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Identification of lactic acid bacteria isolated from Thai fermented sausage for nitrite degradation ability

Wanangkarn, A.[1, 2*], Tan, F. J.[3], Phumthong, N.[1] and Boonsema, P.[1]

[1]Department of Agricultural Science, Faculty of Agriculture, Natural Resources and Environment, Naresuan University, Phitsanuloke 65000, Thailand; [2]The Center for Agricultural Biotechnology (AGBIOTECH-NU), Naresuan University, Phitsanuloke 65000, Thailand; [3]Department of Animal Science, College of Agriculture and Natural Resources, National Chung Hsing University, Taichung 402, Taiwan.

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Abstract Many traditional Thai fermented meat products were naturally fermented, relying on the natural contamination by house flora resulting in products with inconsistent qualities and unsafe products. The lactic acid bacterial species in Thai fermented sausage (E-sarn sausage) was identified by using molecular techniques and investigated their potential to reduce the nitrite content. The isolated strains were identified by sequencing 16S ribosomal DNA as *Enterococcus durans*, *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Pediococcus pentosaceus*. The nitrite degradation capacity in MRS broth, *E. durans*, *L. plantarum*, *L. brevis*, and *P. pentosaceus* could degrade 89.35%, 79.11%, 86.22% and 72.87% of the initial nitrite concentration, respectively. The nitrite concentration decreased throughout fermentation time and exhibited the lowest final value as 12.78-32.28 μg/ml at the third day. These results suggested that lactic acid bacteria isolates from E-sarn sausage had showed a potential as starter culture to reduce the residual nitrite in Thai fermented meat products.

Keywords: Fermented sausage, Identification, Lactic acid bacteria, Nitrite degradation

Introduction

Many traditional fermented meat products in Thailand, including E-sarn sausage (pork fermented sausage) were naturally fermented by house flora, which occurs during animal slaughtering and sausage manufacturing (Ammor *et al.*, 2005). Furthermore, this natural fermentation may result in products with inconsistent qualities and even unsafe products (Wanangkarn *et al.*, 2012). For this reason, fermented sausages are generally manufactured with food preservatives (Paik and Lee, 2014). Nitrate and nitrite are widely used as curing agents by combining with potassium or sodium salts in meat products to extend the shelf life (Goswami *et al.*, 2014) due to their preserving action on color, flavor, and the reduction of lipid oxidation (Alahakoon *et al.*, 2015). Moreover,

[*]**Corresponding Author**: Wanangkarn, A.; **Email**: Amornrat.w@nu.ac.th

nitrite is recognized as an antimicrobial agent to inhibit the growth of pathogenic bacteria, which cause food poisoning, such as *Clostridium botulinum*, *Salmonella* spp. and *Listeria* spp. (Hospital *et al*., 2014; Tompkin, 2005). However, the addition of a high level of nitrite has toxicological risks due to the transformation of nitrite to N-nitrosamine (Mirvish, 1995; Cammack *et al*., 1999).

N-nitrosamines are formed by the reaction between the nitrosating agents, which originate from nitrite and amines derived from protein (De Mey *et al*., 2017). These substances are considered carcinogens and mutagens that can cause gastric and colorectal cancer in humans (Oostindjer *et al*., 2014; Zhu *et al*., 2015). Due to the health concerns, the maximum level of sodium nitrite permitted by the Ministry of Public Health of Thailand is 125 ppm of sodium nitrite for meat products. The current approved levels of sodium nitrite in meat products may be revised in the near future due to the increasing demand of additive-free and healthy food products by consumers. However, the addition of nitrate and nitrite should be limited to the necessary minimum levels, which provide enough protection against food poisoning (Hospital *et al*., 2012). The residual nitrite level in meat products is dependent on many factors, including heat treatment, storage condition, food additives, pH value, and packaging type (Boci *et al*., 2014). Furthermore, previous studies demonstrated that some lactic acid bacteria (LAB) in meat products are highly efficient in both nitrate and nitrite reductase activities (Ammor and Mayo, 2007).

Therefore, the purpose of study was identified LAB species in Thai fermented sausage (E-sarn sausage) and investigated their potential as starter culture for fermented sausages with nitrite reducing capacity.

Materials and Methods

Sausage sampling and isolation of lactic acid bacteria

Freshly-manufactured E-sarn sausages were obtained from a local meat factory (Phitsanuloke, Thailand) and transported to the lab. After collection, the sausages were stored at 4°C until analyzes. A 25-g sample was homogenized with 225 ml of 0.85% NaCl solution (w/v). Serial dilutions of the samples were made and plated onto the Man Rogosa Sharpe (MRS) agar supplemented with 1% $CaCO_3$ to distinguish the acid-producing bacteria and incubated at 37 ℃ for 48 h (APHA, 2001). LAB isolates were selected and streaked on MRS agar (Harrigan, 1998). The purified colonies were stored as liquid cultures in MRS broth with 30% (v/v) glycerol at -80 ℃ before being subjected to molecular identification (Papamanoli *et al*., 2003).

Depletion of sodium nitrite by LAB culture in MRS broth

The nitrite reducing capacity of LAB was determined as described by Esmaeilzadeh *et al*. (2012). The LAB strains were activated in MRS agar and incubated at 37°C for 24 h. Freshly cultured LAB strains were inoculated at 10^8 CFU/ml into 10 mL MRS broth containing 120 μg/ml sterilized sodium nitrite solution. The control samples were made without any inoculums. The mixture tubes were incubated at 37°C for 3 days. The supernatant was collected by centrifugation at 3000 g for 15 min and used to measure nitrite. The degree of microbial depletion of nitrite in broth cultures was analyzed using colorimetric nitrite assay. The optical density (OD) value of colored mixtures was performed at 538 nm. A standard curve in the range 0-3,000 μg/ml of $NaNO_2$ solutions was subjected to similar colour development and OD measurement steps. The above test was analyzed in triplicates.

DNA extraction and 16S ribosomal DNA amplification of LAB isolates

LAB isolates were activated in MRS broth at 30 ℃ for 12 h before analysis, and then genomic DNA of the isolates was extracted using a DNA extraction kit. The genomic DNA was quantified using a nanodrop 2000 spectrophotometer and checked for integrity by horizontal gel electrophoresis (Bio-RAD, California, USA) with 1.5% (w/v) agarose pre-stained with SYBR® safe DNA gel (Invitrogen, UK) in 1X TBE using 100 V for 20 min. The 16S rDNA was amplified by polymerase chain reactions (PCR) and using the primer set of BSF8/20 (5’-AGAGTTTGATCCTGGCTCAG-3’) and REVB (5’-GGTTACCTTGTTACGACTT-3’). The amplification program was performed with an initial denaturation at 95 ℃ for 3 min, followed by 35 cycles include a denaturation at 94 ℃ for 1 min, annealing at 50 ℃ for 1 min, elongation at 72 ℃ for 2 min, and a final extension at 72 ℃ for 10 min. The amplified product was checked using electrophoresis in a 1.5% (w/v) agarose gel and photographed via an UV illumination.

16S rDNA gene sequencing analysis

Prior to sequence analysis of 16S rDNA, the 16S rDNA was purified by PureLink™ PCR Purification Kit (Thermo Fisher Scientific Inc., MA, USA.). Sequencing reactions were evaluated by the Biodesign company (Pathumthani, Thailand). The identities of these isolates were determined through the GenBank DNA database using the basic local alignment search tool (BLAST).

Results

Genotypic techniques have a high discriminatory power to classify LAB isolates from foods and other materials. In this study, all genomic DNA samples were extracted and amplified 16S rDNA by using the universal primer set. As shown in Fig.1, LAB isolated and purified from Thai fermented sausage were successfully amplified in the above PCR amplification procedures, and samples showed a single band without any non-specific amplification. The lengths of PCR amplification product were approximately 1,600 bp.

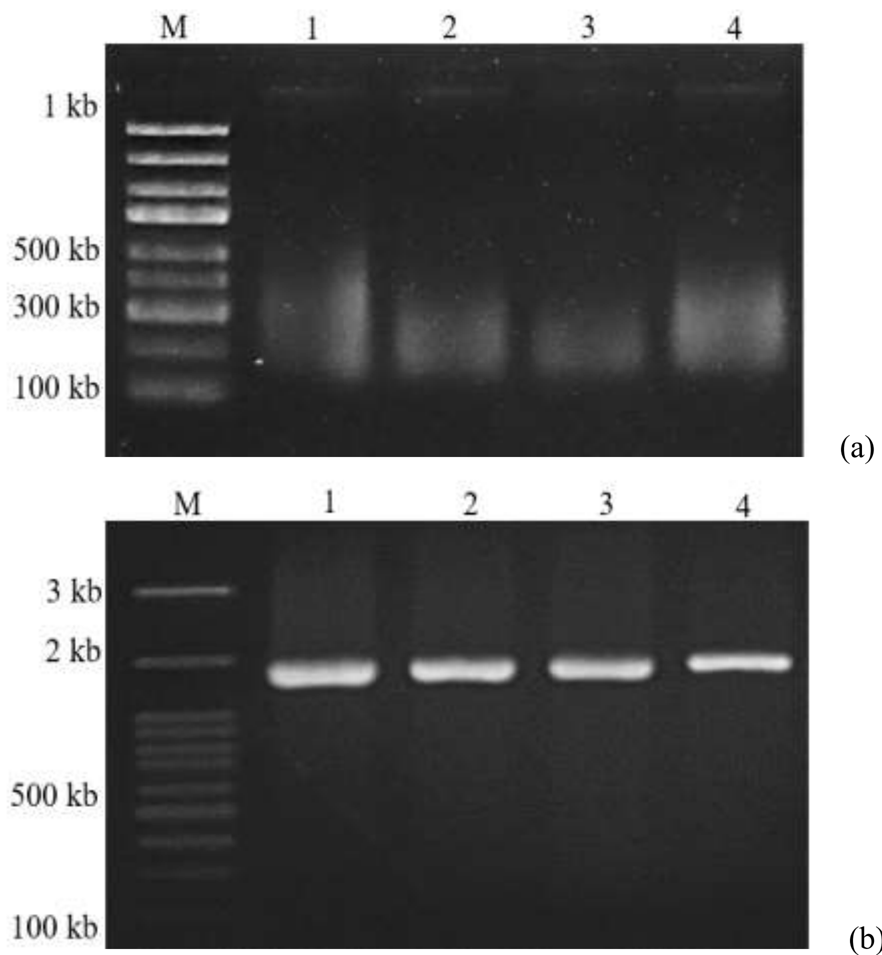

Figure 1. The gel electrophoresis analysis of (a) DNA extracts and (b) 16S rDNA gene on 1.5% agarose gel from LAB. Lane M: MW marker; lane 1: *E. durans*; lane 2: *L. plantarum*; lane 3: *L. brevis*; lane 4: *P. pentosaceus*

The 16S rDNA sequence of four LAB were compared with the nucleotide sequence deposited in GenBank DNA database by the National Center for Biotechnology Information (NCBI). The results showed that from 16S rDNA sequencing which indicated that lanes 1, 2, 3, and 4 were matched with the species of *E. durans* (98% identity) with accession number HQ603862.1, *L. plantarum* (97% identity) with accession number MF369875.1, *L. brevis* (95% identity) with accession number KM495930.1, and *P. pentosaceus* (97% identity) with accession number NC_008525.1, respectively (Table 1).

Table 1 Identification of lactic acid bacteria isolated from Thai fermented sausage

Isolate no.	*The closest relative species*	*Similarity (%)*[*]	*Accession no.*[*]
1	*Enterococcus durans*	98	HQ603862.1
2	*Lactobacillus plantarum*	97	MF369875.1
3	*Lactobacillus brevis*	95	KM495930.1
4	*Pediococcus pentosaceus*	97	NC_008525.1

[*] The similarity percentage and accession numbers of the 16S rDNA sequences obtained from NCBI.

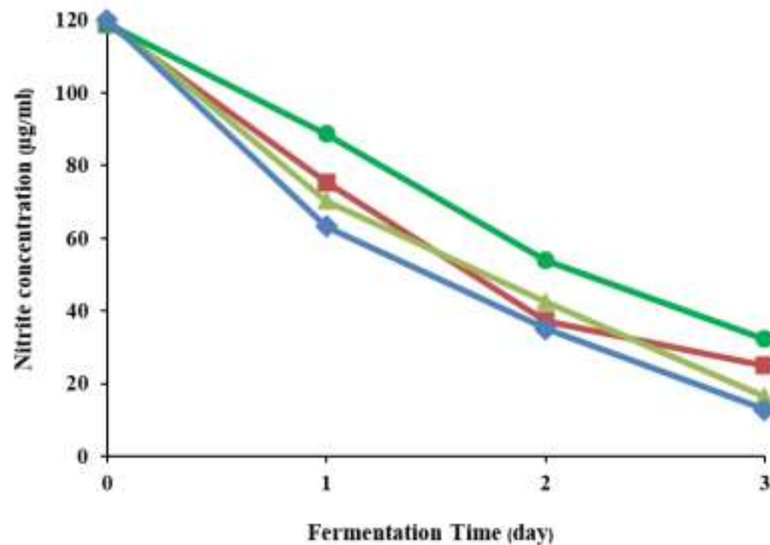

Figure 2. Nitrite depletion by pure LAB in MRS-sodium nitrite solution during fermentation period. Symbol: *E. durans* (◆), *L. plantarum* (■), *L. brevis* (▲) and *P. pentosaceus* (●)

The change of nitrite concentration during fermentation period is shown in Fig. 2. The initial nitrite concentration was 120 μg/ml, and the addition of pure LAB in MRS-sodium nitrite solution was capable of reducing the nitrite concentration. At the end of fermentation, *E. durans, L. plantarum*, *L. brevis, and P. pentosaceus* depleted 89.35%, 79.11%, 86.22% and 72.87% of the initial nitrite, respectively. The nitrite concentration decreased throughout fermentation time and exhibited the lowest final value as 12.78-32.28 μg/ml at third day. Additionally, MRS broth inoculated with *P. pentosaceus* exhibited the highest nitrite concentration throughout fermentation time. However, there was no significant difference ($P>0.05$) among all treatments.

Discussion

In this study, the LAB isolates were identified as *E. durans, L. plantarum*, *L. brevis, and P. pentosaceus*. Ammor *et al*. (2005) reported that the most common LAB isolated from dry fermented sausages are *Lactobacillus*, *Pediococcus*, and *Enterococcus*. According to the study of Thai fermented sausage, *L. plantarum* and *L. sakei* were identified as the major LAB species isolated from Mum sausages, whereas the minority species, such as *L. brevis, L. fermentum, P. pentosaceus, Leuconostoc mesenteroides,* and *Lactococcus lactis* were also found (Wanangkarn *et al*., 2014). Tanasupawat *et al.* (2015) also reported that the dominant LAB species in Thai fermented sausages, including Mum (fermented beef), Nham (fermented pork), and Sai-krog-prieo (fermented sausage) were identified as *Lactobacillus* sp., *L. pentosus*, and *P. pentosaceus*. A strain of lactobacillus is usually present as a dominant species (Thiravattanamontri *et al.,* 1998). It may be due to the lactobacillus showing more acid tolerance than other LAB species and continuous growth throughout the fermentation process (Lee *et al.*, 2006; Wu *et al.*, 2009). Hugas and Monfort (1997) mentioned that the LAB originating from fermented meats should be well adapted to the ecological condition during meat fermentation and used as starter cultures. The addition of starter cultures in sausage production can improve the safety, stability, and shelf life of products (Leroy *et al*., 2006). Klingberg *et al.* (2006) showed that inoculation of *L. plantarum* and *L. pentosus* to ferment Scandinavian-type sausage could inhibit pathogens and spoilage bacteria. During the fermentation process, LAB can inhibit the growth of undesirable bacteria by competing for nutrients and produce various specific inhibitory substances, including carbon dioxide, diacetyl, hydrogen peroxide, organic acids, and bacteriocins (Caplice and Fizgerald, 1999; Ghalfi *et al*., 2010). Moreover, LAB degrade carbohydrates into a variety of organic acids (lactic acid, acetic acid, and propionic acid), which contribute to a pleasant taste

in fermented products. Organic acid can also enhance the flavor of fermented sausage by interacting with alcohols and aldehydes (Liu *et al*., 2001).

Furthermore, to guarantee the fermented sausage products are safe and good quality, manufacturers use not only a starter culture, but also add curing agents, such as nitrite to develop colour, inhibit pathogenic bacteria, and retard the rancidity of products (Maryuri *et al*., 2012). In the current study, we investigated the ability of pure LAB to deplete nitrite concentration in MRS broth during the fermentation period. It was observed that LAB were able to reduce nitrite, which was in agreement with previous studies. *L. plantarum* (Esmaeilzadeh *et al.,* 2012) and *P. pentosaceus* (Wu *et al*., 2012) are the active species for nitrite degradation. Dodds and Collins-Thompson (1984) compared different strains of LAB, including *Lc. lactis*, *L. acidophilus*, *L. viridescens*, and *L. plantarum* and found that these LAB can rapidly deplete 61.4-92.7% of the original nitrite levels (200 ppm) in broth medium after 24 hours at 30 ℃ under anaerobic conditions. However, in order to inoculate the meat product with LAB, residual nitrite was decreased by the action of LAB for 30% in the bologna. Several researchers have mentioned that the reduction of nitrite during the fermentation period was due to the destruction of nitrite to nitrous acid, nitric oxide (NO) and nitrates (Dodds and Collins-Thompson, 1984; Honikel, 2004).

The LAB isolated from E-sarn sausages were identified as *E. durans, L. plantarum*, *L. brevis, and P. pentosaceus*. The addition of these pure LAB in MRS-sodium nitrite solution was capable to reduce 70-80% of the original nitrite levels (120 ppm) after 72 hours at 37 ℃. These results were provided data to support these LAB strains as a starter culture for Thai fermented sausages with potential to reduce nitrite of the final products. However, the amount of nitrite reduction depends on the condition of sausage production, including raw materials, incubation temperature, and time.

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