Characterization and detection of *Lactococcus lactis* subsp. *lactis* Sb 2 as probiotic starter in beef Nham

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Abstract— **The probiotic agent designated as Sb 2, isolated from seabass (*Lates calcarifer*), was identified as *Lactococcus lactis* subsp. *lactis* by 16s rDNA. Bacteriocin gene of this strain was identified as *Nisin A* by PCR technique using specific primer. This strain was applied as starter culture in beef Nham. Survival of *L. lactis* subsp. *lactis* Sb 2 during Nham fermentation at 30°C for 3 days was detected by PCR-RAPD fingerprint technique. The 3 distinct DNA bands about 1200, 2000 and 3000 bp, which corresponded to DNA fingerprint of this strain, were found from day1 to day 3 of fermentation. This confirmed the survival of *L. lactiss* subsp. *lactis* Sb 2 during Nham fermentation process**.

Keywords— **Probiotic, Starter cultures, PCR-RAPD**

I. INTRODUCTION

Nham is a traditional Thai fermented meat which has been popular among consumers in Thailand. It is normally eaten without cooking and consider being ready-to-eat after 3-4 days of fermentation process [1, 2]. It has been reported that Nham sold in retail markets contaminated *Salmonella* Anatum, *Listeria monocytogenes* and *Staphylococcus* [3, 4]. However, pathogen contamination can be reduced by using starter culture to achieve an acidic pH of ≤ 4.6 during fermentation [5]. Therefore, recent studies were aimed on inhibition of pathogen during product fermentation [6, 7].

L. lactis subsp. *lactis* Sb 2 was isolated from gastrointestinal tract of seabass (*Lates calcarifer*). This strain was able to produce bacteriocin, which inhibit several bacteria including *Staphylococcus aureus*, *Listeria innocua*, *Enterococcus feacalis*, *Leuconostoc mesenteroides* and *Pseudomonas fluorescens* ect. In addition, this strain has been shown probiotic properties as it was able to grow and produce bacteriocin when culture in pH 3-10, NaCl 1-5% and survive in gastrointestinal tract model [8, 9, 10]. Thus, the objective of this study was to determine the survival of *L. lactis* subsp. *lactis* Sb 2 during beef Nham fermentation, in order to use this strain as probiotic starter culture.

Materials and Methods

A. Nham preparation

Nham formulation : containing cooked rice, grinding fresh garlic, sodium nitrite, sugar, mixed all ingredients and hand kneaded for 10 min before adding 10^6 cfu/g of *L. lactis* subsp. *lactis* Sb 2 and mixed thoroughly again. This mixture was filled tightly in a long thin edible skin and tied both ends. Then, Nham were left fermented at 30 °C for 3 days.

B. Identification and detection of bacteriocin gene

L. lactis subsp. *lactis* Sb 2 was tested for bacteriocin gene using specific primer for Nisin A Forward (5'- CCGGAATTCATAAGGAGGCACTC AAAATG -3') and Nisin A Reverse (5'-CG GGGTACCTACTATCCTTTGATTTGGTT -3') [11]. Analysis of DNA sequences was searched by BLAST program (Genbank). Moreover, randomly selected lactic acid bacteria isolated from beef Nham, which showed similar DNA finger print pattern to *L. lactis* subsp. *lactis* Sb 2 were also detected for Nisin A with the same specific primer.

C. ***Polymerase Chain Reaction-Random Amplified Polymorphic DNA (PCR-RAPD)***

Lactic acid bacteria isolated from beef Nham were randomly selected at least 20 colonies everyday from day 1 to day 3 of fermentation for DNA fingerprint by RAPD technique using specific lactic acid bacteria Primer ; OPA – 3 (5'-AGTCAGCCAC-3') (Biodesign, U.S.A.) [12]. Synthesis of DNA products, using single lactic acid bacteria colony as DNA template, was performed in 25 µl reactions of Taq DNA polymerase (Vivantis, Malaysia) for DNA amplification. Reaction mix and PCR cycle program were prepared as suggested by the manufacturer except denaturing and annealing step for PCR was done at 94 ℃, 5 min and at 36 ℃, 1 min. PCR product were electrophoresed on 1.5% agarose gels stained with ethidiumbromide (1 µg/ml). The band pattern was visualized using gel documentation system (GeneGenius, U.S.A.).

Results and Discussion

A. Identification of bacteriocin gene in L. lactis subsp. lactis Sb 2

L. lactis subsp. *lactis* Sb 2 were tested for bacteriocin gene using specific primer for Nisin A. The PCR results demonstrated that 200 bp products was bacteriocin (Fig 2). Further, this PCR products was sequenced and data searched by BLAST program (Genbank) from NCBI (http://www.ncbi.nlm.nih.gov/) with 99% homology to Nisin A. (Accession no. AF46535.1 on 24/09/2009). This result was similar to [13] that characterized Nisin Z from *Lactococcus lactis* N 12. Moreover, Noonpakdee [14] detected 227 bp DNA products from *L. lactis* WNC 20 isolated from Nham using the same specific primer for Nisin A.

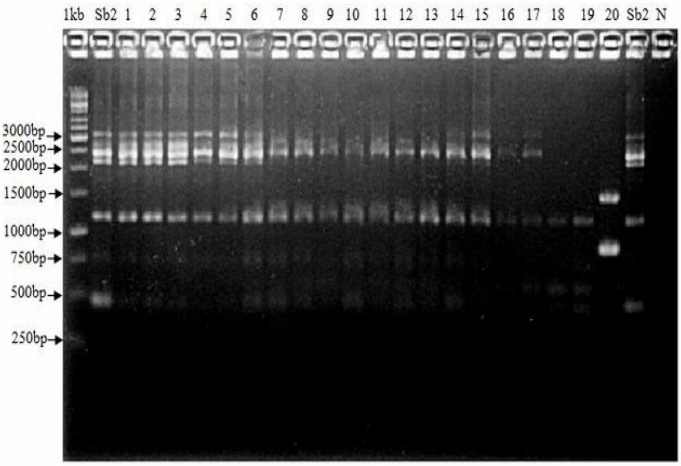
B. Detection of Lactic acid bacteria using Polymerase Chain Reaction-Random Amplified Polymorphic DNA (PCR-RAPD)

The results showed that there were 3 distinct DNA bands products at 1,200, 2,000 and 3,000 bp from randomly selected lactic acid bacteria colonies from day 1 to day 3 of Nham fermentation. These DNA products were corresponded to *L. lactis* subsp. *lactis* products. However, there were 17, 20 and 2 isolates out of 20 that showed DNA products matching from day1 to day 3 of Nham fermentation (Fig.1 A, B, C). Therefore, *L. lactis* subsp. *lactis* Sb 2 could survive fermentation process in beef Nham with pH 4.5 in high salt condition. As a result of this, *L. lactis* subsp. *lactis* Sb 2 has a good potential to act as probiotic starter culture. This was similar to *in vitro* work done by Pilasombut [8] reported that *L. lactis* subsp. *lactis* Sb 2 can be survive in pH 3-10 and NaCl at 1-5%.

C. Detection of bacteriocin gene; Nisin A

Random lactic acid bacteria colonies no. 1-18, 21-36 and 41-42 from day 1 to day 3 of fermentation respectively, were tested for *Nisin A* bacteriocin gene in order to confirm that these colonies were *L. lactis* subsp. *lactis* Sb 2. All tested colonies presented 200 bp DNA products after PCR reaction with Nisin A specific primers (Fig. 2).

A)



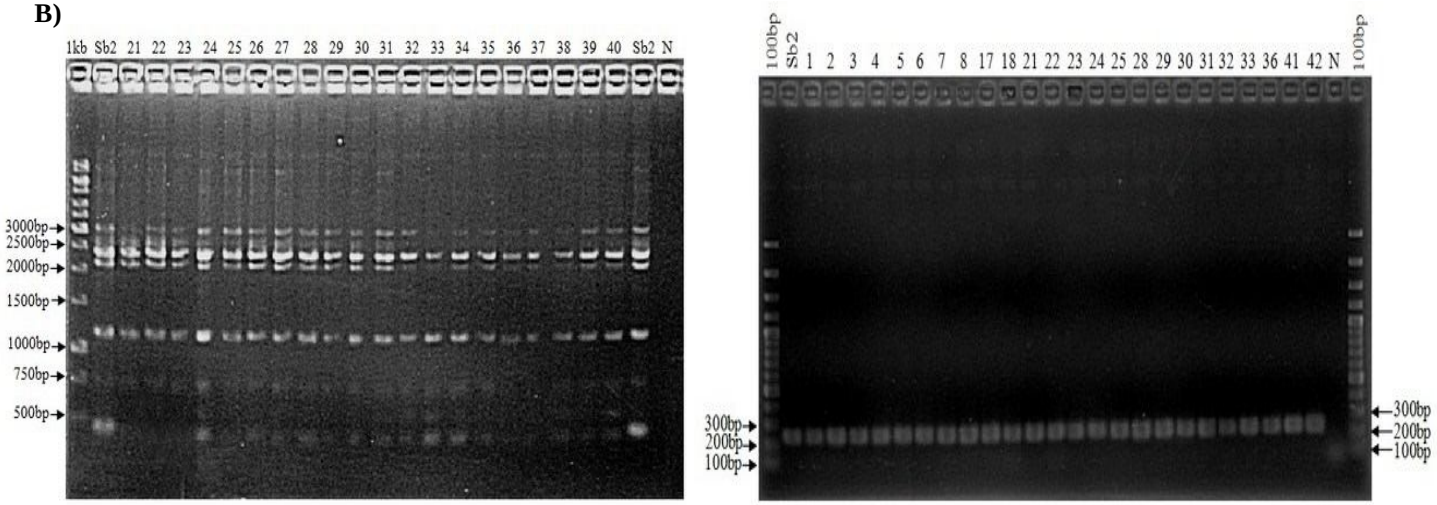


Fig. 1 DNA fingerprint of amplified single colony of Nham inoculated with *L. lactis* subsp. *lactis* Sb 2 using OPA-03 primer at day 1-3 of fermentation process. Lane M = DNA standard 1 kb Ladder; Sb 2 = DNA fingerprint of with *L. lactis* subsp. *lactis* Sb 2; N = Negative control. (A) Lane 1-20 = colony at day 1 of fermentation; (B) lane 21-40 = colony at day 2 of fermentation; (C) lane 41-60 = colony at day 3 of fermentation.

Fig. 2 Showing the DNA product of *L. lactis* subsp. *lactis* Sb 2 from PCR reaction, using specific primer for *Nisin A* gene. Lane M = DNA standard 100 bp Ladder; Sb 2= *Nisin A* gene of *L. lactis* subsp. *lactis* Sb 2, lane N = Negative control**,** lane 1-18, lane 21-36 and lane 41-42 = colony isolated at day 1 to day 3 of fermentation, respectively.

II. CONCLUSIONS

Survival studied of *L. lactis* subsp. *lactis* Sb 2 in beef Nham fermentation process by PCR-RAPD fingerprint technique revealed similar DNA fingerprint patterns of *L. lactis* subsp. *lactis* Sb 2 marker from day 1 to day 3. In addition to, *Nisin A* gene was also detected from these isolates. Therefore, Sb2 could survive through fermentation process and have a potential to apply this *L. lactis* subsp. *lactis* Sb 2 as a probiotic starter culture in beef Nham.

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