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# Isolation and identification of lactic acid bacteria from different stages of traditional Malaysian tempeh production

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## ABSTRACT

**Aims:** There are many methods of soybean tempeh production as they vary according to the producers. Isolation of lactic acid bacteria (LAB) from tempeh was carried out at different stages of the tempeh production to examine the occurrence of LAB and to identify the isolates.
**Methodology and results:** Morphological, physiological and chemical characteristics with the use of API 20 Strep, API 50 CHL kit and 16S rRNA gene sequences were employed to identify LAB. By using API 20 Strep and API 50 CHL kit, fourteen LAB were obtained and twelve isolates have been successfully identified: seven coccus LAB isolates as *Enterococcus faecium*, four cocci-bacilli as *Leuconostoc mesenteroides* ssp. *mesenteroides*, one bacilli as *Lactobacillus delbrueckii* ssp. *delbrueckii*. Meanwhile, two bacilli isolates were categorised as unidentified strain. On the other hand, molecular identification using 27f and 1429r primer had revealed that *L. mesenteroides* and *L. delbrueckii* were identified as *Leuconostoc lactis* and *Leuconostoc* sp. respectively. Whereas, two previously unidentified bacilli isolates were identified as *Alicyclobacillus* sp.
**Conclusion, significance, and impact of study:** This result shows that various types of LAB was detected at every stages of tempeh production and had been identified by using two different techniques. The unique characteristics of LAB offer their potential towards food and pharmaceutical industry.

*Keywords:* Soybean, tempeh, lactic acid bacteria, isolation, identification.

## INTRODUCTION

Tempeh is a patty of cooked soybean being fermented by mould starter culture *Rhizopus* spp., most commonly *Rhizopus oligosporus*. Soybean tempeh is a famous fermented food in Malaysia and this food gains its popularity from its taste, digestibility, nutritional value and well-known as high-quality meat replacers (Nout and Aidoo, 2010). Yellow seeded soybean (*Glycine max*) is the most preferred type, use as the raw material although many other types of soybean and legumes can be used such as red kidney bean (Srapinkornburee *et al*., 2009), *mucuna* (Sri Handajani, 2001), chickpea (Abu-Salem and Abou-Arab, 2011), and barley (Feng, 2006).

The group of lactic acid bacteria (LAB) is responsible to accelerate the fermentation process of tempeh. LAB are Gram positive, bacilli and cocci shape, catalase negative and produce lactic acid as their main product. Aside from being well known for its GRAS (Generally Regarded As Safe) status, this type of microorganisms has a long history of application prior to their advantageous influence on nutritional values, organoleptic, and shelf-life characteristics of the fermented food (Leroy and De Vuyst, 2004). Occurrence of lactic acid bacteria (LAB) have been investigated from tempeh and other famous Malaysian fermented foods such as *tempoyak*, *tapai*, *budu* and *cincalok* (Kormin *et al.*, 2001; Moreno *et al*., 2002; Liasi *et al*., 2009; Belal and Zaiton, 2011; Kalavathy *et al.*, 2012; Hajar and Hamid, 2013). Ability of these microorganisms to produce variety of antimicrobial compounds and combat pathogenic microorganisms (Menssens and De Vugst, 2002), makes them bacteria of interest for probiotic purposes.

In this study, the method used for tempeh production is different than most of the methods described by other authors as this method involved double soaking process. The soaking process is very important as it functions to increase the moisture content of the beans, make the beans edible, facilitate microbial activity during the fermentation, and to naturally extract antimicrobial (saponins) and bitter compounds (Nout and Aidoo, 2010). Although additional time is required, soaking process is

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still the only inexpensive and simple method considering its multi-effects in enhancing nutritional value and lowering the anti-nutritional factor (Mumba *et al.*, 2004). This includes significant reduction of phytate (Karkle and Beleia, 2010), increase of total soluble solid (Arbianto, 1978) and decrease of oligosaccharides, sucrose content and also tannins (Egounlety and Aworh, 2003). Other steps of tempeh production are the same as the method used by most producers except some might varies in terms of incubation time, temperature and packaging materials. Hence this study was conducted with the aim to isolate LAB from tempeh production that involved both double soaking process and over-fermented tempeh.

Awareness on importance of healthy foods contributes to the high interest and demand for natural fermented foods.LAB has the advantages to offer the solution for this problem. However, the number of local LAB strains isolated from tempeh is still scarce. As a result, isolation of LAB from local resources like Malaysian tempeh is necessary. The aim of this study is to isolate and identify the natural population of LAB from traditional tempeh processing. The significant of this study is to make use of local fermented product and its waste to its best use. Efforts to understand LAB isolated from local fermented food may be of value to the Malaysian economy.

## MATERIALS AND METHODS

### Tempeh production

Production of soybean tempeh was prepared according to a local producer in Klang, Malaysia. Yellow seeded soybean (Country Farm Organics) was used to make the tempeh. In this study, 100 g soybean was soaked in 1 L water for 24 h at room temperature (RT) and then drained. It was then cooked at 100 °C for 10 min in 1 L water. After boiling, half cooked soybean was left soaked in the boiled water and left for another 24 h at RT. Next, the water was drained and washed several times to remove the hulls. Soybean was further cooked at 100 °C for another 30 min and the water was drained subsequently. The cooked soybean was cooled at RT for 20 min and then mixed thoroughly with 0.03 g inoculums (Raprima Inokulum Tempeh, PT Aneka Fermentasi Industri, Bandung, Indonesia). Finally, soybean was packed into perforated plastic bag and incubated at 37 °C for 7 days. The overall production of soybean tempeh and stages where samples were taken is shown in Figure 1. All samples were freshly analysed on the same day they were produced.

### Isolation of LAB

Each sample of 25 g was aseptically added into 225 mL of sterile peptone water (0.1% w/v) solution and was mixed thoroughly. Isolation of LAB was performed as method described by Muhialdin and Hassan (2011) with slight modification. Serial dilutions were performed and 1 mL aliquots of appropriate dilution were directly inoculated in duplicate on the de Man Rogosa Sharpe (MRS) agar (Oxoid Ltd, Basingstoke, UK) with 0.8% calcium carbonate ($CaCO_3$) using pour plate method and were incubated for 48 h at 37 °C. The identified colonies were counted and total numbers of viable cells were reported as colony-forming units (CFUs). White small colonies with clear zone around them were randomly isolated using sterilized toothpicks and streaked on fresh MRS agar.

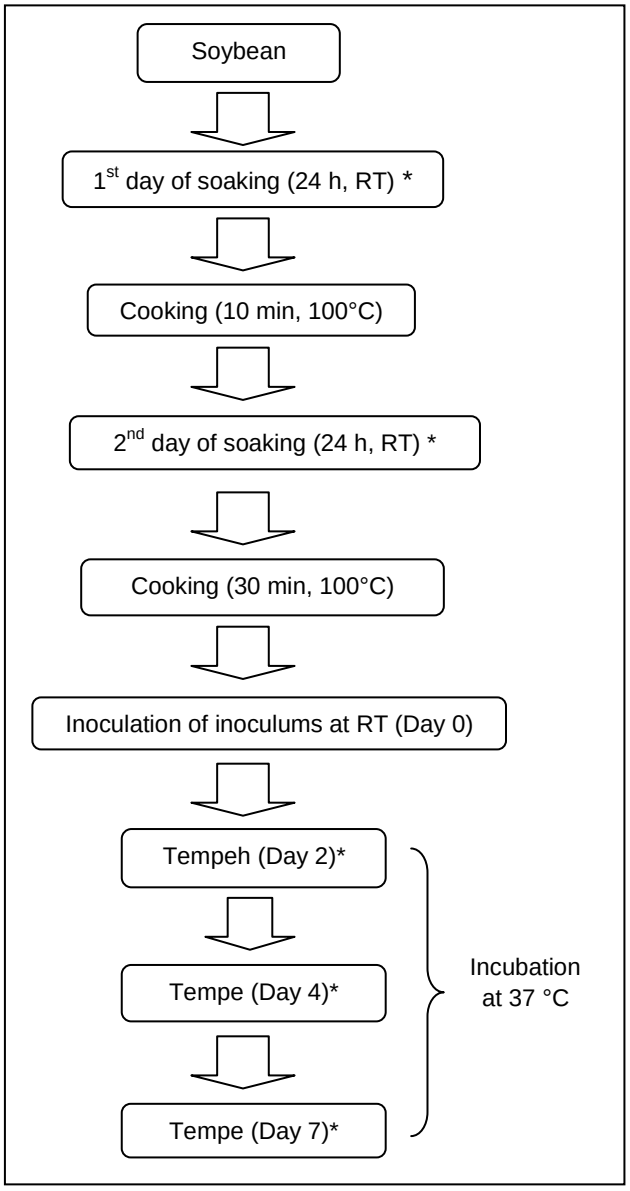


**Figure 1:** Flowchart of tempeh production (* Stage where sample was taken).

### Purification

Single colony was picked from the plates and subculture to purify. Pure bacterial strains were obtained after five times successive transfer of individual colony on the plates and incubated for 24 h at 37 °C. Culture preservation was performed on all the isolates obtained.


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Fresh overnight cultures of each isolate were grown in broth and stored in the freezer at −80 °C in 20% glycerol (Kheadr, 2006).

**Identification of LAB**

Identification of LAB was done based on morphology and catalase reaction. Morphological observations of LAB with gram staining were carried out. Based on previous description, it is known that LAB is gram-positive with rod or cocci shape. Catalase test was performed using freshly prepared 3% hydrogen peroxide ($H_2O_2$).

**Identification of LAB using API 20 Strep and API 50 CHL**

All isolates with gram positive and catalase negative characteristic were tested for the production of acids from carbohydrates and related compounds by using API 50 CHL and API 20 Strep (API System, Bio-Merieux, France). The API 50 CHL system was used for the identification of *Lactobacillus* and related genera while API 20 Strep was used for the enzymatic and carbohydrate fermentation patterns of most streptococci and enterococci strains. All tests were conducted according to the manufacturer's instructions. All data were obtained from the kit after incubation for a period 4 to 48 h. Data were recorded and analysed using API LAB Plus, version 5.1. Interpretations of the respective bacterium were compared with information from the database.

**Identification using 16S rDNA**

All 14 LAB isolates were identified using 16S rRNA gene sequencing. LAB isolates were initially grown in MRS broth for 24 h at 37 °C. The DNA was extracted using the DNeasy Blood and Tissue kit (QIAGEN, German), according to the manufacturer's instructions. The 16S rRNA gene was amplified with universal primers 27f (5'-AGAGTTTGATCATG CCTCAG -3') and 1429r (5'-TACGGTTACCTTAC CTTGTTACGACTT-3') (Kalavathy *et al.*, 2012). Amplification was performed in a 25 µL volume containing 12.5 µL TopTaq Master Mix (QIAGEN, German), 9.5 µL $dH_2O$, 10 µM 27f primer, 10 µM 1429r primer and 10 µM template DNA. Polymerase chain reaction (PCR) was run using Bio-Rad, MJ Mini Thermal Cycler under following conditions: one cycle of initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C (45 sec), annealing at 47 °C (30 sec), elongation at 72 °C (45 sec) and final extension at 72 °C for 5 min (Kalavathy *et al.*, 2012). The PCR products were loaded in 0.7% agarose gels for electrophoresis and visualised by GelStar staining (Lonza, USA) under UV transilluminator. The PCR products were sent for purification and sequencing to My TACG Bioscience Enterprise (Selangor, Malaysia). Sequences were manually edited and aligned using DNA Baser version 4.16.0 and matched with DNA sequences from GenBank using the BLAST software (BLASTN) at the National Centre of Biotechnology Information, NCBI (http://www.ncbi.nlm.nih.gov). A definite confirmation of the strain was obtained on the basis of these results.

**RESULTS AND DISCUSSION**

**Isolation and purification of LAB**

After the purification process, a total of 14 strains of LAB which produce clear zone around their colonies were isolated from different stages of tempeh production. The appearance of clear zone was due to dissolution of $CaCO_3$ in MRS medium by acid production. All isolates were Gram positive, catalase negative and have the shape of bacilli, cocci and cocci-bacilli. From the isolation process, it was observed that LAB present at all stages of tempeh production (Table 1).

**Table 1:** Enumeration of LAB at different stages of tempeh production.

| Stage | Number of LAB at different stage of tempeh production (log CFU/mL) |
|---|---|
| Day 1 soaking water | 7.35-7.56 |
| Day 2 soaking water | 7.40-7.79 |
| Tempeh (day 2) | 8.11-8.92 |
| Tempeh (day 4 ) | 7.34-8.45 |
| Tempeh (day 7) | 7.74-8.00 |

The values are expressed as minimum and maximum.

From Table 1, it was observed that the number of LAB from day 1 soaking water were in the range of 7.35-7.56 log CFU/mL. It is in agreement with the range of 7.9- 9.3 log CFU/mL as reported by Moreno *et al.* (2002). As for day 2 soaking water, the number of LAB were in the range of 7.40- 7.79 log CFU/mL. This showed that the number of LAB increased with the time of soaking. According to Nuraida *et al.* (2008) the value of LAB was decreased (-1.63 log CFU/mL) from soaking water from day 1 to day 2 while in this study the number of LAB was slightly increased (+ 0.14 log CFU/mL). The study also reported that as time increased from soaking day 1 to day 2, the pH of soaking water became more acidic with changes from pH 5.25 to pH 4.84 (Nuraida *et al.*, 2008). The number of LAB at all stages was between 7.34-8.92 log CFU/mL throughout the whole process. This finding was also in agreement with Moreno *et al.* (2002) and Balqis *et al.* (2013) who reported that the numbers were in the range of 6.8-9.9 and 6.88-8.98 log CFU/mL respectively. The numbers of LAB obtained in this study was in agreement with findings by Liu *et al.* (2012) who reported that the range was between 6.0-9.3 log CFU/mL. Occurrence of LAB at all stages of tempeh making is very important because during soaking and fermentation, growth of LAB helps in acidifying the soaking water and tempeh itself which in turn inhibits the growth of potential pathogenic bacteria (Nout *et al.*, 1987; Ashenafi and Busse, 1992).

The presence of yeast and mould were also observed at all stages of tempeh processing except during soaking. Isolation by Moreno *et al.* (2002), also reported the

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presence of mould and yeast at different stages of tempeh production although they were cultured on different media. This finding was also supported by Balqis *et al.* (2013) who reported that the presence of mould in the final product was predicted since the mould was intentionally incorporated into the tempeh as an inoculum.

It was observed that as the fermentation time increased, the number of LAB present also increased except for tempeh at day 4 as the sample had already spoiled and dominated by spoilage causing bacteria. Research by Fardiaz *et al.* (1990) on over-fermented soybean tempeh excellently discussed on the physical and chemical changes of tempeh during the prolonged fermentation. In his work, it was proven that there was significant increase in gram negative bacteria and *Staphylococcus spp*. even though the count was below the numbers that could cause food poisoning. Some of the bacteria reported to be obtained from over-fermented tempeh were; *Escherichia coli*, *Enterobacter aerogenes*, *Staphylococcus*, *Vibrio cholerae*, *Proteus morganii* and *P. vulgaris* (Fardiaz *et al.*, 1990).

**Identification of LAB**

The Gram staining technique is a very crucial test as it will assist in identifying the LAB. Only Gram positive, rod and cocci shape bacteria were selected and tested for catalase. Therefore, as many as 14 bacteria isolates were used for further identification (Table 2).

**Table 2:** Isolation and identification of LAB.

| Source | Number of isolate | Gram stain | Catalase | Morphology | API identification |
|---|---|---|---|---|---|
| Day 1 soaking water | 6 | + | - | Cocci | A, B, C, D, E, F- *Enterococcus faecium* |
| Day 2 soaking water | 2 | + | - | Cocci-bacilli | G, H- *Leuconostoc mesenteroides ssp. mesenteroides* |
| Tempeh (day 2) | 2 | + | - | Cocci<br>Bacilli | 1-*Enterococcus faecium*<br>J-*Lactobacillus delbrueckii ssp. delbrueckii* |
| Tempeh (day 4 ) | 1 | + | - | Bacilli | K- Unidentified |
| Tempeh (day 7) | 3 | + | - | Bacilli<br>Cocci-bacilli | L- Unidentified<br>M, N- *Leuconostoc mesenteroides ssp*. *mesenteroides* |

**Identification of LAB using API 20 Strep and API 50 CHL**

A total of 12 strains out of 14 isolates from different stages of tempeh production were satisfactorily identified using API 20 Strep and API 50 CHL kit as shown in Table 2. API 20 Strep was used for the enzymatic and carbohydrate fermentation for enterococci strains. Seven cocci isolates were excellently identified as *Enterococcus faecium* with % ID between 99.3-99.7%. All bacilli and cocci-bacilli shape bacteria were identified using API 50 CHL which was identified based on carbohydrate fermentation with respect to sugar utilisation and acid production of each species. Four other isolates were identified as *Leuconostoc mesenteroides* ssp. *mesenteroides* (% ID: 95.7-99.9%), one isolates as *Lactobacillus delbrueckii ssp. delbrueckii* (% ID: 97.8%).However, two isolates from tempeh at day 4 and day 7 could not be identified with the result appeared as doubtful profile. According to Bill *et al.* (1992) and Klinger (1992), some commercial identification systems often yield good result with respect to genus but not sufficient at species level.

**Identification using 16S rDNA**

For more accurate result, identification at molecular level was performed. All PCR products were shown to produce 1.5 kb fragment (Figure 2). The BLAST result analysis had revealed that *Leuconostoc mesenteroides* and *L. delbrueckii* were reidentified as *Leuconostoc lactis* (100% identity*)* and *Leuconostoc sp.* (99% identity*),* respectively. The unidentified isolated were identified as *Alicyclobacillus* sp. (100% identity). The remaining LAB isolates were confirmed as *Enterococcus faecium* with 99-100% identity (Table 3).

Based on the results obtained, it was clearly shown that there was significant different in types of LAB from day 1 and day 2 soaking water. As for day 1 soaking water, the type of LAB dominating this sample was the cocci shaped LAB identified as *Enterococcus faecium* while day 2 soaking water was dominated by cocci-bacilli shaped LAB identified as *Leuconostoc sp*. and *Leuconostoc lactis*.

Absence of *Enterococcus faecium* from day 2 soaking water may be due to the boiling temperature during cooking of the soybean. Generally, the species identified in this study were in good agreement with other work by previous researchers. Kalavathy *et al.* (2012), isolated *Lactobacillus sp*. and *Pediococcus acidilactaci* from tempeh with the antibacterial properties against *Micrococcus luteus.* Meanwhile Belal and Zaiton (2011)

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discovered *Lactobacillus brevis* and *Pediococcus pentosaceus* whereas Kormin *et al.* (2001) identified *Lactobacillus plantarum* BS2 from tempeh also with similar characteristics. Furthermore, *Enterococcus faecium* was successfully obtained from 7 days old fermented tempeh (Moreno *et al.* 2002). *Lactobacillus spp., Weisella* spp., *Pediococcus* spp. and *Enterococcus faecalis* were LAB isolated from similar fermented soybean, *douchi* (Liu *et al*., 2012). Findings from this study were also supported by Balqis *et al.* (2013), with the identification of heterofermentative *Lactobacillus* spp. at different stages of tempeh production along with *Streptococcus* non-enterococci bacteria from 2 days old tempeh.

**Table 3:** Identification of LAB isolates using BLAST software.

| Isolate | Species | Identity (%) | Accession number |
|---|---|---|---|
| A | *Enterococcus faecium* | 99 | CP006030.1 |
| B | *Enterococcus faecium* | 99 | CP006030.1 |
| C | *Enterococcus faecium* | 99 | KJ728981.1 |
| D | *Enterococcus faecium* | 100 | KJ728981.1 |
| E | *Enterococcus faecium* | 99 | CP006030.1 |
| F | *Enterococcus faecium* | 99 | CP006030.1 |
| G | *Leuconostoc lactis* | 100 | AB904777.1 |
| H | *Leuconostoc sp.* | 99 | JX026036.1 |
| I | *Enterococcus faecium* | 99 | KJ728981.1 |
| J | *Leuconostoc sp.* | 99 | JX026035.1 |
| K | *Alicyclobacillus sp.* | 100 | KC893645.1 |
| L | *Alicyclobacillus sp.* | 100 | KC893647.1 |
| M | *Leuconostoc lactis* | 100 | KJ095665.1 |
| N | *Leuconostoc lactis* | 100 | KJ095665.1 |

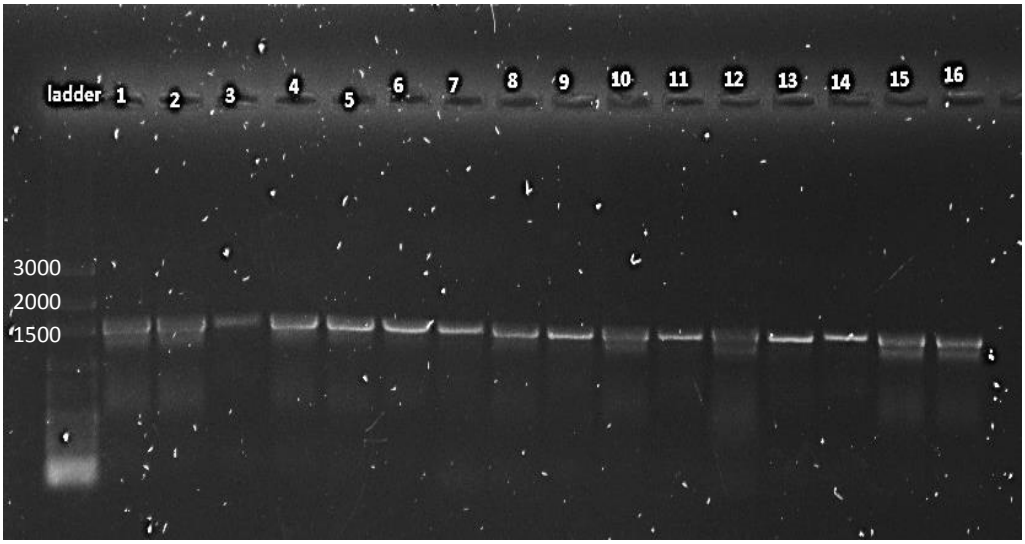


**Figure 2:** The PCR amplification of ribosomal RNA gene using 27f and 1429r primer. Lanes 2-16 corresponds to genomic sample of the LAB isolates except lane 7. Lane 1 is control in this case is *L. plantarum*.

## CONCLUSION

In this study, LAB has been successfully isolated from different stages of tempeh production with 50% cocci shape, 35.7% cocci-bacilli shape and 14.3% bacilli shape LAB. Overall, all 14 LAB was successfully identified as: *Enterococcus faecium* (7), *Leuconostoc lactis* (3), *Leuconostoc* sp. (2) and *Alicyclobacillus* sp. (2). Day 1 soaking water was dominated by cocci shape LAB identified as *Enterococcus faecium* while day 2 soaking water was dominated by cocci-bacilli shaped LAB identified as *Leuconostoc* sp. and *Leuconostoc lactis*. This

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shows that different types of microorganisms were involved during prolonged soaking and tempeh production. LAB isolates obtained will be further characterised for probiotic purposes before it can be applied in food and pharmaceutical products.

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