

## Original article

# Lactic acid bacteria isolated from Thai fermented pork, pickled mustard greens and pickled spring onions and their lifespan-extending potentials in *Caenorhabditis elegans*

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## Abstract

**Background:** Fermented foods are natural sources of probiotics, especially lactic acid bacteria (LAB). LAB provide health benefits through modulating gut microbiota, improving immune function, boosting antioxidative capability, and extending lifespan.

**Objectives:** To isolate LAB from Thai fermented foods and examine their lifespan-extending effects in *Caenorhabditis elegans* (*C. elegans*) model.

**Methods:** Bacteria were isolated from Thai fermented pork (Neam), pickled mustard greens (Pak Gard Dong), and pickled spring onions (Ton Hom Dong). LAB were screened by the production of clear zone on CaCO<sub>3</sub>-containing MRS agar. One isolate was selected from each food source for further testing. Gram stain and 16S rRNA sequencing were carried out for LAB identification. The lifespan-extending effect of each LAB strain was investigated in the *C. elegans* model.

**Results:** Three colonies of LAB, with obvious clear zone formation, were isolated and selected from Thai fermented pork, pickled mustard greens, and pickled spring onions. Gram stains showed that they were gram-positive bacilli. 16S rRNA sequencing revealed that LAB isolates from fermented pork, pickled mustard greens, and pickled spring onions were *Lactiplantibacillus pentosus* (99.72%), *Lactiplantibacillus pentosus* (99.79%), and *Lactiplantibacillus argentoratensis* (99.24%), respectively. Lifespan of *C. elegans* fed with OP50 and LAB isolates, particularly *Lactiplantibacillus pentosus* from Neam, significantly increased compared with *C. elegans* fed with OP50.

**Conclusion:** *Lactiplantibacillus pentosus* isolated from Thai fermented pork (Neam) could significantly extend the lifespan of *C. elegans*. This probiotic LAB strain could be beneficial for promoting longevity in humans.

**Keywords:** lactic acid bacteria, lifespan extension, Probiotic, Thai fermented food.

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Thai fermented foods have been shown to contain probiotics, primarily lactic acid bacteria (LAB).<sup>(1)</sup> According to literature, *Lactococcus lactis* was found in fermented shrimp (Kung Som) and fermented pork.<sup>(2, 3)</sup> *Enterococcus faecalis* was isolated from fermented fish (Pla Ra) and fermented bamboo shoots.<sup>(4, 5)</sup> *Lactobacillus pentosus* was identified in pickled mustard greens (Pak Gard Dong).<sup>(6)</sup> It is well recognized that dietary probiotics are essential for maintaining healthy condition. Several health benefits of LAB have been suggested including immune system stimulation, intestinal flora balance, serum cholesterol reduction, antioxidant function, anti-ageing, and lifespan extension.<sup>(7)</sup> Searching for dietary LAB that could delay ageing and extend lifespan has gained attention. Study by Hu et al. showed that *Pediococcus acidilactici* isolated from fermented pickles significantly increased (16.7%) lifespan of *Caenorhabditis elegans* (*C. elegans*).<sup>(8)</sup> *Lactiplantibacillus plantarum* strain A72 isolated from fermented vegetables could increase lifespan of *C. elegans* by 25.1%.<sup>(9)</sup> *Propionibacterium freudenreichii* isolated from dairy products (milk and cheese) increased *C. elegans* lifespan by 13.0%.<sup>(10)</sup> Additionally, *Lactiplantibacillus plantarum* strain PFA2018AU isolated from fermented carrots could increase *C. elegans* lifespan up to 30.0%.<sup>(11)</sup>

Hitherto, lifespan-extending effect of LAB isolated Thai fermented food have not been reported. Therefore, in this study, we aimed to isolate LAB from Thai fermented pork (Neam), pickled mustard greens (Pak Gard Dong), and pickled spring onions (Ton Hom Dong) and explore whether they could extend lifespan in *C. elegans* model.

## Materials and methods

### Isolation of LAB from Thai fermented food

Three types of Thai fermented foods, including Neam (fermented pork), Pak Gard Dong (pickled mustard greens), and Ton Hom Dong (pickled spring onion) were selected for the study. Food samples were blended, and 25 g of each homogenized sample was added to 225 ml of 0.1% peptone and then vortexed to ensure uniformity. Bacteria were then isolated using the dilution-plate method on de Man, Rogosa, and Sharpe (MRS) agar and incubated at 37°C for 48 hours under anaerobic conditions. The isolated colonies were stored in 10.0% skim milk at -20°C for further study.

For LAB isolation, 0.2 mL of bacterial stock in skim milk were placed and streaked onto MRS agar supplemented with 0.3% CaCO<sub>3</sub> and incubated at 37°C for 48 hours under anaerobic conditions. The size of the clear zone that formed was measured. A single colony with obvious clear zone formation (CaCO<sub>3</sub> dissolution) was selected for each food sample.

### Gram staining

The selected LAB were streaked onto an MRS agar plate and incubated for 2 days at 37°C. After incubation, a single colony was picked and placed on a glass slide. The slide was heated by passing it over a Bunsen flame three times for fixation. Crystal violet solution was added and quickly incubated for 30 seconds. The slide was washed under running water to remove any unbound violet crystals. An iodine solution was added for 10 seconds to enhance the violet crystals' adherence to bacterial cell walls. After a 10-second rinse with alcohol, excess iodine solution was removed under running tap water. The slide was then incubated with safranin solution for 30 seconds, and excess safranin was rinsed off for no more than five seconds under running water.

### Identification of LAB from Thai fermented foods

The 16S rRNA sequencing analysis was employed for LAB identification. Genomic DNA was used as a template for PCR amplification of a segment of the 16S rRNA gene. Two PCR primers used in this study were: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CTACGGCTACCTTGTACGA-3'). The PCR product size was 1500 base pairs (bp). The PCR conditions were as follows: initial denaturation at 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 2 minutes, and extension at 72°C for 2 minutes. A final extension was performed at 72°C for 7 minutes.

The amplification products obtained from the PCR reaction were analyzed by 1.5% agarose gel electrophoresis before sending out for sequencing. All 16S rRNA gene sequences were analyzed using the EzBiocloud database.

### Lifespan assay by *C. elegans*

Fresh colonies of selected LAB were prepared on MRS agar. After that, a single colony was selected and transferred to MRS broth and incubated at 37°C

for 48 hours under anaerobic conditions. Then, propagated LAB in MRS broth were diluted with PBS to obtain an OD<sub>600</sub> of 10<sup>8</sup> CFU/mL.

The longevity experiments were performed in young adults (L4 stage) of wild-type N2 *C. elegans* worms. The LAB at a concentration of 10<sup>8</sup> CFU/mL in MRS broth was added to a 24-well plate containing 485 µL of M9 buffer mixed with 5 µL of 5-fluoro-2-deoxyuridine (FUDR). Subsequently, *Escherichia coli* OP50 (OP50) was added at a 1:1 ratio with LAB (5 µL each). Each well contained 10 nematodes. The worms were monitored daily until all of them died. All lifespan assays were conducted with four independent biological replicates.

### Statistical analysis

Survival curves were constructed using the Kaplan–Meier method. Difference between survival curves was tested using the Log-rank test. GraphPad Prism version 10.4.1 (GraphPad Software, San Diego, CA, USA) was employed for graphs and computations. A *P* < 0.05 was considered statistically significant.

## Results

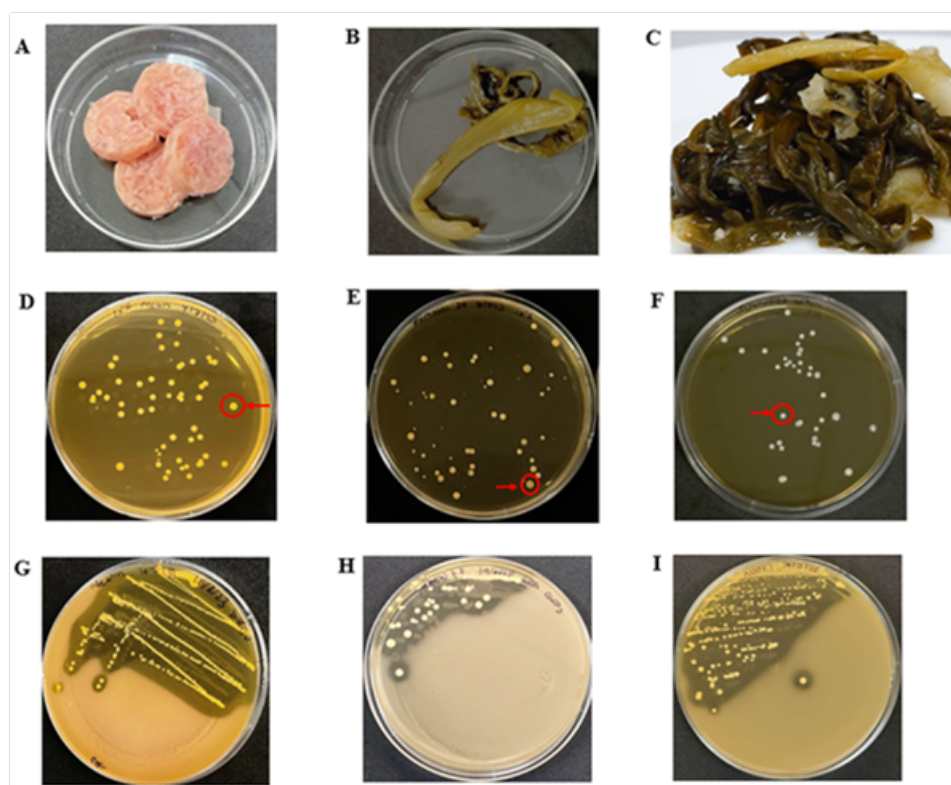
### Isolation of LAB from Thai fermented foods

Bacteria were isolated from three types of Thai fermented foods, including Neam, Pak Gard Dong, and Ton Hom Dong (**Figure 1A–1C**). Each type of food exhibited colonies with distinct morphologies, as shown in **Figure 1D–1F**.

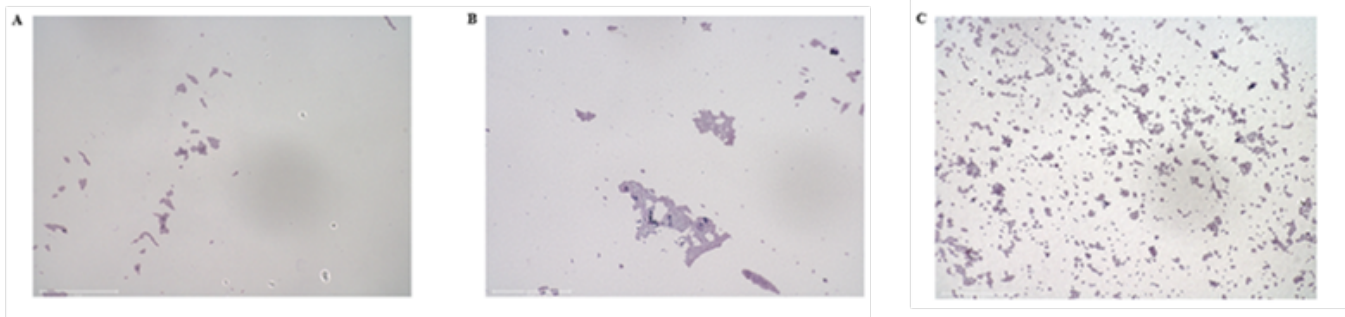
For LAB screening, single colonies formed in **Figure 1D–1F** were selected (in circle) and tested for their ability to produce lactic acid by screening on CaCO<sub>3</sub>-supplemented agar. The presence of a clear zone suggested the dissolution of CaCO<sub>3</sub> due to lactic acid production. As shown in **Figure 1G–1I**, the clear zone diameters for Neam, Pak Gard Dong, and Ton Hom Dong were 1 mm, 10 mm, and 8 mm, respectively.

### Gram staining

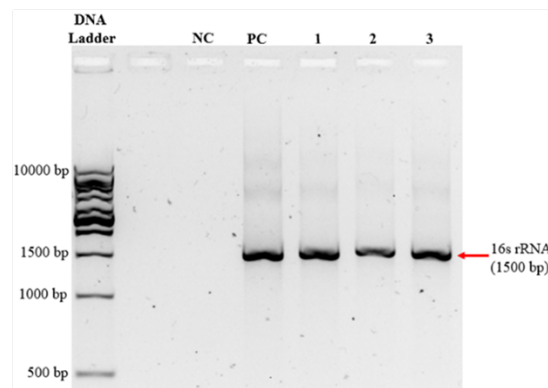
Gram stain analysis of the LAB revealed that they had bacilli-shaped morphology, with cells exhibiting purple coloration after crystal violet staining. This indicated that the isolated LAB were gram-positive bacilli, as shown in **Figure 2**.



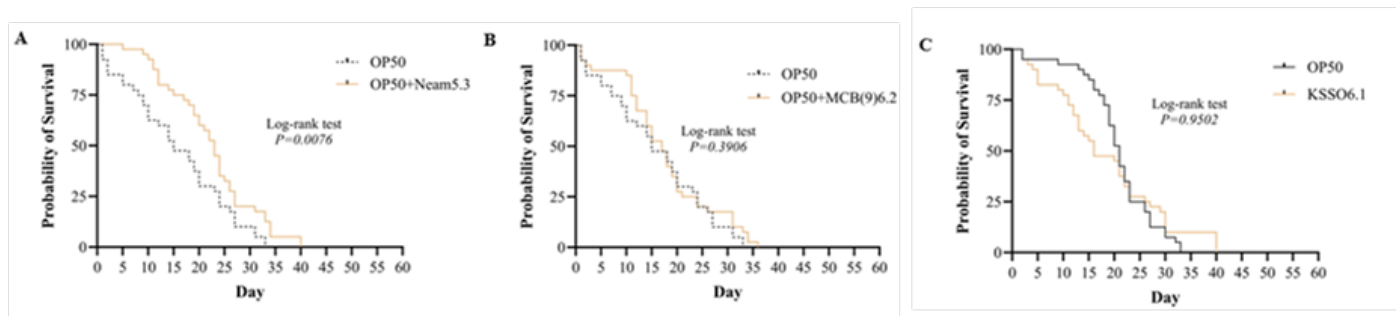
**Figure 1.** (A–C) Representative images of Thai fermented foods: (A) Neam, (B) Pak Gard Dong, and (C) Ton Hom Dong. (D–F) Bacterial colonies isolated from Thai fermented foods. The single colonies circled are those selected for further experiments: (D) Neam, (E) Pak Gard Dong, and (F) Ton Hom Dong. (G–I) Formation of clear zones due to Ca-lactate production by LAB: (G) Neam, (H) Pak Gard Dong, and (I) Ton Hom Dong.



**Figure 2.** Microscopic images of isolated LAB. All three LAB were Gram-positive, bacilli-shaped bacteria. (A) Neam, (B) Pak Gard Dong, and (C) Ton Hom Dong. Magnification: 600x.



**Figure 3.** Agarose gel electrophoresis of PCR products amplified from the 16S rRNA gene of LAB samples. NC: negative control, PC: positive control, (1) Neam5.3, (2) MCB(9)6.2, (3) KSSO6.1.



**Figure 4.** The survival curve of worms fed with LAB in combination with OP50 at a 1:1 ratio, compared with control worms fed with OP50 alone. Kaplan–Meier survival curves were plotted over time (days), and the log-rank (Mantel–Cox) test was used to compare the experimental groups: (A) Worms fed with OP50 (median survival: 15 days) compared with those supplemented with Neam5.3 and OP50 (median survival: 23 days),  $n = 40$ . (B) Worms fed with OP50 (median survival: 15 days) compared with those receiving MCB(9)6.2 and OP50 (median survival: 18 days),  $n = 40$ . (C) Worms fed with OP50 (median survival: 21 days) compared with those supplemented with KSSO6.1 and OP50 (median survival: 16 days),  $n = 40$ .

### Identification of the LAB isolates

The three LAB strains that were isolated and selected from Neam, Pak Gard Dong, and Ton Hom Dong were designated as Neam5.3, MCB(9)6.2, and KSSO6.1, respectively. PCR product of each LAB isolate is shown in **Figure 3**. The 1500 bp PCR fragments were cut, purified and sent for sequencing. Based on sequencing results, Neam5.3, MCB(9)6.2, and KSSO6.1 were identified as *Lactiplantibacillus pentosus* (99.7%), *Lactiplantibacillus pentosus* (99.7%), and *Lactiplantibacillus argentoratensis* (99.2%), respectively.

### Lifespan assay in *C. elegans*

For lifespan testing, *C. elegans* were fed with OP50 and tested LAB isolate at a ratio of 1:1. Worms fed with OP50 alone were served as control. The results showed that Neam5.3 significantly increased nematode lifespan ( $P < 0.05$ ), whereas MCB(9)6.2 and KSSO6.1 did not ( $P > 0.05$ ), compared with controls (**Figure 4**).

### Discussion

Probiotic LAB are commonly found in various fermented foods. <sup>(12)</sup> Thai fermented foods such as fermented tea leaves, fermented crab, pickled fish, and fermented fish intestines, which are widely consumed in Thailand, also contain a variety of LAB. <sup>(6)</sup> In our experiment, we isolated bacteria from Thai fermented foods and selected three colonies from three different kinds of fermented food. Initially, we tested the LAB properties of these colonies by examining the formation of clear zones in MRS agar containing  $\text{CaCO}_3$ . MRS media contains glucose, which provides carbon and energy for bacterial growth, particularly acid-producing ones. The lactic acid produced by LAB reacts with  $\text{CaCO}_3$  in the media, producing calcium lactate and creating a clear zone. <sup>(13, 14)</sup>

In this study, all isolated LAB were identified as lactic acid bacilli, which were rod-shaped gram-positive. The identification process led to the classification of these lactic acid bacilli strains as *Lactiplantibacillus*. One of the LAB that are commonly used in fermentation is *Lactiplantibacillus* (formerly known as *Lactobacillus*) strains, which are known for their probiotic and functional qualities as well as their functions in promoting health. <sup>(15)</sup> LAB are commonly identified and characterized by

amplifying and sequencing a specific section of their 16S rRNA gene using primers 27F and 1492R, which are known to encompass a wide variety of bacterial taxonomy. <sup>(16)</sup>

*C. elegans* is a widely used model organism for aging research due to several advantageous characteristics. It is a free-living, non-parasitic nematode with a short lifespan of approximately 2–3 weeks, making it ideal for lifespan studies. Additionally, it shares a high degree of genetic similarity with humans (approximately 60.0–80.0%), is easy to maintain under laboratory conditions, and has a rapid reproductive cycle. <sup>(17)</sup> In this study, we compared worms fed only with OP50, a typical food source, and those fed with both OP50 and LAB (1:1). The worm's growth and development are supported by the nutrients provided by OP50. <sup>(18)</sup> The findings showed that worms fed with OP50 combined with LAB from Neam (*Lactiplantibacillus pentosus*) significantly extended the lifespan of *C. elegans*, while LAB from Pak Gard Dong, and Ton Hom Dong did not show the lifespan-extending effect. This was consistent with other studies that found that LAB could extend the lifespan of *C. elegans*. For examples, *Pediococcus acidilactici* from fermented pickles extended lifespan by 16.7% <sup>(8)</sup>, *Lactiplantibacillus plantarum* strain A72 from fermented vegetables extended lifespan by 25% <sup>(9)</sup>, and *Lactobacillus plantarum* strain As21 from yak milk extended lifespan by 34.5%. <sup>(19)</sup>

Limitations of the present study should be mentioned. There was no mechanistic investigation in this study. The gut of *C. elegans* differs significantly from that of humans in terms of structure, nutrient absorption, and interactions with microbes. As a result, the worm's response to LAB may not fully represent the effects observed in humans. Additionally, the development and overall function of the *C. elegans* gut are also distinct from those in humans, which may further limit the applicability of findings from this model. <sup>(17)</sup>

### Conclusion

Thai fermented foods were good natural source of LAB. *Lactiplantibacillus pentosus* isolated from Neam exhibited the lifespan-extending effect in *C. elegans* model. This LAB could be a promising probiotic for promoting longevity in humans.

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## Conflicts of interest statement

The authors declare that there are no conflicts of interest regarding the publication of this paper.

## Data sharing statement

The processed 16S rRNA sequencing data generated in this study have been deposited in the Ezbiocloud database under accession numbers AZCU01000047, AZCU01000047, and CP032751. Additional supporting data, including bacterial strain information and lifespan assay results, are available from the corresponding author upon reasonable request.

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