DISCUSSION

The goal of this study was to monitor changes of microbial population in fermented Nham with added starter culture compare with natural fermentation by molecular method based on PCR-techniques. Nhams were sampled at specific time interval during fermentation, microbial counts were determined and chemical values were measured. In chemical analysis, the final pH of Nham was ranging from 4.2 to 4.4 and the lactic acid concentration was found to be between 2.1-2.4 %, which was typical characteristic of Nham. This was resulting from typical microbial growth in Nham fermentation where LAB is increasing in numbers by utilize sugar, producing acids consequence decrease in pH of the system. In microbiological analysis, no significant differences in the growth patterns of microbial population were observed in the both types of Nham fermentation except those of *Enterobacteriaceae*. The populations of *Enterobacteriaceae* were fairly different in both type of Nham at the early stage (0-12 h) of fermentation, which abruptly increase in natural while gradually decrease in starter cultured Nham. After 24 h, the counts of *Enterobacteriaceae* gradually decrease toward until the end of fermentation in both type of Nham since these microorganisms are inhibited by pH decrease, high acidity and the antimicrobial substance produced by lactic acid bacteria (Antara *et al*., 2002 and García-Fontáin *et al*., 2007). However, the numbers of *Enterobacteriaceae* in starter cultured Nham were significantly lower at the end of fermentation. This may be due to Nham inoculated with *Lb. plantarum* accelerated the fermentation process approximately 12 h faster than natural. In additional, starter cultured Nham had higher lactic acid than natural Nham. The reduction of *Enterobacteriaceae* by decreasing pH is in agreement with the results previously demonstrated (Antara *et al*., 2004). From these results showed the important potential of starter culture to acidify its environment rapidly, as the acid production and the accompanying pH decrease are well-known to extend the lag phase of sensitive organisms including food-borne pathogen (Kostinek *et al*., 2007). Although, the number of LAB population as determined by plating analysis was not significantly different in both types of Nham fermentation, the LAB population in starter cultured Nham were higher than those in natural Nham during the first stage 12 h of fermentation prior to be being slightly

lower throughout the later part of fermentation. Our results were fairly similar to those observed by Opaswatcharanon (2004) and Valyasevi *et al*. (2006). No noticeable difference in initial LAB cell counts despite the inoculation was performed with *Lb. plantarum* at value 10^4 cfu/g. Since the initial LAB population was near 10^7cfu/g and therefore, the number of starter could be considered as negligible.

A total of 1,529 bacterial isolates from both types of Nham fermentation were subjected to molecular identification using combination of ITS-PCR and 16S rDNA sequencing. Dynamics changes in the ecology of the major bacterial species during fermentation were observed. At the early stage (0-6 h) of fermentation, the microbial diversity was similar in both types of Nham fermentation. At the start of fermentation, *Lc. garvieae* were the predominant species in both types of Nham. The high proportion of *Lc. garvieae* isolates present in Nham may be come from the raw materials, especially pork meat that is contaminated during slaughtering. Since *Lc. garvieae* has been described as an emerging zoonotic pathogen responsible for mastitis in cows and buffalos (Ammor *et al*., 2005). Therefore, cross-contamination from the surface that contact with the meat, the environment, the manufacture equipment, the manipulation, etc., can also contribute to the contamination of pork during slaughtering. Other predominant species were *M. caseolyticus* and *Lc. lactis*. The other bacterial species identified at lower proportion were *K. oxytoca*, *K. pneumoniae*, and *Klebsiella* sp., *B. cereu*s, *S. gallinarum*, and *Enterococcus* sp. The variety of species detected in Nham could be due to several aspects that go from contamination in the processing plant and in the fermentation rooms, to contamination derived from the presence of a higher number of personnel handling the products. Although, the pathogenic bacteria found in low proportion of isolates, they were abundant enough in Nham to be isolated from countable plates. These species presence in Nham at the beginning of fermentation demonstrates the low hygienic conditions of Nham production in this study. Surprisingly, no *Lb. plantarum* species were isolated in starter cultured Nham despite these species were inoculated in Nham at start of fermentation. It may be due to the inoculation were small amounts when comparing between natural LAB microflora and therefore, *Lb. plantarum* species were not detected at this sampling time. After 6 h of fermentation, *Lc. garvieae*

remained dominant species in both types of Nham. *Lc. lactis* remained constant while *M. caseolyticus* disappeared in both types of Nham fermentation. *M. caseolyticus* was found only once at 0 h of fermentation. It might be expected that the use of local types of spices, especially garlic which may be inhibited the growth and distribution of this microorganism during the 6 h of Nham fermentation.

After 12 h of fermentation, the significant difference in species composition was observed between natural and starter cultured Nham. In starter cultured Nham, the dominant species were *Lc. lactis* followed by, *Lb. plantarum* and *Lc. garvieae*. In contrast to the natural fermentation, *Lc. lactis* were the predominant species followed by *Lc. garvieae*. The genetic diversity of LAB species in natural fermentation was demonstrated the low hygienic of Nham which the species *K. oxytoca* and *K. pneumoniae* remain found at this sampling point. In addition, these species could also be related to the counts of *Enterobacteriaceae* that appeared at highest value of 9.6 x 10^6cfu/g at this fermentation time. It should be noted that *Lc. lactis* became the predominant species, while *Lc. garvieae* remained involved but dramatic decreasing of population in both types of Nham fermentation. Moreover, it is interesting to notice that *Lb. plantarum* has only been found in significant numbers of total isolates obtained from starter cultured Nham.

At 24 h of fermentation, the LAB species from natural Nham displayed the higher diversity, with 9 species identified while the species composition of starter cultured Nham were comprised of 5 species. *Lb. plantarum* became the predominant species followed by *Lc. lactis* and *Lc. garvieae* in starter cultured Nham. In contrast to natural Nham, *Lc. lactis* remained a dominant species followed by *P. pentosaceus, Lb. plantarum* and *Lc. garvieae*. It is a noteworthy that *Lb. plantarum* became the predominant species in starter cultured Nham, while these species were only found at a small percentage in natural Nham at this sampling time. The rapid increased of *Lb. plantarum* in starter cultured Nham, possibly due to the use of *Lb. plantarum* to initiate the fermentation. In addition, Valyasevi *et al*. (2006) had suggested the ability to use polyfructose founded in garlic of this *Lb. plantarum* starter culture. Therefore, these reasons could be used to explain the presence of *Lb. plantarum* as a dominant

species in starter cultured Nham during fermentation. Furthermore, it should be noted that *P. pentosaceus* was found to be the second dominant species at much lower proportion in natural Nham. This also agrees with previous studied on the LAB species associated with the commercial Nham (Tanasupawat and Daengsubha, 1983, Tanasupawat *et al.*, 1992 and Kunawasen, 2000). It should be mentioned that the use of *Lb. plantarum* BCC 9546 as starter culture may plays an important role to inhibit the population of *P. pentosaceus* in Nham fermentation and therefore, starter culture had a role in decreasing diversity of the LAB species which resulting in less heterogeneous LAB than in natural fermentation.

At 36 h of fermentation, the natural fermentation remained higher heterogeneous microbial diversity than those in starter cultured Nham. The dominant species remained *Lc. lactis*, followed by *P. pentosaceus* and *Lc. garvieae* in natural Nham. In comparison to starter cultured Nham, *Lb. plantarum* remained the dominant species while *Lc. Lactis* rapidly decreased at this sampling time. Moreover, it is interesting to notice that *Lc. garvieae* disappeared in both types of Nham fermentation at this period. The absent of *Lc. garvieae* might resulted from high lactic acid production and a decrease in pH in both types of Nham at this sampling time.

At the last stage (48-72 h) of fermentation, differences in LAB species composition were clearly observed between Nham inoculated with and without starter culture. The natural fermentation showed a higher level of microbial diversity than those founded in starter cultured Nham. The majority of LAB species remained constant in both type of Nham. At 48 h of natural fermentation, *Lb. plantarum* remained a dominant species followed by *P. pentosaceus*. On the contrary, *Lb. plantarum* remained dominant species followed by *Lc. lactis* in starter cultured Nham. From 60 h through the end of fermentation, in natural Nham, *Lb. plantarum* species were continually increased while *P. pentosaceus* species were not dramatically reduced. In contrast to starter cultured Nham, *Lb. plantarum* was the only dominant species continually increased until the end of fermentation. Furthermore, it should be mentioned that *Lb. plantarum* species were an absolute predominant species in starter inoculated Nham which could be explained not only by a better adaptation to the

complex environment of Nham but also by inhibition the growth of the other LAB species, especially *P. pentosaceus* in Nham fermentation.

Our results indicating that the main bacterial species found in Nham were member of LAB group. Isolates of *Lb. plantarum* were obtained from both types of Nham fermentation. In natural fermentation, *Lb. plantarum* was initially detected in small proportion when fermentation reached 24 h and became predominant species after 36 h until the end of fermentation period as described by previous studied (Techapinyawat, 1975 Tanasupawat and Daengsubha, 1983, Tanasupawat *et al.*, 1992 Kunawasen, 2000, Valyasevi *et al*., 2001 and Phoonsawat, 2005), our results confirm the important of these *Lb. plantarum* species for natural Nham fermentation. Moreover, *Lb. plantarum* strains are involved in many fermented food products for acid production during fermentation such as cassava, vegetable and sausages fermentation. (Wiriyacharee *et al*., 1990, Antara *et al*., 2002, Tamang *et al*., 2005 and Kostinek *et al*., 2007). This agrees well with the results on the finding of this study. On the other hand, in starter culture inoculated Nham, *Lb. plantarum* was detected in significant numbers when fermentation reached 12 h and became predominant species after 24 h until the end of fermentation period. This might reflect in the more rapidly decline of pH and more lactic acid were produced in Nham with the starter culture especially when the fermentation reached 24-36 h since *Lb. plantarum* is very efficient acid producer (McDonald *et al*, 1990). In our study, sugar analysis was determined by Plengvidhaya *et al.* (2005). The result obtained from this research showed that a large increase in availability of free fructose when fermentation reached 24 h which can contribute to the significantly lower pH of Nham may be correlated with the starter culture *Lb. plantarum* domination in the fermentation. This information also agrees with the finding by Valyasevi *et. al*. (2006) and might suggest the ability to use of polyfructose founded in garlic of *Lb. plantarum* starter culture.

The rep-PCR fingerprinting technique using $(GTG)_5$ primer is a genotypic tool for rapid and reliable speciation and typing of lactobacilli and other LAB in food-fermentation products (Gevers *et al*., 2001, Tamang *et al*., 2005, Ouadghiri *et al*., 2005, and Kostinek *et al*., 2007) including fresh sausages (Cocolin *et al.*, 2004).

Concerning the genotypic characterization of the 4 *Lb. plantarum* reference strains, rep-PCR was shown in our study to be a suitable method for trace of *Lb. plantarum* strain BCC 9546. The characterization by rep-PCR was performed on the *Lb. plantarum* strains isolated from starter cultured inoculated Nham. The results obtained underlined how distributive *Lb. plantarum* BCC 9546 strain was in Nham fermented with this type of starter culture. In addition the role of unmarked *Lb. plantarum* BCC 9546 in the fermentation of Nham could help in deciding the use of this strain in Nham processes for the manufactory. To our knowledge this is the first time that rep-PCR was used to tracking changes of unmarked *Lb. plantarum* starter culture during Nham fermentation. Subsequence characterization of the 413 *Lb. plantarum* isolates by rep-PCR fingerprinting using $(GTG)_5$ primer revealed that the majority of the isolates generated identical fingerprint patterns as that of the starter culture strain, indicating successful growth and persistent of the starter culture in the fermentation process. A small number of *Lb. plantarum* isolates generated unique and different fingerprints from the starter culture, showing diversity within the species naturally occurred in Nham. During fermentation of *Lb. plantarum* BCC9546-inoculated Nham, the starter culture was rapidly grown and dominated in the fermentation process during the first 12 h and maintained its high prevalence throughout the fermentation period. Although other strains of *Lb. plantarum* were detected, the starter culture strain was the most predominant representing higher than 90% of total *Lb. plantarum* isolates. Starter or BCC 9546 predominated over other strains during Nham fermentation. This suggests that they are well adapted to the Nham environment. Our results are confirming the importance of this strain as responsible for Nham fermentation.