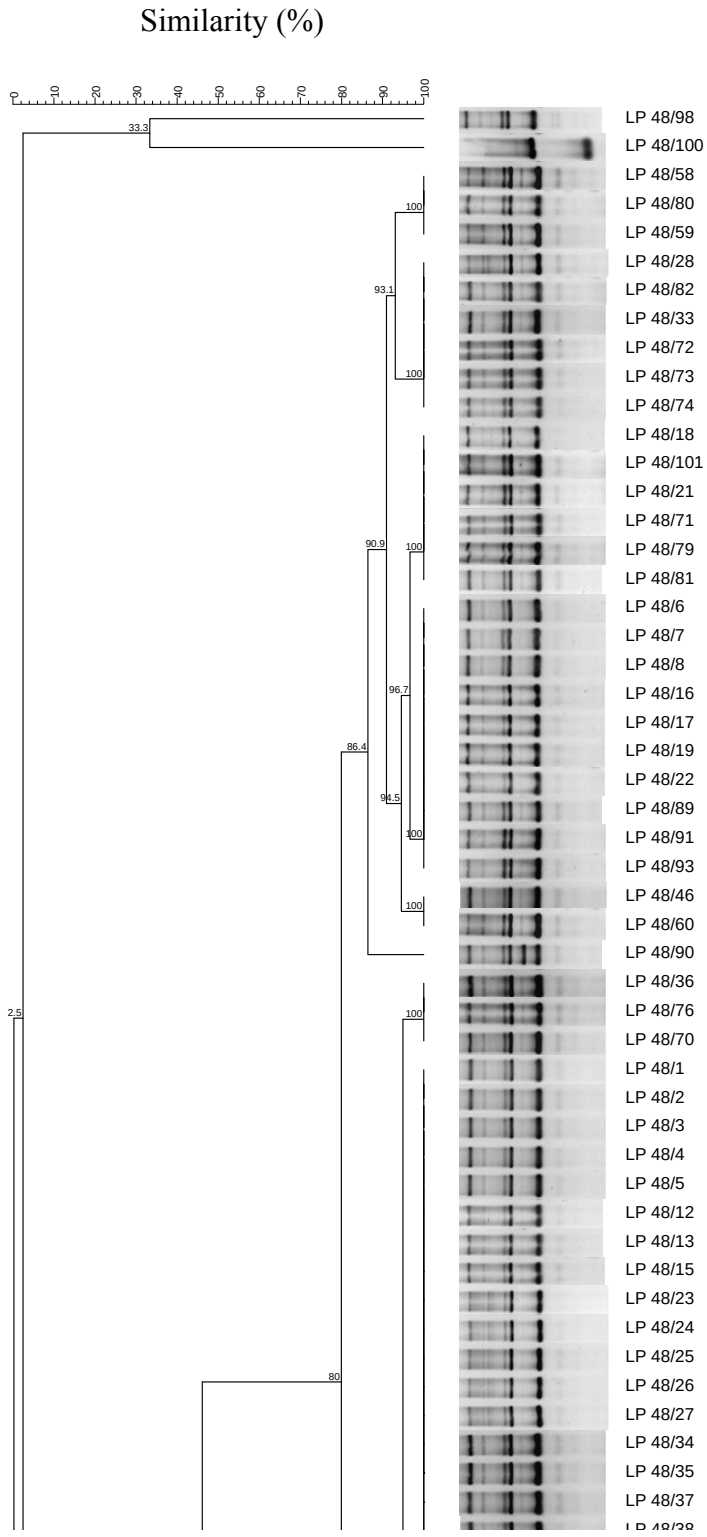
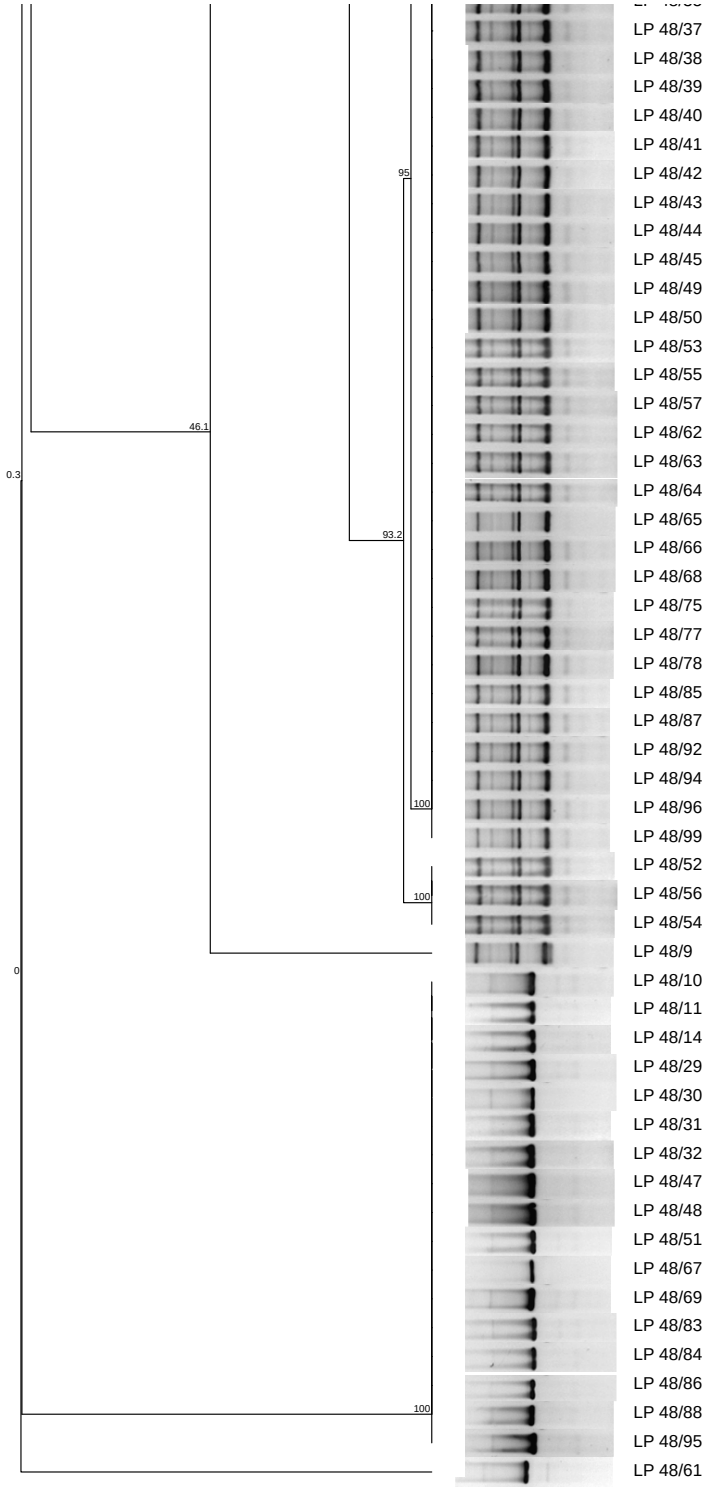


Appendix Figure E12 Dendrogram and corresponding restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from starter cultured Nham at 48 h of fermentation.



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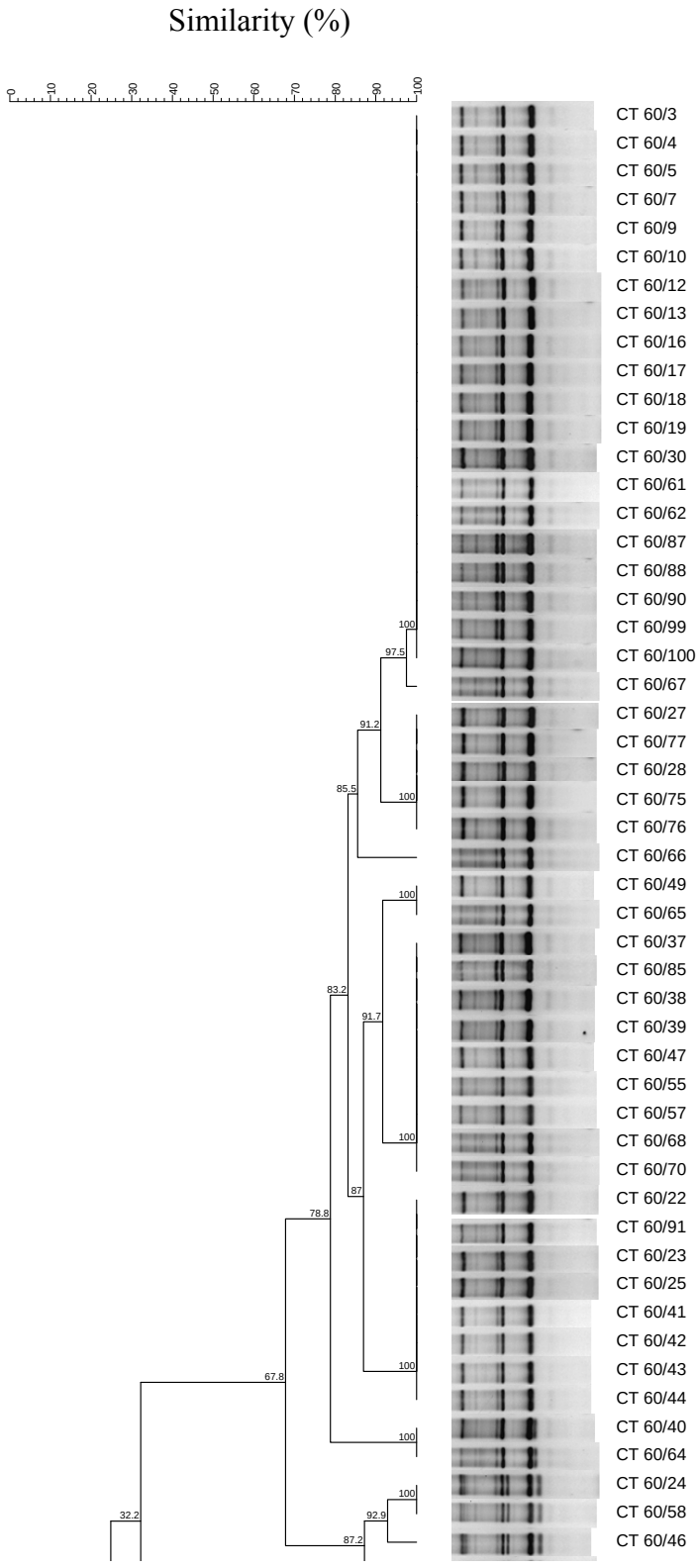
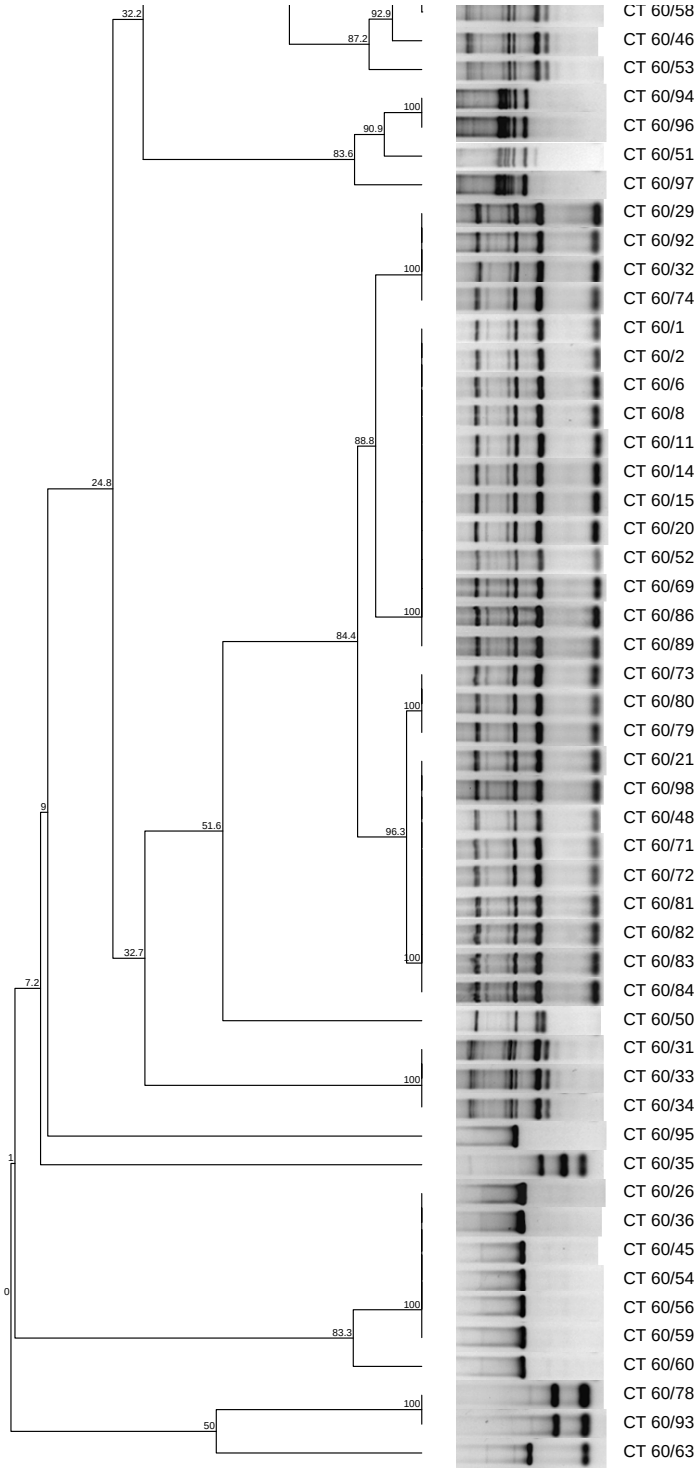


Appendix Figure E13 Dendrogram and corresponding restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from natural Nham at 60 h of fermentation.



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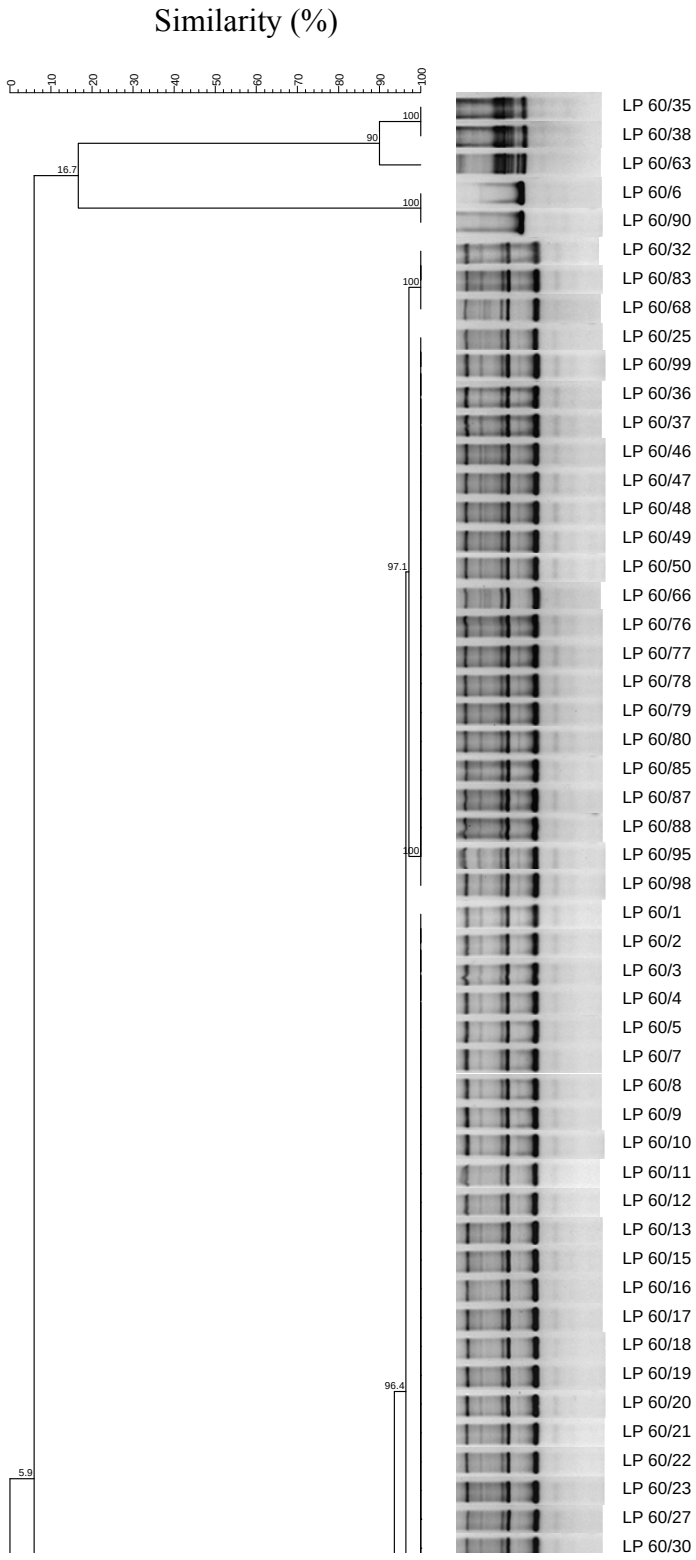
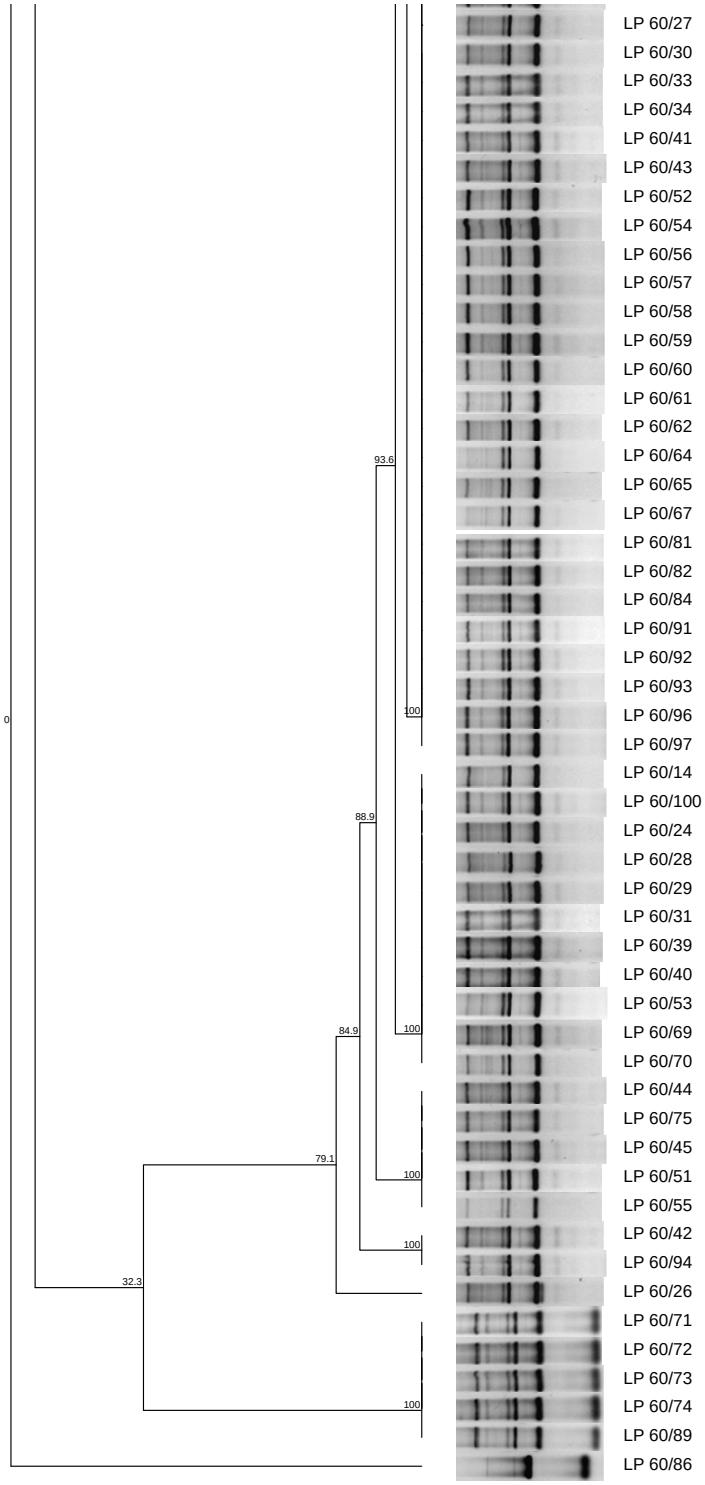


Appendix Figure E14 Dendrogram and corresponding restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from starter cultured Nham at 60 h of fermentation.



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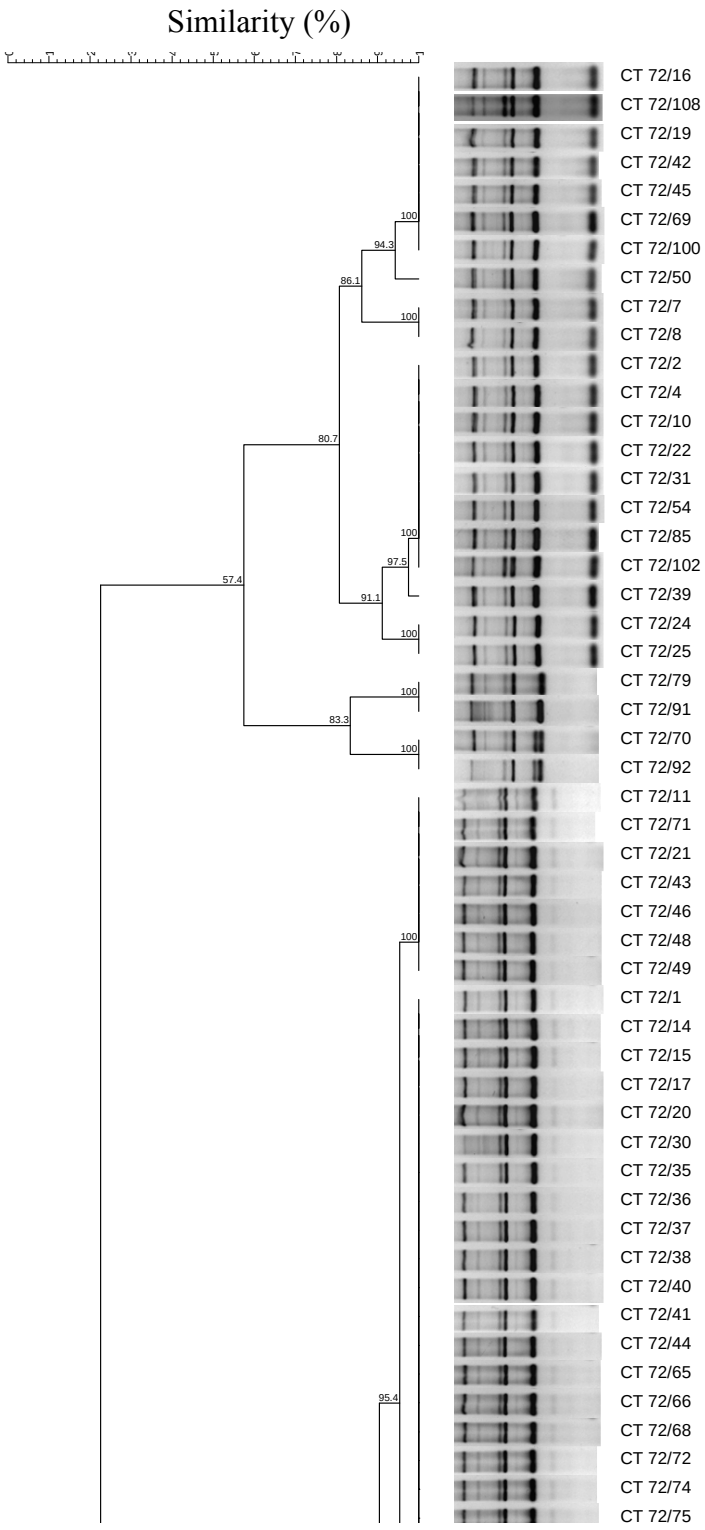
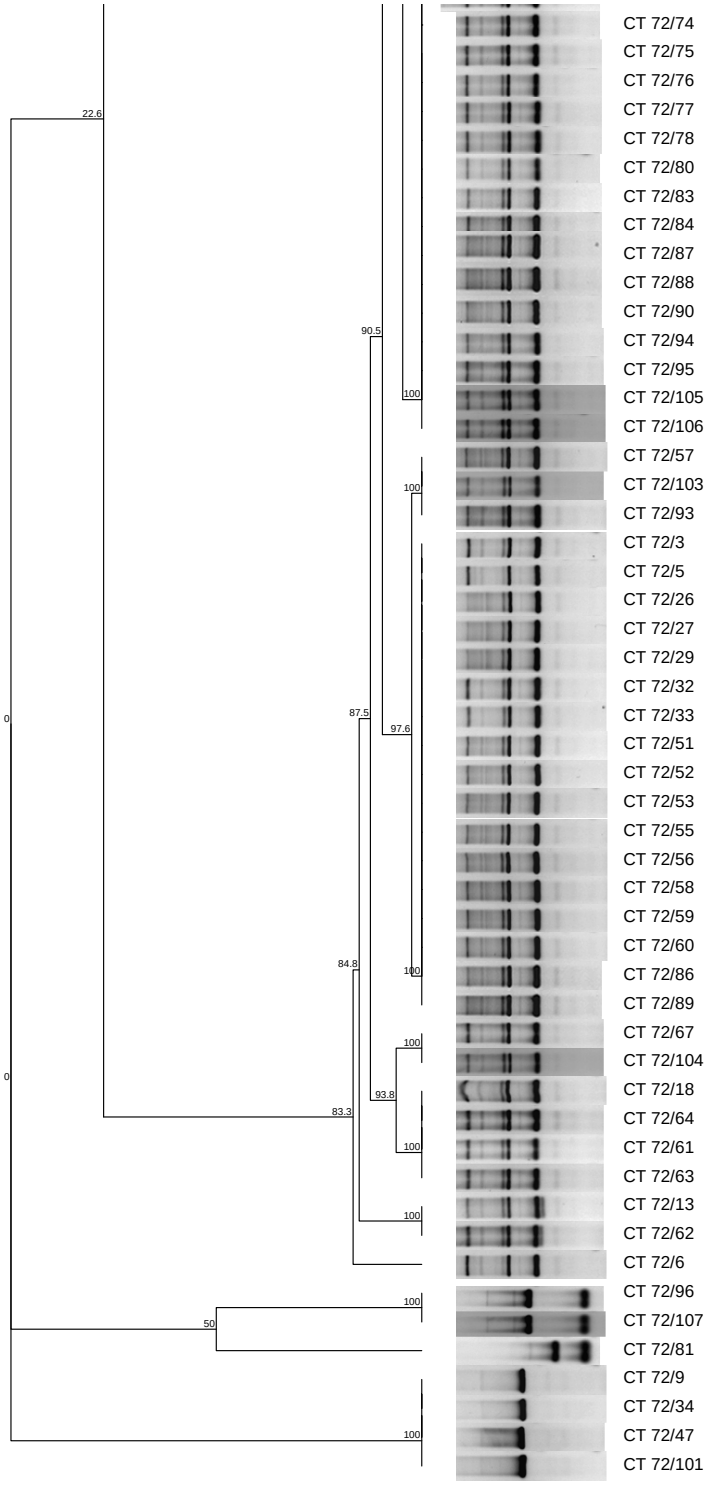


Appendix Figure E15 Dendrogram and corresponding restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from natural Nham at 72 h of fermentation.



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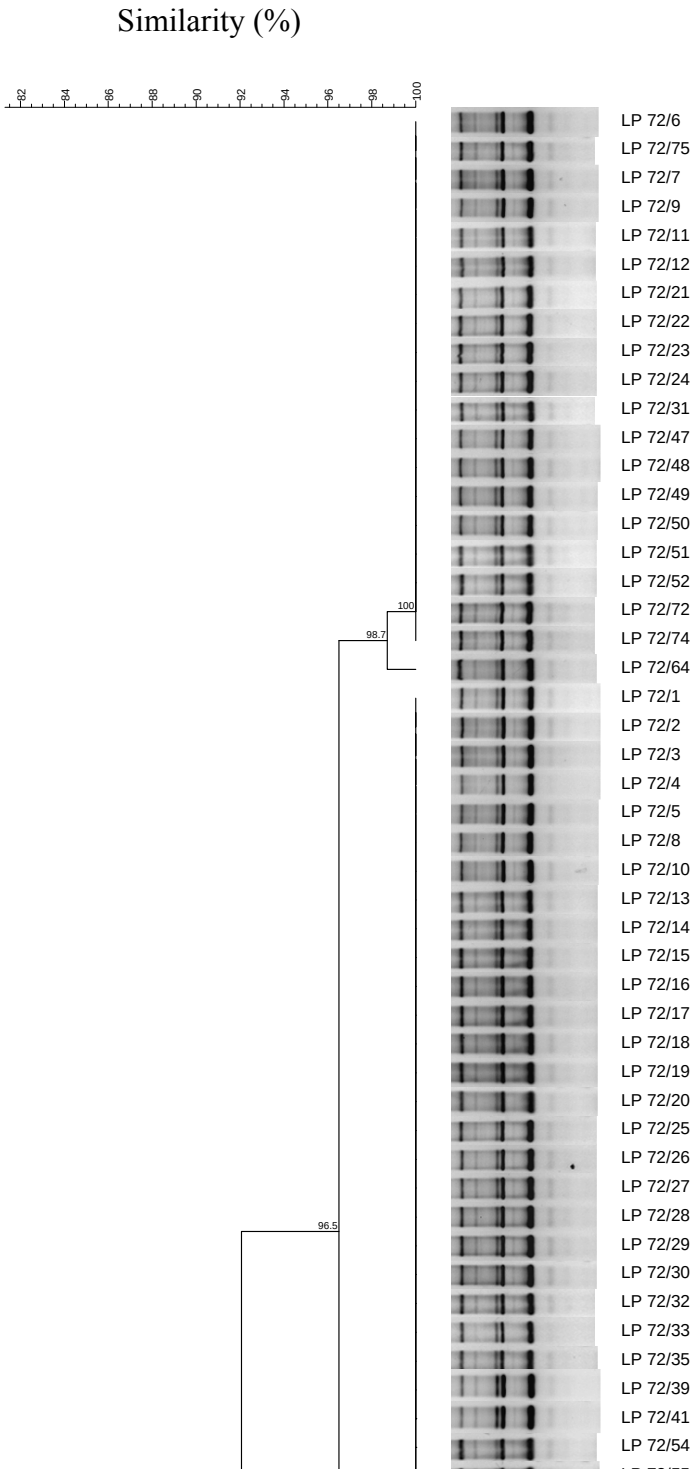
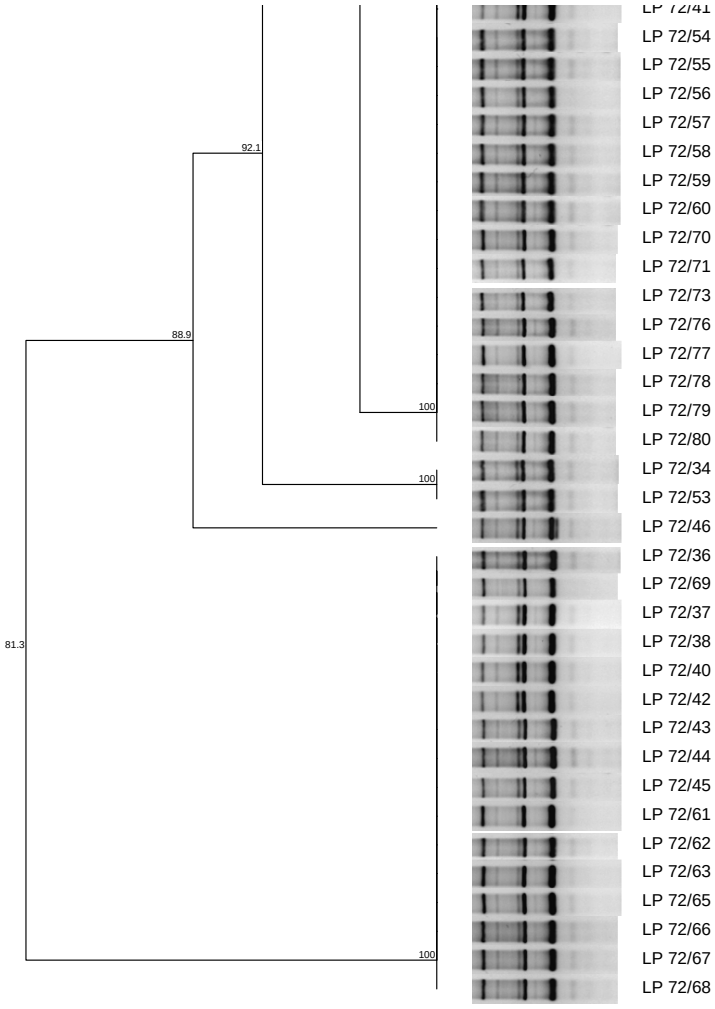


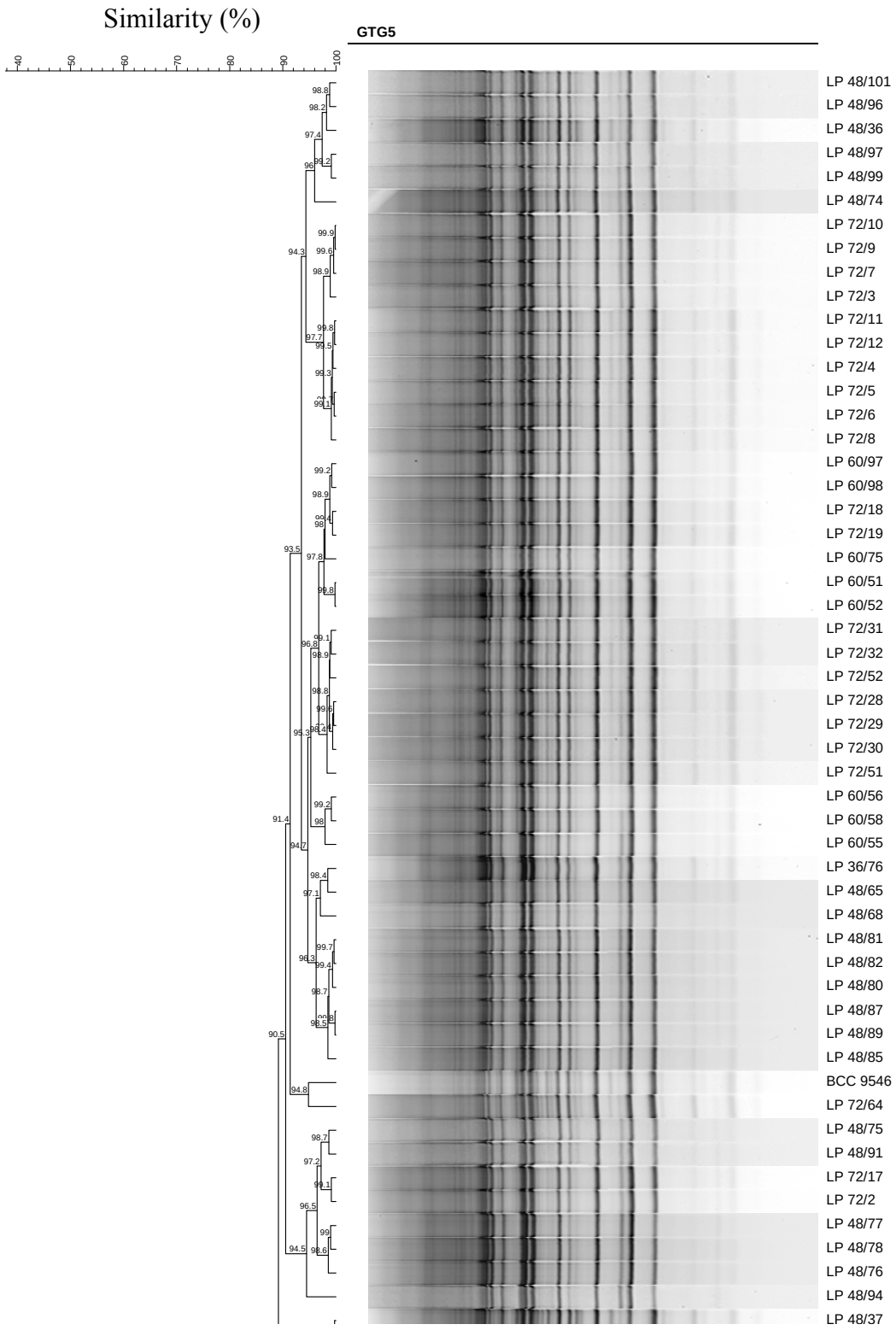
Appendix Figure E16 Dendrogram and correspondin restriction pattern of ITS with *Rsa*I of bacterial isolates obtained from starter cultured Nham at 72 h of fermentation.



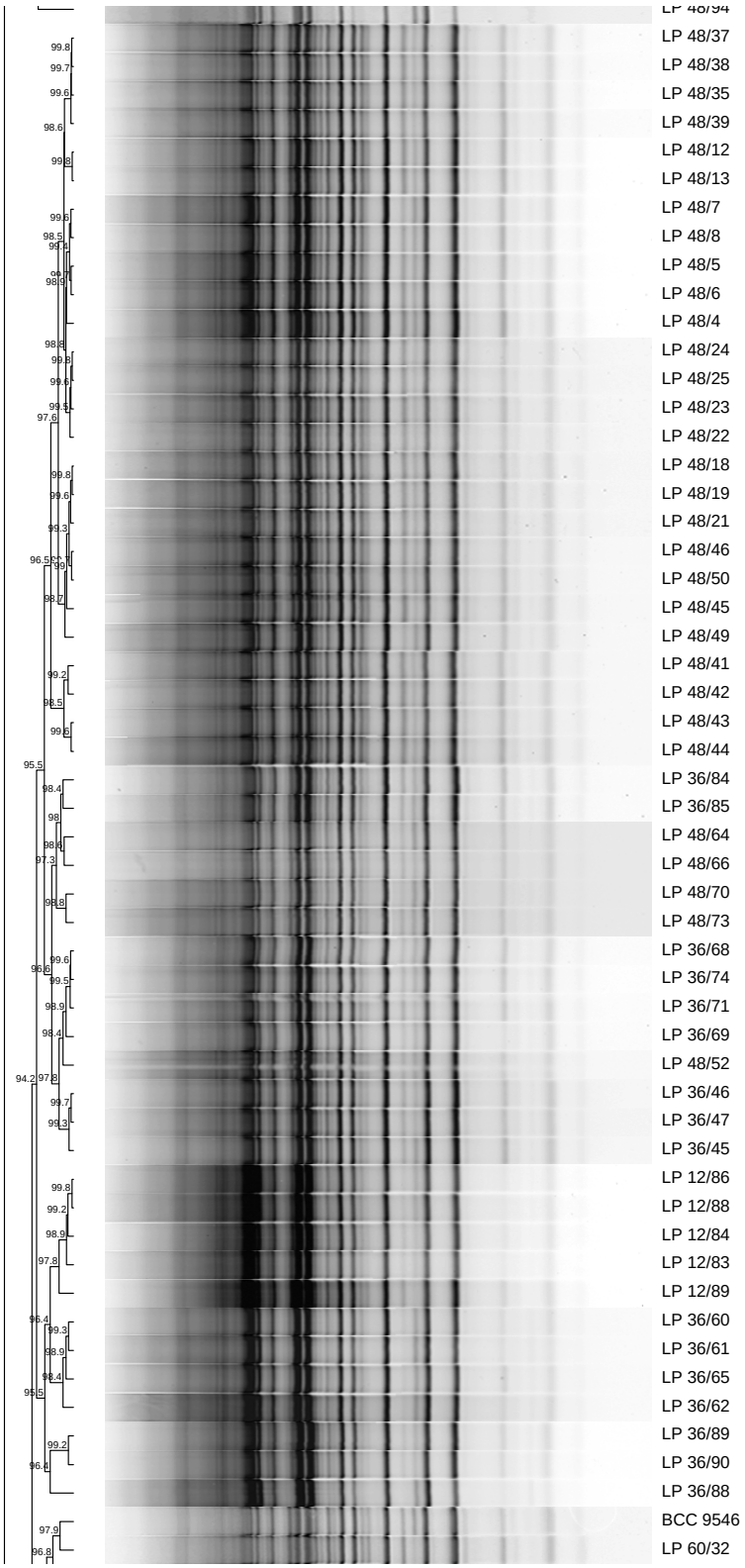
Appendix Figure E16 (Continued)

Appendix F

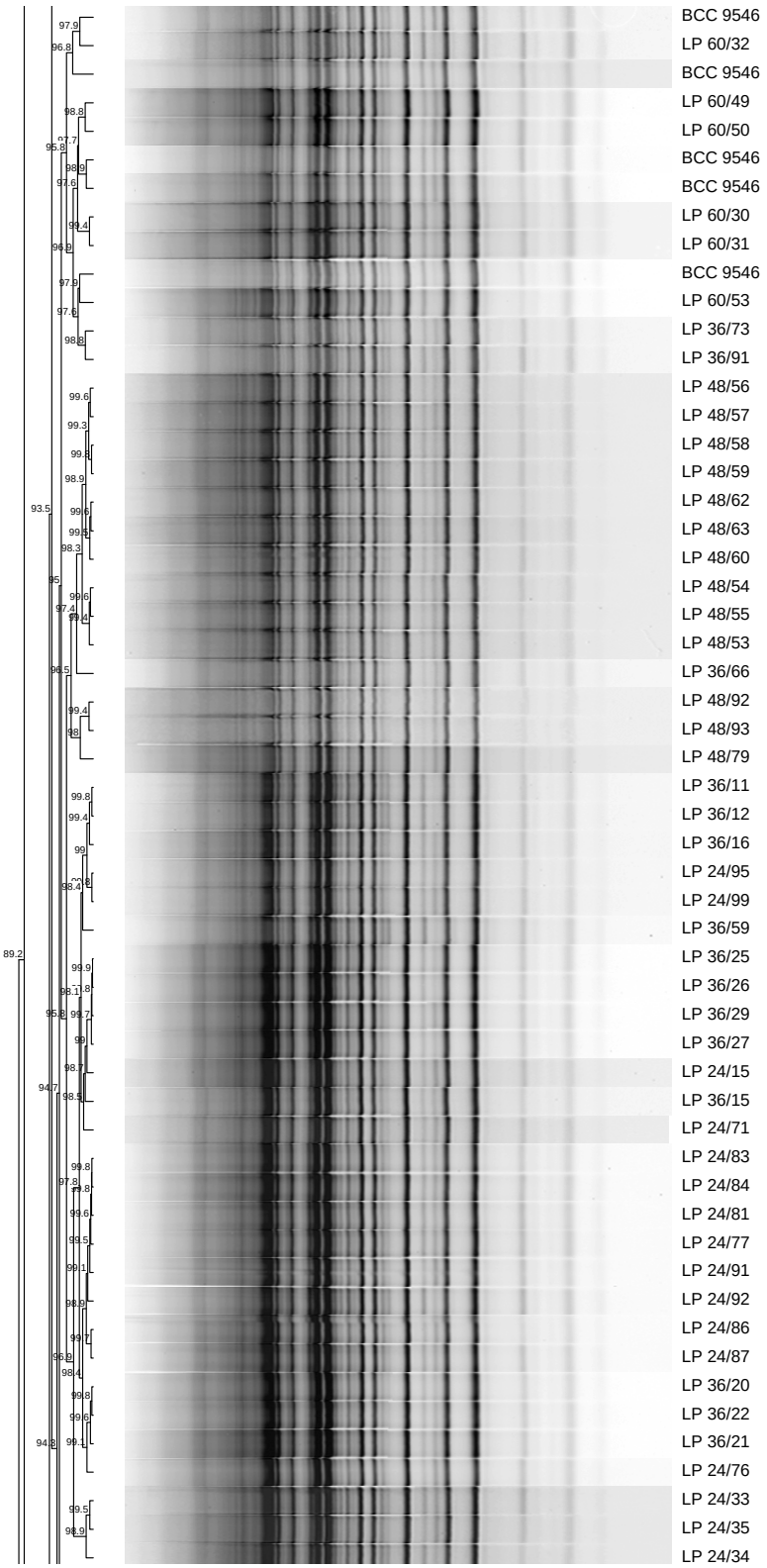
Dendrogram of $(GTG)_5$ -PCR genomic fingerprint



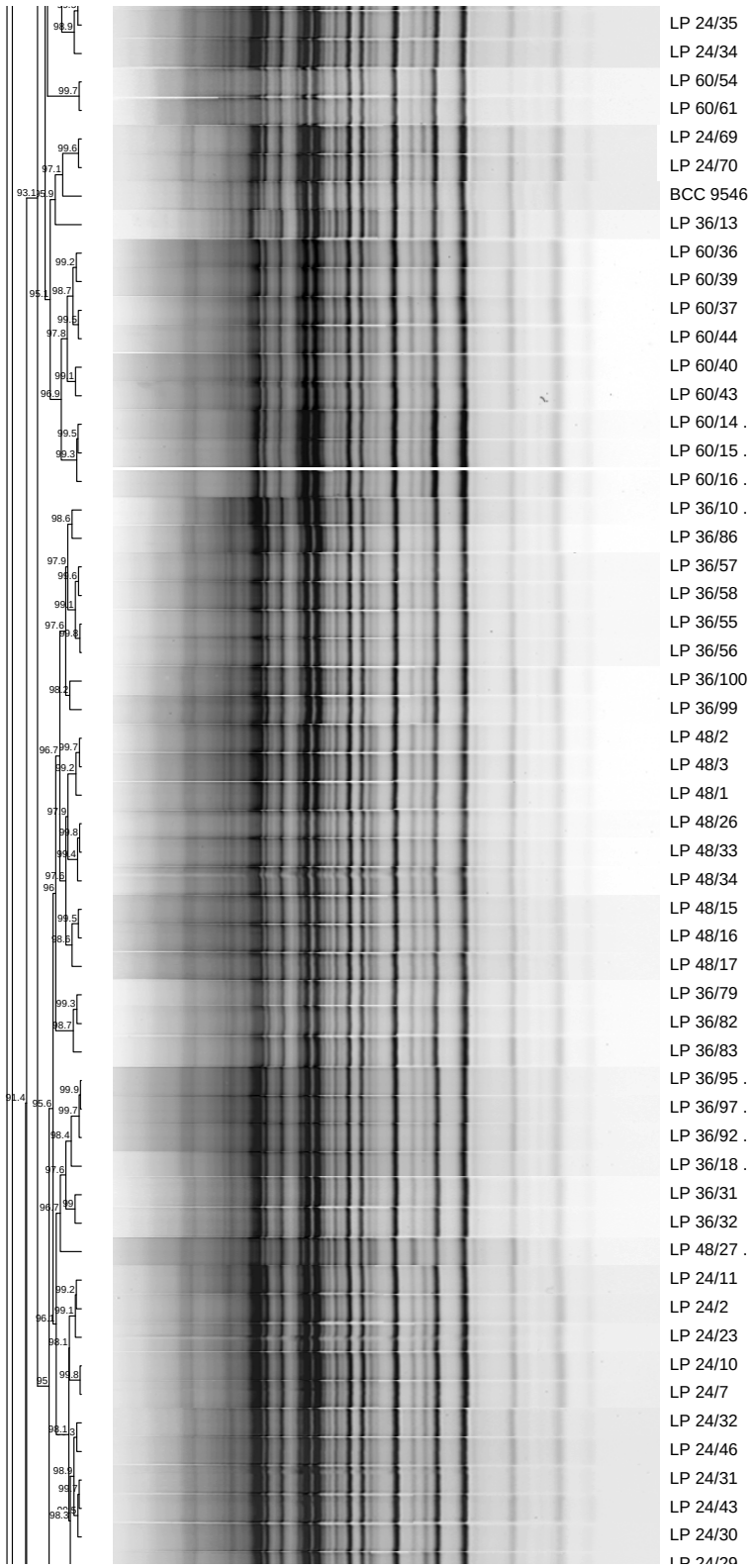
Appendix Figure F17 Dendrogram and corresponding of the $(GTG)_5$-PCR genomic fingerprint of *Lb. plantarum* isolates obtained from starter cultured Nham.



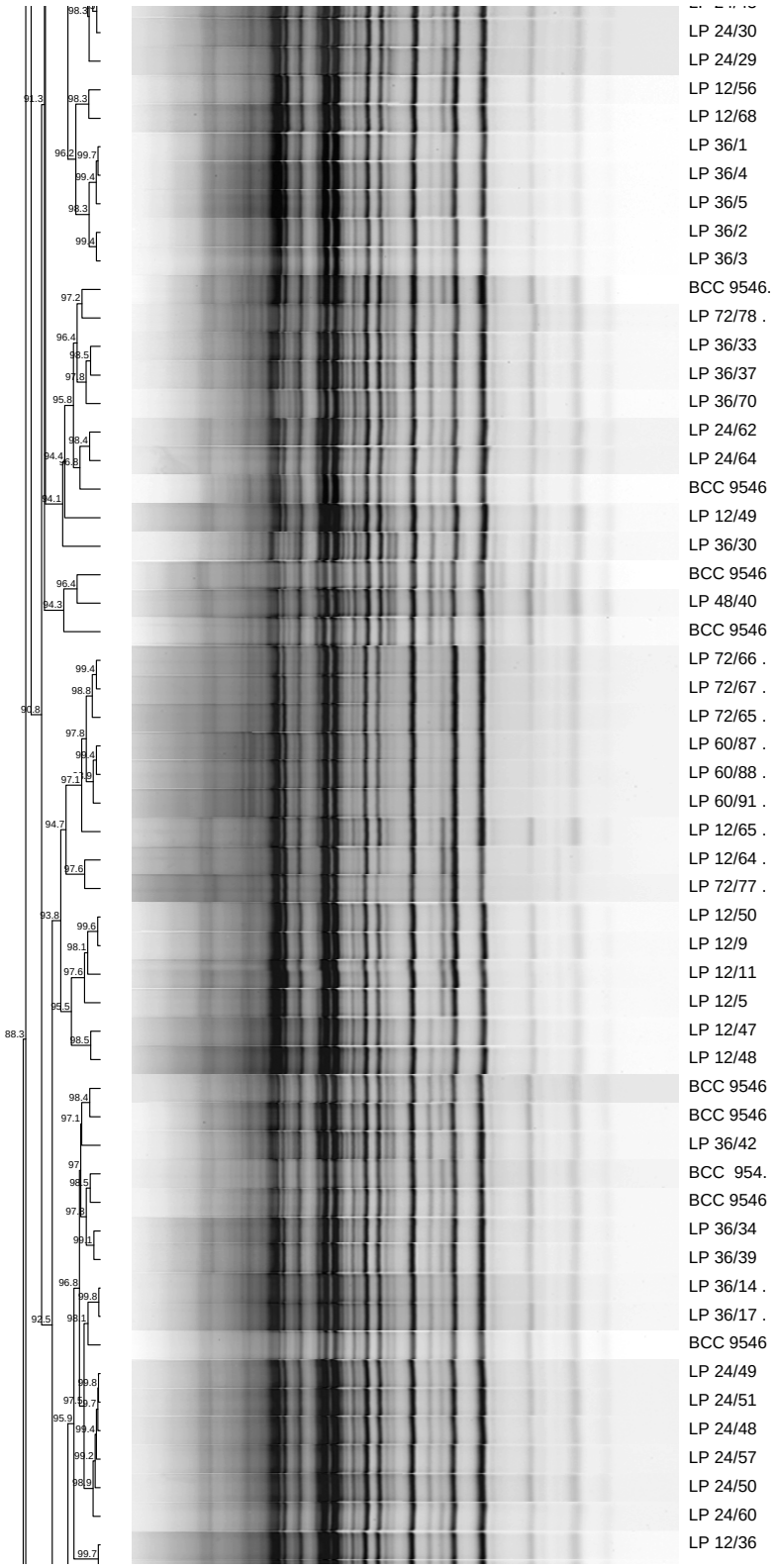
Appendix Figure F17 (Continued)



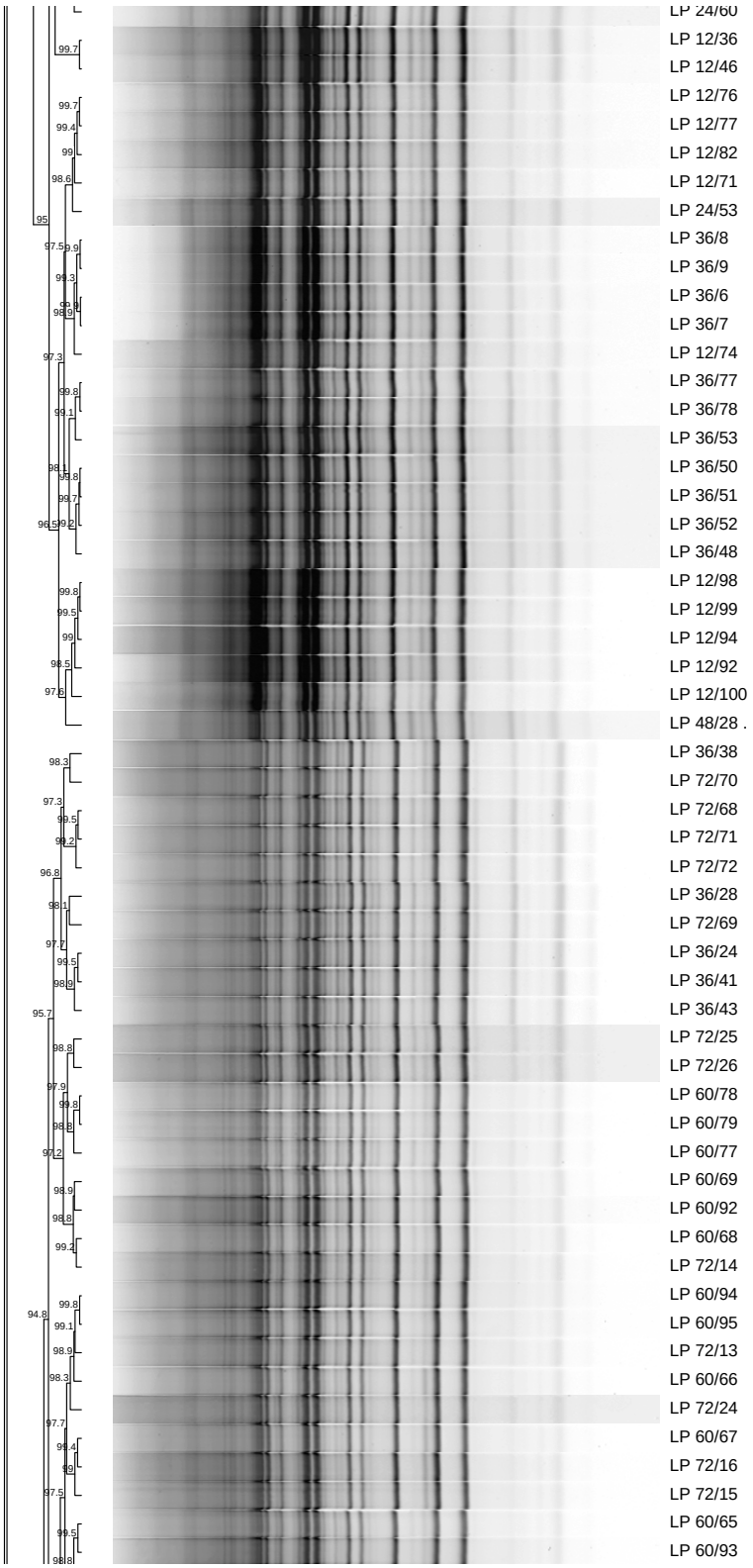
Appendix Figure F17 (Continued)



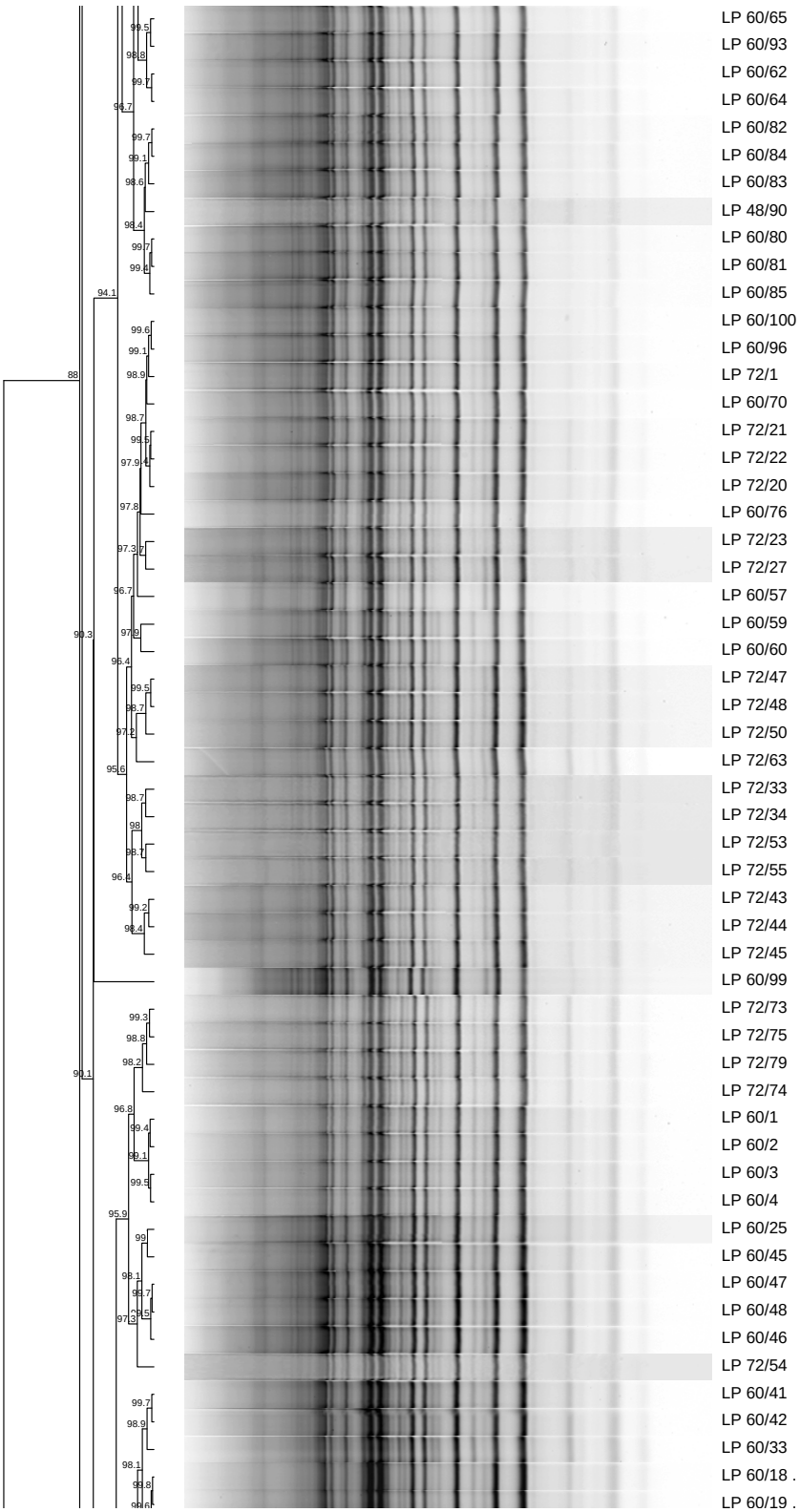
Appendix Figure F17 (Continued)



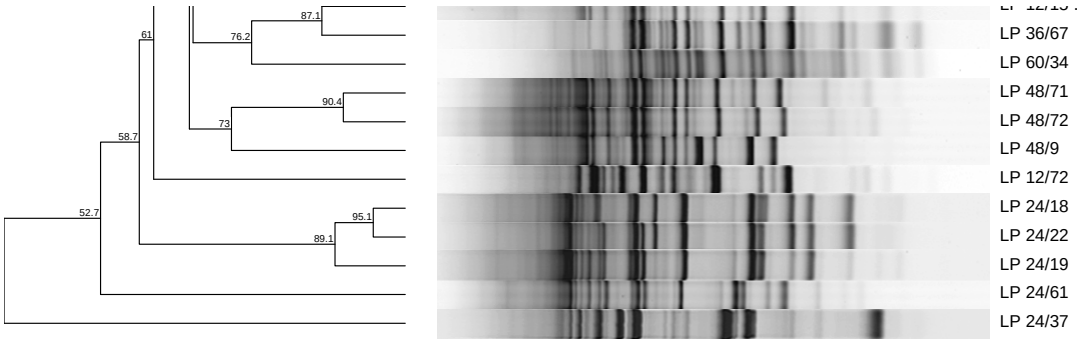
Appendix Figure F17 (Continued)



Appendix Figure F17 (Continued)



Appendix Figure F17 (Continued)



Appendix Figure F17 (Continued)

CIRRICULUM VITAE

NAME : Ms. Kittima Kongtong

BIRTH DATE : January 27, 1978

BIRTH PLACE : Lopburi, Thailand

EDUCATION :	**YEAR**	**INSTITUTE**	**DEGREE/DIPLOMA**
	2000	Mahidol Univ.	B.Sc. (Biotechnology)

SCHOLARSHIP LGS Scholarship for Graduate Student, National Center for Genetic Engineering and Biotechnology National Science and Technology Development Agency (BIOTEC), Thailand. (2005-2007)

ORAL PRESENTATION Comparison of microbiological and chemical changes in spontaneous and starter culture inoculated Nham during the early stage of fermentation, The 18 th Annual Meeting of Thai Society for Biotechnology, November 2-3, 2006, Thailand

POSTER Characterization of *Lb. plantarum* in starter cultured Nham fermentation using repetitive sequence-based PCR**,** The 19 th Annual Meeting of Thai Society for Biotechnology, October 9-12, 2007, Thailand