INHIBITORY EFFECT OF *Lactobacillus* FROM FERMENTED FOODS AND DRINKS ON *Porphyromonas gingivalis*

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Abstract

Lactobacillus spp. are potential lactic acid bacteria in improving periodontal health. In the present study, *Lactobacillus* spp. were isolated from cheese, commercial milk, yogurt, pickle and tempeh. A total of 41 isolates were identified with biochemical tests and were used to antagonize a periodontal pathogen, *Porphyromonas gingivalis* ATCC 33277. The inhibitory study was investigated through agar well diffusion method. All isolates were able to inhibit the growth of *P. gingivalis*. One-way ANOVA shows that there was statistical significance difference among the isolates *Lactobacillus* species where the p=0.02. Isolate *L. plantarum* showed the highest inhibition (12.33±0.88 mm) whereas isolates *L. reuteri* and *L. suebicus* exhibited the least inhibitions on *P. gingivalis* (9.83±0.17 mm). All isolates were further identified using 16S rDNA gene identification. Based on the comparison of biochemical tests and 16S rDNA identification, the 41 isolates can be known as *L. rhamnosus, L. plantarum, L. paracasei*, *L. fermentum* and *L. suebicus*. In conclusion, this inhibitory study proved that the presence of *Lactobacillus* in foods and drinks may be an alternative for the prevention of oral disease.

Keywords: *Lactobacillus*, *P. gingivalis*, inhibitory effects, 16S rDNA, oral disease

Introduction

Oral subgingival plaque is a very complex ecosystem holding more than 500 bacterial species (Paster et al., 2001). *Porphyromonas gingivalis* is one of the causative agents of periodontal disease (Ximenez-Fyvie et al., 2000). It is viewed as a pathogenic bacterium that leads to the apical migration of the junctional epithelium and accelerates the collapse of the structure of periodontal ligament and the alveolar bone (Pejcic et al., 2006). Competency of host immune response is associated with the advancement of aggravation, abrogation and dormancy of this periodontitis (Schenkein, 2006). Lactic acid bacteria such as *Lactobacillus* are used extensively as a starter culture in the fermentation process. Fermentation is a traditional method to preserve foods where the starter culture is used to induce the sugars converted into alcohol and lactic acid (Chelule et al., 2010). This preservation action promotes the quality of foods in term of their flavour, texture and aroma (Balqis et al., 2013). Besides that, it produces antimicrobial metabolites that are capable of suppressing oral pathogens from causing periodontal diseases (Ahola et al., 2002). The common antimicrobial metabolites produced are organic acid (lactic, acetic and propionic acids), hydrogen peroxide, diacetyl and bacteriocin (Otero & Nader-Macias, 2006; Ito et al., 2003; Dobson et al., 2012). However, the type and amount of the metabolites produced can be affected by the conditions such as temperature, type of substrate, co-factors existence and adaptation of the culture (Laref et al., 2013). The release of organic acids lowers the level of the pH and precedes the killing of unwanted microorganism. Since the organic acids are liposoluble, the cell membrane of the pathogen can be broken off and the cytoplasm can be reached easily

(Djadouni & Kihal, 2012). Most *Lactobacillus* spp. display similar patterns from biochemical methods causing the identifications become unreliable (De Vries et al., 2006). The modern technique of 16S rDNA gene sequencing is fast with an accurate result in determining the *Lactobacillus* species (Balcázar et al., 2007). The stress of environmental conditions can alter the stability between oral health and upcoming diseases (Marsh, 2002) as the normal microbiota can protect the oral cavity from oral pathogens (Stingu et al., 2008). A previous study found that consuming products containing *Lactobacilli* depress the risk of caries and mutant *Streptococci* (Kang et al., 2011). However, the capabilities of bacteria administered in foods to fight the development of oral pathogens remain questionable. Therefore, the present study was to investigate the ability of *Lactobacillus* isolated from foods and drinks to kill the oral pathogen, *P. gingivalis.*

Materials and Methods

Isolation and Identification of *Lactobacillus* spp.

Cheese, yoghurt, fermented milk, tempeh and pickled samples were purchased from a local supermarket. All samples were homogenized in 0.1% sterile buffered peptone water (Saranya & Hemashenpagam, 2011). Diluted samples were plated on the de Man Rogosa and Sharpe (MRS) agar and were incubated at 37°C for 48 hours under anaerobic conditions (Hoque et al., 2010). Further identification of *Lactobacillus* includes the Gram reaction, catalase test, gas production from glucose, carbohydrate fermentation pattern, ammonium production from arginine, and growth at 45°C and 10°C as described in the Bergey's Manual of Systematic Bacteriology (2009).

Inhibitory Study of *Lactobacillus* spp. on *Porphyromonas gingivalis*

The isolated *Lactobacilli* were grown in MRS broth at 37°C for 16 hours. Cells were separated by centrifugation at 5000 rpm for 10 min at room temperature. *P. gingivalis* were pre-inoculated in the Brain Heart Infusion agar and 6 mm diameter wells were made after the agar had solidified. A 100 µl of *Lactobacilli* supernatants were placed into the wells. The *Lactobacillus casei* ATCC 393 and sterile MRS broth were used as the positive and negative control respectively. The assay was performed in triplicates. Inhibition zones around the wells were measured and recorded after 24 hours of incubation (Mohankumar & Murugalatha, 2011).

DNA Isolation and Polymerase Chain Reaction (PCR)

The isolated *Lactobacillus* was harvested by centrifugation at 5000 rpm for 10 min. The genomic DNA was extracted using an Easypure Bacteria Genomic DNA kit (Beijing, China) according to the manufacturer's protocol. The isolated DNA was further amplified by PCR by using genus-specific primers LbLMA1-rev (5'-CTCAAAACTAAACAAAGTTTC-3') and R16-1 (5'-CTTGTACACACCGCCCGTCA-3') designated by Dubernet et al. (2002). The PCR master mix used was 5x Firepol Master Mix Ready to Load (Solis Biodyne) containing: 5x Reaction buffer (0.4M Tris-HCl, 0.1 M $(NH_4)_2SO_4$, 0.1% w/v Tween-20), 12.5mM $MgCl_2$, 2mM deoxynucleoside triphosphates (final concentration 200 µM) and PCR primers (final concentration, 40 µM each), in a hot lead thermocycler (Eppendorf). The PCR assay was performed according to the manufacturer's protocol. The reaction mixture was subjected to an initial heating at 95°C for 13 min. The temperature was cycled through 94°C for 15 min, 51°C for 1 min, and then a final extension at 72°C for 2 min. The cycle was repeated 30 times. PCR products were detected by 2% (w/v) agarose gel electrophoresis in TBE buffer stained with gel stain. A 100 bp DNA ladder was used to identify the molecular sizes of the

bands.

16S rDNA Sequencing
All PCR products were sent to the First Base Laboratories Sdn Bhd for sequencing. The sequence was compared with the NCBI database using the BLAST algorithm.

Analysis of Data
All quantitative data were expressed by their mean ± SEM (standard error of the mean). The results were also analysed by IBM SPSS Statistics software (version 21, 2012). Analysis of variance (ANOVA) was used to determine the significant differences between these groups. A *p*-value of $p \leq 0.05$ was considered to be statistically significant.

Result and Discussion

A total of 41 *Lactobacillus* colonies from all the samples were isolated. The characteristics of *Lactobacillus* colonies were whitish color, small diameter, entire margins, convex and smooth surface. The *Lactobacillus* colonies were identified based on the morphological and biochemical methods. From the Gram staining, all isolates showed positive reactions with long bacillus and short coccobacillary shape. All isolates were also found to be catalase-negative, and unable to produce ammonia from arginine. The biochemical results are shown in Table 1. Silvia & Medana (2011) stated that the *Lactobacillus* isolated from fermented Romanian vegetables were bacilli, cocci, coccibacillary and diplococci in shape. Some of the *Lactobacillus* from milk products were found to be from long to very short rods in chains (Priya et al., 2011). Besides that, the isolates which were cocci in morphology were able to grow at 15°C but unable to withstand temperature at 45°C (Parvathy & Puthuvallil, 2005). However, some strains such as the thermophilic microorganism grow well at 45°C but were unable to grow at 10°C (Zarnea, 1984). *L. casei* and *L. bulgaricus* from cheese and yoghurt are Gram positive, catalase-negative and producing no gas from glucose (Ozlem and Feryal, 2006).

The organic acids produced by sugars are always associated with the caries process but *Lactobacilli* are able to act in balancing the microecology of oral cavity (Simark-Mattsson et al., 2007). In a complex community of cells, the metabolic activity between the tested isolates showed massive differences (Hedberg et al., 2008). In addition, the sugar fermentation allows a preliminary identification of *Lactobacilli* at species level. About 63% of the isolates were able to ferment all ten types of sugars while 27% of the isolates were unable to ferment arabinose and 10% of the isolates were unable to ferment trehalose only. Based on the patterns, they can be identified as *Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus paracasei, Lactobacillus reuteri* and *Lactobacillus suebicus* (Table 2).

In vitro assay was carried out to determine the antagonistic potential of *Lactobacilli* on *Porphyromonas gingivalis*. The antagonistic effects of isolates were compared among the probable species. Although all isolates showed inhibitions on *P. gingivalis*, one-way ANOVA indicates a statistical significance difference among the *Lactobacillus* isolates where the p=0.02. *L. plantarum* C6 showed the highest inhibition (12.33$\pm$0.88 mm). On the other hand, the isolates *L. rhamnosus* Y5 and *L. paracasei* M11 exhibited the inhibition zones with the diameters were 11.67$\pm$0.33 and 11.00$\pm$0.58 respectively. *L. reuteri* T2 and *L. suebicus* T9 were the least effective in combating the growth of *P. gingivalis* where the diameter of inhibition zone was 9.83$\pm$0.17 mm.

Table 1 Biochemical characterization of *Lactobacillus* isolated from the tested samples

Isolate	Gram Stain	Catalase Test	CO2 from Glucose	Arginine Hydrolysis	Growth at 45°C	Growth at 10°C
C1,C3,Y1, Y2, Y3, Y4,Y5, P1, P2, P3, P4, P5, P6, P7, P8, P9	G +ve	-	-	-	+	+
C2, C4, C5, C6, C7	G +ve	-	-	-	-	+
M1, M2, M3, M4, M5, M6, M7, M8, M9, M10, M11	G +ve	-	-	-	+	+
T1, T4, T7, T8	G +ve	-	+	-	+	-
T2, T3, T5, T6, T9	G +ve	-	+	-	+	+

+ indicates positive reaction, - indicates negative reaction

Table 2 Sugar Fermentation Patterns of *Lactobacillus* isolated from the tested samples

Isolate	Ar	Fru	Gal	Glu	Lac	Mal	Mn	Sor	Suc	T	Probable Identity
C1, C3, Y1, Y2, Y3, Y4, Y5, P1, P2, P3, P4, P5, P6, P7, P8, P9	+	+	+	+	+	+	+	+	+	+	*L. rhamnosus*
C2, C4, C5, C6, C7	+	+	+	+	+	+	+	+	+	+	*L. plantarum*
M1, M2, M3, M4, M5, M6, M7, M8, M9, M10, M11	-	+	+	+	+	+	+	+	+	+	*L. paracasei*
T1, T4, T7, T8	+	+	+	+	+	+	+	+	+	-	*L. reuteri*
T2, T3, T5, T6, T9	+	+	+	+	+	+	+	+	+	+	*L. suebicus*

Ar= Arabinose; Fruc= Fructose; Gal= Galactose; Glu= Glucose; Lac= Lactose; Mal= Maltose; Mn= Mannitol; Sor= Sorbitol; Suc= Sucrose; T= Trehalose; (+)= able to ferment; (-)= not able to ferment

Our findings were similar to the research by Azizah et al. (2013) where the *Lactobacillus* spp. inhibited periodontal pathogen with small zones of inhibition (10.67 mm $\pm$ 3.4 mm). Chen et al. (2012) also found that *L. fermentum* exhibited a moderate inhibitory effect against *P. gingivalis* ATCC 33277 where the inhibition zone was 14.00 $\pm$ 1.0 mm. However, a study by Rawee et al. (2011) found that *L. paracasei* SD1*, L. casei* SD2*, L. plantarum* SD4 and *L. rhamnosus* SD5 displayed strong inhibitions on *P. gingivalis* ATCC 33277 (23.00 mm-30.00 mm). *Lactobacilli* spp. produced organic acid such as acetic acid and lactic acid which are involved in the inhibitory activity of probiotic bacteria on pathogens (Zhang et al., 2011). Besides that, *Lactobacilli* sometimes produced hydrogen peroxide, diacetyl, bacteriocins, and antimicrobial peptides (Cortes et al., 2014).

Table 3 The growth inhibition of selected isolates of *Lactobacillus* against *P. gingivalis*

Isolate	Mean diameter of inhibition zone (mm)
L. reuteri T8	9.83±0.17[a]
L. suebicus T2	9.83±0.17[a]
L. paracasei M11	11.00±0.58[ab]
L. rhamnosus Y5	11.67±0.33[ab]
L. plantarum C6	12.33±0.88[b]
L. casei ATCC 393 (Positive control)	10.83±0.44

Note: Including 6 mm of well

[a, b] Means with the same letter are not significantly different

From the DNA extraction, the average size of DNA was ~20kb (Figure 1-5). However, some of the DNA were found to be intact and in an undegraded condition and some were found to be in degraded conditions. A study by Sachinandan et al. (2011) reported that the DNA of *Lactobacilli* isolated from curd have high molecular weight where the sizes were ~20kb in all the samples. The degradation of DNA might be due to the incomplete lysis of the *Lactobacillus* cell wall. This is because of the high level of peptidoglycan in the *Lactobacillus* cell wall (Braun & Hantke, 1974). The covalent cross link in the peptidoglycan can be disrupted by using enzymes such as lysozyme, mutanolysin and labiase (Sachinandan et al., 2011). Lysozyme is one of the best enzymes to cleave the peptidoglycan (Touch et al., 2003) but the use of lysozyme alone usually will yield a low quality of DNA (Sachinandan et al., 2011). Due to this, Moore et al. (2004) suggested the lysozyme was used in combination with chemicals such as EDTA to effectively break the peptidoglycan.

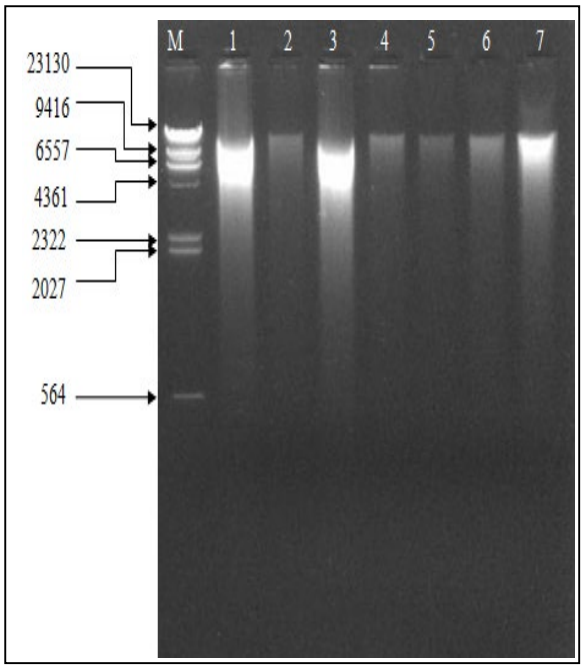

Figure 1 Agarose electrophoresis pattern of isolated *Lactobacillus* DNA on 1% agarose gel. Lanes 1-7: *Lactobacillus* DNA isolated from cheese sample; Lane M, *Hin*dIII digested Lambda DNA fragments

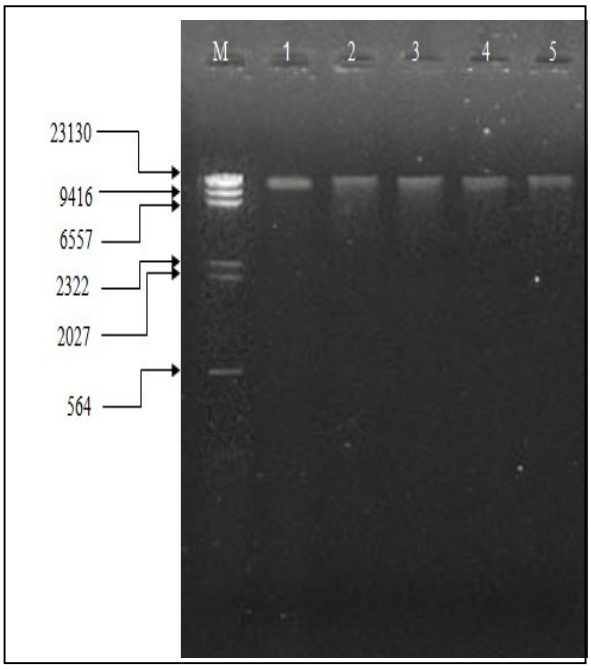

Figure 2 Agarose electrophoresis pattern of isolated *Lactobacillus* DNA on 1% agarose gel. Lanes 1-5: *Lactobacillus* DNA isolated from yoghurt sample; Lane M, *Hin*dIII digested Lambda DNA fragments

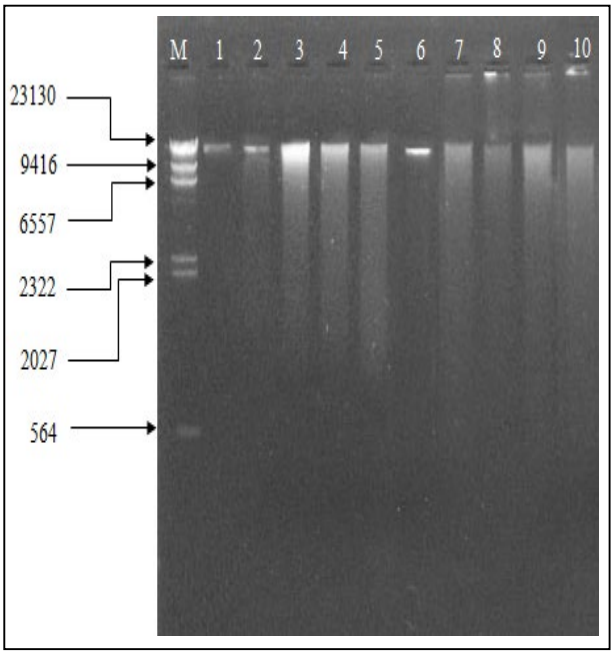

Figure 3 Agarose electrophoresis pattern of isolated *Lactobacillus* DNA on 1% agarose gel. Lane 1: *Lactobacillus casei* ATCC 393; Lanes 2-10: *Lactobacillus* DNA isolated from pickle sample; Lane M, *Hin*dIII digested Lambda DNA fragments

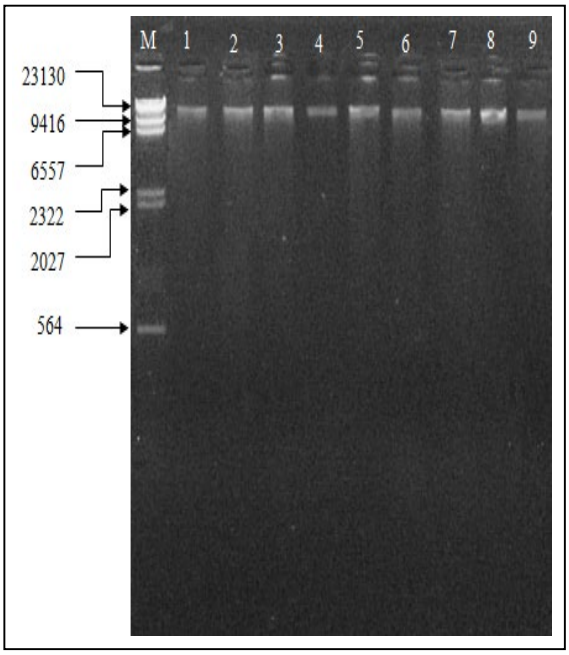

Figure 4 Agarose electrophoresis pattern of isolated *Lactobacillus* DNA on 1% agarose gel. Lanes 1-9: *Lactobacillus* DNA isolated from tempeh sample; Lane M, *Hin*dIII digested Lambda DNA fragments

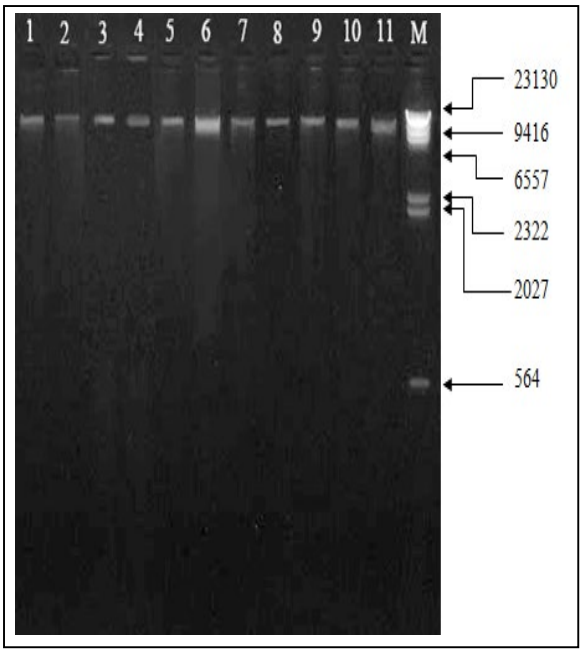

Figure 5 Agarose Electrophoresis pattern of isolated *Lactobacillus* DNA on 1% agarose gel. Lanes 1-11: *Lactobacillus* DNA isolated from fermented milk sample; Lane M, *Hin*dIII digested Lambda DNA fragments

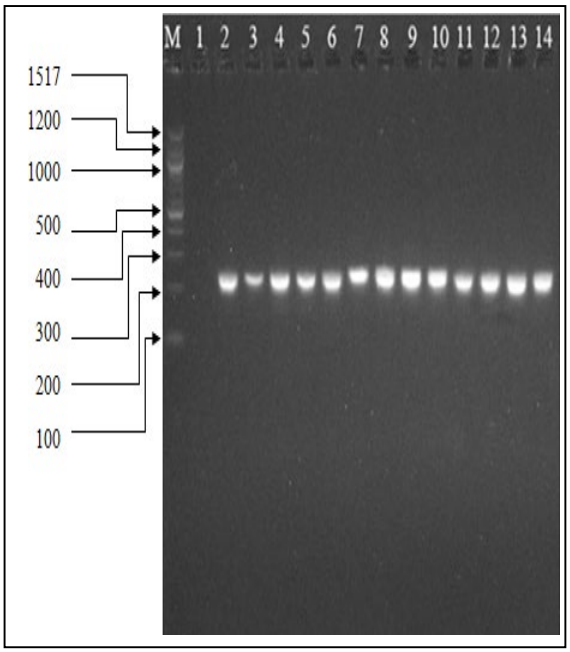

Figure 6 Example of agarose electrophoresis pattern on 2% agarose gel. Lane 1: Negative control; Lanes 2-13: *Lactobacillus* isolates' PCR products; Lane 14: *Lactobacillus casei* ATCC 393; Lane M, 100 bp ladder.

The sequencing results exhibited a significant similarity with the 16S rDNA gene sequences of known *Lactobacillus* species (Table 4). Most isolates showed high similarity (more than 97%) except T2, T3, T5, T6, T7 and T9 that showed 88-94% of the maximum identity. From the Genbank comparison, only 37 isolates showed a significant similarity of species as identified by biochemical works. Isolates T1, T4, T7, and T8 had been misidentified. The isolates T1, T4, T7 and T8 displayed 94-98% similarity with *L. fermentum*. *L. fermentum* is occasionally mistaken as *L. reuteri*. For example, *L. fermentum* NCDC77 (S8) was first identified as *L. reuteri* but later 16S rRNA sequencing confirmed it as *L. fermentum* (Kamna and Sudhir, 2017).

From the result, several constraints have been observed between the phenotypic test identification and molecular tool identification. According to Bulut *et al*. (2005), the identification of lactic acid bacteria (LAB) by phenotypic methods such as sugar fermentation may be ambiguous and problematic due to the increase of characters variability among the species. Besides that, phenotypic methods may also be unstable owing to the cultural conditions such as the expression of genes which may not show or loss of some characteristics during culturing (Mohania et al., 2008). In contrast, 16S rDNA sequencing method is more advantageous as the sequence can be carried out not only on the bacterial culture but also on the sample so as to study the diversity of the organisms without culturing (Gill et al., 2008).

Table 4 Comparison of *Lactobacillus* strains identified by biochemical tests and 16S rDNA gene identification

Isolate	Biochemical Test Identification	16S rDNA Gene Identification (% identity)	Accession Number
C1, C3, Y1, Y2, Y3, Y4, Y5, P1,P2, P3, P4, P5,P6, P7, P8, P9	*L. rhamnosus*	*L. rhamnosus* GG	NC_013198.1
C2, C4, C5,C6,C7	*L. plantarum*	*L. plantarum* WCFS1	NC_004567.2
M1, M2, M3, M4, M5, M6, M7, M8, M9, M10, M11	*L. paracasei*	*L. paracasei* subsp. *paracasei* JCM 8130	NZ_AP012541 .1
T1, T4, T7, T8	*L. reuteri*	*L. fermentum* IFO 3956	NC_010610.1
T2, T3, T5, T6, T9	*L. suebicus*	*L. suebicus* DSM 5007	NZ_AZGF010 00056.1

Conclusion

From the comparison of biochemical tests and 16S rDNA gene identification, the isolated *Lactobacillus* were *L. plantarum, L. rhamnosus, L. paracasei, L. fermentum* and *L. suebicus.* The *Lactobacilli* were investigated for their inhibitory activity. The result showed that all isolated *Lactobacillus* able to inhibit the periodontal pathogen, *P. gingivalis*. This study also proves that *Lactobacillus plantarum* C6 has the highest inhibition zones among the isolates. It is hoped that a details investigation will be conducted in the future about the structure, adhesion and aggregation abilities of *Lactobacillus plantarum* C6 on the oral mucosa.

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Conflict of Interest

The authors declare no conflict of interest

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