CHAPTER IV
RESULTS AND DISCUSSION

1. Sampling, Enumeration and Isolation of the Microorganisms Derived from Mhom Produced Locally in the Northeastern of Thailand

The fermented Mhom are common products throughout the Northeastern part of Thailand. Differences in the composition, ripening and fermentation conditions exist within different provinces. Mhom fermentation is a well-known microbial process. In this part of the study, traditional and naturally fermented Mhom products manufactured locally in four provinces from Northeastern Thailand were followed to detect, quantify and isolate microorganisms involved in the manufacturing process.

1.1 Mhom sampling

Samples (5 g) of the traditional Thai fermented dry sausage, Mhom, produced from meat and collected from each of different local markets in four provinces in the Northeastern part of Thailand, i.e., Chaiyaphum (CP), Khon Kaen (KK), Mahasarakham (MK), and Kalasin (KS) in which the local people commonly produce in large scale for the commercial purpose, were compared in this study. The sampling points, from different locations of Northeastern Thailand, were selected on the basis of different traditional technologies used for production, without the use of starter cultures. Samples were collected at 20-to-30-day-ripening periods, and were then kept under refrigeration until chemical analysis.

1.2 Physicochemical analysis

The physical-chemical parameters, i.e., pH and total acidity, were examined in all samples using the methods cited by Franco et al. (2002), and the results are summarized in Table 4.1. Mhom is formally characterized by the acidity with the final pH of about 4 to 6. In the present study, the average pH values of the ready-to-eat Mhom samples, such as CP1, CP2, KK, MK and KS, which have similar fermentation, or ripening periods of 20 to 30 days were virtually similar, i.e., 4.74, 4.94, 4.77, 4.30 and 4.76, respectively. Also, there were no significant difference in the pH values of all samples of different sources of the manufacturing process. The lowest and highest pH values were observed, respectively, in the MK and CP2

samples. The overall average pH value was 4.70, and this value was within the range of the standard pH value of Mhom products provided and accepted by the Community Product Standard No. 146/2546 issued by the Thai Industrial Standards Institute, Ministry of Industry, Thailand. This result indicated that Mhom samples after stuffing and ripening with the average pH value of 4.70 is suitable for consumption. Also, the overall average pH value of 4.70 was lower than the pH value of some fermented meat products reported previously (Comi et al., 2005, Fontana, Cocconcelli and Vignolo, 2005). However, the average pH value of Mhom was similar to those found in various sausages (Salgado et al., 2005; Visessanguan et al., 2005). It was found that the lowered pH greatly extends its shelf life by inhibiting the growth of many food spoilage and some pathogenic bacteria. Since Mhom is normally uncooked, but cured and ready to eat, so in order to ascertain the risk and safety of Mhom consumption from the survival and growth of some naturally occurring pathogenic microorganisms in which their contamination in the meat and meat products are always happened, it is recommended that the fermented sausages with the pH lower than 4.6 are safe for the human consumption (Paukatong and Kunawasen, 2001, Valyasevi and Rolle, 2002). The lowered pH values observed in any Mhom samples in spite of their source of production revealed that they may have certain organic acids, such as lactic acid, acetic acid and oxalic acid, produced by some type of bacteria, such as lactic acid bacteria (LAB), during the fermentation period of Mhom production. Thus, the determination of total acidity and quantification analysis of different bacterial groups were further determined in the next step.

Two main microbial populations that are involved in the fermentation of sausages and their main transformations that lead to the final product involve the activity of two microbial groups, i.e., LAB and micro/staphylococci (Rantsiou et al., 2005). It has been reported previously that LAB are responsible for the acidification, while the micro/staphylococci produce lipases, eventually releasing short-chain fatty acids that are responsible for the aroma of the naturally fermented sausage (Rantsiou et al., 2005; 2006). In the current study, the measurement of total acidity resulted from the most common and predominant acid produced by LAB during the fermentation period, i.e., lactic acid, was determined in all Mhom samples. With similar time period of fermentation, i.e., 20 to 30 days, the percentage values of total acidity found

in CP1, CP2, KK, MK and KS samples were 1.16, 0.78, 1.56, 0.94 and 0.86, respectively. The lowest and highest acidity were observed, respectively, in the CP2 and KK samples. The average percentage of total acidity of all samples was 1.06. It is noticeable that total acidity, mainly from lactic acid was related to the decreased pH values. It has been long known that LAB are the major producers of lactic acid responsible for the decrease in pH and increase in acidity during the fermentation of the traditional Thai-fermented sausage, called Nham (Visessanguan et al., 2004). Therefore, the lowered pH values found in any samples observed in the present study were probably due to the increased amount of lactic acid, produced by LAB during the fermentation process of Mhom. However, the level of decreased pH values in any sample were not completely correlated to the percentage of total acidity observed from each source of Mhom production. To explain this issue, the prominent examples were the MK samples in which the lowest LAB count was found, but however, the pH value and percentage of total acidity was lowest. From this point of view, there may be another organic acids produced during the fermentation period. To support this hypothesis, Visessanguan et al. (2004) reported that among the organic acids detected during the time of fermentation of the Nham, lactic acid was found the most dominant followed by acetic acid and oxalic acid. The other organic acids, such as acetic, butyric and succinic acids were found with inconsiderable amount. Thus, the results from the previous study of Visessanguan et al. (2004) may explain why in the present study the values of total acidity, pH and LAB counts were not correlated to each other. Therefore, identification of the other organic acids produced during the fermentation period of Mhom production warrants further study.

As described above, the inverse relationship between the values of total acidity and pH in the final products was observed in the present study. This phenomenon was prominent in the CP2 samples. In general, total acidity increased significantly during the first seven days of the ripening period, then more and more slowly afterwards until the 14^{th} day, when it gradually decreased until the end of the ripening period. The decreased values of total acidity could be related to the growth and development of other micro flora. Of these, molds and yeasts are of primary relevace as they are capable of consuming lactic acid in the presence of an organic nutrient for the bacteria (Salgado et al., 2005).

1.3 Microbiological analysis

The analysis of microbiological load of Mhom samples, i.e., counts of total aerobic bacteria, LAB, yeasts and molds, *Micrococcaceae*, Enterococci, *Staphylococcus aureus*, Coliform bacteria and *Escherichia coli* strain O157:H7 was carried out and the results are summarized in Table 4.1.

Table 4.1 Chemical and microbiological analyses of Mhom samples (n = 5) from five different locations in the Northeastern Thailand

Microorganism (cfu/g)	Sources					
	CP1	CP2	MK	KK	KS	Average
pH	4.74 b	4.94 a	4.30 c	4.77 b	4.76 b	4.70
Total acidity (%)	1.16 b	0.78 c	0.94 c	1.56 a	0.86 c	1.06
Aerobic plate count	6.0×10^7 b	6.1×10^6 d	1.4×10^8 a	2.0×10^7 c	3.2×10^7 c	5.0×10^7
Yeast and Mold	7.3×10^6 e	3.9×10^7 b	1.2×10^7 d	2.6×10^7 c	1.6×10^8 a	4.9×10^7
LAB	6.0×10^6 b	7.8×10^6 b	5.4×10^6 b	6.4×10^6 b	1.0×10^7 a	7.1×10^6
Micrococcaceae	2.7×10^4 d	7.5×10^5 a	2.8×10^4 d	3.7×10^4 c	3.9×10^5 b	2.5×10^5
Enterococci	4.6×10^4 d	6.8×10^4 c	1.9×10^4 e	9.6×10^4 b	5.5×10^5 a	1.6×10^5
Staphylococcus aureus	3.3×10^3 d	2.7×10^6 a	9.8×10^3 d	6.3×10^4 c	8.5×10^4 b	5.7×10^5
Coliforms	2.4×10^2 d	9.6×10^2 b	2.2×10^2 d	6.2×10^3 a	3.3×10^2 c	1.6×10^3
E. coli O157:H7	ND	ND	ND	ND	ND	

LAB: Lactic acid bacteria, CP: Chaiyaphum, KK: Khon Kaen, MK: Maha Sarakham, KS: Kalasin.

Total acidity (% acidity as lactic acid), ND: not detected.

Subscript letters with different letters in the same row indicate significant difference ($p \leq 0.05$) analyzed by Scheffe test.

The results of detection and enumeration of total aerobic bacteria from all Mhom samples by using the aerobic plate counts showed high bacteria counts with the values ranging from 10^6 and 10^8 cfu/g (Table 4.1). The results indicated the presence of high levels of microbial contamination in raw materials and ingredients used in Mhom production by the manufacturers. While the MK samples had the highest average aerobic plate count of 1.4×10^8 cfu/g, the CP2 samples had the lowest count of 6.1×10^6 cfu/g. The overall average count of total aerobic bacteria was 5.0×10^7 cfu/g (Table 4.1).

Over the last few years, the novel means and benefits to develop and add the specific LAB strains for our food products have been discovered. LAB display several important roles in the ripening of fermented meat, to improve safety and stability of the sausages and of providing health benefits with its probiotic properties and characteristics to balance out the bacteria in the body by keeping a steady supply of the "friendly" bacteria so that one is destroyed by potential illness, another is present to take its place, as reported in a recent review by Lucke (2000). In agreement with the results obtained from previous studies on the other meat sausages (Gonzalez and Diez, 2002), LAB were the major microbial groups. In the present study, the total LAB counts were 6.0×10^6, 7.8×10^6, 5.4×10^6, 6.4×10^6 and 1.0×10^7 cfu/g for CP1, CP2, KK, MK and KS, respectively (Table 4.1). The average count of LAB observed in this study was 7.10×10^6 cfu/g. This average value was similar to those observed in the traditional Spanish pork sausages, such as Androlla (Fontan et al., 2007) and Botillo (Maria et al., 2007) and Greek fermented sausage (Drosinos et al., 2005), but this average value was slightly higher than those observed in sausages produced locally in Northeast Italy (Comi et al., 2005), dry-cured lacon, a Spanish traditional meat product (Vilar et al., 2000) and buffalo sausage (Sachindra et al., 2005).

It has been reported that the fermented sausages are considered good substrates for the growth of yeasts and molds. Yeasts also play an important role in sausage fermentation and bring about characteristics flavors and surface appearance (Romano et al., 2006). Previous studies carried out with different yeast starters, mainly Debaryomyces hansenii and Candida famata, have shown the contribution of yeasts to the development of color by removing the oxygen, and flavor (Juan-Pablo

et al., 2000) due to their ability to degrade peroxides, lipolytic activity and, to a lesser extent, proteolytic activity (Luecke, 1985 cited in Romano et al., 2006). Furthermore, yeasts are capable of protecting fermented sausages from the adverse effects of light. The results of yeast and mold counts in Mhom products collected from different locations in Northeast Thailand varied with the values ranging from the highest level of 7.3×10^6 cfu/g to the lowest level of 1.6×10^8 cfu/g, with the average count of 4.9×10^7 cfu/g (Table 4.1). The results obtained from the Scheffe test showed that yeasts and molds counts were reached the highest ($p \leq 0.05$) in the KS samples. The results indicate differences in sanitation practices of different manufacturing houses. In fact, it was believed that yeasts delay rancidity and protect the red nitrosomyoglobin from breakdown by degrading peroxides and consuming oxygen, thus stabilizing the appealing red color of fermented sausages, indicating that the product is fully cured (Romano et al., 2006). Yeast and mold counts found in the current study were really quite similar to that observed in sucuk, a Turkish dry-fermented sausage (Aksu and Kaya, 2004). Nevertheless, the average yeast and mold counts in Mhom were slightly higher than those found in the Spanish traditional pork sausage, androlla (Fontana et al., 2007) and the sausages produced in Northeast Italy (Comi et al., 2005). The evidence of yeasts and molds contamination in the fermented meat products is commonly found due to the initial microbial contamination of raw materials used in the manufacturing process, and the other contamination produced during shipping, handling, or storage. Although yeasts and molds are not totally pathogenic, The present study further provides state-of-the-art technology for controlling contamination during manufacturing process, shipping, handling, or storage of Mhom locally produced in Northeast Thailand.

The numbers of yeast colonies also help to explain the amount of lactic acid and pH level found in the fermented meat products (Juan-Pablo et al., 2000). In general, yeasts are potent consumers of lactic acid in foods, and this leads to the increase in pH (Juan-Pablo et al., 2000, Walker, 1977). This was most evident in the CP2 and KS samples.

As has been previously reported, the sausages with a short ripening time have more lactobacilli from the early stages of fermentation, and at the end of ripening, an acid flavor with little aroma predominates. In contrast, sausages with

longer maturation times contain higher numbers of Micrococcaceae in the early stages of fermentation (Comi et al., 2005; Rantsiou et al., 2005). Micrococcaceae and LAB are the most important microorganisms used as starter cultures in the processing of dry-fermented sausages. Micrococcaceae participate in the preservation of meat products, avoiding rancidness and developing the typical red color due to the catalase and nitrate reductase reactions (Rantsiou et al., 2005Martin et al., 2007). Some Micrococcaceae strains are able to produce antimicrobial substances. It was found that Micrococcaceae have a low rate of acidification and produce protease and lipase and thus release various aromatic substances and organic acids (Rantsiou et al., 2005). In this study, Micrococcaceae were also found at high numbers ranging from 2.70×10^4 to 7.50×10^5 cfu/g, with the average value of 2.50×10^5 cfu/g (Table 4.1). This average value was slightly to be higher than the ones that observed in Northeast's Italian sausages (Comi et al., 2005).

The enterococci are also the important components of microbes involved with the ripening of sausages by which probably through their biochemical properties, such as glycolytic, proteolytic and lipolytic activities (Hugas et al., 2003). Therefore, enterococci possess several indispensible benefits contributing to the typical taste and flavor and color stability, and preventing the rancidity and release of various aromatic substances (Papamanoli et al., 2003). Enterococci have also the ability to produce the so-called bacteriocins, enterocins, which are small peptides providing with antimicrobial activity towards the closely related gram-positive bacteria, including spoilage or pathogenic bacteria, such as Listeria. Moreover, enterococci are used in some countries as probiotics (Franz et al., 2003). In the present study, the enterococci counts of Mhom samples from different locations were not statistically significant. The highest enterococci count of 5.5×10^5 cfu/g was found in the samples from KS (Table 4.1). The average enterococci count was 1.60×10^5 cfu/g, and this value was slightly to be lower than those reported in Greek fermented sausages (Papamanoli et al., 2003), Northeast Italian sausages (Comi et al., 2005) and Spanish sausages (Fontan et al., 2007). Enterococci may be used as starter cultures in some foods and are commercially available as probiotic cultures for preventing and treating intestinal disorders in animals and humans (Stiles and Holzapfel, 1997). However, this genus may also be negatively associated with foods

due to its possible implication as an indicator of faecal contamination (Franz, Holzapfel and Stiles, 1999).

Recently, it has been reported that the persistence of enterococci during ripening can be attributed to their wide range of growth temperatures and their high tolerance to salt. It is very likely that in sausages, no hurdles are found for their inhibition, allowing them to coexist with lactobacilli as the dominant populations during the fermentation process. Also, the growth of LAB in meat products can provide an ecological niche advantageous for the enterococci population (Hugas et al., 2003; Rantsiou et al., 2005). The information described above may help to explain the presence of the highest numbers of both LAB and enterococci in the KS samples.

As have been given by the previous report, the decreased pH caused by the high acidifying capability of the endogenous LAB micro flora results in the reduction of Micrococcaceae and Enterococci counts (Raccah, 1992). Similar results were observed in this study. The Micrococcaceae and Enterococci counts were found to be reduced and reached the lowest numbers in the MK samples that possess the lowest average pH value (Table 4.1).

Staphylococcus aureus is an important foodborne pathogen in fermented meat products (Kang and Fung, 2000). For S. aureus to grow, production methods play a very important role in dry and semi-dry sausage. For example, fermentation temperature applied at 30–40 °C allows possible growth of S. aureus and its enterotoxin production (Kaban and Kaya, 2006). Since S. aureus, which is salt and nitrite tolerant, is also able to grow under anaerobic conditions, there is an increased risk that it will grow and produce toxins. The first hours and days of fermentation are particularly critical. It has been therefore demonstrated that different types of fermented sausages are implicated in staphylococcal food poisoning outbreaks. In the current study, S. aureus counts varied with the values ranging from 3.3×10^3 to 2.7×10^6 cfu/g, with the average value of 5.7×10^5 cfu/g (Table 4.1). The *S. aureus* count of the CP2 sample was the highest ($p \leq 0.05$), whereas the lowest count was in the CP sample. It is noticeable that the numbers of S. aureus found in all Mhom samples were within the wide range of S. aureus counts reported in the previous literature for different raw-cured sausages (Comi et al., 2005, Fontana et al., 2007). We assume that the contamination of all Mhom samples with S. aureus probably occurred during the

handling of the raw materials and Mhom processing. These results indicate poor food hygiene in the handling of the process and in the manufacturing plants of Mhom samples tested in CP, MK, KK and KS. It is recommended that Mhom products produced locally in such provinces should be consumed as a cooked food.

Coliforms represent one of the most important causes of spoilage in sausages if they can grow to sufficiently high numbers, surviving through the seasoning and manufacturing processes. The pH value and the water activity value (A_w) are the most important preservative factors for slowing and then stopping the growth of these spoilage bacteria. As the seasoning process develops, the sausages become increasingly dehydrated, and the lower the water content of the products the higher is the NaCl concentration. In seasoned sausages the antimicrobial effect of NaCl acts in combination with nitrates and nitrites (Colavita et al., 2003). In the present study, there was present significant difference between sampling locations in coliform counts (Table 4.1). The numbers of coliforms in all Mhom samples were found to be in the range of 2.2×10^2 to 6.2×10^3, with the average value of 1.6×10^3 cfu/g. The highest number was present in the KK samples, whereas the lowest number was found in the MK samples. These results were caused probably by the different time period of the final drying of Mhom what was done at ambient temperature. It has been recently found that higher temperature favoured development of coliforms and heat resistant bacteria (Walczycka et al., 2009).

It has been long known that *E. coli* O157:H7 is the most common and important foodborne pathogen identified in humans. Dry fermented sausages have been identified as a source of *E. coli* O157:H7 in eastern as well as western countries, like the US and Canada. Laboratory confirmed cases of *E. coli* O157:H7 have been linked to the consumption of dry cured sausage. Studies have shown that *E. coli* O157:H7 can tolerate acidic conditions and survive many of the typical dry fermentation processing conditions (Williams et al., 2000). These findings have led to significant changes in the industry and in the manufacturing of dry fermented sausage because the infective dose of *E. coli* O157:H7 is very low and any survival in a ready-to-eat product has the potential to cause illness. *Escherichia coli* O157:H7 may be also present in raw ground beef that is used in the manufacturing of other meat products.

Nevertheless, *E. coli* O157:H7 was not detected in any of the Mhom samples tested in the present study.

In summary, the physicochemical and microbiological quality of Mhom samples obtained in a traditional way, i.e., bought at the direct sale stalls, differed significantly concerning the source of manufacturers. These results may be possibly due to the raw materials and ingredients used, and the manufacturing process. Also there is possible bacterionic influence of acidifying micro flora on pathogens found in Mhom during the time period of fermentation. However, the results obtained concerning the detection, quantification and isolation of microorganisms derived from Mhom produced locally in Northeastern Thailand allow for the conclusion that those products generally are consistent with the Community Product Standard issued by the Thai Industrial Standards Institute as far as their physicochemical composition, but the micrological load is somewhat not appropriate. Because Mhom products are normally scalded at home after purchase, most of the vegetative microorganisms present are eliminated so the product is rather safe for consumption. It can be generally concluded that the directly sold Mhom products are rather microbiologically unstable, but they are still bought and consumed for their traditional sensory characteristics. From the nutritional point of view, Mhom products have some valuable physicochemical composition, i.e., decreased pH and increased acidity, thereby preventing the growth and development of other naturally occurring pathogenic bacteria.

2. Biochemical, Molecular Characterization and Identification of the LAB Isolated from Mhom Produced Locally in the Northeastern of Thailand

Recently, molecular tools, based on the identification of carbohydrate fermentation profiles derived from the API 50 CH strips and API CHL medium and sequencing of 16S rDNA, have been developed and adapted for identification of LAB spp. isolated from the fermented meat sausages (Cocolin et al., 2001; Rantsiou et al., 2005; 2006). In this paper, traditional, natural fermentations performed in three countries from Central and Southern Europe, Hungary, Italy and Greece, were followed to study.

In this part of the study, the main purpose was to molecular characterize and identify the LAB strains isolated from naturally fermented Mhom, produced in five different locations in Northeastern Thailand, in order to investigate the differences of strains coming from different producing areas and to study the dynamics of LAB populations involved in the manufacturing process. Seventy one strains were isolated throughout the fermentation periods on MRS agar. They were identified by carbohydrate fermentation profiles derived from the API 50 CH strips and API CHL medium, RAPD-PCR and sequencing of the bacteria cell have mitochondrial 16S rRNA gene.

2.1 Characterization and identification of the LAB isolates utilizing the carbohydrate fermentation profiles derived from the API 50 CH strip and API CHL medium

71 isolated LAB strains were obtained from MRS agar, and identified to the species level by using the API 50 CH strip and API CHL medium. The strains isolated were tested for cell morphology, gram reaction. Catalase was detected by dropping a 3% H_2O_2 solution directly onto each agar plate. The most abundant LAB in Mhom were *Lactobacilli* spp. (*L. plantarum*, *L. curvatus*, *L. delbrueckii*, *L. acidophilus*, *L. paracasei*, *L. brevis 1*, *L. brevis 3*, *L. pentosus* and *L. mesenteroides*), followed by *Pediococcus pentosaceus 1*, *Pediococcus pentosaceus 2* and *Carnobacterium divergens*, respectively. Homofermentative lactobacilli accounted for 88.73% of the isolates obtained from MRS agar.

The results from these biochemical tests were obtained by the computer program APILAB Plus (API system, Bio-Merieux, France). The results from computer analysis are shown in Table 4.2. Our results revealed that the first group (99.9% identity) consisted of 45 strains identified as *Lactobacillus plantarum*. Second group (99.7 %) consisted of 3 strains isolated and identified as *Lactobacillus cuvatus*. Third group (97.3%) consisted of 4 strains isolated and identified as *Lactobacillus delbrueckii*. Fourth group (99.0%) consisted of 2 strains isolated and identified as *Lactobacillus acidophilus*. Fifth group (98.3%) consisted of 4 strains isolated and identified as *Lactobacillus paracasei*. Sixth group (87.3%) consisted of 2 strains isolated and identified as *Lactobacillus brevis 1*. Seventh group (99.5%) consisted of 2 strains isolated and identified as *Lactobacillus brevis 3*. Eighth group

(98.0%) consisted of 1 strain isolated and identified as *Lactobacillus pentosus*. Ninth group (96.9%) consisted of 1 strain isolated and identified as *Lactobacillus mesenteroides*. Tenth group (99.5%) consisted of 1 strain isolated and identified as *Carnobacterium divergens*. Eleventh group (99.9%) consisted of 5 strains isolated and identified as *Pediococcus pentosaceus 1*, while the last group (99.9%) consisted of 2 strains isolated and identified as *Pediococcus pentosaceus 3*.

Table 4.3 shows the geographical distribution among LAB populations isolated from Mhom products collected from five different sources in the Northeastern part of Thailand. The molecular identification was based on API 50 CH strip. The results showed that *Lactobacillus plantarum* was mostly found in the CP1, CP2 and MK samples, and was the main species (61.97%) of *Lactobacillus* in Mhom, especially in the sample from CP1 (50%), whereas *Pediococcus pentosaceus 1* was found in the KS samples. Interestingly, our results demonstrated that Mhom samples collected from each sampling site consisted much more than one species of LAB. The total number of LAB species was the most abundant in CP1, followed by MK, CP2, KK and KS, respectively. In CP1 region Mhom samples comprised of seven different LAB species. In contrast, in KS region there were only three different species. *L. plantarum*, together with *L. curvatus*, were the two lactobacilli species most commonly isolated from meat and meat products, including sausages elaborated with different technologies (Schillinger and Lucke, 1987). Recently, *L. plantarum* has also been isolated in the other raw-cured sausages (Coppola et al., 2000), but in larger proportion to that observed in this study. *L. delbrueckii* was present with a similar proportion to that of *L. paracasei*, accounting for 5.63% of the isolates purified on MRS agar.

Table 4.2 LAB strains isolated from Mhom and characterized using the API 50 test.

Strain number	API 50 CH identification	ID (%)
CP101, CP102, CP105, CP106, CP107, CP108, CP109, CP110, CP111, CP112, CP113, CP115, CP116, CP117, CP118, CP120, CP121, CP122, CP124, CP125, CP127, CP129, CP201, CP202, CP203, CP204, CP205, CP207, CP208, CP211, CP214, MK101, MK104, MK105, MK106, MK107, MK108, MK110, MK111, MK113, MK114, KK102, KK105, KS114	*Lactobacillus plantarum*	99.9
CP103, KK103, KK104	*Lactobacillus curvatus*	99.7
CP104, KK101, MK102, MK112	*Lactobacillus delbrueckii*	97.3
CP114, CP123	*Lactobacillus acidophilus*	99.0
CP126, CP128, CP206, MK119	*Lactobacillus paracasei*	98.3
CP119, CP131	*Lactobacillus brevis 1*	87.3
CP209, CP213	*Lactobacillus brevis 3*	99.5
KK114	*Lactobacillus pentosus*	98.0
CP130	*Lactobacillus Mesenteroides*	96.9
MK103	*Carnobacterium divergens*	99.5
CP212, MK120, KS101, KS105, KS115	*Pediococcus pentosaceus 1*	99.9
KS103, KS113	*Pediococcus pentosaceus 2*	99.9

Table 4.3 Distribution and species identification based on API 50 CH of LAB isolated from Mhom products collected from five different manufacturing sites in Northeastern Thailand.

Species	Numbers of isolates identified						
	CP1	CP2	MK	KK	KS	Total isolates	(%)
Lactobacillus plantarum	22	9	10	2	1	44	61.97
Lactobacillus curvatus	1	-	-	2	-	3	4.23
Lactobacillus delbrueckii	1	-	2	1	-	4	5.63
Lactobacillus acidophilus	2	-	-	-	-	2	2.82
Lactobacillus paracasei	2	1	1	-	-	4	5.63
Lactobacillus brevis 1	2	-	-	-	-	2	2.82
Lactobacillus brevis 3	-	2	-	-	-	2	2.82
Lactobacillus pentosus	-	-	-	1	-	1	1.41
Lactobacillus Mesenteroides	1	-	-	-	-	1	1.41
Carnobacterium divergens	-	-	1	-	-	1	1.41
Pediococcus pentosaceus 1	-	1	1	-	3	5	7.04
Pediococcus pentosaceus 2	-	-	-	-	2	2	2.82
Total isolates	31	13	15	6	6	71	100
(%)	43.66	18.31	21.13	8.45	8.45	100	

2.2 Characterization and identification of LAB by using RAPD-based molecular markers

The API-50 CH strip and API CHL medium was firstly employed for the *in vitro* identification of the isolated LAB strains derived from 5 different sources of Mhom manufacturer, and 71 isolated LAB strains, i.e., 30 isolated strains from CP1 (CP101, CP102, CP103, CP104, CP105, CP106, CP107, CP108, CP109, CP110, CP111, CP112, CP113, CP114, CP115, CP116, CP117, CP118, CP119, CP120, CP121, CP122, CP123, CP124, CP125, CP126, CP127, CP128, CP129, CP130 and CP131), 9 isolated strains from CP2 (CP201, CP202, CP203, CP204, CP205, CP206, CP207, CP208, CP209, CP211, CP212, CP213, and CP214), 10 isolated strains from MK (MK112, MK113, MK114, MK115, MK116, MK117, MK118, MK110, MK111, MK112, MK113 MK114, MK119 and MK120), 6 isolated strains from KK (KK101, KK102, KK103, KK104, KK105 and KK114), and 6 isolated strains from KS (KS101, KS103, KS105, KS113, KS114 and KS115) were identified based on their biochemical profile without knowing the information of their genetic background. Therefore, the main purpose of this experiment was to characterize and identify the genetic background of 71 isolated LAB strains by using Rapid Amplification of Polymorphic DNA (RAPD)-PCR technique and DNA sequencing of 16S rDNA as molecular markers.

The pattern obtained from the biochemical profile analysis revealed that 71 isolated strains were divided into distinct three major groups, i.e. *Lactobacillus, Pediococcus* and *Enterococcus*. RAPD-PCR analysis was then employed to confirm the affiliation of 71 isolated LAB strains. As the first test of three random primers, two RAPD primers of known sequence, i.e., LMPB1 5′-GGAACTGCTA-3′ and LMPB4 5′-AAGGATCAGC-3′, were used in PCR amplification of genomic DNA extracted from of 71 isolated LAB strains.

Our results demonstrated that the RAPD primer LMPB1 gave the similar pattern of RAPD banding with genomic DNA isolated from CP214, MK102, MK105, MK106, MK107, MK109, MK111 and MK112. In contrast, the KK115 and KK103 isolated strains showed a unique RAPD band pattern (Fig 4.1A). The isolated strains KK104 and KK105 showed exactly the same pattern of RAPD banding (Fig 4.1B; Lane 13 and 14), whereas the isolated strain CP126 showed nearly the

identical RAPD band pattern with those of the CP126 and CP206 isolated strains (Fig 4.1B; Lane 17 and 19). These isolated strains showed the RAPD bands which were distinguishable from the other isolated strains (Fig 4.1B; Lane 12, 13, 16, 17, 18 and 20). The MK113, MK101 and MK103 isolated strains gave the identical pattern of RAPD banding, especially the 800-bp band which observed in these three isolated strains and the 1400-bp band which found in MK113 (Fig 4.1C; Lane 22, 23 and 24). The CP212, KS101, KS102, KS104 and KS106 isolated strains gave rise to the similar pattern of RAPD banding (Fig 4.1C; Lane 26, 27, 28, 29 and 30), whereas the other isolated strains showed the distinguishable RAPD band patterns as already described above (Fig 4.1C). The CP130, MK104 and CP210 isolated strains presented the unique pattern of RAPD banding from each other (Fig 4.1D).

The RAPD primer LMPB4 was then applied for PCR amplification of the genomic DNA extracted from of 71 isolated LAB strains in order to compare and confirm the results obtained from the LMPB1 primer. From representative isolated strains (34 isolated strains) performed by RAPD-PCR and agarose gel electrophoresis analysis, the results revealed that CP214, MK102, MK105, MK106, MK107, MK109, MK111, MK112, and MK103 gave the indistinguishable PCR banding, except for the MK115 isolated strains (Fig. 4.2A; Lane 1, 2, 3, 4, 5, 6, 7, 8 and 10). The CP102, KK104 and KK105 isolated strains gave rise to an identical pattern of RAPD banding (Fig. 4.2B; Lane 11, 12 and 14), while the CP123, CP126, CP128, CP206, and KK101 isolated strains (Fig. 4.2B; Lane 16, 17, 18, 19 and 20) showed nearly the same pattern of RAPD banding. A particular 100-bp band was present in the DNA samples of the CP123, CP206 and KK101 isolated strains, but not present in the samples of KK109 and KK110 isolated strains (Fig. 4.2B; Lane, 16, 19 and 20), while the 1800-bp band was visible in CP123 and CP126 isolated strains (Fig. 4.2B; Lane 16 and 17). The CP104, MK103, KK102, CP212, KS101, KS102, KS104 and KS106 isolated strains showed the same pattern of RAPD banding (Fig. 4.2C; Lane 21, 24, 25, 26, 27, 28, 29 and 30), whereas the MK113, MK101 and KS101 isolated strains (Fig. 4.2C; Lane 22, 23 and 27) revealed a different pattern of RAPD banding from other isolated LAB strains,

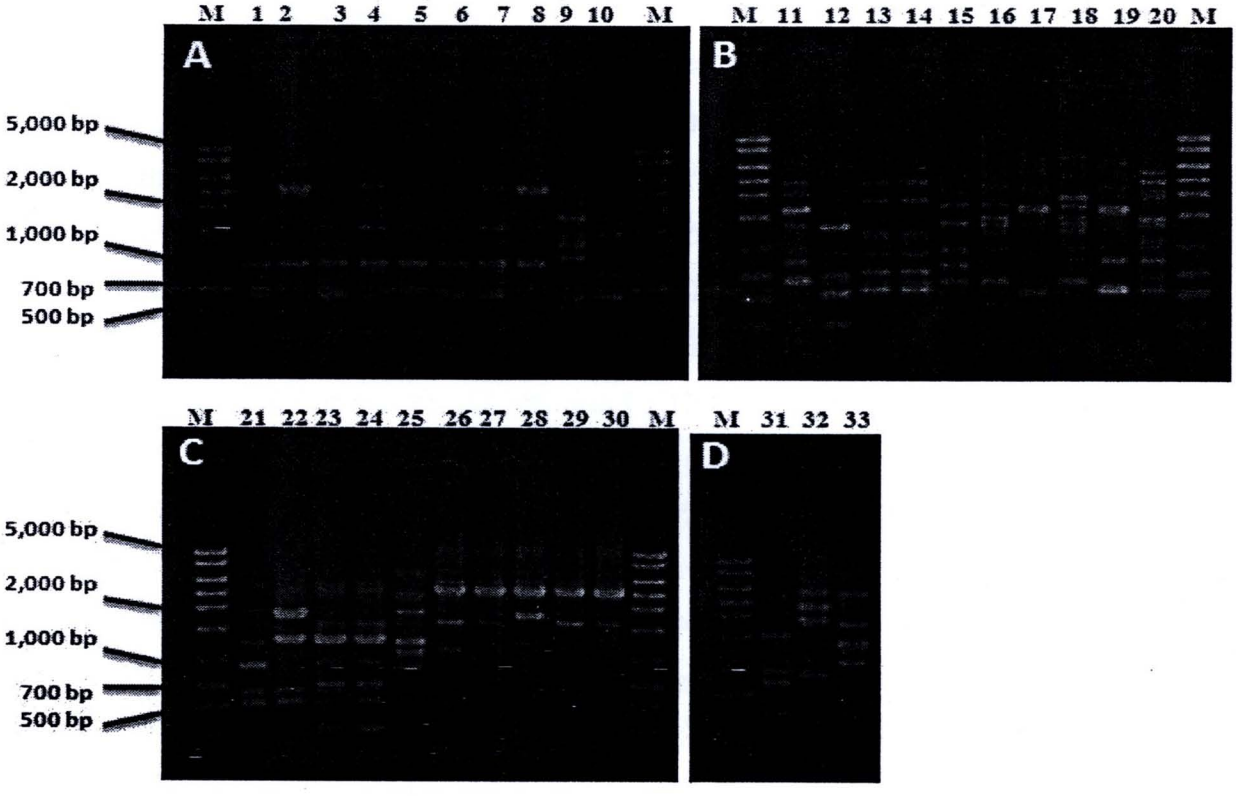

Figure 4.1 RAPD-PCR patterns of amplified DNA fragments obtained by primer LMPB1 from genomic DNA of 33 representative isolated LAB strains. **(A)** *L. plantarum* strains CP214 (Lane 1), MK102 (Lane 2), MK105 (Lane 3), MK106 (Lane 4), MK107 (Lane 5), MK109 (Lane 6), MK111 (Lane 7), MK112 (Lane 8), MK115 (Lane 9) and KK103 (Lane 10); **(B)** *L. curvatus* ssp *curvatus* strains CP103 (Lane 11), MK110 (Lane 12), KK104 (Lane 13), KK105 (Lane 14); *L. acidophilus* 1 strains CP114 (Lane 15) and CP123 (Lane 16); *L. paracasei* ssp *paracasei* 3 strains CP126 (Lane 17), CP128 (Lane 18), CP206 (Lane 19) and KK101 (Lane 20); **(C)** *L. delbrueckii ssp lactis* 2 strains CP104 (Lane 21) and MK 113 (Lane 22); *L. delbrueckii* ssp *delbrueckii* strains MK101 (Lane 23), MK103 (Lane 24) and KK102 (Lane 25); *Pediococcus pentosaceus 1* strains CP212 (Lane 26), KS101 (Lane 27), KS 102 (Lane 28) and KS106 (Lane 30), and KS104 *Pediococcus pentosaceus 2* (Lane 29); **(D)** CP130 *Leuconostoc mesenteroides ssp* (Lane 31); MK104*Carnobacterium divergens* (Lane 32); CP 210 *Lactobacillus fermentum* 1 (Lane 33). Lane M represents band and size of the 300-5000 bp DNA ladder molecular weight marker.

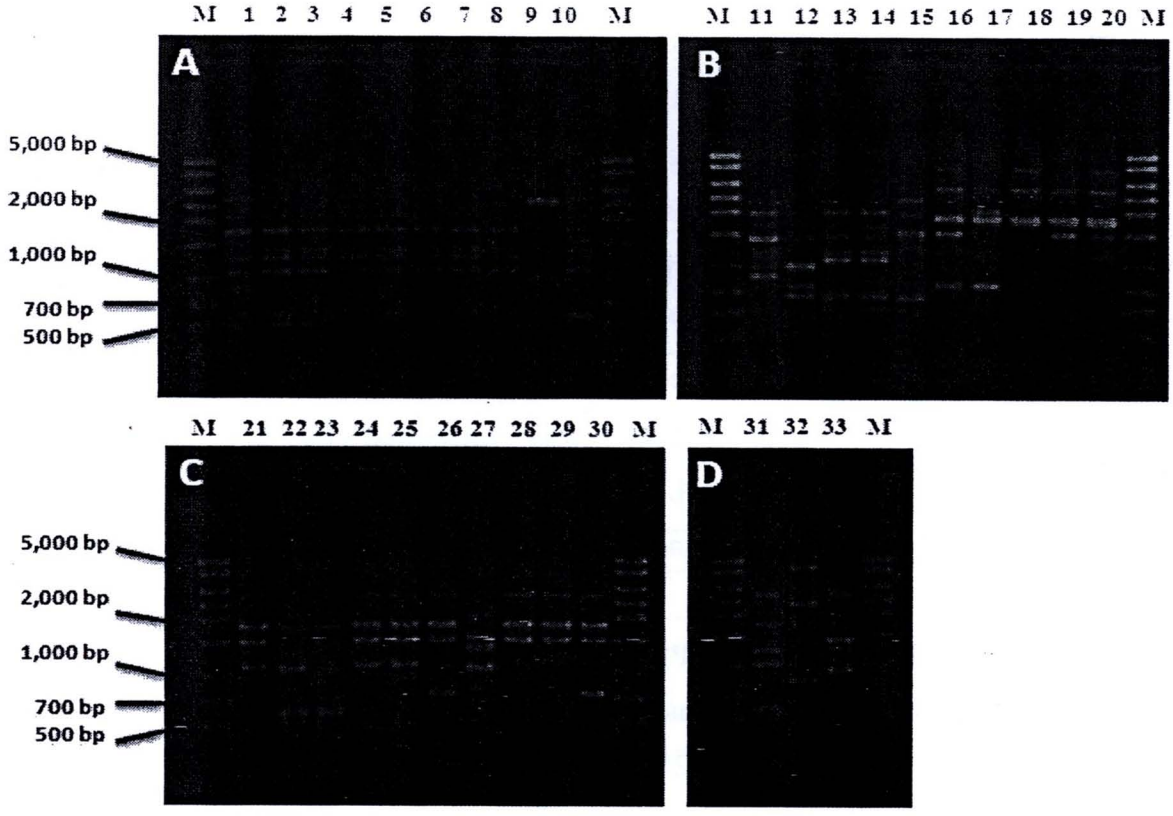

Figure 4.2 RAPD-PCR patterns of amplified DNA fragments obtained by primer LMPB4 from genomic DNA of 33 representative isolated LAB strains. **(A)** *L. plantarum* strains CP214 (Lane 1), MK102 (Lane 2), MK105 (Lane 3), MK106 (Lane 4), MK107 (Lane 5), MK109 (Lane 6), MK111 (Lane 7), MK112 (Lane 8), MK115 (Lane 9) and KK103 (Lane 10); **(B)** *L. curvatus* ssp *curvatus* strains CP103 (Lane 11), MK110 (Lane 12), KK104 (Lane 13), KK105 (Lane 14); *L. acidophilus* 1 strains CP114 (Lane 15) and CP123 (Lane 16); *L. paracasei* ssp *paracasei* 3 strains CP126 (Lane 17), CP128 (Lane 18), CP206 (Lane 19) and KK101 (Lane 20); **(C)** *L. delbrueckii ssp lactis* 2 strains CP104 (Lane 21) and MK 113 (Lane 22); *L. delbrueckii* ssp *delbrueckii* strains MK101 (Lane 23), MK103 (Lane 24) and KK102 (Lane 25); *Pediococcus pentosaceus 1* strains CP212 (Lane 26), KS101 (Lane 27), KS 102 (Lane 28) and KS106 (Lane 30); KS104 *Pediococcus pentosaceus 2* (Lane 29); **(D)** CP130 *Leuconostoc mesenteroides* ssp strain (Lane 31); MK104 *Carnobacterium divergens* (Lane 32); CP 210 *Lactobacillus fermentum* 1 (Lane 33). Lane M represents band and size of the 300-5000 bp DNA ladder.

Cluster analysis was applied to define relations and degrees of similarities between the isolated LAB strains. With respect to the cluster analysis of the profiles obtained by RAPD-PCR from the 71 isolated LAB strains from Mhom samples produced in Northeast Thailand, RAPD bands amplified by a suitable primer (LMPB4) were employed to construct a binomial matrix code on the presence or absence of a non-specific polymorphic DNA bands that appeared at a specific location. From the obtained RAPD–PCR profiles, genetic relationships among the 71 isolated LAB strains were clustered by computer analysis using a NTSYS-PC software package and a dendrogram was generated by UPGMA program. Dendrogram is illustrated in Figure 4.3. Briefly, 71 isolated strains were diverted into several distinct branching trees since their genetic background exhibited a big difference, i.e., not much isolated strains showed identical and similar genetic background to each other. The CP101, CP102, CP105, CP201, CP203, CP210, CP211, CP212 and CP225 were constituted into the same branch, while the CP227, CP229, CP201, CP202, CP204, CP205, CP207, CP211 and CP224 were present in another branch. The MK102, MK105, MK106, MK107, MK108, MK111, MK112, MK115 and KK105 were present in a lower branch. In addition, three distinct branches were closely related and constituted under the same large cluster at coefficient of 0.88 (Fig. 4.3). Most of the isolated strains distributed in several sub-branches of a dendrogram.

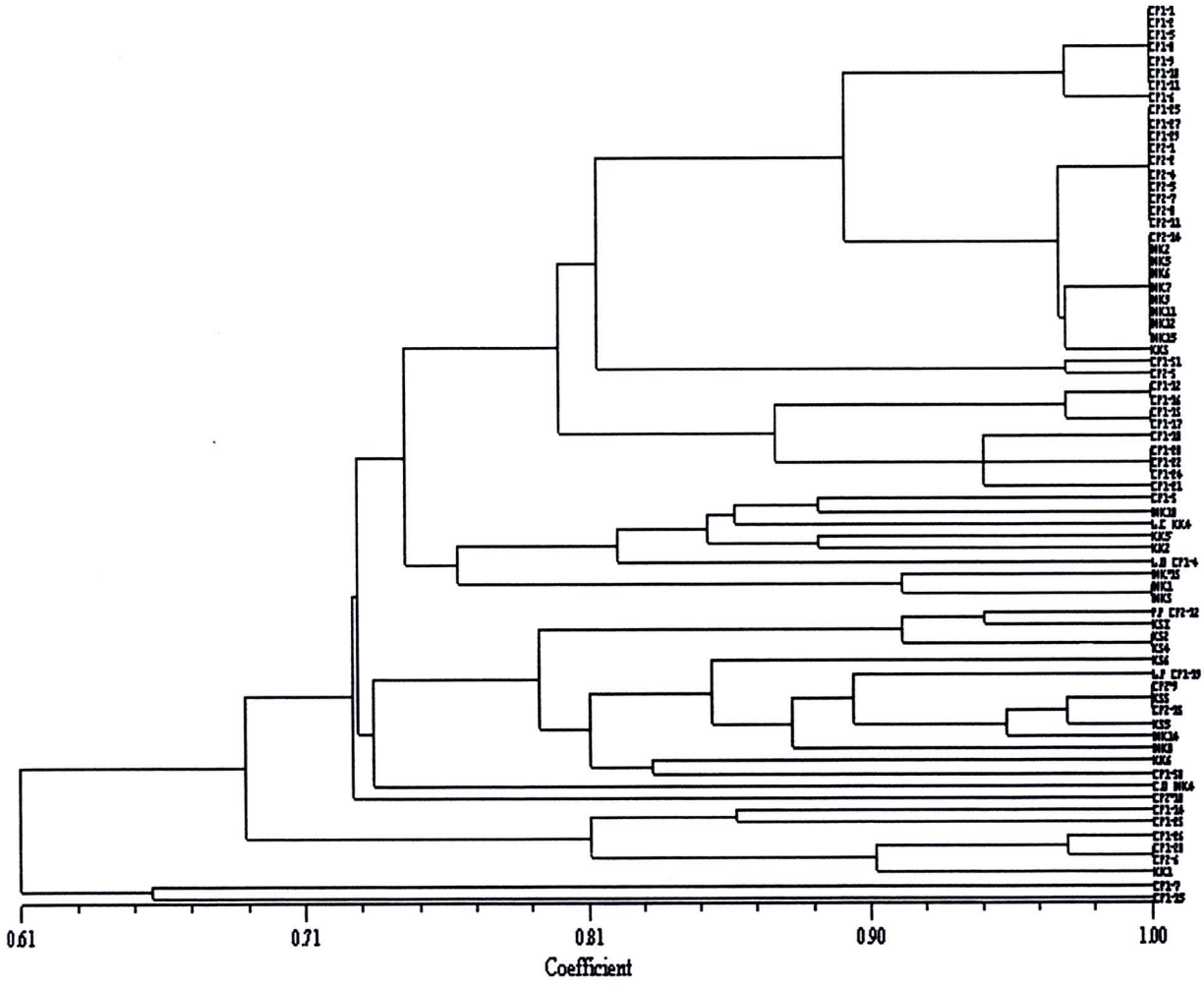

Figure 4.3 Dendogram of 71 isolated LAB strains based on RAPD-PCR analysis with the use of primer LMPB4. The dendogram was constructed from a distance matrix by using an UPGMA program.

2.3 Characterization and identification of LAB by sequencing of 16s rRNA gene

Chromosomal DNAs of 71 isolated strains were purified and amplified at the specific locus of the 16S rDNA gene by using two specific primers LABF and LABR. The sequence of the gene fragments obtained from the 16S rDNA of LABs were aligned for their identity to the other DNA sequences which are deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) by using the BLAST algorithm (Table 4.4). Comparison of the nucleotide sequences of the CP101, CP102, CP105, CP106, CP108, CP109, CP110, CP111, CP112, CP113, CP116, CP117, CP118,

CP120, CP121, CP122, CP124, CP125, CP127, CP129, CP130, CP131, CP201, CP202, CP203, CP204, CP205, CP207, CP208, CP210, CP211, CP214, KK103, MK102, MK105, MK106, MK107, MK109, MK111, MK112 and MK115, indicated 98 to 100% identity, and thereby classified as *Lactobacillus plantarum*. The nucleotide sequence identity of 10 isolated strains, i.e. CP104, CP114, CP123, CP128, KK104, KK105, MK101, MK103, MK110 and MK113, was 98-100%, and thereby classified as *Lactobacillus farciminis*. The other 7 isolated strains, i.e. CP103, CP107, CP115, CP126, CP206, KK101 and MK108, exhibited 98 to 100% nucleotide identity, and thereby classified as *Enterococcus*.

Table 4.4 List of the isolated LAB strains and degree percentage of their nucleotide identity compared to other sequences of 16S rDNA in the GenBank database

Isolated strains	**% Nucleotide identity**
CP101, CP102, CP105, CP106, CP108, CP109, CP110, CP111, CP112, CP113, CP116, CP117, CP118, CP120, CP121, CP122, CP124, CP125, CP127, CP129, CP130, CP131, CP201, CP202, CP203, CP204, CP205, CP207, CP208, CP210, CP211, CP214, KK103, MK102, MK105, MK106, MK107, MK109, MK111, MK112, MK115	(98-100%) *Lactobacillus plantarum*
CP104, CP114, CP123, CP128, KK104, KK105, MK101, MK103, MK110, MK113	(98-100%) *Lactobacillus farciminis*
CP119, CP209, CP212, KK106, KS102, KS103, KS104, KS105, KS106, MK114	(98-100%) *Pediococcus pentosaceus*
CP103, CP107, CP115, CP126, CP206, KK101, MK108	(98-100%) *Enterococcus*

The Clustal X and tree view program was employed to analyze the sequences of the 16S rDNA of the isolated LAB strains and to perform the phylogenetic tree analysis. Our results revealed that the phylogenetic tree of the isolated LAB strains based on their 16S rDNA was divided into eight major clusters. MK109, CP124, CP101, CP109, CP214, MK106, MK107, CP106, CP125, CP108, CP202, CP105, CP205, CP208, CP122, CP120, CP117, MK112, KK102, CP103, CP115, KK101, MK113, CP107 and CP126 were constituted into the first cluster and identified as *L. plantarum*1, whilst the second and third clusters were also identified as *L. plantarum*1. The fourth cluster was constituted by the MK113, CP128, MK101, CP104, CP114, KK105, CP125, KK104, MK103 and MK110. The CP119, KK106, KK103, KK105, KK102, MK114, KK101, KK104 were constituted the fifth cluster.The sixth cluster was constituted by the CP112, CP113 and CP127. The CP201, CP130, CP208, CP102, CP104, MK111, CP131, CP108 and CP211 were constituted the seventh cluster. The CP129, CP111, CP202, CP116, CP105, CP121 and MK102 were in the last cluster (Fig. 4.3)

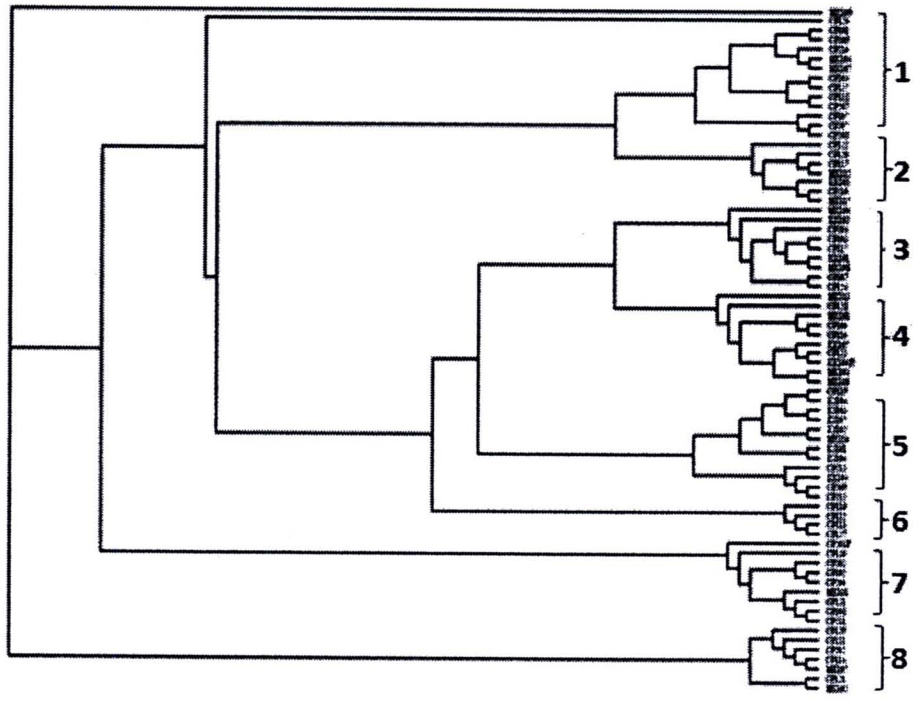

Figure 4.4 Cluster analysis of the profiles obtained by the homologous sequence analysis of 16S rDNA of the isolated LAB strains utilizing the BLAST program.

The RAPD-PCR technique is a molecular tool that has been used for taxonomy and breeding for decades. RAPD-PCR has discriminated the genetic background of several kinds of living organisms. RAPD-PCR was applied for the identification and characterization of LAB (Nigatu et al. 2001) and other bacteria isolated from various foods and food process (Buruzzi et al. 2005; Kastner et al. 2006). In 2006, Khaleda and colleagues identified the phylogetic tree of *Lactobacillus* isolated from the digestive tract of mice using Multiplex RAPD-PCR, and found that generated RAPD profiles were capable of discrimination of all *lactobacillus* strains (Daud Khaleda et al. 2006). In this study, RAPD-PCR was used as a molecular marker for characterization and classification of lactic acid bacteria isolated from 5 different sources of Mhom manufacturing. 71 isolated LAB strains were obtained and grouped based on the carbohydrate fermentation profiles (API-test). It was expected that the isolated LAB strains would carry the different genetic background although the API-test showed similar results and grouped them into the same cluster. Hence, RAPD–PCR was performed to genetically discriminate theses LAB by following the protocol typing of *Listeria* strains (Mazurier and Wernars, 1992). It was found that LMPB1 and LMPB4 were suitable primers for genetically discriminating LAB obtained from Mhoms. Another primer name could not distinguish the strain by RAPD. The particular DNA bands, i.e., 1500-bp, 1900-bp and 2300-bp, were present in *L. plantarum* 1 strains isolated from MK (Fig. 4.1A). These DNA bands might be linked with lactic acid genes. RAPD profiles generated by LMB4 primer were sufficient to construct the dendrogram (Fig. 4.3). A 16S rDNA gene of isolated bacterial strain was amplified and analyzed by Clustal X. 8 clusters were independently separated (Fig. 4.4). Based on the phylogenetic tree, it was divided into 8 major clusters. The first, second and third clusters are represented by *L. plantarum* 1 and theses results were in relative accordance with the results obtained from RAPD profiles and the API 50 test. Interestingly, it was found that *L. plantarum* showed the genetic background both similar, or different among LAB community. Once the LAB isolated strains collected from the same region and their genetic background were analyzed, the results revealed that they might be developed from the same origin, indicating the reliability of the techniques used in this study. RAPD-PCR and 16S rDNA gene sequencing was a powerful tool to discriminate the genetic background of

L.plantarum, *L. farciminis*, *P. pentosaceus* and *Enterococcus*, particularly the results obtained from 16S rDNA gene sequencing were relatively in accordance with the results of carbohydrate fermentation test. Although the CP114 and CP123 were identified as *L. acidophiles* based on the API 50 test, they were characterized as *L. faciminis* based on 16S rDNA gene sequencing. Thus, it is possible that their physiological characteristics are relevant to the genetic background.

3. Screening and Application of LAB as the Starter Cultures for Mhom Production

3.1 Microbiological quality of raw materials and ingredients

Raw materials and ingredients, such as unpeeled and peeled garlic, beef, liver and spleen, were evaluated for their microbiological load, including aerobic plate count, and counts of LAB, *Micrococcaceae*, coliforms, enterococci, *Escherichia coli* O157: H7, *Staphylococcus aureus*, yeasts and molds as previously described (Fontana et al., 2005, Rogosa et al., 1951). The results demonstrated that the initial flora found in Mhom originated mainly from raw materials and ingredients used in the manufacturing process. The data of microbiological load, i.e., total viable count, LAB count, *Micrococcaceae* count, coliforms count, enterococci count, *Escherichia coli* O157: H7 count, *Staphylococcus aureus* count, yeasts and molds count, of the raw materials and ingredients, such as unpeeled and peeled garlic, beef, liver, spleen, and natural casings are presented in Table 4.5.

Table 4.5 Microbiological quality of raw materials and ingredients used in Mhom locally produced in Northeast Thailand ($n = 3$)

Microorganism (cfu/g)	Raw materials/ Ingredients					
	Unpeeled garlic	Peeled garlic	Beef	Liver	Spleen	Natural casings
Total viable count	1.88×10^3 cd	3.67×10^1 d	4.40×10^5 a	8.27×10^4 b	3.17×10^4 bc	5.72×10^5 a
Yeasts and molds	1.40×10^3 c	1.06×10^2 c	7.57×10^4 a	2.29×10^4 b	1.41×10^4 b	1.34×10^2 c
Lactic acid bacteria	ND	ND	2.36×10^4 a	1.18×10^4 b	2.88×10^4 a	2.45×10^4 a
Micrococcaceae	1.20×10^2 c	9.00×10^1 c	2.20×10^4 b	6.03×10^4 a	3.23×10^4 b	2.70×10^4 b
Enterococci	ND	ND	6.63×10^3 b	2.47×10^3 c	1.57×10^4 a	2.26×10^3 c
Staphylococcus aureus	ND	ND	3.10×10^5 a	3.43×10^4 c	6.53×10^4 b	5.69×10^4 b
Coliforms	ND	ND	1.47×10^4 b	1.19×10^4 b	1.58×10^5 a	1.24×10^4 b
E. coli O157:H7	ND	ND	ND	ND	ND	ND

Different letters in the same row denote significant differences according to the LSD test ($p \leq 0.05$).

ND: not detected.

There was a significant difference between raw materials and ingredients in total viable count. The highest of total viable count were present in natural casings and meat. The highest count of 5.72 x 10^5 cfu/g was found in the natural casings, whereas the lowest count was observed in the ingredients used, such as peeled garlic.

Yeasts and molds were mainly found in the raw materials used, such as beef, liver and spleen, with the average count of 7.57 x 10^4, 2.29 x 10^4, and 1.41 x 10^4 cfu/g, respectively.

The high LAB counts of 2.88 x 10^4, 2.45 x 10^4, 2.36 x 10^4, and 1.18 x 10^4 cfu/g were respectively observed in spleen, natural casings, beef and liver.

As it can be seen, the numbers of *Micrococcaceae* found in the unpeeled and peeled garlic were quite low to the similar extent, i.e., 1.20 x 10^2 and 9.00 x 10^1 cfu/g, respectively, but their counts remain relatively high in the liver (6.03 x 10^4 cfu/g), spleen (3.23 x 10^4 cfu/g), natural casings (2.70 x 10^4 cfu/g) and beef (2.20 x 10^4 cfu/g).

The numbers of enterococci were found mainly in the spleen, beef, liver and natural casings, with the average count of 1.57 x 10^4, 6.63 x 10^3, 2.47 x 10^3 and 2.26 x 10^3 cfu/g, respectively.

Staphylococcus aureus and coliforms counts were detected in spleen, natural casings, beef and liver, with their highest counts found in beef.

It is interesting to note that there were no strains of LAB, enterococci, *Staphylococcus aureus* and coliforms detected in the unpeeled and peeled garlic. Likewise, *E. coli* O157:H7 was not detected in any of unpeeled garlic and peeled garlic, as well as beef, liver, spleen and natural casings in all Mhom samples tested in the present study.

3.2 Screening of the already identified LAB

3.2.1 Primary screening

In general, Mhom is characterized by its acidity with the final pH value of about 4.0 to 6.0. It has been previously demonstrated that LAB play a particularly important role and are commonly found in dry-fermented sausages (Coppola et al., 1998). Recently, LAB have been employed as the starter cultures, promoting meat fermentation (Papamanoli et al., 2003). LAB improve the safety and

stability of the meat products, enhance color stability, prevent the rancidity and release of various aromatic substances (Coppola et al., 1998; Hammes et al., 1995; Nychas and Arkoudelos, 1990; Papamanoli et al., 2003). *Lactobacilli* strains are the predominant LAB in the naturally fermented meat products. Among them the most frequently isolated strains are *Lactobacillus curvatus*, *Lactobacillus sakei* and *Lactobacillus plantarum* (Hammes, 1990; Schillinger and Liicke, 1987; Rantisiou et al., 2005; 2006). Therefore, the main aim of this part of study is to evaluate the *in-vitro* antimicrobial activities of LAB isolated from Mhom samples on the growth and survival of some food spoilage and food-borne pathogenic bacteria.

3.2.1.1 Determination of antimicrobial activity of LAB against several pathogenic bacteria

The samples in this study were collected from 5 different manufacturing sites located in Northeastern Thailand with a long tradition in Mhom production, i.e., Chaiyaphum 1 (CP1), Chaiyaphum 2 (CP2), Khon Kaen (KK), Mahasarakham (MK), and Kalasin (KS). Several LAB strains were isolated from Mhom using MRS agar based on their gram-positive staining, and indole-, catalase-, motility- and spore forming-negative, and rod-shaped. LAB isolates were preliminarily examined for the antimicrobial activity using the modified disc diffusion method as previously described (Hamadan and Mikolajcik, 1974). The agar disc diffusion method was selected in this study to test the antimicrobial activities of LAB isolates in order to know that if the antimicrobial compounds produced by LAB were extracellular and released from the cell wall into the medium. The LAB isolates and used in this study are CP102, CP103, CP105, CP106, CP108, CP110, CP111, CP112, CP113, CP114, CP115, CP116, CP117, CP118, CP120, CP121, CP124, CP125, CP126, CP127, CP128, CP129, CP130, CP131, CP136, CP137 and CP138 isolated from local Mhom samples produced from Chaiyaphum 1; CP203, CP204, CP205, CP207, CP208, CP210, CP213, CP214, CP217, CP218, CP220, CP221, and CP222 isolated from local Mhom samples produced from Chaiyaphum 2; MK102, MK103, MK106, MK112, MK113, MK114, MK115, MK118 and MK119 isolated from local Mhom samples produced from Mahasarakham; KK111, KK112, KK113 and KK114 isolated from local Mhom samples produced from Khon Kaen; KS101, KS103,

KS105, KS114 and KS115 isolated from local Mhom samples produced from Kalasin.

The antimicrobial activity of LAB isolates were tested in duplicate against the indicator bacteria, i.e., some food spoilage and food-borne bacteria, including *Staphylococcus aureus* strains ATCC13565, ATCC25923 and ATCC25904, *Bacillus cereus*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhi*, *Enterococcus faecalis*, *Psudomonas* sp. and *Staphylococcus epidermidis* and the results are summarized in Table 4.6 and Fig. 4.5.

The results from the modified disc diffusion method revealed LAB isolates exhibited *in vitro* inhibitory activity against most of the bacterial indicator strains tested in this study. This indicates that the inhibitory compounds produced by the LAB isolates were extracellular and diffusible because inhibition took place by diffusion through a layer of agar.

LAB isolates with the inhibitory effect on the indicator bacteria exhibiting various diameters of inhibitory zones (Table 4.6). Fig. 4.5 illustrate the zones of inhibition of LAB isolates against some types of pathogenic bacteria. Of all indicator bacteria used in this study, *Enterococcus faecalis*, *S. typhi*, *E. coli* O157:H7, *S. aureus* strain ATCC25923, *B. cereus* and *L. monocytogenes* were the most sensitive pathogenic microbes against all LAB isolates, whereas *S. epidermidis*, *S. aureus* ATCC13565 and *Pseudomonas* sp. revealed the weak positive results. All LAB isolates exhibited strong antimicrobial activities when tested against *Ent. faecalis* and *S. typhi* as revealed by their highest diameter of inhibition zone, with the average inhibition zone diameters ranging from 7 to 14 mm. Ten isolates from CP1 (i.e., CP105, CP108, CP114, CP115, CP116, CP120, CP124, CP129, CP130 and CP131), two isolates from CP2 (i.e., CP210 and CP222), four isolates from Mahasarakham (i.e., MK106, MK112, MK113 and MK114), one isolate from KK (i.e., KK111), and three isolates from KS (i.e., KS101, KS105 and KS115) resulted in the highest diameter of inhibitory zone against *E. coli* O157:H7 than the other LAB isolates. CP115, CP116, CP120, CP210, CP213, MK114 and KS103 isolates displayed the highest inhibitory activities than the other LAB isolates on *L. monocytogenes* tested strain.

The isolation of LAB from food products is primarily performed to acknowledge and evaluate their inhibitory effect, or antibacterial potency against the pathogenic and food spoilage bacteria from food. As described previously, most of the LAB strains produced inhibitory acids and bacteriocins, ribosomally synthesized peptides that exhibit antibacterial activity (Michael et al., 2000), and thereby their great and reliable value were recognized as the natural preservatives of acid fermented meat products. The results of the present study show that the cell-free supernatants of LAB cultures exhibited stronger antibacterial activity than the neutralized culture supernatants (pH 6.5) (Fig. 4.5). The inhibitory effect may be due to acids or bacteriocin-like substances produced by LAB cultures or combination of both (Aslim et al., 2005). The results from the recent report of Aroutcheva et al. (2001) have revealed that no correlation was found between bacteriocin activity, lactic acid and hydrogen peroxide production. *Lactobacillus* strains 228, 345, and 431 produced H_2O_2 but did not demonstrate any inhibitory effect. Similar results obtained in the present study, all strains may produce H_2O_2 but not all the LAB isolates exhibited antimicrobial activities against the bacterial indicators. All indicator bacteria were inhibited by the culture supernatants. The highest antimicrobial activity was found to be against *Entercoccus faecalis*, *S. typhi*, *E. coli* O157:H7 and *L. monocytogenes*, respectively. Similar results were reported by Gurira and Buy (2005) who showed that LAB isolate, i.e., *Pediococcus* sp. isolated from South African farm-style cheese showed high inhibitory activity against *E. coli* and *L. monocytogenes* ATCC7644, but exhibited low inhibitory activity against *B. cereus* ATCC1178. The antimicrobial potency in this study could be due to the high accumulation of antimicrobial compounds in the cells, which can be effectively discharged from the cell wall. LAB may exert their antibacterial activity through the production of lactic acid and other metabolites, such as hydrogen peroxide and short chain fatty acids (Rantsiou et al., 2005; 2006). The medium used for the inhibition assay lacked a fermentable carbohydrate, a factor which reproduce false-positive reaction due to acid production. It is most likely that much of the inhibition observed against the indicator bacteria may be due to hydrogen peroxide which is produced majority under aerobic, or microaerophilic conditions by LAB. Also the specific antibacterial compounds, such as bacteriocins or antibiotics, have been identified in

identified in the culture medium of several lactic acid producing bacteria (Rantsiou et al., 2005; 2006).

3.2.1.2 Lactic acid productivity of the LAB isolates

The amount of lactic acid produced in the cell-free supernatants of the LAB isolates were quantified by an HPLC-UV detection system according to the method of Butkhup and Samappito (2008). The concentrations of lactic acid produced by each LAB isolate on the overnight culture supernatant are shown in Fig. 4.6 and Fig. 4.7. HPLC-UV analysis revealed that the amounts of lactic acid production by most of the LAB isolates were higher than 5 g/l. Seventeen LAB isolates, such as CP136, CP129, KK114, KK111, CP117, CP130, CP220, KK113, CP131, CP218, CP114, CP210, CP118, CP222, CP120, MK114, KS103 and CP138, resulted in the high production of lactic acid (>15 g/l) compared to the other LAB isolates. As our LAB were grown in MRS broth media containing glucose as the primary carbon source, the observed inhibition might arise from the acid produced. To find out whether the inhibitory effect was due to acid or any other antibacterial substances, the supernatants of the LAB cultures were neutralized to pH 6.5 and used to detect inhibition. Our data showed that when the pH values of the culture supernatants were adjusted to 6.5 with NaOH, there was a reduction of inhibition activity against some pathogenic bacteria (Fig. 4.5). Taken together, our results indicated that the inhibitory substance may be due to organic acids and/or acid derivatives and/or bacteriocins produced and released by LAB. Similar results were previously observed in which the antimicrobial activity of culture filtrates of LAB against some food-borne pathogens and other bacterial species was removed when the filtrates were neutralized to pH 6.5 (Varadaraj et al., 1993; McLean and McGroarty, 1996).

In bacterial cells, the organic acids, such as formic acid, acetic acid, lactic acid, propionic acid, benzoic acid and free fatty acids, are produced from sugars (Ray and Sandine, 2000), amino acid and/or lipid metabolism (Gumella and Broadbent, 2001). These acids are responsible for inhibition of pathogenic and spoilage organisms, and can be produced by LAB in varying quantities. LAB are found to produce large quantities of lactic acid, which reduces the pH of the media of the environment to hostile levels for other

microorganisms (Amrane and Prigent, 1999). In addition to the pH effect, there are other modes through which the acids inhibit pathogenic and spoilage microorganisms. In this study, 5 LAB isolates, such as CP116, CP120, CP210, MK114 and KS103, were finally characterized.

They exhibited profound antibacterial effects *in vitro* in the antimicrobial activity assay. They resulted in the highest inhibition activity against 7 to 8 test strains and produced large quantities of lactic acid. Therefore, only CP116, CP120, CP210, MK114 and KS103 isolates were further identified in the secondary screening.

In conclusion, LAB isolates involved in Mhom fermentation had antimicrobial activities against pathogenic bacteria. *Enterococcus faecalis*, *S. typhi*, *E. coli* O157:H7, *S. aureus* strain ATCC25923, *B. cereus* and *L. monocytogenes* were the most sensitive pathogenic microbes against all LAB isolates, whereas *S. epidermidis*, *S. aureus* ATCC13565 and *Pseudomonas* sp. revealed the weak positive results. Utilizing the modified disc diffusion method, our results indicated that the inhibitory products are extracellular and diffusible. When the pH value of the culture supernatants was adjusted to 6.5, there was reduction of inhibition activity against some pathogenic bacteria. Taken together, the results obtained from this study revealed that at least the inhibitory substances may be due to organic acids and/or acid derivatives and/or bacteriocin produced and released by LAB into the medium. The glucose composition of MRS medium where LAB grew could have a contribution to the inhibition of some food-borne pathogens introduced into it. 5 LAB isolates, such as CP116, CP120, CP210, MK114 and KS103, were finally isolated and screened in this study by based on their high antimicrobial properties, as well as lactic acid productivity.

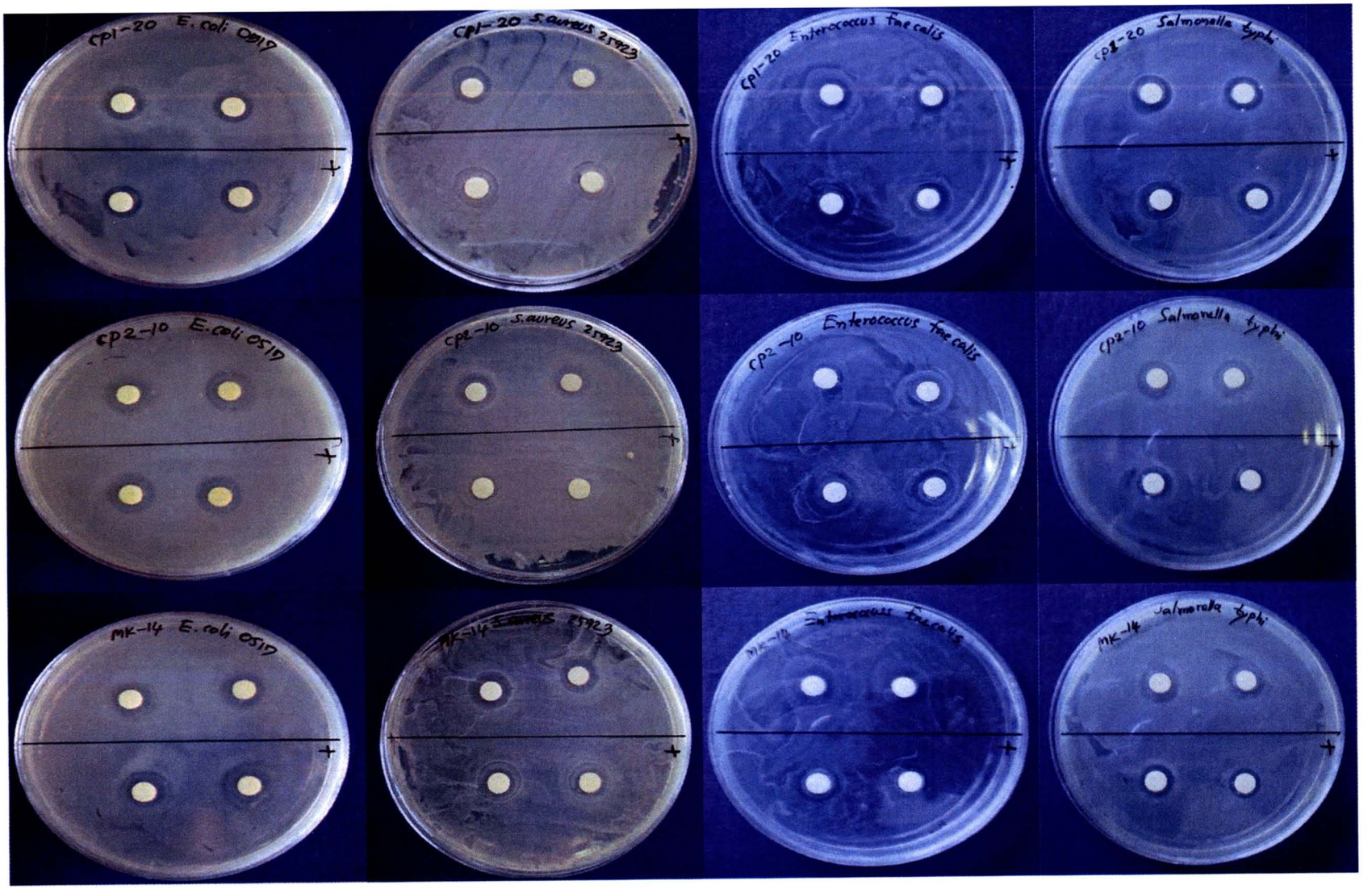

Figure 4.5 Inhibitory zone of cell-free supernatant and neutralized culture supernatant (+) against pathogenic bacteria, including *Staphylococcus aureus* ATCC25923, *Escherichia coli* O157:H7, *Enterococcus faecalis*, and *Salmonella typhi*.

Table 4.6 Diameter of zones of inhibition produced by LAB isolates on the test strains as assessed by the modified disc diffusion method

LAB isolate	Test strains									
	S. aureus 13565	*S. aureus* 25923	*S. aureus* 25904	*B. cereus*	*E. coli* O157:H7	*L. monocytogenes*	*S. typhi*	*En. faecalis*	*S. epidermidis*	*Pseudomonas* sp.
CP1-2	-	-	-	-	-	-	+++	++++	++	-
CP103	-	-	-	-	+++	-	+++	++++	-	-
CP105	-	++	+++	++	++++	-	++++	++++	-	-
CP106	-	-	-	-	+++	+++	++	++	-	-
CP108	-	+++	+++	-	++++	-	+++	+++	++	-
CP110	-	++	-	-	+++	-	+++	+++	-	-
CP111	-	++	-	-	-	-	+++	++++	++	-
CP112	-	++	-	++	-	++	+++	++++	-	-
CP113	++	+++	++	+++	-	-	++++	++	++	-
CP114	-	+++	++	+++	++++	+++	+++	++++	-	-
CP115	-	++	+++	+++	++++	++++	+++	++	-	-
CP116	-	+++	++	+++	++++	++++	+++	++++	++	-
CP117	-	+++	++	++	-	+++	+++	++	++	-
CP118	-	+++	+++	++	++	-	++++	++++	+++	-

Table 4.6 Diameter of zones of inhibition produced by LAB isolates on the test strains as assessed by the modified disc diffusion (Cont.)

LAB isolate	Test strains									
	S. aureus 13565	*S. aureus* 25923	*S. aureus* 25904	*B. cereus*	*E. coli* O157:H7	*L. monocytogenes*	*S. typhi*	*En. faecalis*	*S. epidermidis*	*Pseudomonas* sp.
CP120	-	+++	+++	+++	++++	++++	++++	++++	+++	-
CP121	-	+++	-	++	-	-	++++	++++	+++	-
CP124	-	+++	++	-	++++	-	++++	+++	-	-
CP125	-	+++	-	-	+++	-	+++	++++	-	-
CP126	-	+++	++	+++	-	-	+++	++	-	-
CP127	-	++	++	-	+++	-	++	+++	++	-
CP128	-	++	-	+++	+++	+++	+++	+++	-	-
CP129	-	++	++	++	++++	++	+++	++++	++	-
CP130	-	+++	+++	++	++++	+++	+++	+++	-	-
CP131	-	++	+++	++	++++	+++	+++	+++	-	-
CP136	-	++	-	++	+++	++	+++	++	-	-
CP137	-	++	+++	-	+++	+++	+++	+++	-	-
CP138	-	+++	-	+++	+++	-	+++	+++	-	-
CP203	-	++	++	++	-	-	+++	++	-	-
CP204	-	-	++	+++	++	-	+++	+++	-	-

Table 4.6 Diameter of zones of inhibition produced by LAB isolates on the test strains as assessed by the modified disc diffusion (Cont.)

LAB isolate	Test strains									
	S. aureus 13565	*S. aureus* 25923	*S. aureus* 25904	*B. cereus*	*E. coli* O157:H7	*L. monocytogenes*	*S. typhi*	*En. faecalis*	*S. epidermidis*	*Pseudomonas* sp.
CP205	-	+++	++	-	-	-	+++	++++	++	-
CP207	-	+++	-	++	-	+++	+++	++++	+++	-
CP208	-	+++	++	-	-	+++	++++	++++	-	-
CP210	-	+++	+++	+++	++++	++++	+++	++++	+	-
CP213	-	++	-	++	+++	++++	+++	++++	-	-
CP214	++	+++	++	+++	+++	-	+++	++++	-	-
CP217	-	++	+++	++	+++	++	++	++++	-	-
CP218	-	++	-	++	+++	+++	+++	+++	-	-
CP220	-	+++	-	++	-	-	+++	++++	++	-
CP221	-	+++	++	+++	-	+++	+++	++++	-	-
CP222	-	+++	++	-	++++	-	+++	++++	-	-
MK102	-	-	+++	-	-	+++	++++	+++	++	-
MK103	-	++	++	-	+++	+++	++	++++	-	-
MK106	-	+++	-	+++	++++	+++	++	+++	-	-
MK112	-	-	-	-	++++	-	+++	++++	-	-

Table 4.6 Diameter of zones of inhibition produced by LAB isolates on the test strains as assessed by the modified disc diffusion (Cont.)

LAB isolate	Test strains									
	S. aureus 13565	*S. aureus* 25923	*S. aureus* 25904	*B. cereus*	*E. coli* O157:H7	*L. monocytogenes*	*S. typhi*	*En. faecalis*	*S. epidermidis*	*Pseudomonas* sp.
MK113	-	++	++	++	++++	-	+++	++++	+++	-
MK114	-	+++	-	-	++++	++++	+++	++++	-	-
MK115	-	++	-	++	+++	+++	+++	++	-	-
MK118	-	-	-	+++	-	+++	+++	+++	-	-
MK119	-	-	++	-	+++	+++	+++	++++	-	-
KK111	-	+++	-	-	++++	+++	+++	++++	-	-
KK112	-	+++	-	-	+++	-	+++	++++	-	-
KK113	-	+++	-	++	-	-	+++	++++	-	-
KK114	-	++	++	++	+++	-	++	++++	-	-
KS101	-	++	++	-	++++	+++	+++	+++	-	-
KS103	-	++	++	++	+++	++++	+++	+++	-	-
KS105	-	++	++	-	++++	+++	+++	++++	-	-
KS114	-	+++	-	-	+++	+++	+++	++++	-	-
KS115	-	++	-	-	++++	+++	+++	++++	-	-

(-) no inhibition detected; (+) 0.5-2.0 mm diameter of inhibition zone; (++) 2.1-8.0 mm diameter of inhibition zone; (+++) 8.1-10 mm diameter of inhibition zone; (++++) > 11 mm diameter of inhibition zone

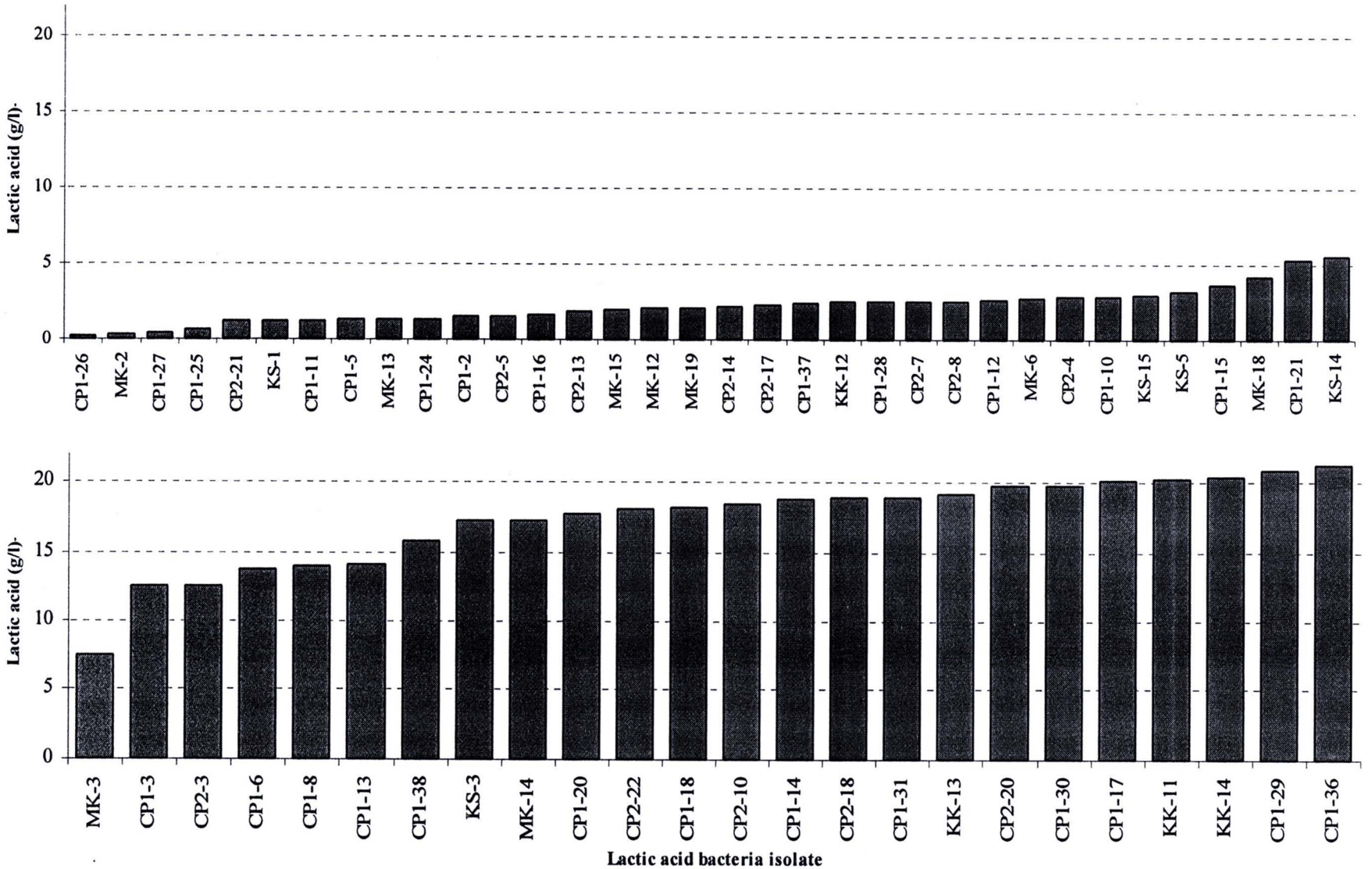

Figure 4.6 Concentrations of lactic acid (g/l) in the supernatants from the overnight-grown cultures of LAB isolates

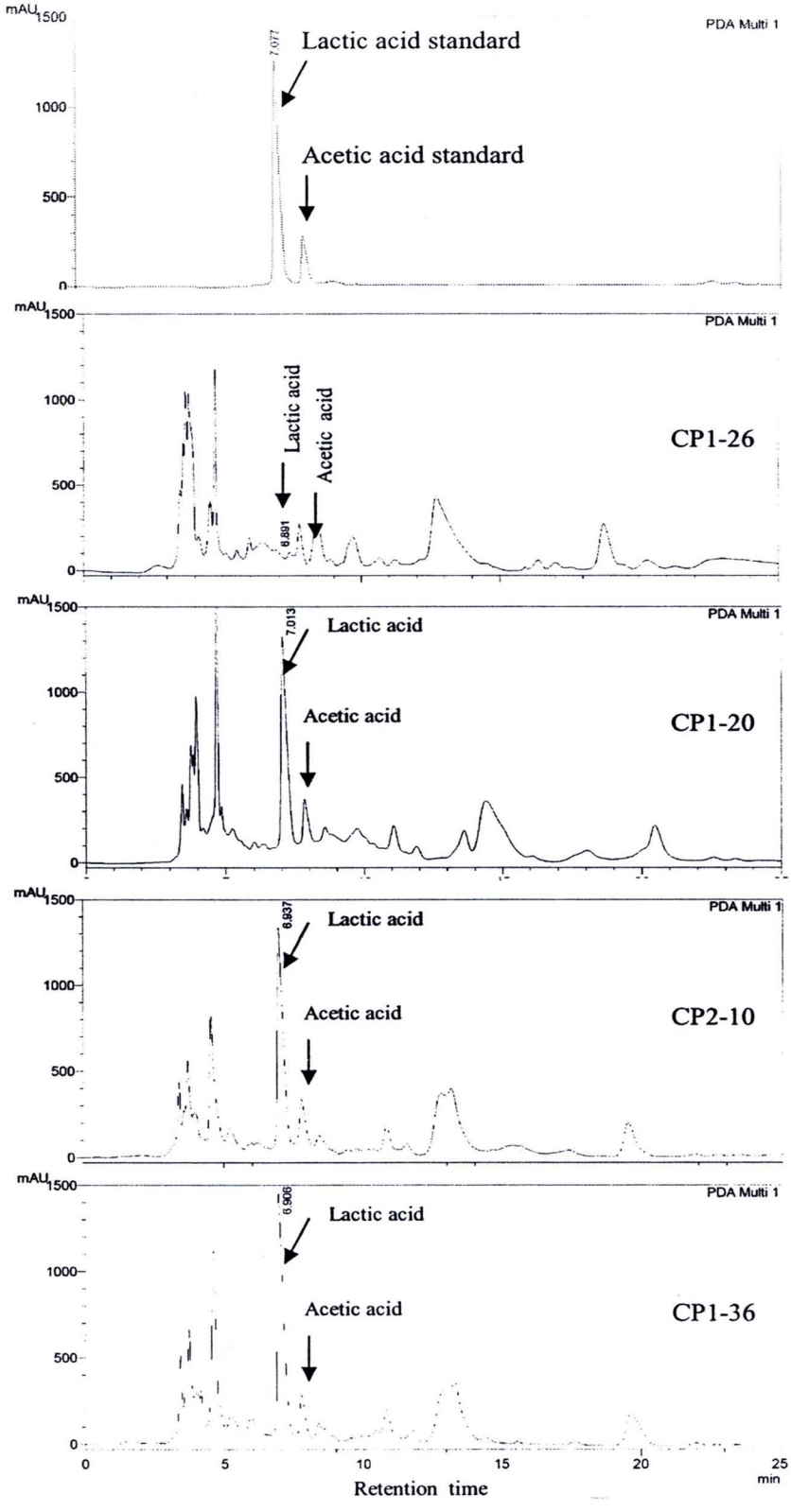

Figure 4.7 An HPLC chromatogram of lactic acid and acetic acid standards, and lactic acid and acetic acid productivity of some LAB isolates

3.2.2 Secondary screening of LAB

The secondary screening for the growth rate and acidifying capacity of the already identified LAB were evaluated utilizing the standard methods as previously described (Buckenuskes, 1993; Holzapfel, 2002).

3.2.2.1 Tolerance of LAB to high temperature, high concentration of lactic acid and sodium chloride and low pH

5 selected LAB isolates, i.e., CP116, CP120, CP210, MK114 and KS103 were further evaluated for their growth rate, acidifying capacity, tolerance to the extreme environmental conditions, such as high temperatures, high concentrations of lactic acid and sodium chloride, and low pH.

Table 4.6 shows the tolerance of each of the five LAB isolates to the extreme environmental conditions tested in the present study. The results demonstrated that 4 isolates could grow at 15–45°C, while the one isolated from CP1 (i.e., CP116) could not grow at 15°C. Wouters et al. (2000) reported that the reduced glycolytic activity leads to the decreased production of lactic acid in *Lactobacillus lactis* at low temperature. The ability of LAB to grow at high temperature is a desirable trait as it could translate to increase rate of growth and lactic acid production. At the same time, a high fermentation temperature reduces contamination by other microorganisms.

CP116, however, was the least tolerant to high concentrations of lactic acid. The other three isolates from CP1 (CP120), CP2 (CP210) and MK (MK114), were the most tolerant to high lactic acid concentrations as they were able to grow at 7.5%. KS103 isolate was the most sensitive to low lactic acid concentrations as its growth was indicated only at 2.5%. None of the five isolates grew at 10% and 15% lactic acid concentrations. A higher tolerance to high lactic acid concentrations is a desirable trait for an industrial strain of LAB as it could produce more lactic acid in the fermentation broth without prematurely affecting itself adversely.

Similar to their high tolerance to lactic acid concentrations, CP120, CP210 and MK114 isolates were the most tolerant to high NaCl concentration compared to the other isolates. The CP120, CP210 and MK114 isolates exhibited the ability to grow at high salt concentrations weven up to the

saturating concentration of NaCl used in this present study, while the other two isolates did not possess the ability to grow at this highest salt concentration. These findings provided an indication of osmotolerance level of the LAB strains tested in this study. It seems likely that the high osmotic strength of LAB is due to the production of biogenic amines, such as proline, in the cells (Colovita et al., 2003). Bacterial cells cultivated in a high salt concentration would experience a loss of turgor pressure, which would then affect the physiology, enzyme activity, water activity and metabolism of the cells (Liu *et al.*, 1998). Some cells could overcome this effect by regulating the osmotic balance between the inside and outside of the cell (Kashket, 1987). Several lines of evidence indicate that the strains of lactococci (Uguen et al., 1999) and lactobacilli (Hutkins et al., 1987; Glaasker et al., 1998) exhibited a decreased growth rate when the osmolarity of the medium was increased. Uguen, et al. (1999) recognized the increased amount of glycine betaine, an osmolyte, in LAB cells when they were grown in high NaCl concentrations. Liu et al. (1998) reported that the uptake of glycine betaine was induced in cells as an adaptive measure to withstand increasing external osmotic pressure. The CP120, CP210 and MK114 could be similarly protected to be able to grow at higher NaCl concentration compared to the other isolates. During industrial fermentation, as lactic acid is being produced by the cells, alkali would be pumped into the broth to prevent excessive reduction in pH. Thus, the free acid would be converted to its salt form which would in turn increase the osmotic pressure on the cells. Therefore, an LAB strain with high osmotolerance would be desirable as an industrial potential.

All LAB isolates could grow at pH 4.5 and pH 7.0. LAB are acidophilic but while that means a tolerance to low pH, the latter should be differentiated from a situation in which a high concentration of free acids (H^+) also exists because the free acids could inhibit growth (Amrane and Prigent, 1999). In contrast, all the isolates could not grow in an alkaline environment at pH 9. Rhee and Pack (1980) observed that *Lactobacillus bulgaricus* also could not tolerate too high a pH.

Table 4.7 Broad range tolerance of the five LAB isolates to ranges of temperatures, lactic acid, NaCl concentrations, and pH

Environmental conditions	Isolate name				
	CP116	CP120	CP210	MK114	KS103
Temperature					
15 °C	–	+	+	+	+
30 °C	+	+	+	+	+
37 °C	+	+	+	+	+
45 °C	+	+	+	+	+
50 °C	–	–	–	–	–
Lactic acid concentration (% w/v)					
2.5	+	+	+	+	+
5.0	–	+	+	+	+
7.5	–	+	+	+	–
10	–	–	–	–	–
15	–	–	–	–	–
NaCl concentration (% w/v)					
1.5	+	+	+	+	+
2.5	+	+	+	+	+
5.0	+	+	+	+	+
7.5	+	+	+	+	+
10	–	+	+	+	–
pH					
2.5	–	–	–	–	–
4.5	+	+	+	+	+
7.0	+	+	+	+	+
9.0	–	–	–	–	–

+ indicates color change from purple to yellow, taken to equate growth.

– indicates no color change from purple, taken to equate no growth.

3.2.2.2 Growth and lactic acid production profiles of LAB isolates during the time-course study

Based on the screening test for the broad range tolerance of LAB to various extreme environmental conditions, the five LAB isolates, i.e., CP116, CP120, CP210, MK114 and KS103 were selected to pursue the time-course study to generate their growth and lactic acid production profiles. The growth characteristic of CP120, CP210 and MK114 isolates and its lactic acid production were investigated in the current study. The results of bacterial cell growth and lactic acid production profiles are shown in Fig. 4.8 to Fig. 4.11. At 6-18 h, the growth of MK114 was in the logarithmic phase and lactic acid was detected in the culture medium. At this time, MK114 grew faster than CP120 and CP210 based on cell concentration (log CFU/ml). After 18 h, the growth of MK114 was observed as the cells entered stationary phase and cell concentration leveled off at about 9.78 log CFU/ml, while CP120 and CP210 tended to increase yield continuously until 30 h before their proliferation leveled off at about 10.27 to 10.28 log CFU/ml, respectively (Fig. 4.8). The pH values of cultures at early stationary phase rapidly dropped in the logarithmic phase to be at around 5.20, 4.33 for MK114, and 3.79 and 3.93 for CP120 and CP210, respectively, and still remained to be the same throughout the incubation time period. The lower cell concentration produced by MK114 with respect to CP120 and CP210 correlated well with the lower amount of lactic acid produced by MK114 (Fig. 4.9), thereby resulting in the higher pH in the medium (Fig. 4.10) and lower consumption of glucose (Fig. 4.11). These findings of the current study are consistent with those of in Table 4.7, whereby the MK114 isolate was found to be unable to tolerate high lactic acid concentrations compared to CP120 and CP210 (Table 4.7). While the growth profiles of CP120 and CP210 did not appear to be different from one another, the amount of lactic acid produced by CP210 was higher than CP120 and MK114 over 60 h. The production of lactic acid by CP210 also peaked faster at 42 h compared to CP120 in which the production of lactic acid peaked at 48 h. These lactic acid production profiles corresponded well to the pH and glucose profiles in the cultures of two LAB strains. The highest lactic acid concentration produced by CP210 was found at 42 h, with the value of 18.69 mg/ml. At this time, the yield of lactic acid from glucose was highest with CP210 at 53.13, followed by CP120 at 33.86. These

results demonstrated that CP210 had the high efficacy in converting glucose to lactic acid. CP210 and CP120 had similar tolerance levels to high temperature (up to 45°C), lactic acid concentration (7.5%), NaCl concentration (10%), and to low pH (4.5), but the faster production of lactic acid by CP210 might give it a slight advantage over CP120. This difference between CP210 and CP120 was apparent under current experimental conditions, i.e., using MRS medium in which glucose was the primary carbon source and the medium was not pH-controlled. A pH controlled medium would probably allow for higher accumulation of lactic acid and would be appropriate for future studies where optimization of the fermentation process was the focus for the industrial purposes. In developing the fermentation process to industrial level, cheaper sources of carbon are necessary and the medium would need to be pH controlled by incorporating neutralizing agents such as calcium carbonate into the medium to reduce the inhibitory effects of free lactic acid on the producer cells (Adnan and Tan, 2007; Ibourahema et al., 2008). Under such conditions, the growth and lactic acid production of CP210 and CP120 would need to be reevaluated.

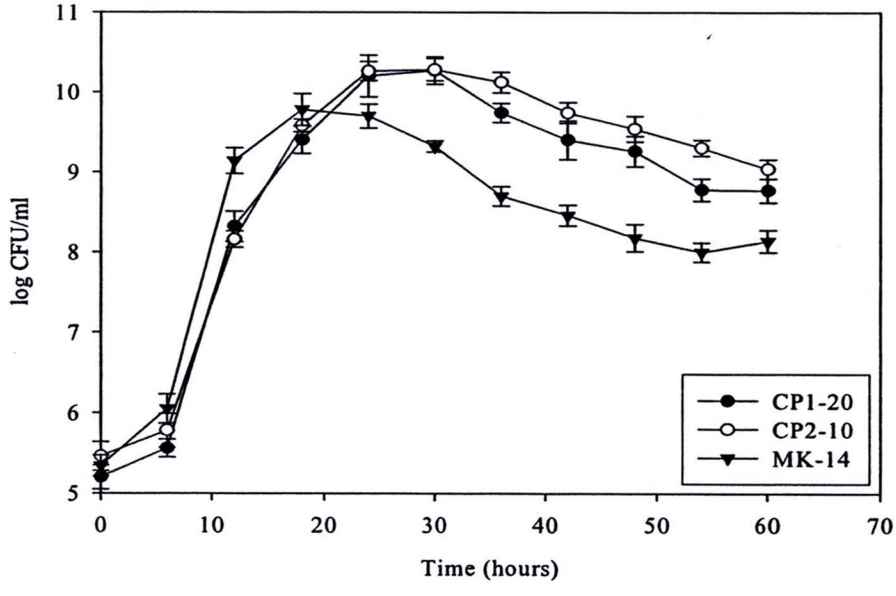

Figure 4.8 Growth of three LAB isolates, i.e., CP120 (•), CP210 (o) and MK114 (▼), over 60 h. The isolates were cultured in the MRS broth (pH 7.0) and incubated at 37°C under non-controlled pH without agitation.

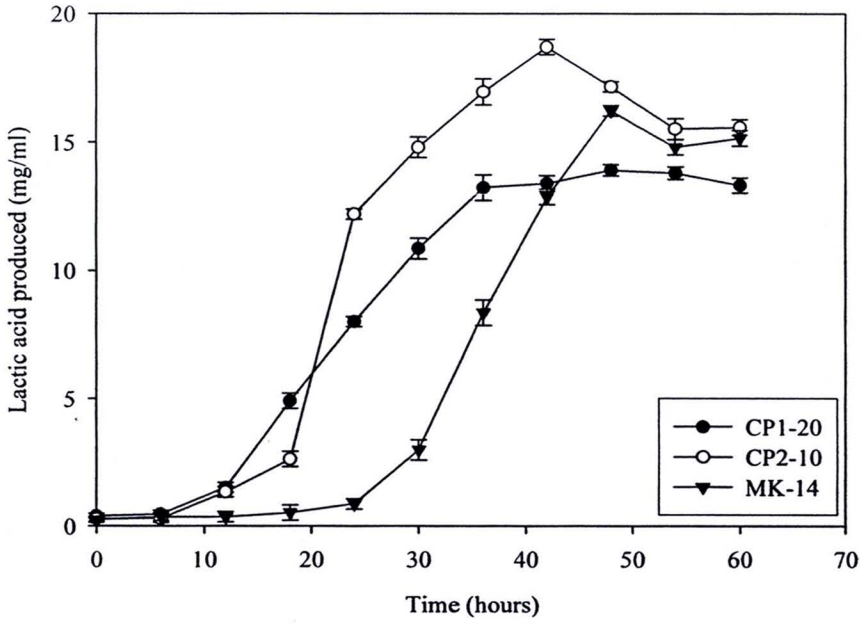

Figure 4.9 Lactic acid produced by three LAB isolates, CP120 (•), CP210 (o) and MK114 (▼), over 60 h. The isolates were cultured in MRS broth (pH 7.0) and incubated at 37°C under non-controlled pH condition without agitation.

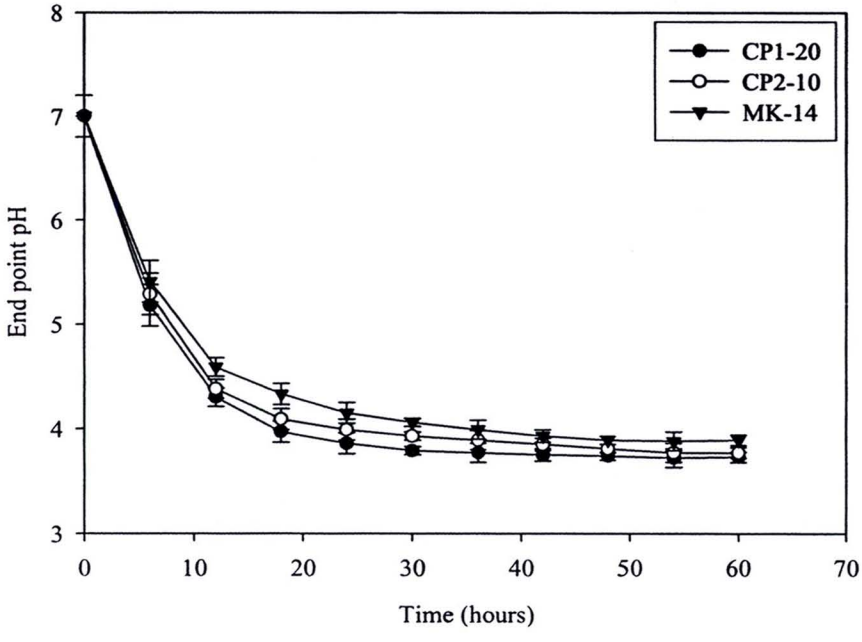

Figure 4.10 pH of the cultures of three LAB isolates, i.e., CP120 (•), CP210 (o) and MK114 (▼), over 60 h. The isolates were cultured in MRS broth (pH 7.0) and incubated at 37°C under non-controlled pH condition without agitation.

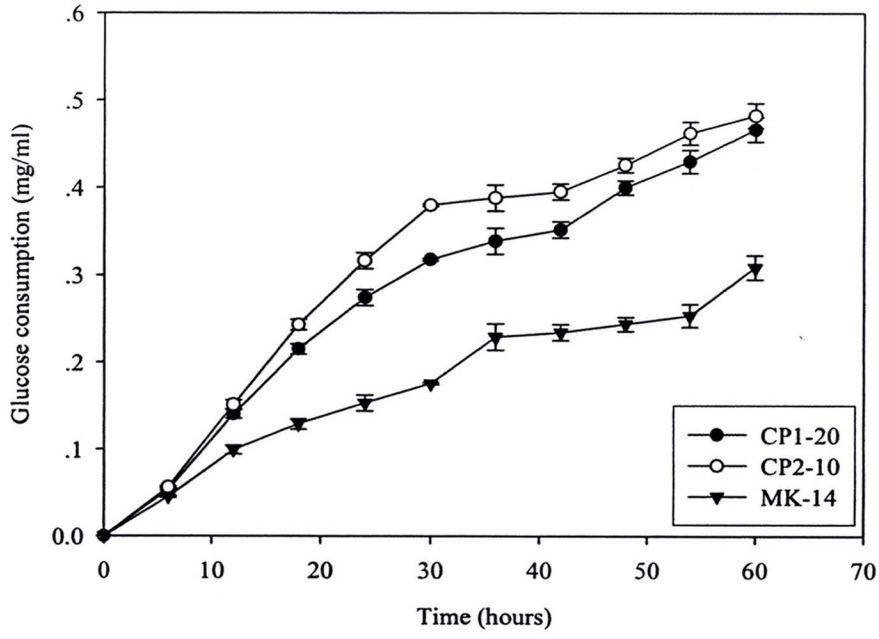

Figure 4.11 Glucose consumption (mg/l) by three LAB isolates, i.e., CP120 (•), CP210 (o) and MK114 (▼) over 60 h.

3.3 Application of LAB as the starter cultures

The already identified and selected LAB, i.e., *L. plantarum* strains CP120 and MK114, were designated as different combinations of starter cultures to the raw materials and ingredients, and were allowed to ferment Mhom products.

3.3.1 Preparation of Mhom

The use of starter cultures for fermented sausage production is becoming increasingly necessary to guarantee safety, to standardize product properties, including consistent flavor and color, and to shorten ripening time (Coppola et al., 1997; Rantsiou et al., 2005). The appropriate starter culture combinations were applied the raw material and ingredient mixture and then microbial profiles were followed and determined for dynamic changes during fermentation (Fontana et al., 2005). Thus, the application of starter culture, i.e., *L. plantarum*, in Mhom could provide an additional tool for enhancing the competitiveness of the starter organisms in favor of the fortuitous flora as well as preventing the outgrowth of food pathogens in Mhom. Therefore, it is essential to optimize the appropriate starter culture combination to be added in Mhom, which can

vary depending on the capability of the starter to grow in the product. The identified LAB selected were assigned as different combinations of starter cultures to the prepared raw material and ingredients and allowed to them ferment to Mhom products.

Table 4.8 Starter cultures and their combination used in Mhom fermentation process

Batch	Starter cultures
I	No starter cultures added (i.e., control)
II	*L. plantarum* 1 CP120
III	*L. plantarum* 1 CP210
IV	*L. plantarum* 2 MK114
V	*L. plantarum* 1 CP120 : *L. plantarum* 1 CP210 (1:1, v/v)
VI	*L. plantarum* 1 CP120 : *L. plantarum* 2 MK114 (1:1, v/v)
VII	*L. plantarum* 1 CP210 : *L. plantarum* 2 MK114 (1:1, v/v)
VIII	*L. plantarum* 1 CP120 : *L. plantarum* 1 CP210 : *L. plantarum* 2 MK114 (1:1:1, v/v/v)

In this study, Mhom products were manufactured according to the following formula: beef 300 g/kg, liver 400 g/kg, spleen 100 g/kg, roasted rice powder 85 g/kg, garlic 85 g/kg, salt 30 g/kg, and glucose 3 g/kg. The minced mixture was divided into seven different batches as shown in Table 4.7. The starter cultures were previously isolated and selected from indigenous fermented Mhom based on their high antimicrobial activity and tolerance to the extreme environmental conditions.

3.3.2 pH measurement

Each Mhom sample (5 g) was suspended with 5 ml distilled water. The mixture was homogenized, and then the pH detected. Table 4.9 and Fig. 4.12 show the pH characteristics of Mhom produced from beef, liver, spleen, roasted rice powder, garlic, salt, and glucose as additional substrate for fermentation. With the use of different combinations of LAB starter cultures, the obtained Mhom products had pH values ranging from 4.62 to 4.66 at the end of the fermentation process. The initial pH of inoculated batches ranged between 5.26 and 5.48,

thereafter, it decreased rapidly to pH 4.49 (CP210), after 5 day. The drop in pH was attributed to the production of acids during bioconversion. According to Frazier and Westhoff (1988), the trend in pH reduction may best be explained as due to the involvement of LAB that were able to ferment glucose to produce lactic acid, which then resulted in the lowering of the pH. However, it took 5 days for the sausages inoculated with different starter cultures, as shown in Table 4.11, to reach a similar pH value (pH 4.49-4.67). From day 9, a slight increase in pH was observed in all inoculated batches. This may be due to the production of ammonia and biogenic amines as a result of enzymatic activity (Hughesa et al., 2002). In addition, the yeast counts help to explain the lactic acid and pH level in the fermented meat products (Juan-Pablo et al., 2000). Yeasts are potent consumers of lactic acid in foods which leads to an increase in the pH (Juan-Pablo *et al.*, 2000; Walker, 1977). This was evident in the pH level at the end of the fermentation process which had a higher level than before ripening. The pH of the samples remained fairly constant after 14 days of fermentation. Statistical analysis of pH values recorded throughout ripening revealed significant differences between CP120 and other inoculated batches on day 1 to 3 (Table 4.12). Differences in pH between samples could be attributed to the availability of fermentable sugars coupled with the population of lactic acid bacteria at the time.

Table 4.9 Changes in pH values during the ripening of Mhom [a]

Day	pH						
	CP120	CP210	MK114	CP120+ CP210	CP120 +MK114	CP210 +MK114	CP120 + CP210 + MK114
0	5.48 a	5.37ab	5.36 ab	5.26 b	5.27 b	5.28 b	5.26 b
1	5.33 a	5.16 b	5.05 b	5.16 b	5.14 b	5.11 b	5.17 b
3	4.95 a	4.64 b	4.85 ab	4.75 b	4.74 b	4.95 a	4.84 ab
5	4.56 a	4.49 a	4.56 a	4.67 a	4.57 a	4.55 a	4.51 a
7	4.59 a	4.57 a	4.55 a	4.58 a	4.48 a	4.50 a	4.52 a
9	4.63 a	4.67 a	4.67 a	4.59 a	4.62 a	4.61 a	4.66 a
11	4.62 a	4.61 a	4.60 a	4.65 a	4.67 a	4.66 a	4.62 a
14	4.64 a	4.63 a	4.64 a	4.67 a	4.64 a	4.66 a	4.63 a
21	4.61 a	4.63 a	4.63 a	4.65 a	4.62 a	4.62 a	4.63 a
28	4.64 a	4.62 a	4.66 a	4.66 a	4.64 a	4.64 a	4.65 a

[a] Values in a row followed by the same letter are not significantly different ($p \leq 0.05$).

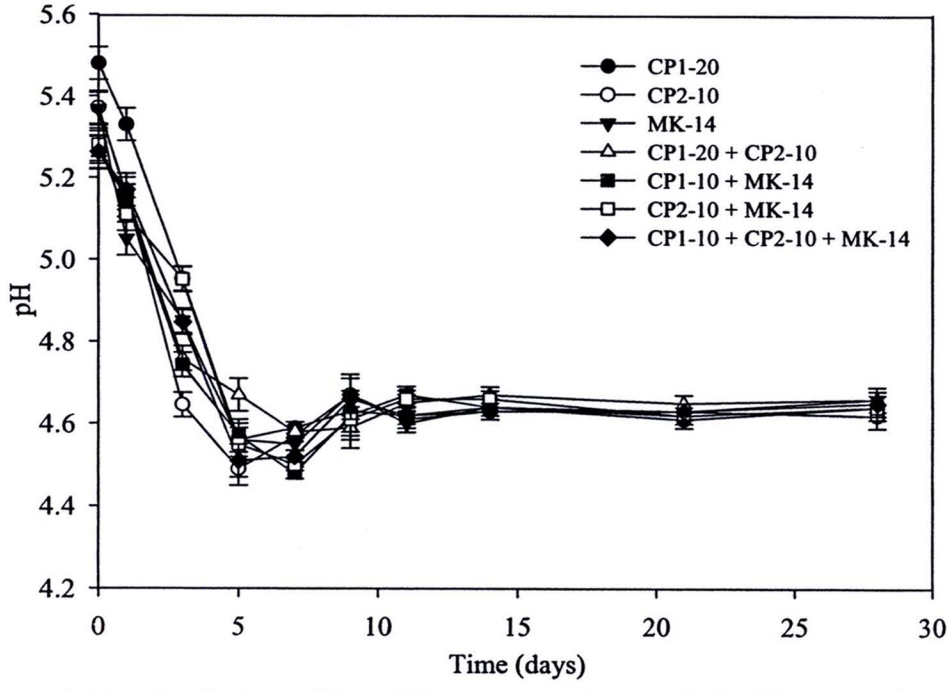

Figure 4.12 Evolution of the pH levels over time period of fermentation of Mhom production using different starter cultures

3.3.3 Weight loss

Weight loss was determined as previously described (Nakao et al., 1991). All Mhom samples with casing (100 g) were weighed before fermentation. After 1, 3, 5, 7, 9, 11, 14, 21 and 28 days of fermentation and ripening period, Mhom samples were weighed. Difference in weight of Mhom samples before and after fermentation and ripening period was referred as the weight loss. Our results revealed that the percentage of weight loss of Mhom samples increased from initial values of 10.18–15.48 (day 1) to 54.36–58.33% during the ripening process. When fermentation was prolonged to 28 days, CP210 exhibited the highest weight loss, with significant differences between CP210 and other inoculated batches (Fig. 4.13). On the basis of final weight loss as described in the previous report of Hughesa et al., 2002, in this study Mhom can be classified as the semi-dry, acid fermented sausage.

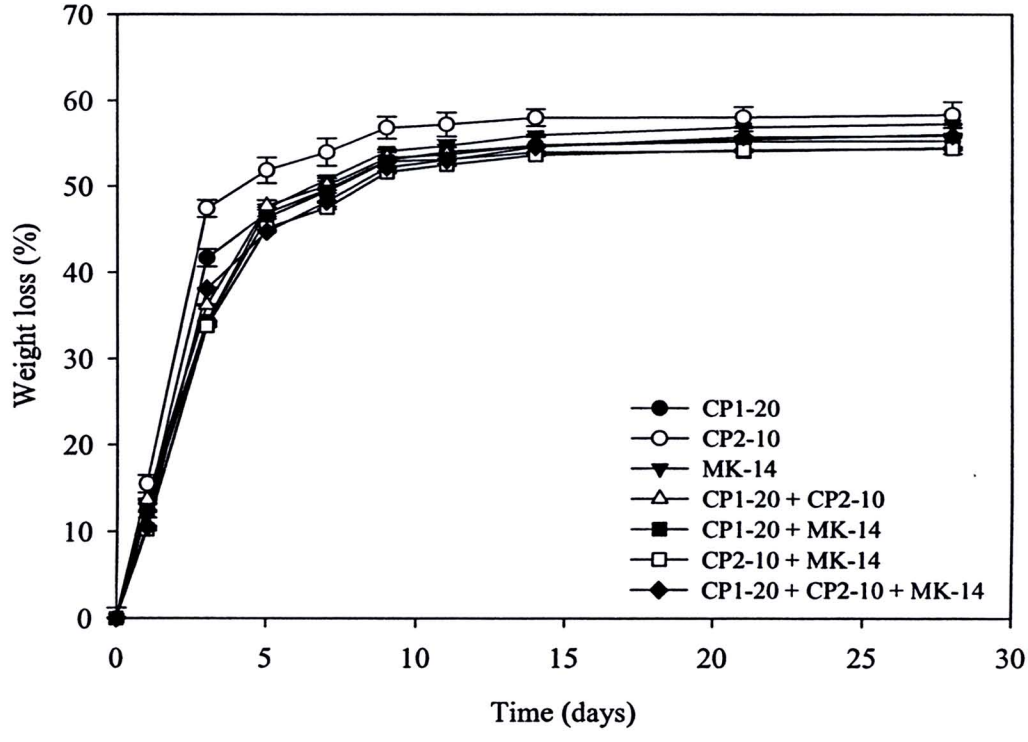

Figure 4.13 Percentages of weight loss over time period of fermentation of Mhom production using different starter cultures

3.3.4 Assay of residual glucose

The ten-fold diluted culture supernatants was used for the assay of residual glucose using dinitrosalicylic acid (DNS) as previously reported (Miller, 1959). Glucose consumption was mainly detected during the stationary phase at pH 4.5–5.9. At the beginning of fermentation, glucose consumption was initiated earlier and maintained during the stationary phase. Maximum and minimum glucose consumption was correlated with microbial growth. Seven of the inoculated batches exhibited possessed similar rates of glucose utilization (Fig. 4.14), and the amount of glucose produced varied according to the inoculated batches.

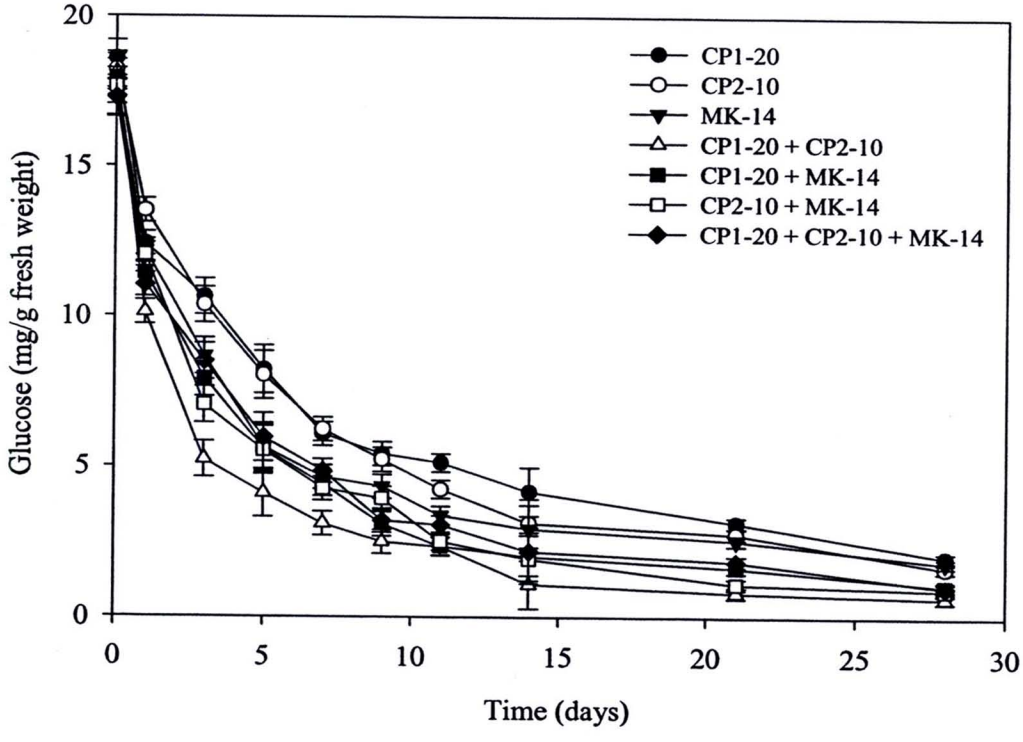

Figure 4.14 Changes in the glucose levels over time period of fermentation of Mhom production using different starter cultures

3.3.5 Lactic acid assay

The lactic acid produced in Mhom samples was analyzed with the HPLC-UV detection system as previously reported (Butkhup and Samappito, 2008). The ability to produce lactic acid is one of the most important characteristics of

the starter cultures. Quantification of lactic and acetic acids after inoculation for 3 and 14 days are summarized in Table 4.9. The concentrations of lactic acid increased gradually up to day 13 (Fig. 4.15 and 4.16). The combination of strains CP120 + CP210 resulted in the highest amount of lactic acid ($p \leq 0.05$). The rates of lactic acid accumulation and the final lactic acid values at 14 days were different for most of the test strains. When fermentation was prolonged to 14 days, all strains exhibited the higher amounts of lactic acid and acetic acid than the 3 days of fermentation ($p \leq 0.05$). Acetic acid represented a small amount of the total acids produced by all strains. For the strains CP210 + MK114, acetic acid represented about half of the total acids produced during growth for 14 days. The production of acetic acid exhibited the lower amounts in the case of strain combination than the single strain ($p \leq 0.05$). Previous reports demonstrated that lactic and acetic acids are often suggested to be major contributors to the acid aromas and tastes of fermented sausage. Also, lactic and acetic acids are likely to play an important role in imparting a tangy acidic character (Rantsiou et al., 2005; 2006). Fermented sausage with high concentrations of lactic and acetic acids generally showed higher sensory acceptance scores (Visessanguana et al., 2004). In addition, the slow decrease in pH may play an important role in the development of the fermented sausage's texture/tactile/mouth feel properties due to acid-induced changes in such proteins as myosin (Fretheim et al., 1985) and collagen (Aktas and Kaya, 2001).

Table 4.10 The amounts of lactic acid and acetic acids produced after growth of LAB isolates for 3 and 14 days during the ripening of Mhom [a]

Inoculated batches	Lactic acid (mg/g fresh weight)		Acetic acid (mg/g fresh weight)	
	3 days	14 days	3 days	14 days
CP120	33.98 bB	39.07 cA	1.83 aB	3.71 aA
CP210	31.21 bB	35.66 dA	1.79 aB	3.52 aA
MK114	33.50 bB	36.29 dA	1.81 aB	3.12 aA
CP120 + CP210	36.17 aB	44.86 aA	1.69 bB	3.88 aA
CP120 + MK114	31.96 bB	38.77 cA	1.37 bcB	1.98 cA
CP210 + MK114	27.01 cB	41.95 bA	0.90 dB	1.99 cA
CP120 + CP210 + MK114	30.49 bB	41.18 bA	1.26 cB	2.29 bA

[a] Values in a column (lower case) and row (upper case) followed by the same letter are not significantly different ($p \leq 0.05$).

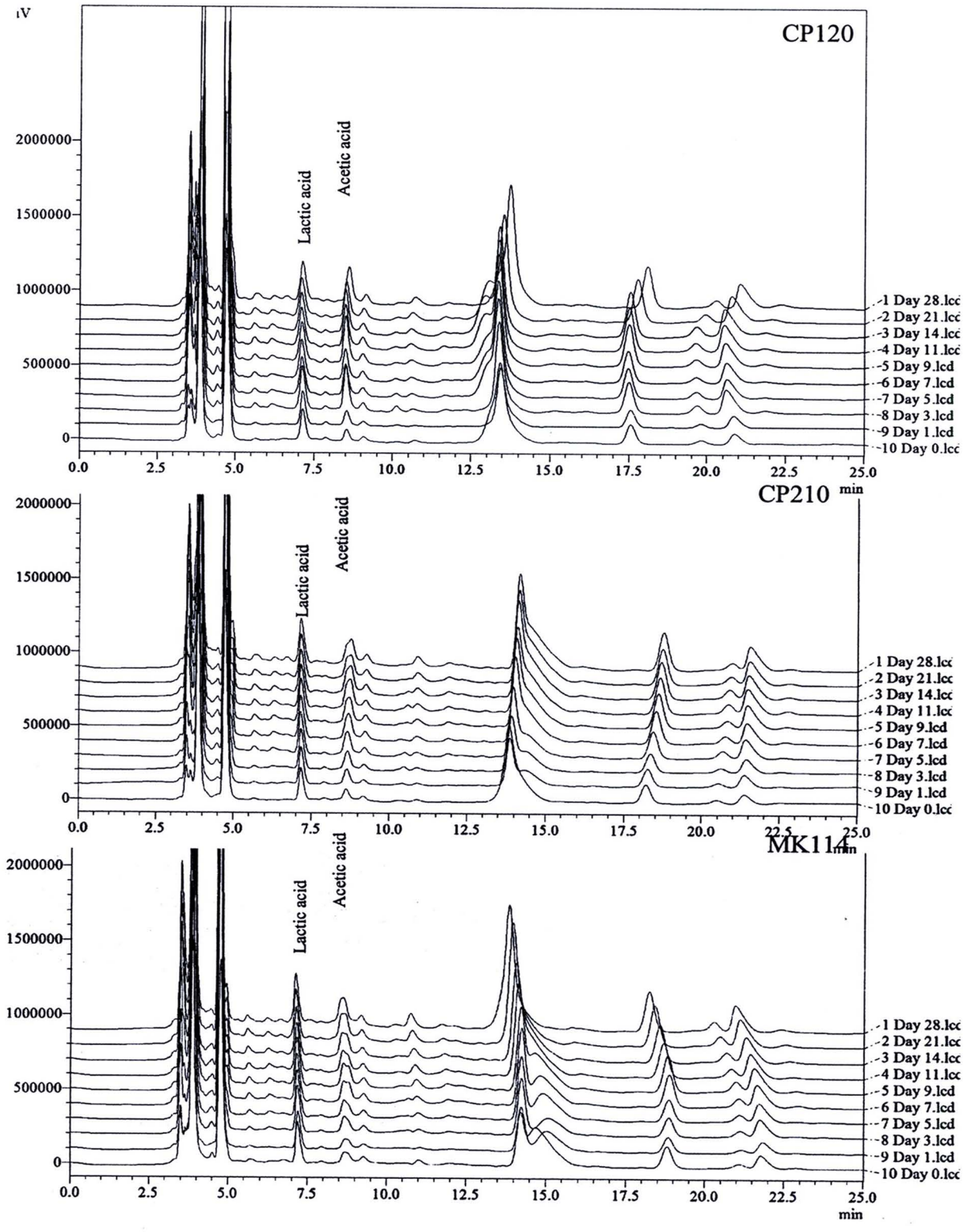

Figure 4.15 The chromatogram of lactic acid obtained during the time period of fermentation of Mhom production using different starter cultures.

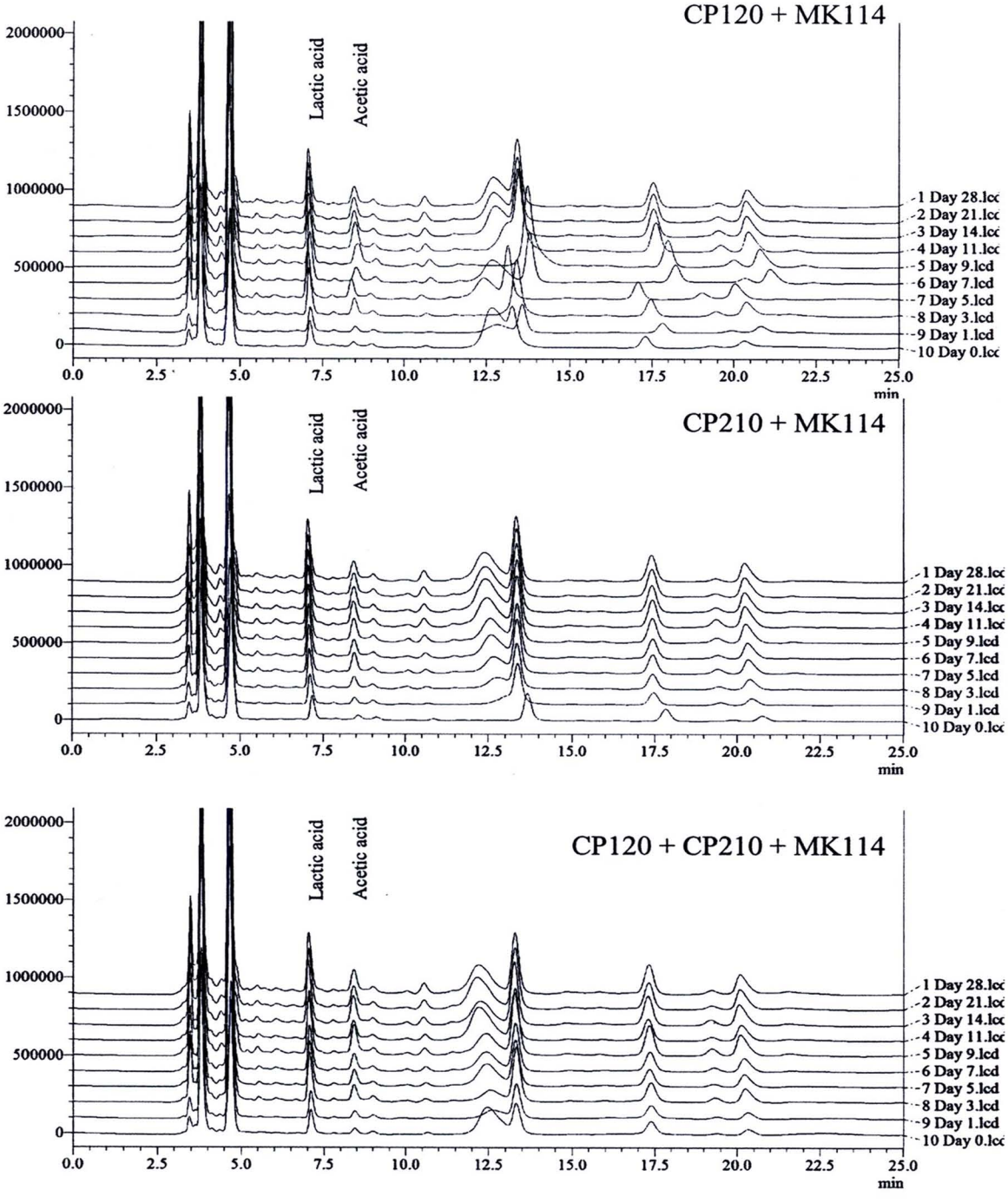

Figure 4.15 The chromatogram of lactic acid obtained during the time period of fermentation of Mhom production using different starter cultures (Cont.).

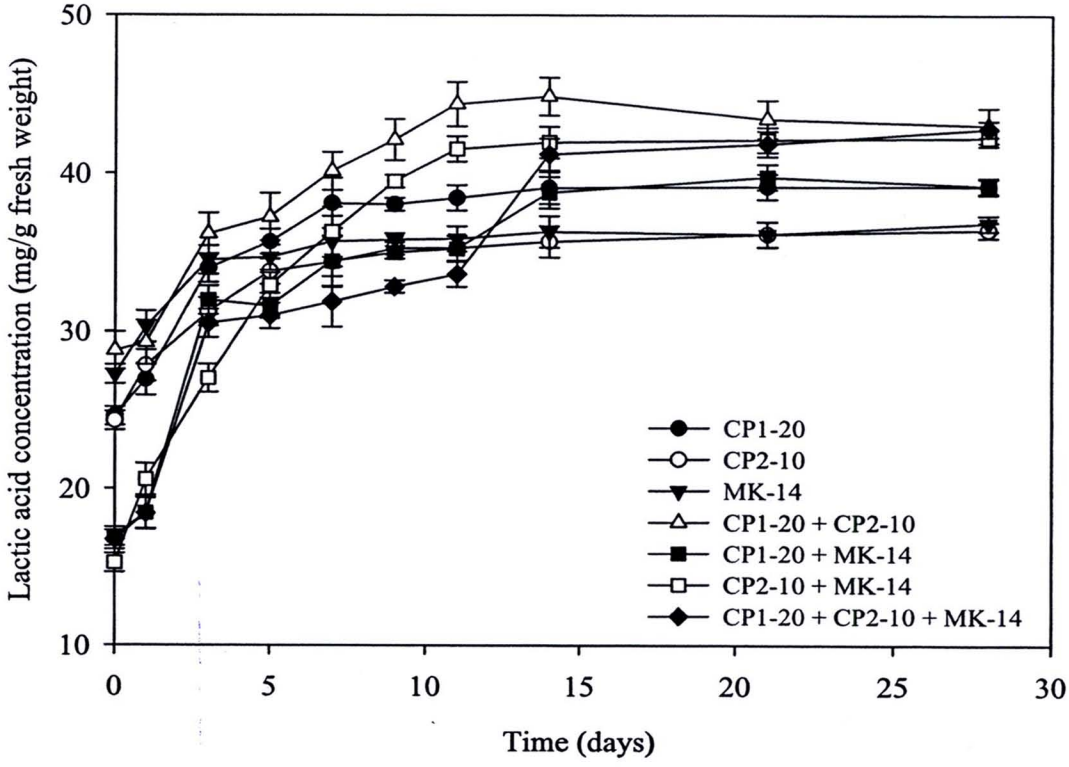

Figure 4.16 The concentrations of lactic acid produced during the time period of fermentation of Mhom production using different starter cultures

3.3.6 Microbiological analysis

In this study, 10 grams of the core of Mhom were aseptically removed, diluted with 90 ml of Saline-Peptone water and homogenized in the Stomacher machine. The homogenate was ten-fold serially diluted, spread in duplicate on the selective agar plates. The numbers of micro flora were determined from plates bearing 25 to 250 colonies. Our results revealed that changes in microbial counts in Mhom inoculated with different starter cultures were generally similar to that of naturally fermented Mhom. Fig. 4.17 demonstrates LAB as the major micro flora mainly constituted in the sausages. Due to relatively high microbial load in Mhom raw mix, inoculation of different starter cultures at the level of 10^6 cfu/g had no significant effects on the initial LAB counts during fermentation. Similar to the results of Visessanguana et al. (2004), initial flora of the fermented sausage derived mainly from the raw materials. At the beginning of fermentation, LAB of inoculated batches; CP120, CP210, MK114, CP120 + CP210, CP120 + MK114, CP210 + MK114 and

CP120 + CP210 + MK114 were 6.30, 6.00, 5.80, 6.85, 6.02, 6.60 and 6.30 log CFU/ml, respectively. The population of LAB exceeded, with the exception of MK114. After 3 days of incubation, the LAB counts in all inoculated batches increased corresponding to the decreases in pH. After 9 days of incubation, the number of LAB continued to decrease. Because of the good adaptation of LAB to the meat environment and their faster growth rates which were displayed during fermentation and ripening of sausages, they became the dominant microflora (Drosinos et al., 2005).

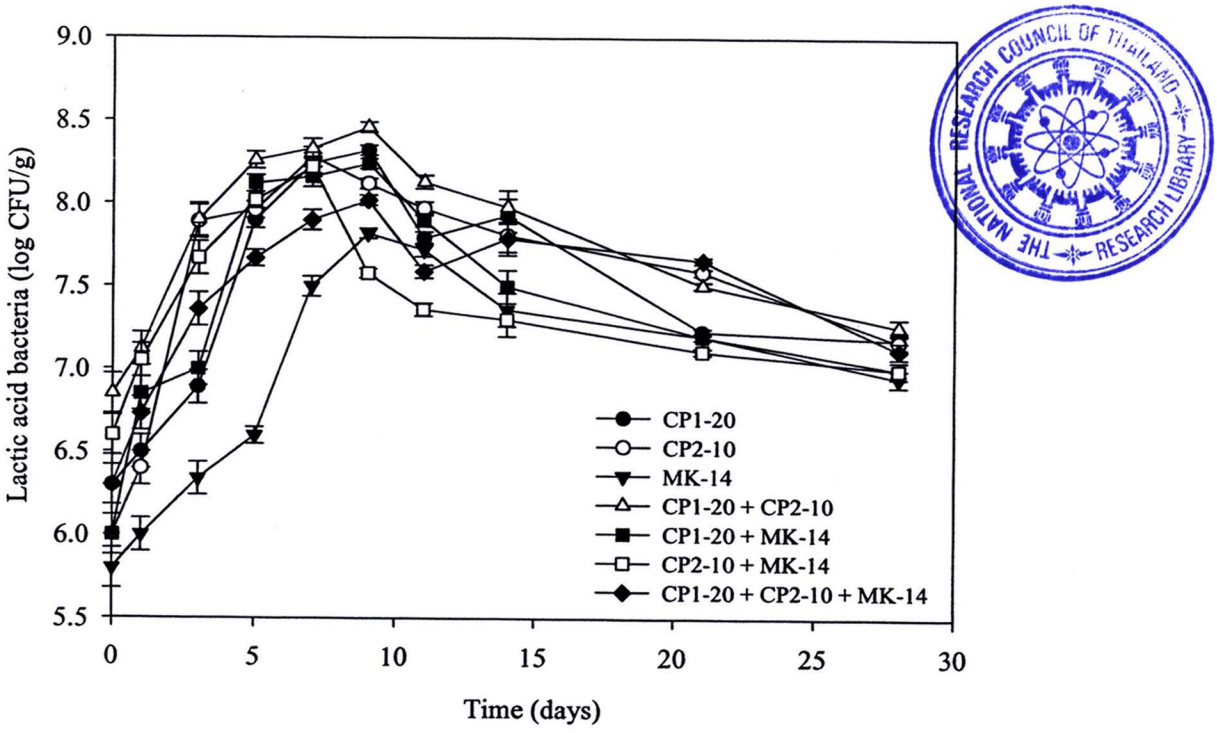

Figure 4.17 LAB count (log CFU/g) during the time period of fermentation of Mhom production using different starter cultures

Total aerobic plate counts showed the same trend with the LAB counts. Thus, fermentation of Mhom involving successive growth of different groups of microorganisms was dominated by LAB. A significant increase in total aerobic counts occurred during the first 9 days and then decreased gradually thereafter in all the inoculated batches. Total aerobic counts of inoculated batches during fermentation are shown in Fig. 4.18. Initial total aerobic plate counts of inoculated batches; CP120,

CP210, MK114, CP120 + CP210, CP120 + MK114, CP210 + MK114 and CP120 + CP210 + MK114 were 6.29, 5.30, 5.31, 5.00, 4.30, 5.05 and 6.01 log CFU/g, respectively. After 3 days of incubation, the number of microorganisms in all inoculated batches increased corresponding to the decreases in pH. After 9 days of incubation, the number of microorganisms continued to decrease. Mhom containing *L. plantarum* 1 CP120 had higher total aerobic counts than other samples at the 28 days of fermentation. The decreases in pH may be due to the production of lactic acid by LAB. In a previous report, it was shown that LAB are important in food flavor development, the presence of LAB in the sample is also expected to contribute significantly to the flavor of Mhom (Gibbs, 1987).

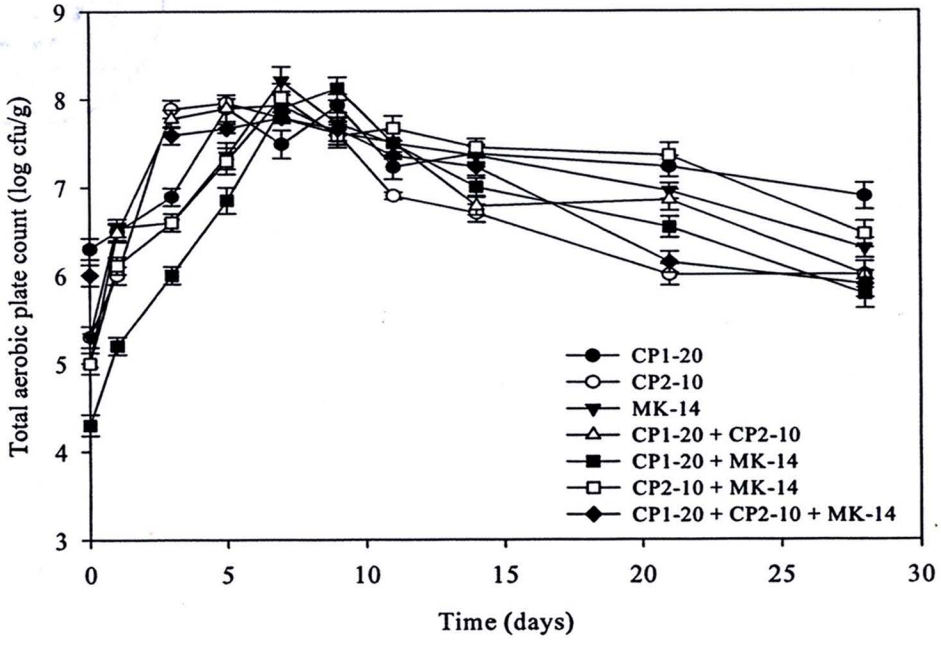

Figure 4.18 Total aerobic plate count (log cfu/g) obtained during the ripening period of Mhom production using different starter cultures

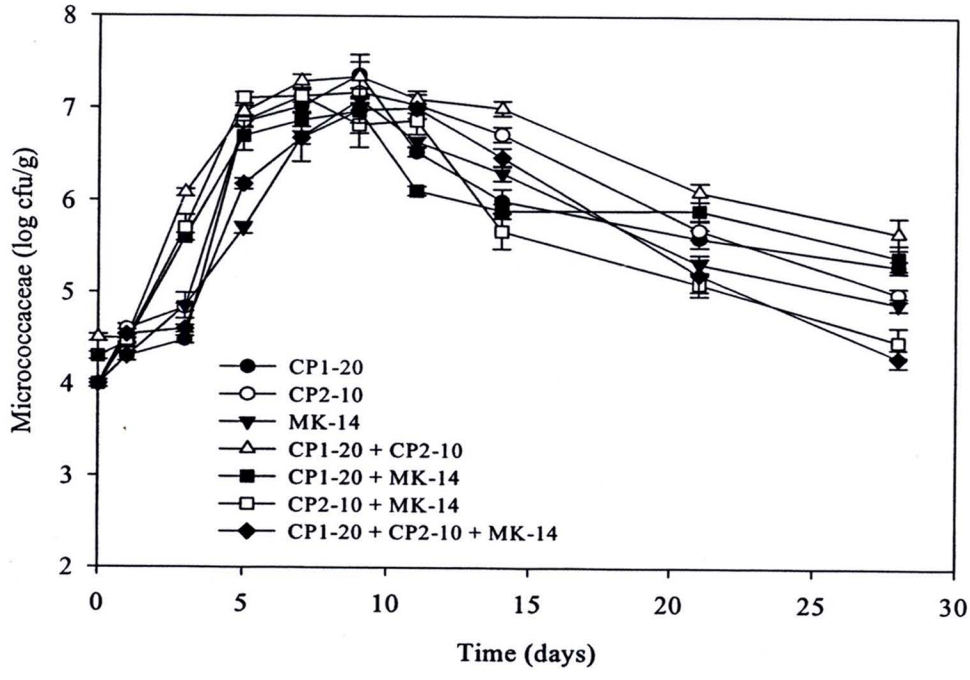

Figure 4.19 Micrococcaeae count (log CFU/g) obtained during the ripening period of Mhom production using different starter cultures

Sausages with a short ripening time have more LAB right from the early stages of fermentation. In contrast, sausages with longer maturation times contain higher numbers of *Micrococcaceae* (Comi et al., 2005). *Micrococcaceae* and LAB are the most important microorganisms used as starter cultures in the processing of dry-fermented sausages. *Micrococcaceae* participate in the preservation of meat products, avoiding rancidness and developing the typical red color due to catalase and nitrate reductase (Martin et al., 2007). The catalase produced by *Micrococcaceae* cells degrades hydrogen peroxide, which is formed particularly by aerobes and LAB, and thereby helps prevent discoloration of the product. The nitrate reductase of the *Micrococcaceae* reduces nitrate to nitrite. Nitrite can be further reduced by nitrite reductase or by chemically means at pH 5.4-5.5, giving nitric oxide as the end product. Nitric oxide binds to metmyoglobin to form nitrosomyoglobin (nitric oxide-metmyoglobin) resulting in bright pink color. Therefore, Micrococcaceae is likely to play a major role in color development of fermented meat sausages (Khieokhachee et al., 1997). Some *Micrococcaceae* strains are able to produce antimicrobial

substances. Therefore, the normal activity of this microbial group reduces spoilage, decreases processing time and contributes to flavor development (Montel et al., 1998). The addition of different strains combination to batches had no effect on *Micrococcaceae* counts, and their counts showed the same trend with total aerobic plate counts (Fig. 4.19). The *Micrococcaceae* counts started to decrease in fermented meat after 9 days of ripening. The maximum *Micrococcaceae* counts were determined at day 7 to 9 of ripening in all the inoculated batches. Aksu and Kaya (2004) found that the maximum level of *Micrococcaceae* counts was reached at day 7 of ripening was reached in Turkish dry-fermented sausage with or without a starter culture.

Enterococci are gram-positive bacteria and fit within the general definition of lactic acid bacteria. They can be used as indicators of faecal contamination, and they have been implicated in outbreaks of foodborne illness, and they have been ascribed a beneficial or detrimental role in foods. In processed meats, enterococci may survive heat processing and cause spoilage (Franz, Holzapfel and Stiles, 1999). Various metabolic products of LAB, such as short-chain organic acids, carbon dioxide, hydrogen peroxide, diacetyl, and bacteriocin, have been reported as antimicrobial agents (Rowan, Anderson and Smith, 1998). Accumulation of organic acids also resulted in the decrease in pH. Thus, the dominance of LAB is likely to contribute to the inhibition of other microorganisms. In the present study, it was found that initial counts of enterococci (5.47-6.40 log CFU/g) gradually decreased during the ripening period by more than one logarithmic unit, ending up at less than 3 log CFU/g for combination strains batches (CP120 + CP210, CP120 + MK114, CP210 + MK114 and CP120 + CP210 + MK114) and less than 4 log CFU/g for single strain batches (CP120, CP210 and MK114), possibly due to the decrease in pH and oxygen limitation (Fig. 4.20). This was a typical decrease due to environmental conditions which could make enterococci grow difficult.

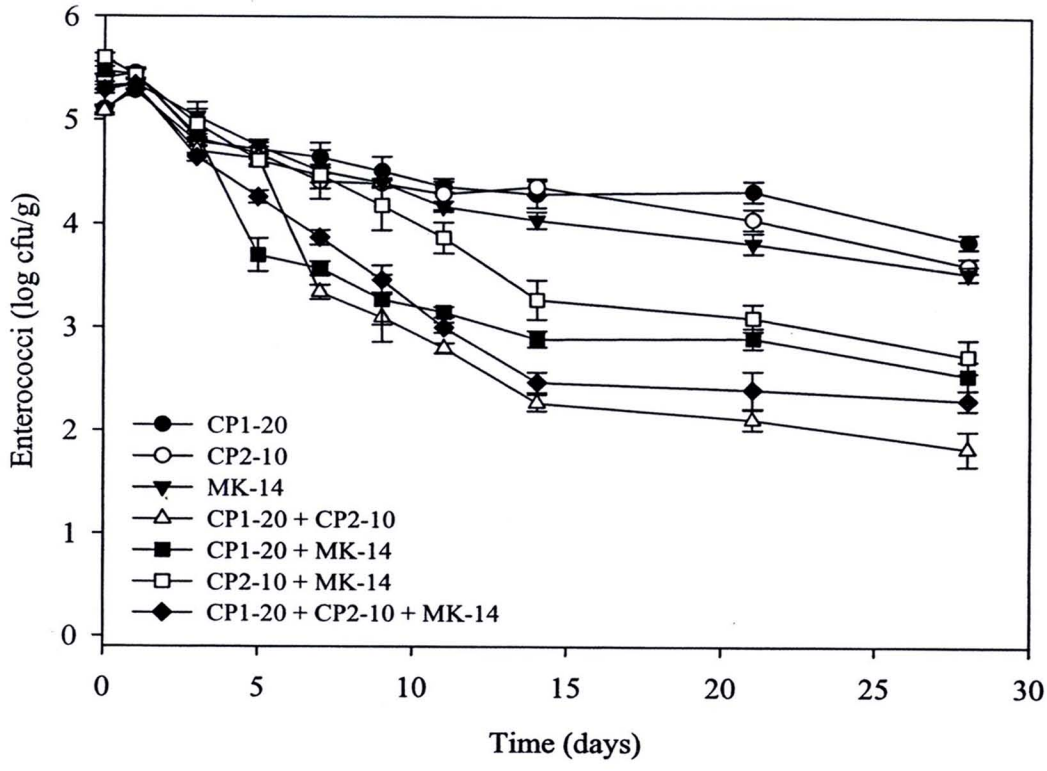

Figure 4.20 Enterococci count (log cfu/g) obtained during the ripening period of Mhom production using different starter cultures

Fermented sausages are considered good substrates for the growth of yeast and molds. The amount of yeast and mold had an effect on the development of color and flavor (Juan-Pablo et al., 2000). Yeast and mold contamination of the fermented sausage obtained mainly from the raw materials. Yeast and mold counts showed the same trend with total aerobic plate counts. The increasing of yeast and mold counts occurred during the first 7 days and then decreased gradually thereafter in all the inoculated batches. Yeast and mold counts of inoculated batches during fermentation were shown in Fig. 4.21. Initial yeast and mold counts of inoculated batches CP120, CP210, MK114, CP120 + CP210, CP120 + MK114, CP210 + MK114 and CP120 + CP210 + MK114 were 7.91, 6.02, 7.26, 7.29, 7.31, 6.60 and 6.99 log CFU/g, respectively. Thereafter, the numbers of yeast and mould continued to decrease. Yeast and mold, in spite of their lower number compared to LAB, played a significant role in producing the characteristic pigmentation (Vernam and Sutherland, 1995) and the production of flavor compounds (Coretti, 1977). It is believed that yeast delays rancidity and protects the red

nitrosomyoglobin from breakdown by degrading peroxides and consuming oxygen thus stabilizing the appealing red color of fermented sausages (Luecke and Hechelmann, 1987).

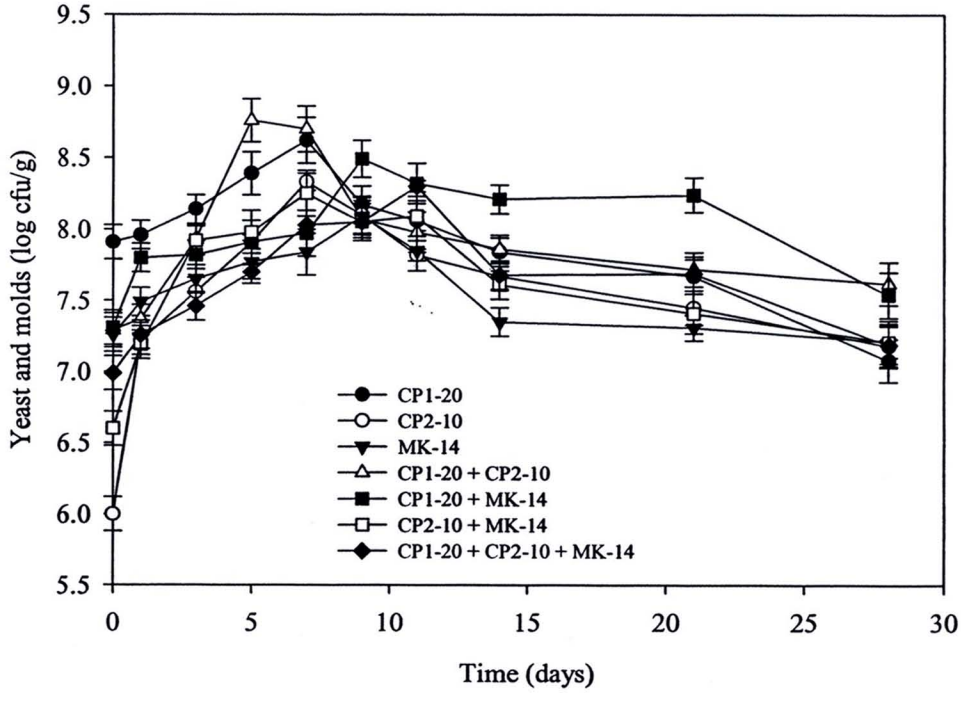

Figure 4.21 Yeast and molds count (log cfu/g) obtained during the ripening period of Mhom production using different starter cultures

Staphylococcus aureus is an important foodborne pathogen in fermented meat products (Kang and Fung, 2000). For *S. aureus* to grow, production methods play a very important role in dry and semi-dry sausages. For example, fermentation temperature applied at 30–40 °C allows possible growth of *S. aureus* and its enterotoxin production (Kaban and Kaya, 2006). In a previous study of Kaban and Kaya (2006), it was found that the starter culture preparation can reduce the growth of *S. aureus*. In present study, the initial counts of *S. aureus* (4.00-4.53 log CFU/g) were gradually decreased during ripening by more than one logarithmic unit, ending up at less than 3 log CFU/g for combination strains batches (CP120 + CP210, CP120 + MK114, CP210 + MK114 and CP120 + CP210 + MK114) and less than 4 log CFU/g

for single strain batches (CP120, CP210 and MK114), possibly due to the decrease in pH and oxygen limitation (Fig. 4.22). This was a typical decrease due to environmental conditions which could make *S. aureus* grow difficult.

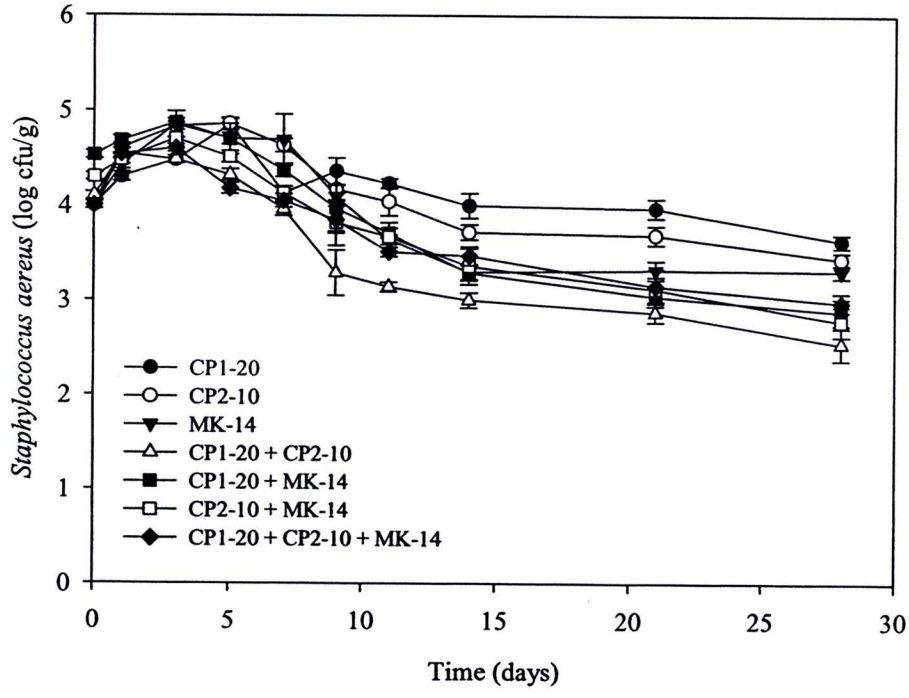

Figure 4.22 *Staphylococcus aureus* count (log cfu/g) obtained during the ripening period of Mhom production using different starter cultures

4. Monitoring the population dynamic of the appropriate starter cultures during the fermentation period

In general, the fermented sausages are prepared from season and raw meat that is stuffed in the casings and allowed to ferment and mature (Lucke, 1987). As have been previously reported, inoculation of the sausage batter with a starter culture composed of selected LAB improved the quality and safety of the final product and standardizes the production process (Hugas and Monfort, 1997; Lucke, 1987). The primary contribution of LAB to flavor generation is ascribed to the production of large amounts of lactic acid and some acetic acid, although they also produce volatiles through fermentation of carbohydrates (Molly et al., 1996). Nevertheless, small manufacturers continue to use the traditional method of spontaneous fermentation

without adding any starter cultures. In the latter case, the required microorganisms originate from the meat itself or from the environment and constitute a part of the so-called "house flora" (Santos et al., 1998). Back-slopping is also used, if material from a successful previous batch is added to facilitate the initiation of a new fermentation process (Alley et al., 1992). The most promising bacteria for starter cultures are those which are isolated from the indigenous micro flora or house flora of traditional products. These microorganisms are well adapted in the meat environment and are capable of dominating the micro flora of products. The aim of starter culture use is being able to catch and standardize products made by natural fermentation, suitable to local habits and preference for each country. Cultures added to sausage mix, grow up together with the beginning flora. That is why, producers have to take in hygiene care and proceed with safe raw materials if they would control fermentation. Appropriate cultures must be selected according to the specific formulation of Mhom fermentation since environmental factors would interact to select only the strains that have high potency to dominate the process.

In the present study, *L. plantarum* was used as a starter culture for Mhom processing and compared to control (no inoculated culture added). The starter cultures were isolated and selected from commercial Mhom for their high antimicrobial activity and could be tolerance to the environmental conditions. Freeze-dried starter cultures of *L. plantarum* 1 CP120, *L. plantarum* 1 CP210, and *L. plantarum* 2 MK114 added at the level of 10^6 colony forming units (CFU)/g of meat batter were used as inocula in the meat fermentation experiments (Mhom). Several multi-combinations of the appropriate starter cultures as shown in Table 4.7 were applied into the mixture of raw materials and ingredients. The microbial profiles were then followed and evaluated for the dynamic changes during the fermentation period. Mhom samples prepared with the defined combination of the appropriate starter cultures were determined for the proximate analysis. Total aerobic plate count, LAB, *Micrococcaceae*, Enterococci, *S. aureus*, yeast and mold counts were determined during ripening, and pH, lactic acid, acetic acid, glucose consumption and weight loss were also analyzed.

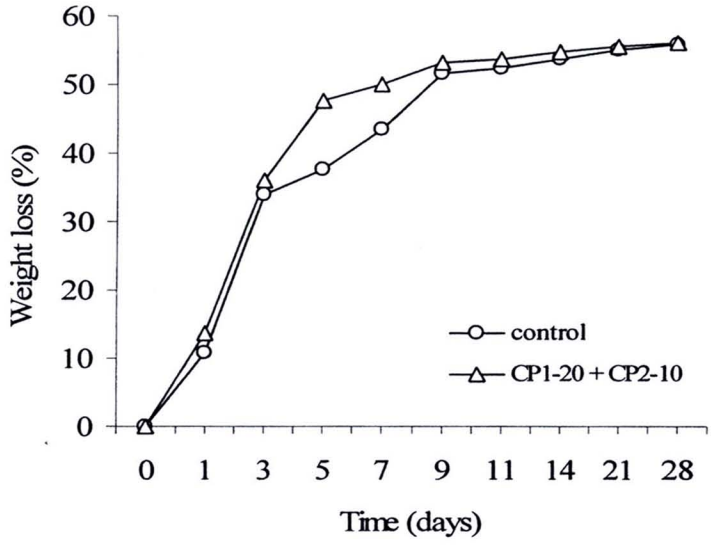

Figure 4.23 Percentage of weight loss observed during the time period of fermentation of Mhom production with and without using starter cultures (*L. plantarum* CP120 + *L. plantarum* CP210)

Increases in weight loss were observed as the fermentation proceeded (Fig. 4.23). Weight loss of fermented Mhom generally decreased as the fermentation time increased. As the fermentation proceeded, Mhom inoculated with *L. plantarum* CP120 + *L. plantarum* CP210 had a greater weight loss than the control. This study produced results which corroborate the findings of a great deal of the previous work in the field that were carried out with Nham. It was concluded that weight loss in meat products is mainly associated with loss in water and water-holding capacity of meat (Visessanguan et al., 2004).

The pH values of Mhom products generally decreased with increasing fermentation time (Fig. 4.26). A rapid decrease in pH during the first 7 days was probably due to an increased amount of organic acids, mainly lactic acid (Fig. 4.25) produced by LAB. LAB are the major producers of lactic acid responsible for the decrease in pH and the increase in acidity during the fermentation (Visessanguana et al., 2004). The average pH value of the sausage mix before stuffing was virtually similar in both systems of Mhom studied, with the average values of 5.40 for the control Mhom and of 5.38 for inoculated batch CP120 + CP210. These initial pH values were lower than some of the values found in the literatures (Salgado et al.,

2005; Coppola et al., 1997; Lizaso et al., 1999). With respect to the evolution of pH during the ripening process of Mhom, substantial differences were found between the results of two inoculated batches. In both cases, significant decrease was observed up to 3 days of ripening, which coincides with the maximum consumption of sugar (Fig. 4.25), the time at which average pH values of 4.95 and 4.75 were observed, in the control and CP120 + CP210, respectively. From this time, the drop in pH of the control and CP120 + CP210 was decreased gradually to the lowest levels, with the average pH values of 4.66 and 4.58, respectively, as LAB reached their late exponential phase. The reduction in pH value may best be explained as due to the involvement of LAB growth that were able to ferment sugars (glucose in particular) to produce lactic acid, which then resulted in the lowering of the pH. From day 14, a slight increase in pH was observed in all treatments and reached an average value of 4.71 (control) and 4.65 (CP120 + CP210) at the end of the process (28 days). This may be due to the production of ammonia and biogenic amines as a result of enzymatic activity.

From day 14, a slight increase in lactic acid content was observed in all treatments and reached an average value of 34.53 (control) and 43.02 mg/g fresh weight (CP120 + CP210) at the end of the process (28 days). This may be due to the consumption by microbial groups that are present, and to the formation of low molecular weight nitrogen compounds, i.e., ammonia and biogenic amines (Salgado et al., 2005; Coppola et al., 1997; Lizaso et al., 1999). However, at the end of fermentation (pH~4.6), Mhom inoculated with starter cultures (*L. plantarum* CP120 + *L. plantarum* CP210) had lower acetic acid than the control (Fig. 4.24 and 4.25). Lactic and acetic acids are often suggested to be major contributors to the acid aromas and tastes of the fermented sausage. Also, lactic and acetic acids are most likely to play an important role in imparting a tangy acidic character. Fermented sausage with high concentrations of lactic and acetic acids generally showed the high sensory acceptance scores (Visessanguana et al., 2004).

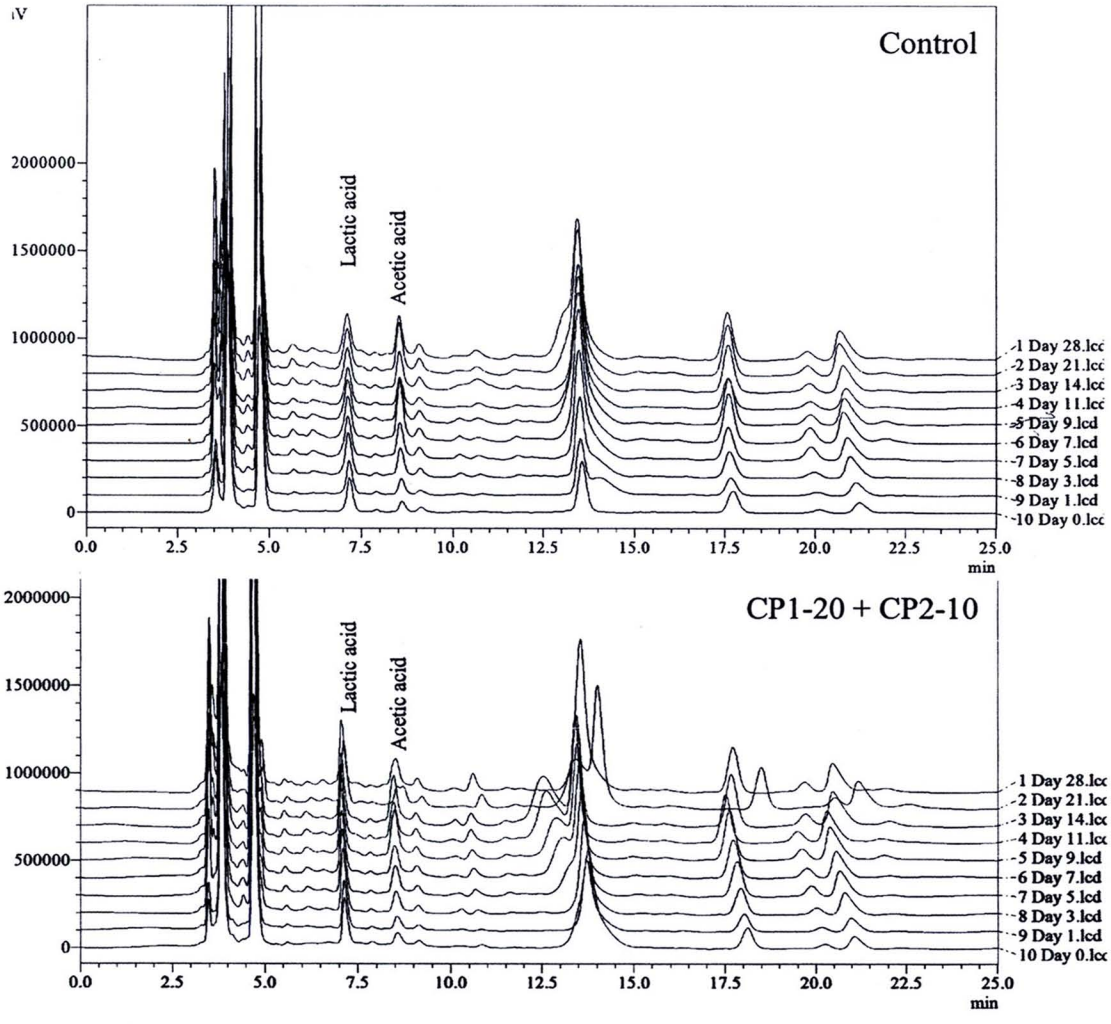

Figure 4.24 HPLC Chromatogram of lactic acid obtained during the time period of fermentation of Mhom production with and without using the starter cultures (*L. plantarum* CP120 + *L. plantarum* CP210)

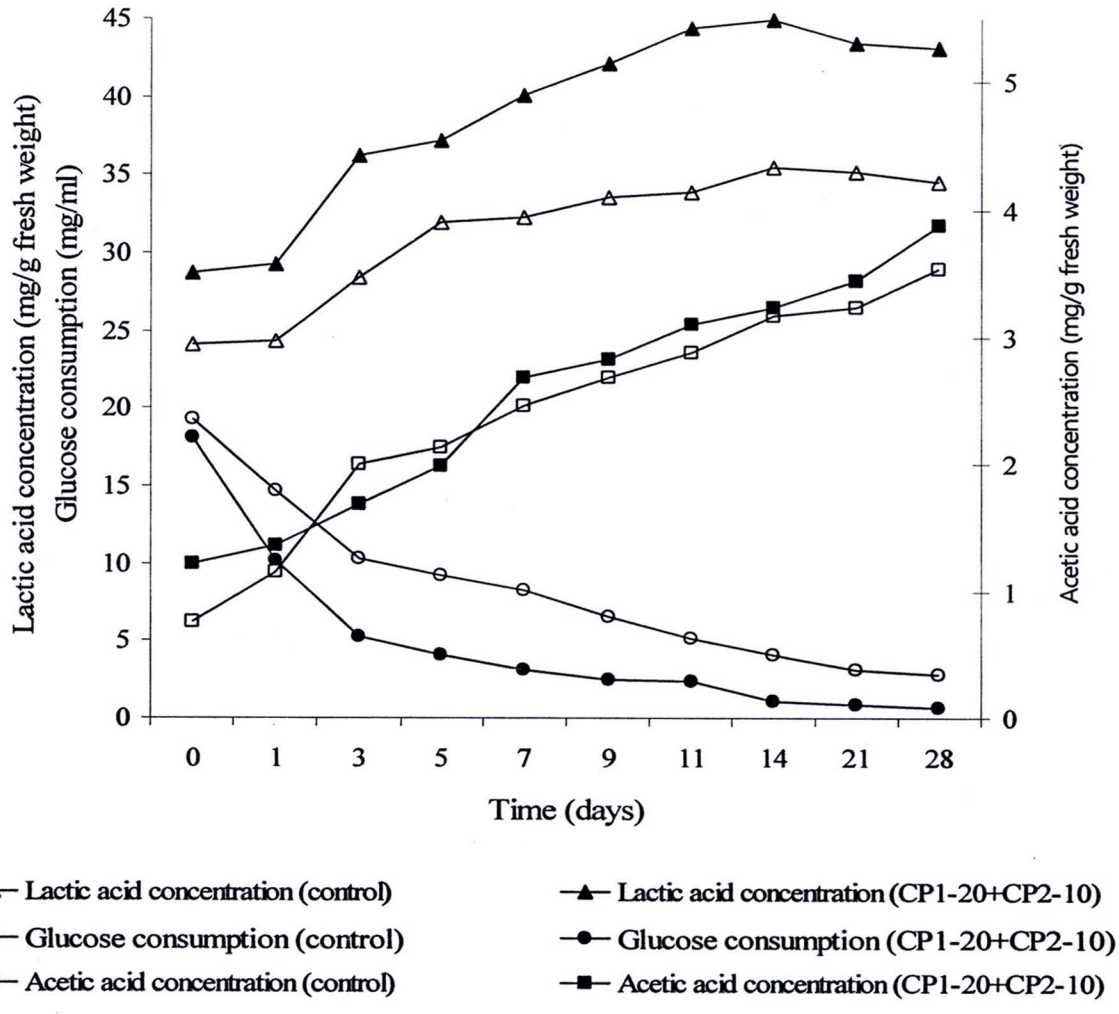

Figure 4.25 Changes in lactic acid concentrations and glucose consumption over the time period of fermentation of Mhom production with and without using the starter cultures (*L. plantarum* CP120 + *L. plantarum* CP210)

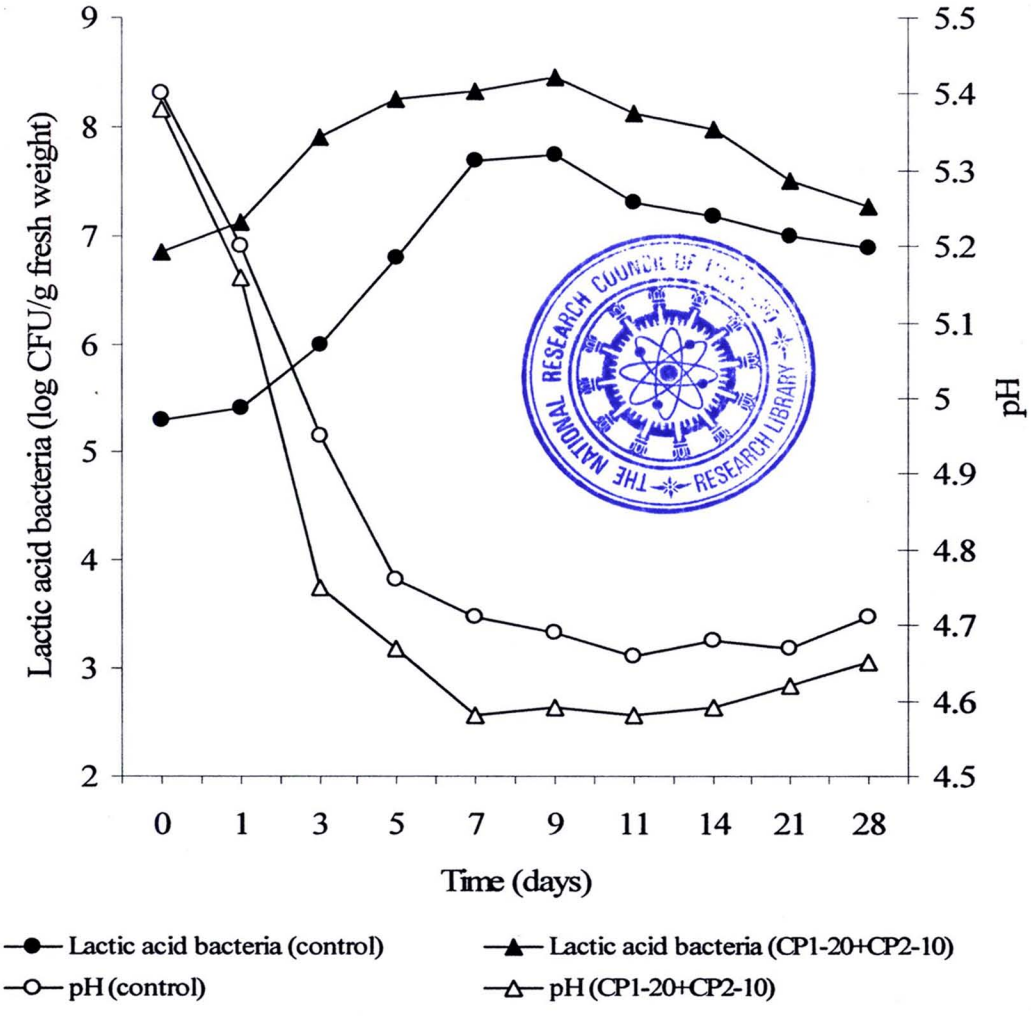

Figure 4.26 Changes in pH values and LAB counts over the time period of fermentation of Mhom production with and without using the starter cultures (*L. plantarum* CP120 + *L. plantarum* CP210)

Our results demonstrated that Mhom fermentation was characterized by a rapid increase in the number of LAB which increased from an initial value of 5.30 log CFU/g to 7.74 log CFU/g (control) and 6.85 log CFU/g to 8.46 log CFU/g (CP120 + CP210) within the first at 9 days of ripening and decreased gradually for the rest of the fermentation period (Fig. 4.2). LAB profiles showed significant differences ($p \leq 0.05$) between the control and the inoculated batches, which reflected the starter culture growth.

Changes in total aerobic plate count, enterococci, *Micrococcaceae*, *S. aereus*, yeast and mold counts during fermentation of Mhom with starter cultures (*L. plantarum* CP120 + *L. plantarum* CP210) and control (no starter culture added) are shown in Fig. 4.27. At the starting time, total aerobic plate counts of the control and inoculated batch (CP120 + CP210) were 5.93 and 5.68 log CFU/g, respectively. The highest numbers of total aerobic plate counts of the control (7.12 log CFU/g) and inoculated batch (7.93 log CFU/g) occurred at day 9 and day 5. In the initial samples, *Micrococcaceae* profiles showed significant differences ($p \leq 0.05$) between the control and the inoculated batch, which reflected the starter culture growth. The initial numbers of *Micrococcaceae* in the control and inoculated batch (CP120 + CP210) were 4.50 and 5.30 log CFU/g. The highest numbers of *Micrococcaceae* count of the control (7.26 log CFU/g) and inoculated batch (3.4 log CFU/g) observed at days 7 and 9, respectively, then gradually decreased until the end of the ripening process and remained at level of 5.12 and 5.65 log CFU/g for the control and inoculated batch, respectively. At the end of the fermentation, the numbers of *Micrococcaceae* in the inoculated batch were higher than those of the control, which contained only micrococci derived from raw materials. Enterococci decreased steadily in numbers at the beginning from 5.43 and 5.33 log CFU/g of the control and inoculated batch (CP120 + CP210), respectively, to 3.44 and 1.83 log CFU/g at 28 days of ripening. Starter culture had an effect on the *S. aureus*. The highest average number of *S. aureus* was determined in the control and this value was statistically different from that of the inoculated batch ($p \leq 0.05$). The duration of the ripening period also had a significant effect on the *S. aureus* count (Fig. 4.27). At the beginning of the fermentation, the numbers of *S. aureus* in Mhom products were 5.28 and 5.31 log CFU/g, respectively, for the control and inoculated batch (CP120 + CP210). These values gradually decreased after 5 days until the end of the ripening process. In contrast, yeasts and molds were detected along the fermentation period. These results indicated that the starter culture preparation can reduce the growth of *S. aureus* when compared to the control. These results are consistent with those of other studies of Kaban and Kaya (2006).

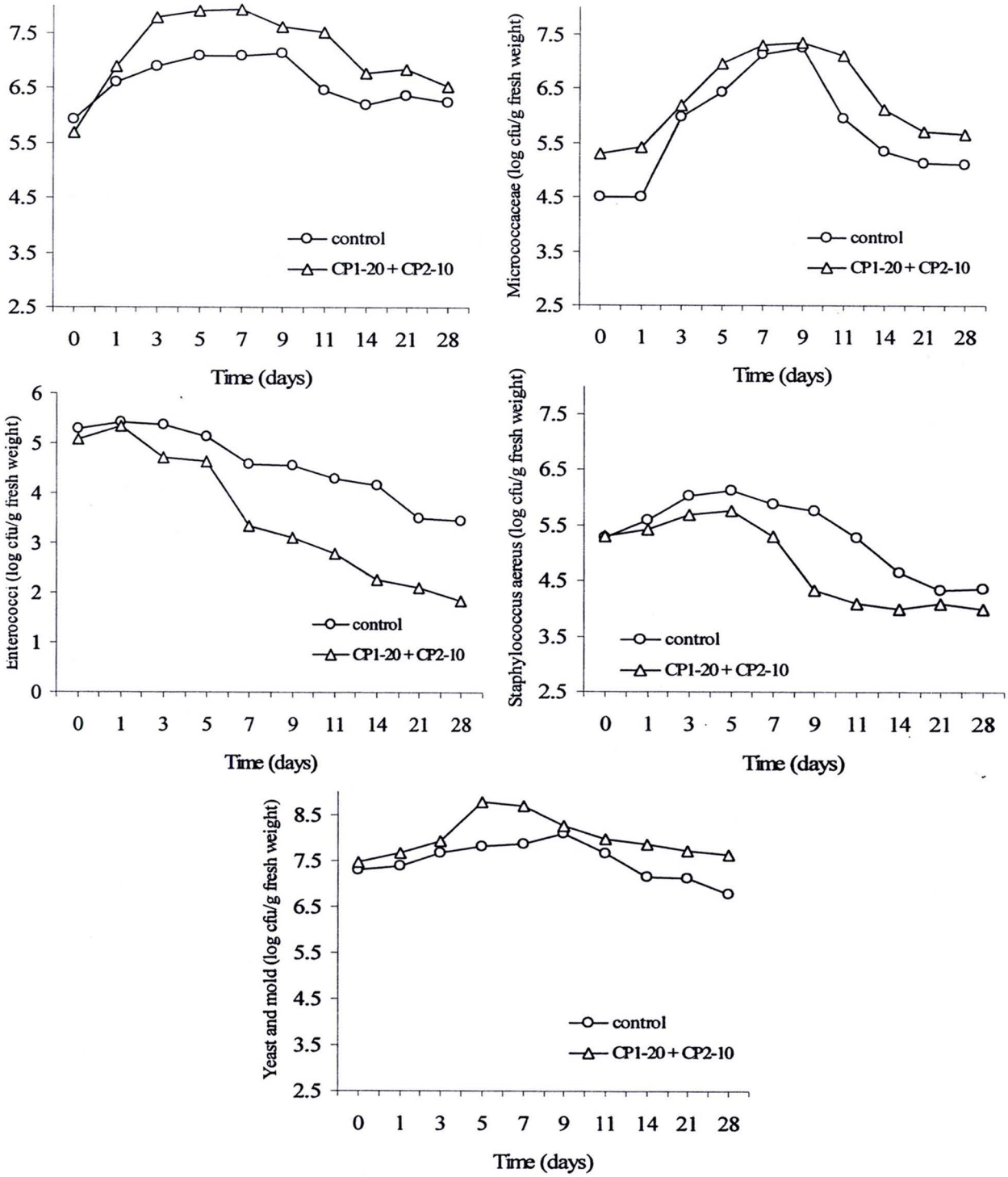

Figure 4.27 Changes in total aerobic plate count, enterococci, *Micrococcaceae*, *Staphylococcus aureus*, yeast and mold counts during fermentation of Mhom with starter cultures (*L. plantarum* CP120 + *L. plantarum* CP210) and control (no starter cultures added).

Organoleptic analysis

Several lines of evidence demonstrated that in the production of fermented sausages, one of the major groups of organisms commonly isolated and believed to be responsible for the organoleptic characteristics of the final product are LAB, and mainly to be *Lactobacillus* spp. They are isolated throughout the fermentation and possess the enzymatic activities that may improve the texture, taste and odor of the sausage (Rantsiou et al., 2005; 2006). In this study, at the end of fermentation (pH~4.6), Mhom manufactured with the LAB as starter culture was evaluated by a panel of ten members. The results were compared with un-inoculated naturally fermented sausage served as the control. Mhom samples were slice in halved with 1 cm thickness. Samples were coded using the random numbers with three digit, and were presented at the same time in randomized order. The panelists were asked to assess samples for their appearance as well as their appreciation was scaled from 0, low intensity or absence, to 10, high intensity. The ratings of each attribute were converted to numerical scores for further statistical analysis. For further characterization of the acceptance of Mhom product a 'just-about-right', or JAR scale with five categories (1 = too weak, 5 = too strong, and 3 = just-about-right) was presented for the evaluation of color, flavor, sourness, odor, saltiness and texture (Meilgaard et al., 1991).

Mhom products for organoleptic analysis were made as already described above. They were presented to a taste panel at the end of the study. Mhom fermentation with *L. plantarum* CP120 + *L. plantarum* CP210 as starter culture were compared to those performed without the starter culture, i.e., control. To get more information about the reasons for liking, a just-about-right scale was attached for twelve attributes including color, uniform color, crustiness, sweetness, saltiness, acid taste, bitterness, glutamate, mustiness, hardness, cohesiveness and fat melting. Qualitatively, similar results were obtained with both types of Mhom (Table 4.10). Fermentation with the LAB starter resulted in a less sweet, musty flavor and more acidic taste compared to an un-inoculated one. These properties were highly appreciated by the consumers.

Table 4.11 Sensory evaluation of Mhom manufactured with or without the starter culture at the end of the fermentation period [a]

Attribute	Lot numbers	
	Without starter	CP120 + CP210
Color	5.61	5.64
Uniform color	5.57	5.62
Crustiness	4.20	4.23
Sweetness	1.34 b	0.72 c
Saltiness	3.45	3.42
Acid taste	3.44 c	4.62 b
Bitterness	1.22	1.26
Glutamate	1.69	1.74
Mustiness	5.69 b	2.34 c
Hardness	6.25	6.21
Cohesiveness	6.37	6.33
Fat melting	1.38	1.42

[a] The appreciation was scaled from 0, low intensity or absence, to 10, high intensity.

[b] and [c], means within the same row with different superscript differ ($p \leq 0.05$).