

Isolation and Antimicrobial Activity of Lactic Acid Bacteria against *Streptococcus Mutans*

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Abstract

The aim of this study was to isolate lactic acid bacteria (LAB) from fermented food, to determine its antibacterial activity, and to identify the LAB in order to select candidate probiotic strains for preventing caries.

The probiotic strains were isolated from eight fermented food which are tapai ubi, tapai pulut, rebung, tauchu, kimchi, cinalok, tempeh and tempoyak, taucu. Ten-fold serial dilutions of the fermented food samples were made in sterile peptone water before plating on de Man Rogosa and Sharpe (MRS) agar. The pure cultures were then randomly picked and biochemically identified. Then, the antibacterial activity of LAB against *Streptococcus mutans* was assessed by using the disk diffusion method.

A total of 120 LAB were isolated from eight different fermented foods. The morphologies of the isolates were circular, convex, dull opaque white or translucent white. Gram staining identification showed that the isolates were Gram-positive rods. Of the 120 LAB isolates, five strains displayed moderate to strong antibacterial activity against *S. mutans* with the inhibition zones ranging from 7-12 mm.

The antibacterial activity demonstrated from LAB isolated from fermented foods suggests that the isolates from fermented foods possess antibacterial properties against a pathogen which responsible for causing dental caries. These strains are currently investigated in depth to assess whether they can be fully characterized as probiotics.

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Introduction

Probiotics can be defined as live microorganism which when administered in adequate amount, it will confer a health benefit to the host.¹ The development of probiotics during the past decade has contributed important advances in the field of food technologies which provides beneficial impacts on consumer health. Increasing evidence of beneficial effects attributed to probiotics, including improvement of intestinal health, enhancement of the immune response, reduction of serum cholesterol, and

cancer prevention.²

Moreover, there is substantial evidence to support probiotic use in the treatment of acute diarrhea diseases, prevention of antibiotic-associated diarrhea, and improvement of lactose metabolism.³ In recent years, there is increasing interest in finding alternative treatment for oral health⁴⁻⁵ due to emergence of bacterial resistance to the antibacterial treatment that has been considered as global problem. Thus, there is urgency to find alternative treatment which are safer but efficient as compared to commercial antibiotics. Recent reviews have reported on the use of probiotic strains for the prevention of oral diseases, including caries.⁶

A few probiotic, namely *Lactobacillus rhamnosus GG*, *L. casei*, *L. reuteri*, *L. plantarum*, *L. brevis CD2*, *Bifidobacterium spp.* has been tested and evaluated on dental caries and they were proposed to reduce caries, *Streptococcus mutans* and lactobacilli count change, plaque pH control and root caries lesions reversal.⁷ However,

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since probiotic activities are strain related, newly found strain could have potential to possess a good probiotic strain. Therefore, strain identification is recommended in order to establish their suitability and performance for food and industrial application. Moreover, only strains classified as lactic acid bacteria are of significance and based on literature, *Lactobacillus* and *Bifidobacterium* constitutes the most bacterial genus used as probiotic.⁸

Thus, this research is carried out to isolate probiotic strains that could have potential applications in the oral cavity that possess antimicrobial properties against cariogenic bacteria, *Streptococcus mutans*.

Materials and methods

Bacterial strains

The bacterial strain used in this study was bought from ATCC (*Streptococcus mutans* ATCC 25175), which was grown in Brain Heart Infusion (BHI) broth (Oxoid, England) at 37 °C. Glycerol stock of the bacteria was kept at -80 °C.

Isolation of lactic acid bacteria

The samples were isolated from eight sources of fermented food which are bamboo shoot, *tapai pulut* (fermented sticky rice), *tapai ubi* (fermented tapioca), *tempoyak* (fermented durian paste), *tempeh* (fermented soybeans), *cencalok* (fermented small shrimps) and *taucu* (fermented yellow soybeans) and kimchi. Serial dilutions of the samples were made with sterile peptone water. Aliquots of 100 µL from appropriate dilutions were spread-plated on de Man Rogosa and Sharpe (MRS) agar and LAB colonies were observed after incubation at 37°C for 48 h. The colonies that were grown in the agar were then identified and classified based on their Gram reaction, morphologies, surface characteristics, margin as well as their color. Colonies with different morphologies were then sub-cultured for 18-24 h into a new MRS agar to obtain a pure homogenous colony. The pure homogenous colonies were then sub-cultured into a sterile MRS broth.

Antibacterial activity of lactic acid bacteria

Antibacterial activity of LAB against *S. mutans* was assessed by the disc diffusion method and the cell-free supernatant (CSF) was used in the assay. To obtain the CSF of LAB, the broth containing pure homogenous colony were

centrifuged (4000x g, 20 min, 4 °C) and the supernatant was filter-sterilized (0.45 µm pore size; Millipore, Burlington, MA, USA). The CSF was then overlaid on a 6 cm disc that put onto Brain Heart Infusion (BHI) agar that was seeded with 100 µL of *S. mutans*. The cells were incubated overnight at 37°C. The LAB that possess inhibition against the *S. mutans* were then repeated in triplicate and identified genetically. Chlorhexidine and MRS broth served as positive negative control respectively.

Identification of lactic acid bacteria

The bacterial 16S rRNA, was amplified full-length 1.5 kb using universal primers 27F and 1492R. The total reaction volume of 25 µl contained DNA purified using in-house extraction method, 0.3 pmol of each primer, deoxynucleotides triphosphates (dNTPs, 400 uM each), 0.5 U DNA polymerase, supplied PCR buffer and water. The PCR was performed as 1 cycle (94 °C for 2 min) for initial denaturation; 25 cycles (98 °C for 10 sec; 53 °C for 30 sec; 68 °C for 1 min) for annealing and extension of the amplified DNA. The PCR products were purified by standard method and directly sequenced with primers 785F and 907R using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Statistical analysis. The data were analyzed using One-Way ANOVA Statistic (SPSS).

Results

Sources	Number of isolated bacteria
Kimchi	11
Tapai pulut	28
Tempoyak	4
Cencalok	3
Tauchu	4
Tempeh	3
Rebung	30
Tapai ubi	37

Table 1. Sample sources and number of isolated bacteria.

Bacterial isolation and biochemical identification

A total of 120 strains were selected based on differences morphology on the plate and due to excellent growth. These sample sources are *kimchi*, *tapai pulut*, *tempoyak*, *cencalok*, *tauchu*, *tempeh*, *rebung*, and *tapai ubi*. The highest number of isolates are from sample *tapai ubi*

which was 37 number of isolated bacteria, while the lowest number were from cencalok which were only three number of bacteria isolated (Table 1).

Antibacterial activity against *S. mutans*

The antibacterial activity against *Streptococcus mutans* was done through disc diffusion methods. The result shows that there were five lactic acid bacteria which were LAB 5, LAB 6, LAB 9, LAB 12 and LAB 16 that possess the antibacterial activities against the *Streptococcus mutans* with a marked zone of inhibition (Figure 1, 2, 3, 4, 5). LAB 12 exhibited the highest inhibition zone which was 11 mm. The antibacterial activity of LABs are significant as compared to the negative control ($P < 0.05$). The size of zone of inhibition of the negative control was 6mm.

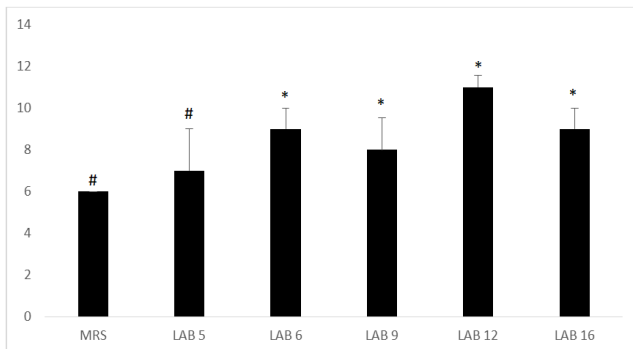


Figure 1. Antimicrobial activity of LAB against *Streptococcus mutans*. MRS is MRS broth served as negative control. * and # is significant at $P < 0.05$.

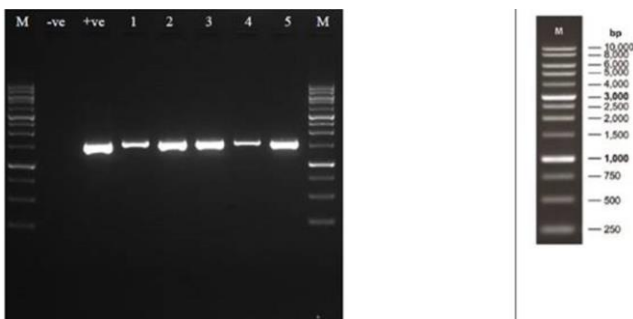


Figure 2. PCR Product of genetic identification 16S rRNA full length identification.

Sample	Similarity Index	Identification
LAB 5	99%	<i>Lactobacillus pentosus</i>
LAB 6	98%	<i>Lactobacillus brevis</i>
LAB 9	100%	<i>Lactobacillus brevis</i>
LAB 12	98%	<i>Lactobacillus plantarum</i>
LAB 16	99%	<i>Lactobacillus brevis</i>

Table 2. Identification strain of LAB through genetic identification.

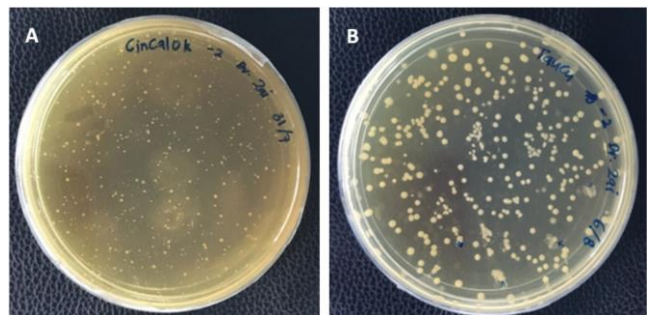


Figure 3. The differences in the bacterial growth isolated from (A) water based sample (*cencalok*) and (B) solid based sample (*taucu*).

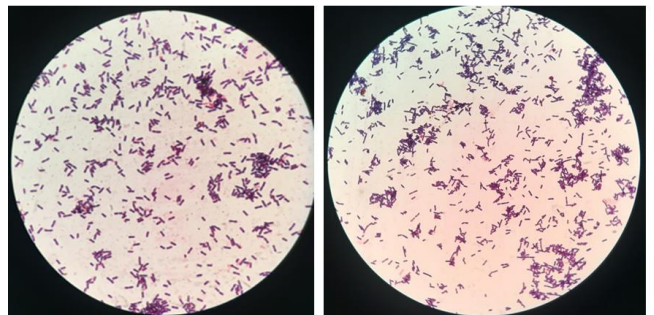


Figure 4. Gram staining images of isolated lactic acid bacteria.

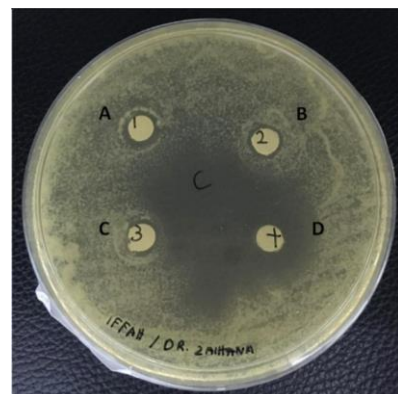


Figure 5: Representative photo of antimicrobial screening of LAB against *S. mutans*. (A) LAB 5 (B) LAB 6 (C) LAB 12 (D) Positive control.

Genetic bacterial identification and safety

The identification of the LAB strain of LAB 5, LAB 6, LAB 9, LAB 12 and LAB 16 were done through two methods which were through conventional identification which was Gram staining, and subsequently through genetic identification. From Gram staining, all LAB isolated appeared to be Gram-positive rod or coccobacilli. For genetic identification, it was made through 16s rRNA gene sequencing.⁹ The result of the genetic identification was reported as Table 2 and the result summary of all positive LAB in Table 3.

Sample	Sources	Morphology	Gram staining	Identification
LAB 5	Rebung	Circular, raised, entire margin, opaque white,	Purple, Gram positive, rod	<i>Lactobacillus pentosus</i>
LAB 6	Rebung	Circular, raised, entire margin, translucent white,	Purple, Gram positive, rod	<i>Lactobacillus brevis</i>
LAB 9	Rebung	Circular, raised, entire margin, translucent white,	Purple, Gram positive, rod	<i>Lactobacillus brevis</i>
LAB 12	Tapai Ubi	Circular, convex, entire margin, opaque white	Purple, Gram positive, rod	<i>Lactobacillus plantarum</i>
LAB 16	Tapai Ubi	Circular, raised, entire margin, translucent white,	Purple, Gram positive, rod	<i>Lactobacillus brevis</i>

Table 3. Result summary of all positive LAB.

Discussion

Fermented food has been shown to be a good source of lactic acid bacteria that has potential to possess probiotic characteristic.¹⁰ Similarly, in this study, 120 lactic acid bacteria were isolated from fermented food. LAB that possessed antibacterial activity in this study were isolated from sample *rebung* and *tapai ubi*. However, very few isolates obtained from *cencalok*. These differences in the number of bacteria isolated could be understood through their nature which *tapai ubi* is in the form of solid whilst *cencalok* is in the form of liquid. It can be postulated that the concentration of the bacteria grows is higher in the solid form rather than liquid form. This can be supported by the number of days and concentration of colonies grown on the MRS agar for *cencalok* took more days which up to 3 to 4 days to have a good colony grow rather than 1 to 2 days for colonies on *tapai ubi*. This results support previous finding that shown most of probiotics were isolated from solid form fermented food.¹¹

The LAB strains identified from the fermented food have the potential to become the

candidates of probiotics as they have the antibacterial effect on *Streptococcus mutans*. This finding agrees with a previous study that shown probiotic able to inhibit *Streptococcus mutans* isolated from children with active caries.¹²

LAB strains (*L. fermentum* 20.4, *L. paracasei* 11.6, *L. paracasei* 20.3 and *L. paracasei* 25.4) were also able to produce bioactive substances that caused a significant reduction in *S. mutans* biofilms.¹³ Similarly, a study by Stecksens-Blicks *et al*¹⁴ found that milk with LAB (*L. rhamnosus* LB21) reduced caries occurrence in school children. Among the isolated LAB, isolates from *tapai ubi* shown to possess a good antibacterial activity. This finding is similar to previous studies by Ramasamy *et al*.¹⁵ which shown LAB isolated from *tapai ubi* possess a good antibacterial activity.

LAB isolated from this study were identified as *Lactobacillus pentosus*, *L. brevis* and *L. plantarum*. In a commercial strain, *Lactobacillus brevis* species have been used commercially in lozenges which had a beneficial effect in a reduction of salivary *Streptococcus mutans*.¹⁶ Moreover, LAB strains from *Lactobacillus rhamnosus* GG, *L. casei*, *L. reuteri*, *L. plantarum*, *L. brevis* CD2, *Bifidobacterium spp.* *etc.* were proposed to reduce caries incidence.⁷ This shown this type of species is a promising candidate as a probiotic strain for caries prevention. Apart from that, the selected strains identified belong to Qualified Presumption of Safety (QPS) status strain by the European Food Safety Authority (EFSA) which is based on their long history of apparent safe use.¹⁷ However, further researches are needed to be conducted to understand its properties, safety and usage of the strain still need to be assessed.

Conclusions

In conclusion, lactic acid bacteria isolated from fermented food in this study possess antibacterial properties against *Streptococcus mutans* and belong to QPS status strain. The strains show promising properties to be used as potential probiotics and useful treatment in oral modalities. However, full characterization and safety assessment of the strains need to be further investigated.

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Declaration of Interest

There is no conflict of interest in this study.

References

1. Food and Agricultural Organization of the United Nations and World Health Organization. Joint FAO/WHO working group report on drafting guidelines for the evaluation of probiotics in food. Food and Agricultural Organization of the United Nations. 2002.
2. Nagpal R, Kumar A, Kumar M, Behare PV, Jain S, Yadav H. Probiotics, their health benefits and applications for developing healthier foods: A review. *FEMS Microbiol. Lett.* 2012;334(1): 1–15.
3. Kechagia M, Basoulis D, Konstantopoulou S, Dimitriadi D, Gyftopoulou, K, Skarmoutsou N, Fakiri, EM. Health Benefits of Probiotics: A Review. *ISRN Nutr.* 2013:481651.
4. Fauzi AF, Indiana SK, Wicaksono RH, Adiningrat A. 2018. A challenge in ethanolic propolis utilization from *Apis Trigona* as an oral antimicrobial agent. *J Int Dent Med Res.* 2018; 11(2): 682-86.
5. Othman TAM, Mohd Hanafiah R, Nam NA, Mohd-Said S, Adnan SNA. Chemical composition and in vitro antimicrobial properties of *Phyllanthus columnaris* stem bark tannins against oral pathogens. *J Int Dent Med Res.* 2019; 12(3): 848-53.
6. Saha S, Tomaro-Duchesneau C, Tabrizian M, Prakash S. Probiotics as oral health biotherapeutics. *Expert Opin Biol Ther.* 2012;12(9):1207-20.
7. Cagetti MG, Mastroberardino S, Milia E, Cocco F, Lingstr P, Campus G. The use of probiotic strains in caries prevention: A systematic review. *Nutrients.* 2013; 5(7): 2530–50.
8. Holzapfel WH, Haberer P, Geisen R, Björkroth J, Schillinger U. Taxonomy and important features of probiotic microorganisms in food and nutrition. *Am J Clin Nutr.* 2001;73(2 Suppl):365S-373S.
9. Suzuki, MT and Giovanoni, SJ. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl Environ Microbiol.* 1996; 62(2):625-30.
10. Ren D, Zhu J, Gong S, Liu H, Yu H. Antimicrobial characteristics of lactic acid bacteria isolated from homemade fermented foods. *Hindawi Biomed Research International.* 2018; 5416725.
11. Zhao W, Liu Y, Latta M, Ma W, Wu Z, Chen P. Probiotics database: a potential source of fermented foods. *Int J Food Prop.* 2019. 22:1, 198-217.
12. Lin X, Chen X, Tu Y, Wang S, Chen H. Effect of probiotic lactobacilli on the growth of *Streptococcus mutans* and multispecies biofilms isolated from children with active caries. *Med Sci Monit.* 2017; 23:4175–81.
13. Rossoni RD, Santos Velosso, MS, Barros PP, Alvarenga JA, Santos Prado ACC, Camargo Ribeir FD, Anbinder AL, Junqueira JC. Inhibitory effect of probiotic *Lactobacillus* supernatants from the oral cavity on biofilms. *Microb Pathog.* 2018; 123:361-367.
14. Steckslen-Blicks C, Sjöström I, Twetman S. Effect of long-term consumption of milk supplemented with probiotic lactobacilli and fluoride on dental caries and general health in preschool children: a cluster-randomized study. *Caries Res.* 2009; 18:374-81.
15. Ramasamy K, Abdul Rahman NZ, Chin Chin SC, Alitheen, NJ, Abdullah N, Wan HW. Probiotic potential of lactic acid bacteria from fermented Malaysian food or milk products. *Int J Food Sci Tech.* 2012; 47:2175–83.
16. Caglar E, Cildir SK, Ergeneli S, Sandalli N, Twetman S. Salivary mutans streptococci and lactobacilli levels after ingestion of the probiotic bacterium *Lactobacillus reuteri* ATCC 55730 by straws or tablets. *Acta Odontol Scand.* 2006; 64:314–8.
17. Koutsoumanis K, Allende A, Alvarez-Ordóñez A, Bolton D, Bover-Cid S, Chemaly M, Herman L. Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 10: Suitability of taxonomic units notified to EFSA until March 2019. *EFSA J* 2019;17(7):5753, 79 pp.