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Anticariogenic Potential of *Lactiplantibacillus plantarum*-Isolated Kimchi in suppression of *Streptococcus mutans*' Growth

(Potensi Antikariogenik Kimchi Pencilan *Lactiplantibacillus plantarum* dalam Merencat Pertumbuhan *Streptococcus mutans*)

MALATHI GANESON[1], ZALEHA SHAFIEI[1,*], NORAZIAH MOHAMAD ZIN[2], MOHD NIZAM LANI[3], ZAMIRAH ZAINAL ABIDIN[1] & MAZLINA MOHD SAID[4]

[1]*Department of Craniofacial Diagnostics and Biosciences, Faculty of Dentistry, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia*
[2]*Centre of Diagnostics and Applied Health Sciences, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia*
[3]*Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, 21030 Kuala Nerus Terengganu, Terengganu, Malaysia*
[4]*Centre for Entrepreneurship and SMEs Development (UKM-CESMED), Faculty of Pharmacy, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia*

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ABSTRACT

Kimchi, a lactic acid-fermented vegetable product, is widely recognised for its popularity and health benefits. It contains antimicrobial microorganisms. This study aimed to isolate, characterise, and test antimicrobial activity of kimchi isolates against *Streptococcus mutans*. Kimchi samples were mixed in MRS broth (1:10 w/v) and incubated overnight at 37 °C. Then, serially diluted 10-folds and spread onto MRS agar to isolate lactic acid bacteria (LAB). Gram staining, catalase, and oxidase tests were performed, followed by API 50 CHL and 16S rRNA sequencing analysis to identify the most potent strain. Four selected strains, K1, K2, K3, and K4 were identified and determined for their antibacterial activity against *S. mutans* using agar spot and agar well diffusion methods. Strain K2 with the diameter of the inhibition zone (33.50 ± 5.47 mm), and strain K3 (33.50 ± 3.62 mm) are the strains with the most potential to inhibit *S. mutans*. However, the cell-free supernatant of strain K2 (9.92 ± 0.58 mm) exhibited higher inhibitory activity than strain K3. Therefore, strain K2 was selected as the most potential strain, identified as *Lactobacillus* (currently known as *Lactiplantibacillus*) *plantarum* 1 in the API web database and confirmed by 16S rDNA gene sequencing as *Lactiplantibacillus plantarum* strain AMT744129 with 99.3% similarity. Thus, *Lactiplantibacillus plantarum* strain K2-isolated kimchi has a cariogenic effect and could be potential as an alternative agent in treating dental caries.

Keywords: Antibacterial; API 50 CHL kit; *Lactiplantibacillus plantarum*; *Streptococcus mutans*; 16S rDNA gene sequencing

ABSTRAK

Kimchi, produk sayuran yang ditapai asid laktik, diiktiraf secara meluas untuk populariti dan manfaat kesihatannya. Ia mengandungi mikroorganisma antimikrob. Penyelidikan ini bertujuan untuk mengasing, menciri dan menguji aktiviti antimikrob pencilan kimchi terhadap *Streptococcus mutans*. Sampel kimchi dicampur dalam kaldu MRS (1:10 w/v) dan diinkubasi semalaman pada suhu 37 °C. Kemudian, dicairkan secara bersiri 10 kali ganda dan disebarkan ke atas agar MRS untuk pengasingan bakteria asid laktik (LAB). Ujian pewarnaan Gram, katalase dan oksidase telah dilakukan, diikuti API 50 CHL dan analisis penjujukan 16S rRNA untuk mengenal pasti strain yang paling berpotensi. Empat strain terpilih, K1, K2, K3 dan K4 telah dikenal pasti dan ditentukan aktiviti antibakterianya terhadap *S. mutans* menggunakan kaedah *spot* agar dan resapan telaga agar. Strain K2 dengan diameter zon perencatan (33.50 ± 5.47 mm) dan strain K3 (33.50 ± 3.62 mm) merupakan strain paling berpotensi merencat *S. mutans*. Namun, supernatan tanpa-sel bagi strain K2 (9.92 ± 0.58 mm) menunjukkan aktiviti perencatan yang lebih tinggi daripada strain K3. Oleh itu, strain K2 telah dipilih sebagai strain yang paling berpotensi, dikenal pasti sebagai *Lactobacillus* (terkini dikenali sebagai *Lactiplantibacillus*) *plantarum* 1 dalam pangkalan data web API dan disahkan dengan penjujukan gen 16S rDNA sebagai strain *Lactiplantibacillus plantarum* AMT744129 dengan 99.3% persamaan. Justeru, strain *Lactiplantibacillus plantarum* K2-pencilan kimchi mempunyai kesan kariogenik dan berpotensi digunakan sebagai agen alternatif dalam merawat karies gigi.

Kata kunci: Antibakteria; API 50 CHL kit; *Lactiplantibacillus plantarum*; penjujukan gen 16S rDNA; *Streptococcus mutans*

INTRODUCTION

Dental caries, commonly known as tooth decay, is a widespread oral health issue that affects individuals across all age groups globally. It is a common and preventable disease caused by bacteria, sugars, and acids. It is a prevalent chronic infectious disease that results from tooth-adherent cariogenic bacteria metabolising sugars to produce acid, which over time demineralises the tooth structure (Meurman & Stamatova 2018; Qiu et al. 2020). If left untreated, the decay process can continue to develop, potentially leading to severe toothache, infection, and tooth loss. Symptoms can include toothache, tooth sensitivity, mild to sharp pain when eating or drinking, visible holes or pits in your teeth, and brown, black, or white staining on any tooth surface.

The World Health Organization (WHO) highlights the strong connection between oral health, overall health, and quality of life. Dental caries' occurrence, intensity, and seriousness differ based on age, gender, ethnicity, socioeconomic background, financial status, and geographical location. As per the WHO Global Oral Health Status Report (2022), approximately 3.5 billion people globally are affected by oral diseases, with three-quarters living in middle-income countries. Around 514 million children worldwide experience primary tooth decay, while it is estimated that 2 billion adults have permanent tooth decay.

Streptococcus mutans is a key player in dental caries development, known for its virulence and acid production (Mulder, Maboza & Ahmed 2020). Additionally, *Streptococcus sobrinus* and *Lactobacilli* are prevalent in advanced caries cases. While *S. mutans* is strongly associated with caries progression, caries still develops without this bacterium. Other factors, such as diet, oral hygiene practices, and genetic predisposition, also play a role in developing dental caries (Zaura & Twetman 2019).

It is crucial to prioritise the prevention of dental caries for the sake of public health. Recent recommendations emphasise the importance of early screening and continuous monitoring of dental caries rather than waiting for the appearance of cavities. Assessing the risk of caries helps identify specific protective factors and determine the need for therapeutic interventions. Factors such as harmful bacteria, inadequate saliva production, and unhealthy dietary habits contribute to the development of caries (Oh et al. 2020). Traditional methods of prevention and treatment, such as fluoride application and dental fillings, are not always effective or accessible to everyone. Chlorhexidine (CHX) mouthwash, with concentrations ranging from 0.1% to 0.2%, has been found effectively reduced plaque when used daily for two weeks, even without mechanical cleaning. It can also serve as a long-term supplement to oral hygiene, recommended at intervals of 4 to 6 weeks and every 6 months (Poppolo-Deus & Ouanounou 2022). CHX demonstrates rapid antimicrobial and antifungal properties, even at low concentrations, and targets aerobic and anaerobic bacteria (Poppolo-Deus & Ouanounou 2022). It is also effective against DNA and RNA viruses, and lipophilic-enveloped viruses like HIV, influenza A, hepatitis B, herpes simplex virus, and cytomegalovirus. However, the therapeutic use of chlorhexidine is limited due to its toxicity and the potential for tooth discolouration (Brookes et al. 2020). Hence, there is a constant search for alternative, more natural methods to prevent and control dental caries.

One promising area of research involves exploring the potential use of probiotics derived from fermented foods. Kimchi, a popular dish in Korean cuisine, is known for its health benefits, largely due to its abundance of probiotics (Zou et al. 2023). Researchers have studied kimchi for its potential impact on dental caries. The lactic acid bacteria (LAB) found in kimchi play a vital role in fermentation and have demonstrated antimicrobial properties that can help prevent dental caries (Qiu et al. 2020; Raghavendra et al. 2023). LAB species such as *Leuconostoc* spp., *Lactobacillus* spp., and *Weissella* spp. are integral to the fermentation process of kimchi (Raghavendra et al. 2023). Consumption of kimchi can indeed influence the gut microbiota and potentially offer health benefits, including anti-inflammatory effects (Marco et al. 2017). Additionally, the kimchi LAB have been associated with the production of beneficial metabolites like bacteriocins, GABA, ornithine, and mannitol, which may have anti-obesity effects and other health advantages (Lee et al. 2021). Thus, this study was conducted to isolate different types of LAB from kimchi, characterise the lactic acid bacteria (LAB), and determine their antimicrobial activity against *Streptococcus mutans*, a major aetiological agent of dental caries.

MATERIALS AND METHODS

ISOLATION OF LACTIC ACID BACTERIA FROM KIMCHI SAMPLE

Kimchi was procured from a vendor named Machisoyo in Melaka, Malaysia, from which lactic acid bacteria (LAB) were isolated. The homemade kimchi was fermented for 43 h including transportation (1 day 19 h) and kept at 4 °C for 2 days. After that, a 10 g kimchi sample was then suspended in 100 mL of de Man-Rogosa-Sharpe (MRS) broth, incubated overnight at 37 °C under microaerophilic condition (in candle jar), serially diluted ten-folds in MRS broth, and each dilution was spread (100 µL) onto MRS agar in triplicates. Plates were incubated microaerophilically at 37 °C for 48 h. Since the LAB strains exhibited similar morphology onto MRS agar, thus, four LAB strains with distinct colony sizes were selected from the resulting plates and subcultured for purity. The four selected kimchi isolates were designated as strains (K1, K2, K3, and K4) and identified using Gram staining, catalase, and oxidase tests according to the basic standard protocol for

bacterial identification. The pure strains were stored in a 20% glycerol-containing MRS broth at -80 °C for further analysis.

STANDARDISATION OF TEST AND INDICATOR STRAINS

Kimchi strains (K1-K4) were standardised using a modified Choeisoongneen et al. (2019) method. Briefly, 10% inocula from stock cultures were grown in MRS broth at 37 °C for 24 h in microaerophilic conditions, then diluted to OD 600 nm = 0.1, re-inoculated into MRS broth (1:10 v/v), and incubated for 48 h (37 °C) until OD 600 nm reached 2.2. The indicator strain, *S. mutans* ATCC 25175 (ATCC, USA), was grown in BHI broth at 37 °C for 24 h and standardised to OD 550 nm = 0.144 (0.5 McFarland), which was equivalent to 1.53×10^7 CFU/mL (Shafiei et al. 2020) before used for subsequent analysis.

ANTIBACTERIAL ACTIVITY OF THE KIMCHI ISOLATES AGAINST *S. mutans*

Agar spot method

The antibacterial activities of four selected kimchi strains (K1, K2, K3 and K4) were assessed using a modified agar spot method (Choeisoongneen et al. 2019). Thirty microliters of each standardised kimchi cell suspension (OD 600 nm = 2.2) were spotted onto MRS agar plates containing a standardised *S. mutans* lawn, in triplicates. Plates were incubated at 37 °C for 24 h and the inhibition zones of *S. mutans* were measured. The experiment was repeated with three biological replicates, each with three technical replicates (n = 9), and data are presented as mean ± SD (mm).

Agar well diffusion method

Kimchi strain cell suspensions were tested against *S. mutans* using the agar well diffusion method. 9-mm wells were created in BHI agar plates spread with standardised *S. mutans* (OD 550 nm = 0.144). Standardised kimchi suspensions (OD 600 nm = 2.2) were added to the wells in triplicate. The experiment was repeated three times (n = 9 total), incubated microaerophilically at 37 °C for 24 h, and inhibition zone diameters were measured and reported as mean ± SD (mm).

IDENTIFICATION OF THE MOST POTENT STRAIN

Analytical profile index (API 50 CHL) identification kit

The most potent strain K2 was identified using the API 50 CHL kit (BioMérieux 2011), a tool specifically designed for identifying Lactobacilli. The inoculum was prepared by a modified manufacturing protocol and the method by Pyar and Kok (2019) with minor modifications. A pure colony of the most potent kimchi strain was transferred into 2 mL of sterile distilled water (Tube 1), and mixed. Then, 350 µL of the mixture was transferred twice to another sterile tube containing sterile distilled water (5 mL) (Tube 2) to reach turbidity equivalent to McFarland standard # 2. The final inoculum was prepared by transferring 700 µL from the initial bacterial suspension (Tube 1) into an API 50 CHL Medium (10 mL). The suspension was mixed and 150 µL was loaded into the well using a sterilised micropipette and covered with 50 µL mineral oil. Then, the strips API kit was incubated at 37 °C for 48 h. The observed colour changes were recorded and analysed using the API web database.

16S rDNA gene sequencing

A 48-h pure colony of the most potent strain K2 on MRS agar plate was cut into pieces measuring (5 mm × 5 mm) in triplicates and sent for analysis of DNA barcoding using 16S rDNA gene sequencing done outsourced by Apical Biosystem Sdn. Bhd., Selangor, Malaysia. A manufacturing protocol from Apical Biosystem was used to amplify the bacterial 16S rDNA gene, which has a full length of 1.5kb. Universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') were used for the amplification process. The total reaction volume of 25 µL mixture contained gDNA purified using an in-house optimised protocol, 0.3 *p*mol of each primer, deoxynucleotides triphosphates (dNTPs, 400 µm each), 0.5 U of thermostable DNA polymerase, supplied with PCR buffer, and water. The PCR was performed as follows: 1 cycle (94 °C for 2 min) for initial denaturation, and 25 cycles (98 °C for 10 s; 53 °C for 30 s; 68 °C for 1 min) for denaturation, annealing, and extension. The PCR products were purified by the standard PCR clean-up method. The purified PCR products were subjected to bidirectional sequencing with universal primers 785F(5'-GGATTAGATACCCTGGTA-3') and 907R(5'-CCGTCAATTCMTTTRAGTTT-3') using the BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems). The bacterial lysis buffer of Bacterial DNA barcoding (1st BASE, KIT-1100-50) was used for DNA extraction (1st BASE, Malaysia). The sequence further analysed in NCBI Blast https://blast.ncbi.nlm.nih.gov/Blast.cgi.

STATISTICAL ANALYSIS

Data are presented as mean ± standard deviation (SD). Nine samples (n = 9) were analysed, comprising three technical replicates and three biological replicates for each experiment. Statistical analysis was performed using IBM SPSS Statistics version 29.0. Since the data violated the assumption of normality assessed using Kolmogorov-Smirnova and Shapiro-Wilk tests, a non-parametric Kruskal-Wallis test was employed to compare the inhibitory zones of kimchi strains against *S. mutans* using both agar spot and agar well diffusion methods. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSIONS

LACTIC ACID BACTERIA ISOLATION AND IDENTIFICATION

Kimchi is recognised for its diverse health benefits attributed to the fermentation process. It possesses properties such as anti-inflammatory, antibacterial, antioxidant, anticancer, anti-obesity, probiotic, cholesterol reduction, and antiaging effects (Patra et al. 2016). The fermentation process promotes the growth of beneficial LAB while inhibiting harmful bacteria, making kimchi a probiotic food (Song et al. 2023). Although there is no specific research on the impact of kimchi on oral pathogens, a study demonstrated that two LAB strains derived from Korean kimchi, *Lactobacillus* sp. M.21 and *Leuconostoc* sp. J.27, can inhibit the growth of foodborne pathogenic biofilms on seafood and food processing surfaces (Toushik et al. 2021). This suggests the potential antibacterial properties of kimchi that may extend to oral pathogens. Another study highlighted the antibacterial activity of LAB isolated from kimchi against bacteria associated with the acidification and hygiene of kimchi, such as *Lactobacillus plantarum*, *Lactobacillus sakei*, *Pediococcus pentosaceus*, and *Escherichia coli* using a deferred method (Park et al. 2014). The findings of the study highlighted the potential antibacterial properties of the LAB strains isolated from kimchi. This suggests that these LAB strains may have the ability to inhibit the growth or activity of certain bacteria, which can be beneficial for the preservation and quality of kimchi. Another research found that the cell-free supernatant (CFS) of *Pediococcus inopinatus* K35, a strain isolated from kimchi effectively inhibited the growth of Multi-Drug-Resistant *Pseudomonas aeruginosa* (Yi & Kim 2023). This suggests that kimchi may have potential antibacterial effects against certain types of bacteria. Another study reported that kimchi-derived LAB from members of *Lactobacillus sakei*, *Lactobacillus plantarum,* and *Leuconostoc pseudomesenteroides* have potential probiotic properties with *Lactobacillus sakei* ADM14 exhibited significant anti-adipogenic effect and could be the potential as a probiotic candidate for use in functional foods (Won et al. 2020). Since the studies do not specifically address oral pathogens, thus, more targeted research is needed to confirm the antimicrobial properties of kimchi on oral pathogens.

Therefore, in this study, we isolated four strains from kimchi from a total of 85 LAB colonies that were counted onto MRS agar at a dilution factor of 10^5, which was equivalent to 0.85×10^8 CFU/mL kimchi sample. Since the morphology of the four selected kimchi-derived colonies were similar, thus, each colony was selected based on different sizes and designated as strains K1, K2, K3, and K4. The colony sizes of kimchi strains K1 were punctiform (< 0.5 mm), strain K2 was small (< 1 mm), strain K3 was medium (1 mm), and strain K4 was large (> 1 mm) (Table 1; Figure 1). All these strains had a circular morphology, a glistering surface, raised in elevation, an even margin, an opaque appearance, and a yellow colour based on the morphology chart (Petersen & McLaughlin 2016). Bacterial morphology plays a crucial role in surface attachment; thus, any variations in their shape can potentially influence their adhesion ability. The LAB colonies have general characteristics, specifically circular white to yellowish, entire margin, convex elevation, and smooth surface.

Gram staining showed strains K1-K4 to be Gram-positive rods-shaped in short chain and small in sizes (Figure 1). This supported the general assumption that all *Lactobacillus* species are Gram-positive bacteria. Gram staining is a crucial method for identifying phenotypic bacteria. This process involves using crystal violet as the primary dye, iodine as a mordant, alcohol acetone as a decolorising agent, and safranin as a counterstaining agent. It is used to differentiate microbes based on the cell ultrastructure, particularly the type of cell wall, where the thick peptidoglycan layer will retain the primary dye, crystal violet, and represent Gram-positive bacteria (Tripathi & Sapra 2023), as exhibited by the four selected kimchi strains.

Furthermore, the catalase test showed no bubble formation in the isolated strain, and the oxidase test showed a purple stain on these kimchi strains, represented as catalase-negative and oxidase-positive. The oxidase test detects cytochrome oxidase, an enzyme involved in bacterial respiration, using tetramethyl-p-phenylenediamine dihydrochloride. When cytochrome c is present, it oxidises the reagent, changing its colour from colourless to dark blue or purple (indophenol blue). However, the selected kimchi strains showed no colour changes, indicating no cytochrome c present, and suggesting they are anaerobic microorganisms. Most of the LAB-derived kimchi strains such as genus Lactobacillus, were characterised as Gram-positive, catalase-negative, aerotolerant anaerobes or microaerophilic, rod-shaped, non-spore-forming bacteria, either homofermentative or heterofermentative (Zheng et al. 2020). According to Abid et al. (2022), *Lactobacillus* species use fermentation to produce their metabolism, and an anaerobic jar with anaerobic jar sachets is the ideal growth environment. Lactobacilli are oxygen-tolerant anaerobes with a fermentative metabolism that cannot produce an active electron transport chain (ETC). However, certain LAB strains can acquire advantageous traits for industrial and biotechnological purposes under conditions, where oxygen is present and respiratory growth occurs (Zotta, Parente & Ricciardi 2017).

The catalase test is another biochemical test used to identify lactic acid bacteria quickly and simply. It is a method used to detect the presence of the catalase enzyme in bacteria. Catalase is responsible for neutralising the harmful effects of hydrogen peroxide, a bactericidal compound. A positive catalase reaction is indicated by the formation of gas bubbles, which indicate the production of oxygen. When catalase is present, it rapidly breaks down hydrogen peroxide into water and oxygen ($2H_2O_2 + O_2$),

TABLE 1. Morphology and biochemical test of kimchi strains (K1, K2, K3 and K4)

Kimchi isolates	Strain K1	Strain K2	Strain K3	Strain K4
Biochemical test				
Gram staining	Gram-positive (purple colour)			
Microscopic structure	Rod-shaped; short chain			
Catalase test	Negative			
Oxidase test	Negative			
Morphological structure of colonies on MRS agar				
Size	Punctiform (< 0.5 mm)	Small (< 1 mm)	Medium (1 mm)	Large (> 1 mm)
Colour	Yellow			
Form	Circular			
Elevation	Raised			
Margin	Even			
Surface	Glistering			
Opacity	Opaque			

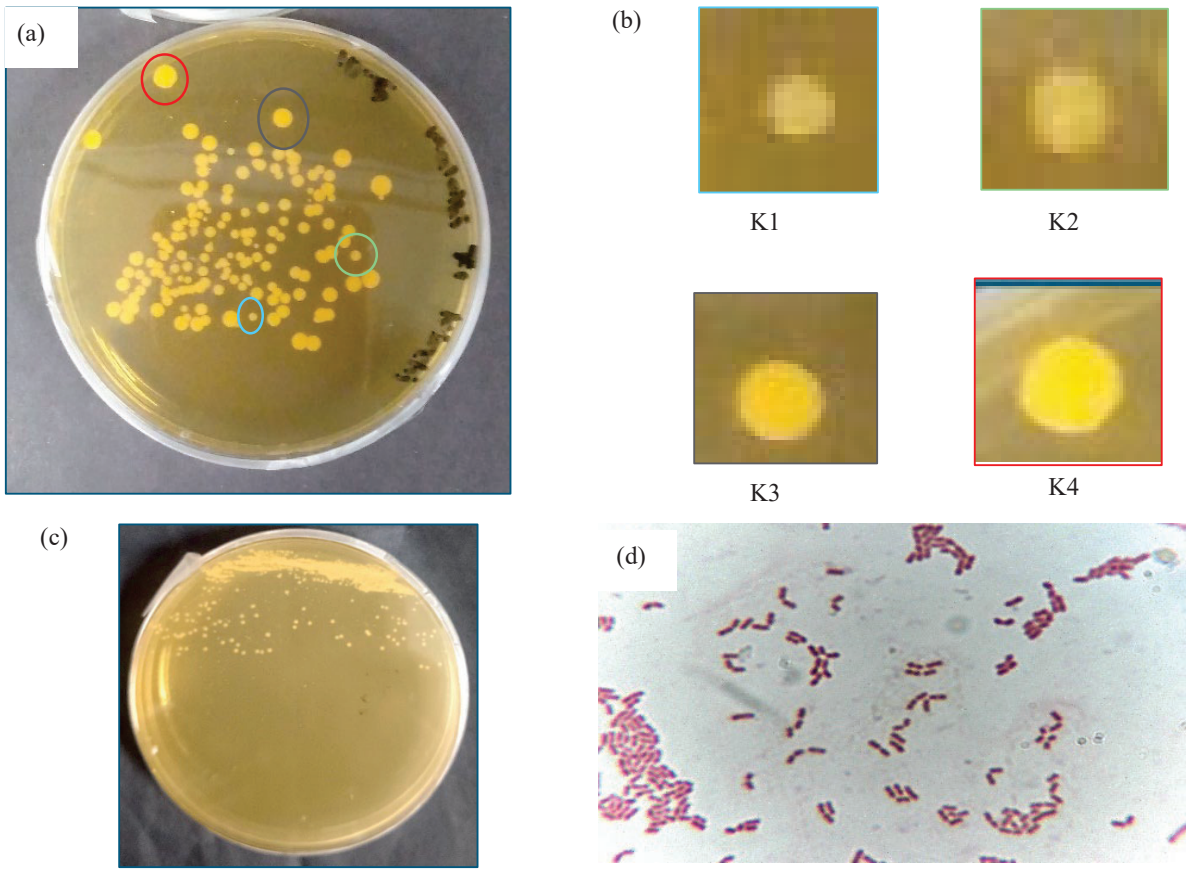


FIGURE 1: Morphology of (a) LAB isolated from Kimchi on MRS agar (dilution 10^{-7}), (b) kimchi strains, K1, K2, K3, K4, (c) pure culture of strain K2 (MRS agar) (d) Gram staining morphology of strain K2 (100X magnification)

resulting in the rapid formation of bubbles. The isolates from kimchi were found to be catalase-negative lactic acid bacteria, as supported by Mokoena (2017).

ANTIBACTERIAL ACTIVITY OF KIMCHI STRAINS

The antimicrobial activity of four kimchi strains (K1-K4) against *S. mutans* ATCC 25175 was evaluated using agar spot and agar well diffusion methods. Strain K4 exhibited the highest inhibitory zones (25.5 ± 0.71 mm), followed by strains K2 (23 ± 2.83 mm), K1 (20.5 ± 2.12 mm), and K3 (20.5 ± 2.12 mm) using agar spot method (Table 2).

The agar spot method involved direct contact between the kimchi strains and *S. mutans*, thus, it is a more sensitive method for determining the antibacterial activity of probiotics compared to the agar well diffusion method. However, due to the small spot size of cell suspension (3 µL) and limited diffusion, the agar spot method showed lower inhibitory zones than that of the agar well diffusion method.

The agar well diffusion method using a larger volume (100 µL) of the standardised kimchi strain cell suspensions (OD 600 nm = 0.22). The results occurred based on the ability of the metabolites in the cell suspensions to adsorb and diffuse through the agar well and inhibit *S. mutans*. Among the four tested kimchi strains, strains K2 and K3 showed the most potential to inhibit the growth of *S. mutans*, with a diameter of inhibitory zones of 33.50 ± 5.47 mm and 33.50 ± 3.62 mm, respectively, followed by strain K1 (33.17 ± 4.36 mm), strain K4 (32.17 ± 2.56 mm), and *Lactiplantibacillus casei* strain ATCC 393 (30.5 ± 2.07 mm) (Table 2). As referred to the size, strain K4 was large (> 1 mm) compared to strain K2 (< 1 mm), this is attributed to the bigger inhibitory zones on the agar spot method and less diffusion of its metabolites affecting the result on the agar well diffusion method. While previous studies have explored various aspects of kimchi, there is still limited information on the ability of probiotic cells in kimchi to inhibit *S. mutans*. Therefore, this study represents the first report demonstrating the inhibitory effect of kimchi strains' cell suspensions on *S. mutans*.

Pairwise comparisons using non-parametric Kruskal-Wallis test for non-normally distributed data showed no significant differences ($p > 0.05$) in antimicrobial activity between kimchi strains using either agar spot or well diffusion methods. This suggests that observed variations are likely due to random error. Although the non-normality and potential high variability of the data may have limited the statistical power, the results support our hypothesis of comparable treatment effects. While both screening agar spot and agar well diffusion methods showed no statistically significant antibacterial activity of the kimchi strains against *S. mutans* ($p > 0.05$), further investigation may be warranted to explore potential limitations of the study design or the sensitivity of the methods. For more precise quantitative analysis, broth microdilution method is recommended.

Therefore, the chosen strains K2 and K3 were processed with a cell-free supernatant (CFS), having its pH adjusted to 6.5 using 1M NaOH to avoid inhibition due to acid lactic produced, and were then evaluated their antibacterial activity against *S. mutans* through the agar well diffusion method. Moreover, the inhibitory function of strain K2's CFS demonstrated a smaller inhibition zone (9.92 ± 0.58 mm) in comparison to strain K3's CFS, which showed no detectable inhibition zone after 24 h of incubation, as well as the positive control (Table3)andpreventstatisticalanalysis.Thisindicatesthatthe cell-free supernatants (CFS) produced by strain K2 retain their inhibitory effects. However, the CFS produced by strain K3 and the positive control strain (strain 393) contained only minimal active metabolites due to the neutralisation and filtration processes, which proved insufficient to inhibit *S. mutans*. This is supported by Wasfi et al. (2018) that *Lactobacillus* sp. strain ATCC 393 demonstrated a large inhibitory zone (23 ± 1 mm) compared to its CFS (18 ± 1 mm) against *S. mutans* in the agar well diffusion method.

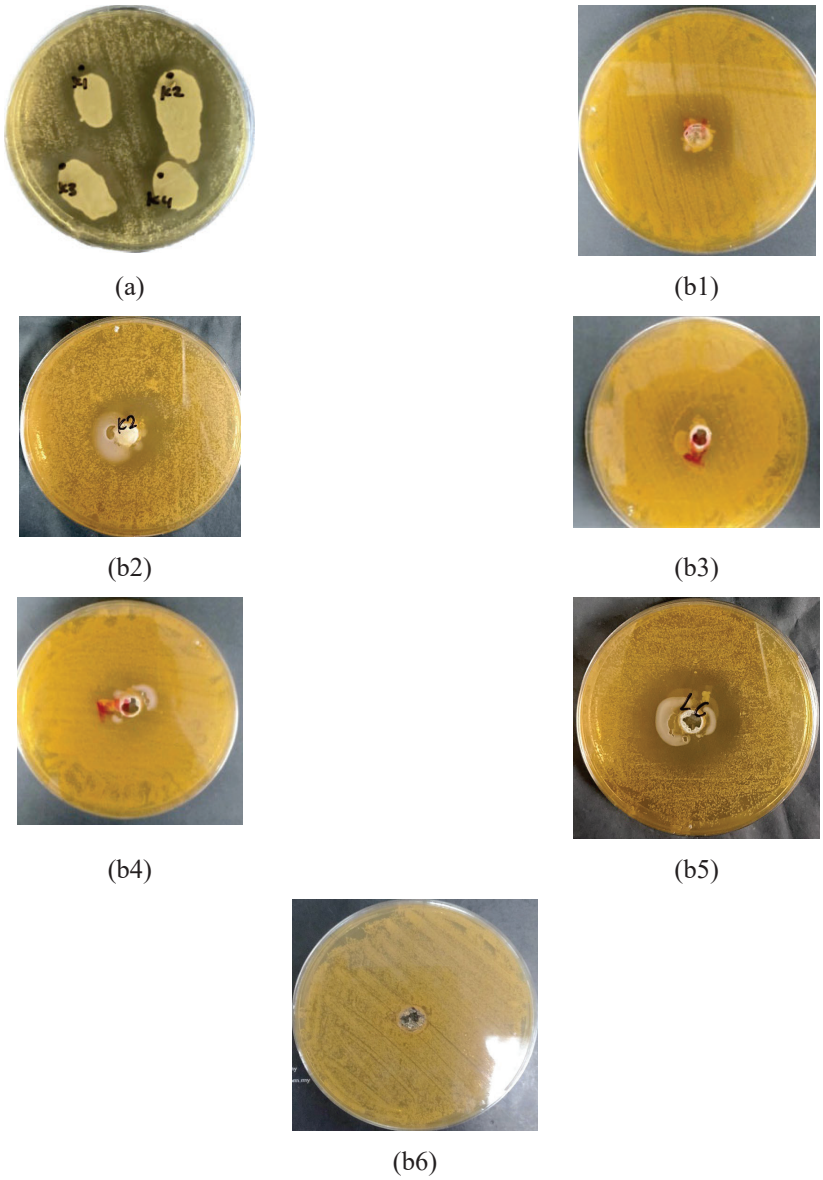
The CFS of LAB is known to contain active substances like bacteriocins and organic acids that can suppress pathogenic bacteria, including those resistant to multiple drugs. Research has proven that LAB-CFS can effectively hinder the growth of various drug-resistant pathogens such as *Escherichia coli*, *Staphylococcus aureus,* and *Porphyromonas gingivalis*, highlighting the strong antimicrobial characteristics of these metabolites (Almohammadi et al. 2022; Qadi et al. 2023; Widyarman et al. 2018). The antimicrobial activity of LAB-CFS is also attributed to a mix of active components like organic acid, hydrogen peroxide, and proteins, which collectively enhance the inhibitory effects against pathogens (Kaewchomphunuch et al. 2022). Hence, even in the absence of living LAB cells, the metabolites in the CFS continue to demonstrate significant inhibitory activity against pathogenic bacteria. This makes CFS a promising candidate for use as a food antimicrobial agent. One major advantage of using CFS is that it prevents fermentation in treated food, which can occur when bacterial cells are present. However, it is important to note that adding CFS to food may lead to changes in the sensory properties of the food. The effectiveness and stability of CFS under different pH values, temperatures, and enzymatic conditions depend on the nature of its antimicrobial compounds. If the antimicrobial activity of CFS is primarily attributed to organic acids, then this activity may be lost when the CFS is neutralised at pH values between 6.0 and 7.0 (Mani-López & Arrioja-Bretón 2022). Therefore, in the case of strain K2, its antimicrobial activity remains unaffected even after adjusting the pH of its cell-free supernatant.

Lactic acid bacteria (LAB) are widespread due to their antibacterial properties, ability to inhibit pathogen growth, and increase foods' nutritional value (Gao et al. 2019). Kimchi strains, which are lactic acid bacteria in the genus Lactobacilli were selected and showed different

TABLE 2. Inhibitory zones of kimchi strains cell suspensions using agar spot and agar well diffusion methods

Bacteria	Diameter zone of inhibition (mean ± SD) (mm)	
	Agar spot method	Agar well diffusion method
Strain K1	20.50 ± 2.12	33.17 ± 4.36
Strain K2	23.00 ± 2.83	33.50 ± 5.47
Strain. K3	20.50 ± 2.12	33.50 ± 3.62
Strain K4	25.50 ± 0.71	32.17 ± 2.56
Lactiplantibacillus casei strain ATCC 393 (Positive control)	20.50 ± 0.71	30.50 ± 2.07
MRS broth (Negative control)	0.00 ± 0.00	0.00 ± 0.00

$p > 0.05$ comparing between kimchi strains and respective negative controls (Kruskall Wallis test; n = 9)



(a) (b1) (b2) (b3) (b4) (b5) (b6)

FIGURE 2: Zone of inhibition of Kimchi strains (K1, K2, K3, K4) against *S. mutans* using (a) agar spot lawn and (b) agar well diffusion methods for strains (b1) K1, (b2) K2, (b3) K3, (b4) K4, (b5) Positive control and (b6) Negative control)

TABLE 3. Inhibitory zone of strain K2's cell-free supernatant against *S. mutans* using agar well diffusion method

Bacteria	Diameter zone of inhibition (mean ± SD) (mm)
	Cell-free supernatant
Strain K2	9.92 ± 0.58
Strain K3	0.00 ± 0.00
Lacticaseibacillus casei strain ATCC 393 (Positive control)	0.00 ± 0.00
MRS broth (Negative control)	0.00 ± 0.00

Insufficient data (zero inhibitory zones in all but one strain) precludes statistical analysis

capabilities in inhibiting *S. mutans* based on agar spot and agar well diffusion methods. The spot method, involving a small volume of the test sample (3 µL) directly applied to a bacterial lawn on agar, offers a quick and easy approach, particularly beneficial when screening numerous samples (Balouiri, Sadiki & Ibnsouda 2016). While this method excels in sensitivity, it may limit the diffusion of active compounds compared to the agar well diffusion method. Both methods are widely used and cost-effective techniques in antimicrobial research. However, the choice between them may depend on the specific requirements of the experiment, such as the nature of the substance being tested and the type of microorganism it's being tested. Moreover, various methods are commonly employed to assess antimicrobial activity, including cross-streaking, co-culture, time-kill kinetics, resazurin assay, and bioautography. In addition, advanced techniques like flow cytometry and bioluminescent techniques offer faster and more sensitive results, providing a deeper understanding of the effects of antimicrobials on microbial viability. However, it is important to note that these advanced techniques may be more expensive and not readily accessible in certain laboratory settings, which can present challenges.

IDENTIFICATION OF THE MOST POTENT STRAINS USING API CHL 50 KIT

The selected potent strain K2 was subjected to the API CHL 50 test to identify lactobacilli based on phenotype characteristics. This test involved analysing the pure isolate and its carbohydrate profile, which was compared to a database using API software (APIWEB™ Biomeriux). The results of the kimchi strain K2 are presented in Table 4. Strain K2 had different fermentation abilities on several types of carbohydrates. Still, it had the same positive results in L-arabinose, D-galactose, D-glucose, D-fructose, mannose, mannitol, sorbitol, Methyl-D-mannoside, N-Acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, sucrose, trehalose, inulin, melezitose, raffinose, B-gentiobiose, and D-turanose. Whereas, the negative results in control, glycerol, erythritol, D-arabinose, ribose, D-xylose, L-xylose, adonitol, Bmethyl-D-xyloside, sorbose, rhamnose, dulcitol, inositol, starch glycogen, xylitol, D-lyxose, D-tagatose, D-fructose, L-fucose, D-arabitol, L-arabitol, 2-keto-gluconate, and 5-keto-gluconate. Some carbohydrates show doubtful carbohydrate profiles such as rhamnose and gluconate (Table 4). The test was repeated two times, yet it shows a doubtful profile. This means the influence factor could be the limitation of the database. Moreover, the API 50 CHL system relies on a reference database. If the strain being tested is not well represented or has unusual metabolic traits, it may not be impossible to generate a confident identification. However, the API database showed that strain K2 shares similar characteristics with *Lactiplantibacillus plantarum* I, with a 99.0% identification match. A previous study reported that the LAB isolates labelled ESCIb which belongs to *Lactiplantobacillus plantarums* had similar positive carbohydrate results in arabinose, galactose, lactose, maltose, mannitose, sucrose, xylose, sorbitol, ribosome, raffinose, and melibiose (Goa et al. 2022).

Lactic acid bacteria, including *Lactobacillus, Pediococcus, Lactococcus, Leuconostoc*, and *Bifidobacterium*, are well-known for their ability to produce lactic acid as the main by-product during carbohydrate fermentation. These bacteria play a crucial role in the natural and spontaneous fermentation of sugars, contributing to the improvement of aroma, flavour, texture, safety, and shelf life of food products. The identification of strain K2 using the API 50 CHL test provides valuable information about its characteristics and further confirms its classification within the *Lactiplantibacillus* genus.

IDENTIFICATION OF STRAIN K2 USING 16S rDNA GENE SEQUENCING

The most reliable method using 16S rDNA gene sequencing was carried out with the most potential strain K2 using a Sanger sequencer version 2.0. The consensus sequences were then blasted in the GenBank of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the results showed significant similarity (99.93%) with 10 different species. Based on this analysis, the bacterial isolate was identified as belonging to the *Lactiplantibacillus* genus,

specifically *Lactiplantibacillus plantarum*. The specific strains within the *Lactiplantibacillus plantarum* species are listed in Table 5, including strain AMT74419, strain SPC-SNU 72-2, strain SRCM 102737, strain SRCM 101518, strain SRCM 101222, strain 101105, strain 100995, strain 8318, plantarum 123-17, and strain TMW 1.1308.

The phylogenetic grouping in Figure 3 further supports the identification of strain K2 as *Lactiplantibacillus plantarum*, as its clusters with the other strains of the same species. The taxa of strain K2 are positioned in the third lane of the branches between *Lactiplantibacillus fabifermentans* strain DSM 21115 and *Lactiplantibacillus paraplantarum* strain DSM 10667.

Lactiplantibacillus plantarum I shows a 99.9% similarity with strain K2. The identification of strain K2 was confirmed through 16S rDNA gene sequencing, and a phylogenetic tree was constructed based on the aligned 16S rDNA gene sequences. The branches of strain K2 are located between *Lactiplantibacillus fabifermentans, Lactiplantibacillus paraplantarum,* and *Lactiplantibacillus plantarum* strains (Figure 4). In the NCBI blast, the majority of *Lactiplantibacillus plantarum* strains exhibit a 99.93% similarity with strain K2. *Lactiplantibacillus plantarum* belongs to the Lactobacillus genus, which is a member of the facultatively heterofermentative group of lactobacilli (Gökmen et al. 2024). Therefore, it has been confirmed that the kimchi isolate strain K2 is identified as *Lactiplantibacillus plantarum* I and belongs to the Lactobacillus genus based on phenotypic and genotypic characteristics using API 50 CHL kit or 16S rDNA gene sequencing methods.

The overall results of biochemical tests and Gram staining indicated that the morphological structures of strain K2 were like the *Lactiplantibacillus* species (formally known as *Lactobacillus* species). According to a recent study, over 300 species in 7 genera and 2 families of lactic acid bacteria have been reclassified into one family

TABLE 4. Identification of strain K2 using API 50 CHL 50 tests kit

No	Substrates	Strain K2	No	Substrates	Strain K2
0	Control	-	25	Esculin	+
1	Glycerol	-	26	Salicin	+
2	Erythritol	-	27	D-Cellobiose	+
3	D-arabinose	-	28	D-Maltose	+
4	L-arabinose	+	29	D-Lactose	+
5	Ribose	-	30	D-Melibiose	+
6	D-xylose	-	31	Sucrose	+
7	L-xylose	-	32	Trehalose	+
8	D-Adonitel	-	33	Inulin	+
9	Bmethyl-D-Xyloside	-	34	Melezitose	+
10	D-Galactose	+	35	Raffinose	+
11	D-Glucose	+	36	Starch	-
12	D-Fructose	+	37	Glycogen	-
13	Mannose	+	38	Xylitol	-
14	Sorbose	-	39	B-Gentiobiose	+
15	L-Rhamnose	?	40	D-turanose	+
16	Dulcitol	-	41	D-Lyxose	-
17	Inositol	-	42	D-tagatose	-
18	Mannitol	+	43	D-fucose	-
19	Sorbitol	+	44	L-fucose	-
20	Methyl-D-mannoside	+	45	D-arabitol	-
21	Metyl-D-glucoside	-	46	L-arabitol	-
22	N-Acetyl- Glucosamine	+	47	Gluconate	?
23	Amygdalin	+	48	2-Keto-Gluconate	-
24	Arbutin	+	49	5-Keto-Gluconate	-

TABLE 5. Description table of strain K2 with accession number

Description	Scientific Name	Percentage identification (%)	Accession number
Lactiplantibacillus plantarum strain AMT74419 chromosome, complete genome	*Lactiplantibacillus plantarum*	99.93	CP052869.1
Lactiplantibacillus plantarum strain SPC-SNU 72-2 chromosome, complete genome	*Lactiplantibacillus plantarum*	99.93	CP050805.1
Lactiplantibacillus plantarum strain SRCM102737 chromosome, complete genome	*Lactiplantibacillus plantarum*	99.93	CP028261.1
Lactiplantibacillus plantarum strain SRCM101518 chromosome, complete genome	*Lactiplantibacillus plantarum*	99.93	CP028241.1
Lactiplantibacillus plantarum strain SRCM101222 chromosome, complete genome	*Lactiplantibacillus plantarum*	99.93	CP028229.1
Lactiplantibacillus plantarum strain SRCM101105 chromosome, complete genome	*Lactiplantibacillus plantarum*	99.93	CP028222.1
Lactiplantibacillus plantarum strain SRCM100995 chromosome, complete genome	*Lactiplantibacillus plantarum*	99.93	CP028275.1
Lactiplantibacillus plantarum strain 83-18 chromosome, complete genome	*Lactiplantibacillus plantarum*	99.93	CP046669.1
Lactiplantibacillus plantarum strain 123-17 chromosome, complete genome	*Lactiplantibacillus plantarum*	99.93	CP046656.1
Lactiplantibacillus plantarum strain TMW 1.1308 chromosome, complete genome	*Lactiplantibacillus plantarum*	99.93	CP021929.1

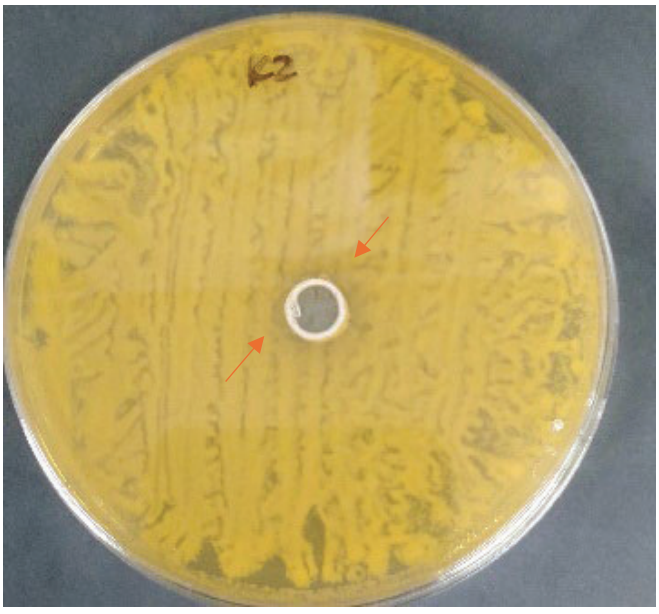


FIGURE 3: Zone of Inhibition of strain K2's CFS against *S. mutans* using agar well diffusion method

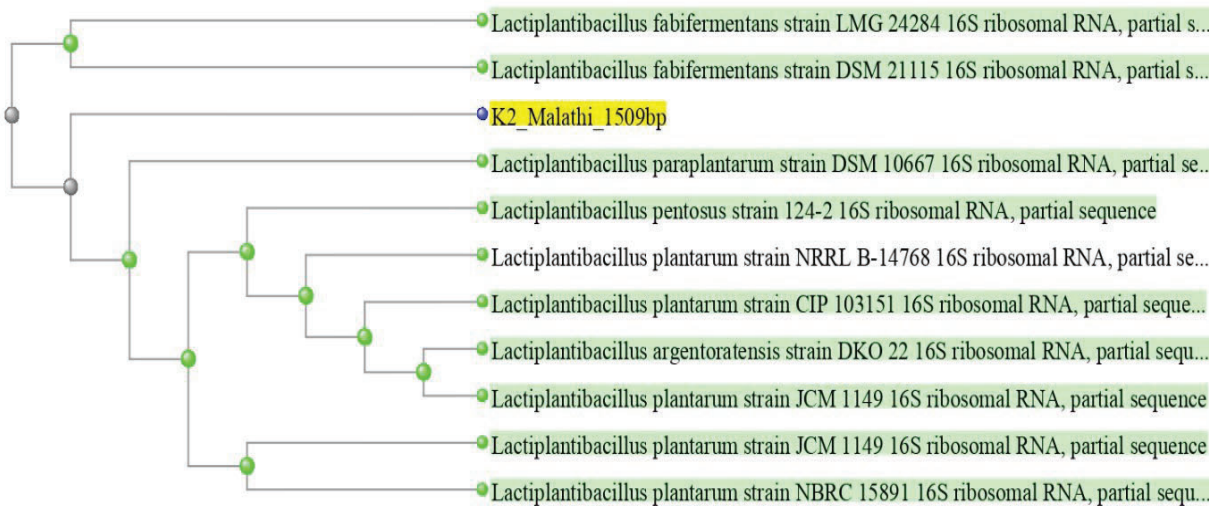


FIGURE 4: Phylogenetic tree of strain K2 group from NCBI blast database.

called *Lactobacillacecae*. This includes 31 genera, such as *Lactobacillus, Paralactobacillus, Pediococcyus, Weisella, Fructobacillus, Convivina, Oenococcus, Leucostonoc*, and 23 new genera that comprise organisms previously classified as *Lactobacillus* species. The study found that using the sequences of 16S rRNA genes was not enough to determine the phylogenetic relationships of *lactobacilli.* It was only after the genome sequences of most type strains of *lactobacilli* became available that the reclassification could be undertaken (Qiao et al. 2022; Zheng et al. 2020, 2015).

CONCLUSION

Four kimchi-derived *Lactobacillus* strains (selected from 85 isolates based on morphology and distinct sizes) exhibited varying antimicrobial activity against *S. mutans*. Strains K2 and K3 showed the strongest activity, with K2's cell-free supernatant (CFS) retaining activity against *S. mutans* at both neutral (pH 6.5) and acidic pH, suggesting the production of stable, effective antimicrobial metabolites. Given its origin in kimchi, its demonstrated efficacy, and its safety profile, *Lactiplantibacillus plantarum* strain K2 represents a promising candidate for development and commercialisation as an anti-caries agent in various oral care and food products.

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*Corresponding author; email: zalehashafiei@ukm.edu.my