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Isolation of *Lactobacillus* from Periodontally Healthy Subjects and its Antimicrobial Activity against Periodontal Pathogens

(Pemencilan *Lactobacillus* pada Subjek Gusi Sihat dan Aktiviti Antimikrobnya Terhadap Patogen Gusi)

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ABSTRACT

Bacteriocin or Bacteriocin like inhibitory substances (BLIS) is a protein antibiotic that has a relatively narrow spectrum of killing activity. It could potentially serve as a natural alternative to antibiotics in reducing the development of multi-drug resistant bacteria. Antimicrobial activity of the strains of Lactobacillus sp. isolated from healthy subjects (test strains) against Aggregatibacter actinomycetemcomitans and other periodontal pathogens (indicator strains) isolated from subgingival plaques of aggressive periodontitis patients were determined by using deferred antagonism test and agar-well diffusion method. Strains of Lactobacillus sp., Aggregatibacter actinomycetemcomitans and black pigmented bacteria were selectively isolated from TJA, TSBV and TSBA agars, respectively. Mean diameter zone of inhibition of at least 10 mm was considered as positive results for both methods. Out of 25 strains of Lactobacillus sp. screened, only eight test strains of Lactobacillus sp. showed the specific antimicrobial activity against certain strains of indicator periodontal pathogens during deferred antagonism test. However, out of eight potential strains, only three strains, which were Lactobacillus sp. strain S, Lactobacillus sp. strain V and Lactobacillus sp. strain W consistently showed positive inhibitory activity against black pigmented bacteria by deferred antagonism test and agar-well diffusion method. Therefore, these three strains should be considered as potential BLIS producer strains for further study.

Keywords: Antimicrobial activity; Lactobacillus sp.; periodontally healthy subjects; periodontal pathogens

ABSTRAK

Bakteriosin atau bahan perencat seakan bakteriosin (BLIS) merupakan protein antibiotik yang mempunyai medan aktiviti pembunuhan yang sempit. Ia berpotensi sebagai produk alternatif antibiotik semula jadi dalam mengurangkan peningkatan bakteria yang rentan terhadap pelbagai dadah. Aktiviti antimikrob strain Lactobacillus sp. yang dipencilkan daripada subjek sihat (strain ujian) terhadap strain Aggregatibacter actinomycetemcomitans dan patogen gusi yang lain (strain penentu) yang dipencilkan daripada plak subgingiva pesakit periodontitis agresif ditentukan dengan menggunakan kaedah ujian penentangan tertangguh dan resapan telaga-agar. Strain Lactobacillus sp., Aggregatibacter actinomycetemcomitans dan bakteria pigmen hitam telah dipencilkan secara terpilih pada agar TJA, TSBV dan TSBA. Purata diameter zon perencatan yang lebih atau sama dengan 10 mm merupakan hasil positif bagi kedua-dua kaedah. Sebanyak 25 strain Lactobacillus sp. telah dikaji, namun hanya lapan strain ujian Lactobacillus sp. telah menunjukkan aktiviti antimikrob yang spesifik terhadap strain patogen gusi tertentu semasa ujian penentangan tertangguh. Walau bagaimanapun, daripada lapan strain yang berpotensi tersebut, hanya tiga strain iaitu Lactobacillus sp. strain S, Lactobacillus sp. strain V and Lactobacillus sp. strain W secara tetap telah menunjukkan aktiviti perencatan yang positif terhadap bakteria pigmen hitam menggunakan kaedah ujian penentangan tertangguh dan resapan telaga-agar. Oleh yang demikian, ketiga-tiga strain tersebut seharusnya dianggap berpotensi sebagai penghasil BLIS untuk kajian lanjut.

Kata kunci: Aktiviti antimikrob; Lactobacillus sp.; patogen gusi; subjek dengan gusi sihat

INTRODUCTION

In Malaysia, as in many other developing nations, periodontal disease is a problem of worrying magnitude (Dental Services Division 1993). In an epidemiological survey of 9,047 adults in Malaysia reported in 1977, 72% had periodontal disease ranging from mild inflammation, intense gingivitis and destructive periodontal disease (Dental Division 1978). Nowadays, increasing evidence

for early onset periodontitis showed that the younger population also has an increase risk of developing periodontitis (Oliver et al. 1998; Taiyeb Ali & Razak 2000). About 4.8% of patients who were referred to Periodontal Department, University of Malaya had early onset periodontitis and 1.9% among these patients were diagnosed with aggressive periodontitis (Taiyeb Ali & Razak 2000). A study done in Nigeria showed the most

frequently lost teeth in periodontal diseased patients were incisors (Dosumu et al. 2003).

The responsible flora in aggressive periodontitis is polymorphic, Gram-negative and microaerophilic or strictly anaerobic bacteria. About 10-20 species were suggested to play a role in the pathogenesis of periodontal destruction (Darby 2001). *Aggregatibacter actinomycetemcomitans* (formally *Actinobacillus actinomycetemcomitans*) has been frequently related to localized juvenile periodontitis (Wilson & Henderson 1995; Zambon 1985). The microbiology of generalized juvenile periodontitis is more complex and is associated with *Porphyromonas gingivalis* (10-15%) and other Gram-negative bacilli (*Eikenella corrodens*, *Capnocytophaga* sp., *Aggregatibacter actinomycetemcomitans*) (Fine et al. 1999). Due to the fact that aggressive periodontitis caused by bacteria such as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and other Gram-negative bacilli, it generally affecting young adults, early elimination of these bacteria will prevent progression of the disease.

Probiotics has been found to be beneficial to the host health which is primarily used for the management of intestinal tract problem. In recent years, probiotics has been used as a treatment to promote oral health. The organisms that were used as probiotics are primarily certain species of Lactobacilli, *Bifidobacteria*, *Saccharomyces* sp. but some Streptococci, Enterococci and commensal *Escherichia coli* which have been claimed to have beneficial effects in certain situations (Caglar et al. 2005; Reid et al. 2003).

The modes of action of probiotics function by preventing adhesion of pathogens to host tissue, stimulation and modulation of the mucosal immune system, modulation of cell proliferation on apoptosis, improvement of intestinal barrier integrity and killing or inhibiting the growth of pathogens through bacteriocins production or other products (peroxide) which are antagonistic towards pathogenic bacteria (Geier et al. 2007; Picard et al. 2005). Bacteriocins are peptide or proteinaceous antimicrobials produced by bacteria capable of inhibiting the growth or killing other bacteria without affecting itself (Tagg et al. 1976). The term BLIS has been adopted and used for bacterial products that have bacteriocin-like inhibitory effects although they may not be completely characterized (Walls et al. 2003).

Increasing use of probiotic bacteria (e.g. *Lactobacillus* and *Bifidobacteria*) to improve gastrointestinal health has prompted interest in the utility of this approach for oral applications. Moreover, the rapid rise of multiresistant bacteria pathogens results in continuous effort to identify alternative methods of combating infection. BLIS could potentially serve as a natural alternative to antibiotics. Unlike classical antibiotics, bacteriocin has a relatively narrow spectrum of killing activity, resulting in a reduction in the intensity of selection for resistance (Bowe et al. 2006). Periodontal disease can be reduced by investigating the potential of BLIS-producing *Lactobacilli* that have the capability to inhibit *Aggregatibacter actinomycetemcomitans* activity and other periodontal

pathogens. Therefore, the aim of this study was to determine the antimicrobial activity of *Lactobacillus* sp. isolated from healthy subjects against *Aggregatibacter actinomycetemcomitans* and other periodontal pathogens isolated from aggressive periodontitis patients.

MATERIALS AND METHODS

CLINICAL EXAMINATION OF THE SUBJECTS

Seven subjects with aggressive periodontitis and 10 volunteers with periodontal healthy between 18 and 35 years of age were recruited. This study was approved by the Research Ethic Committee of Universiti Kebangsaan Malaysia Medical Centre and written consent was obtained from the subjects before obtaining saliva and subgingival plaques.

The periodontally healthy subjects were defined as having at least 24 natural teeth with less than 20% of sites with bleeding on probing (BOP) and plaque score and no probing pocket depth (PD) and clinical attachment level (CAL) of more than 3 mL. The aggressive periodontitis subjects were diagnosed as having at least 20 natural teeth with less than 20% of sites with BOP and plaque score and minimum of 6 teeth with an interproximal site with PD more than 4 mL and CAL between 5 and 10 mL. Both groups did not have any history of systemic disease or medical conditions that would require antibiotic prophylaxis for routine dental procedures. Exclusion criteria include pregnant women, smoking subjects and individuals who had taken antibiotics in the previous 3 months.

ISOLATION OF TEST AND INDICATOR STRAINS

The test strains (*Lactobacillus* sp.) were isolated from the saliva of healthy subjects who chewed a piece of paraffin film for 2 min to stimulate saliva secretion. The indicator strains were isolated from subgingival plaques of patients with aggressive periodontitis. Subgingival plaque samples were obtained from two deepest periodontal pockets of upper incisors where isolation was easily achieved. Isolation of a tooth was done by using sterile cotton rolls and the supragingival region of the tooth surface to be sampled were cleaned and dried with cotton pellets. Subgingival plaque sample from the deepest pocket of a subject was obtained by using fine sterile paper point which was inserted in a periodontal pocket for 60 s. Then, the paper point was transferred into a test tube containing 2 mL of sterilized phosphate buffered saline solution (pH 7.3). The next paper point that was inserted into the second site of the deepest pocket was transferred into a test tube containing 2 mL of 0.9% sodium chloride (Sigma, USA) (pH 7.3). The stimulated saliva and both of the subgingival plaque samples were processed within 2 h of sample collection.

All samples collected were vortex mixed for one min. A 10-fold from 10^{-1} to 10^{-5} dilution of each sample was made. About 0.1 mL of diluted stimulated saliva was

plated on a tomato juice agar (pH 5.0) (TJA) (Sigma, USA), a selective medium for *Lactobacillus*. The triplicate of the tomato juice agar plates were incubated aerobically at 37°C for 72 to 96 h (Charlton & Spies 1956). Ten-fold serially diluted subgingival plaque samples (0.1 mL) from aggressive periodontitis patient was plated on tryptic soy bacitracin vancomycin (TSBV) (Oxoid, USA) and tryptic soy blood agar (TSBA) (Oxoid, USA) to selectively isolate *Aggregatibacter actinomycetemcomitans* (Slots 1982) and black pigmented colonies, respectively. The distinct black colour colonies formed on the surface of TSBA were known as black pigmented bacteria. The plating was done in triplicates and all agar plates were incubated anaerobically at 37°C for 48 to 72 h. Different patterns of colonies on selective media were characterized morphologically according to configuration, margin, elevation, diameter and colour. Different colonies of *Lactobacillus* sp. were selected as test strains. Different colonies of *Aggregatibacter actinomycetemcomitans* and black pigmented bacteria were selected as indicator strains for deferred antagonism test and agar-well diffusion methods. The potential strains of BLIS-producing *Lactobacillus* and the indicator strains were identified by Gram staining.

IN VITRO ANTIMICROBIAL ACTIVITY TESTING

Antimicrobial activity of *Lactobacillus* against *Aggregatibacter actinomycetemcomitans* and black pigmented bacteria were assessed using deferred antagonism test and agar-well diffusion methods. Deferred antagonism test (Balakrishnan et al. 2001; Modified Tagg & Bannister 1979) was performed on TSYCa media prepared by a mixture of tryptic soy broth (Becton Dickinson, USA) with 1.5% bacteriological agar (Difco Laboratories, USA), 2% yeast extract (Difco Laboratories, USA) and 0.25% CaCO₃ (Difco Laboratories, USA). Briefly, a standardized 0.5 McFarland of the test strain culture was inoculated in a 1 cm wide diametric streak across the surface of TSYCa agar using a sterile cotton swab. Then, the plate agar containing the test strain was incubated at 37°C for 24 h. Macroscopically visible growth was removed by scraping with the edge of a glass slide. Then, the plate was inverted over a circle of chloroform-soaked filter paper in the lid of a petri dish. After 30 min, the plate was removed from the lid and exposed to the air for 15 min. After that, a standardized 0.5 McFarland of overnight (18 h, 37°C) purified culture of indicator strain was streaked at right angle to the line of original producer growth with a cotton swab. The same procedure was done in triplicates and all the TSYCa agar plates with the test and indicator strains were incubated at 37°C for 24 h. The reduced growth of indicator strain in the vicinity of the area originally occupied by the growth of producer strain provided evidence of bacteriocin like inhibitory substance production (Tagg & Bannister 1979). The inhibition zone width of the indicator strain with at least 10 mm was considered significantly positive and was further investigated by agar-well diffusion method.

For the agar-well diffusion method, 0.1 mL of standardized 0.5 McFarland individual indicator strain was inoculated and incubated at 37°C for 24 h onto a brain heart infusion (BHI) (Oxoid, USA) agar. Then, four wells of 7 mm diameter were filled with 100 µL of culture supernatant. The plates were incubated at 37°C for 24 h and zones of inhibition were measured in mm (Gaurav et al. 2010). The culture supernatant was prepared by propagating purified test strain in Todd Hewitt broth and incubated at 37°C for 48 h. The cells were separated by centrifuging at 5,000 rpm for 10 min. Cell free supernatant was passed through 0.22 µm membrane filter and evaluated for antimicrobial activity (Aslim et al. 2005). The experiment was done in triplicate and the inhibitory activity of each test strain against each clinical sample of periodontal pathogens was measured and reported as mean diameter (mm) ± standard deviation (SD). The inhibitory activity of test strain was considered significantly positive if the zone inhibition produced by the test strain against the indicator strain (periodontal pathogen) was at least 10 mm (Balakrishnan et al. 2001).

RESULTS AND DISCUSSION

A total of 25 test strains and 21 indicator strains were isolated from 10 selected periodontal healthy and seven aggressive periodontitis subjects, respectively. The distinct strains were selected based on different culture morphologies, sizes and colours. The test strains (*Lactobacillus* sp.) as well as the indicator strains of *Aggregatibacter actinomycetemcomitans* and black pigmented bacteria were obtained from serial dilution suspensions which were grown on selective medium of TJA, TSBV and TSBA, respectively. *Aggregatibacter actinomycetemcomitans* and black pigmented bacteria were chosen as indicator strains as they were generally accepted to be the principal etiological agent of aggressive periodontitis. All of 25 selected test strains (*Lactobacillus* sp.) obtained from TJA were tested for the BLIS activity against *Aggregatibacter actinomycetemcomitans* and black pigmented bacteria. The results obtained from deferred antagonism test showed that out of 25 *Lactobacillus* sp. strains tested, only eight test strains showed positive BLIS activities against six indicator strains. The other 17 test strains did not show any inhibitory effect against any of the periodontal pathogens.

Overall, the results showed that the eight isolated test strains of *Lactobacillus* sp. showed specific antimicrobial activity against certain indicator strains. *Lactobacillus* strain L, N and V only inhibited black pigmented strain 2C with mean diameter inhibitory zones (mm ± SD) of 11.67 ± 0.58 mm, 14.33 ± 4.04 mm and 15.33 ± 4.41 mm, respectively. *Lactobacillus* sp. strain S was capable of inhibiting the growth of black pigmented strain 2A and 2B with mean diameter zone of inhibition (mm ± SD) of 16.67 ± 1.53 mm and 10.67 ± 3.46 mm, respectively. Moreover, *Lactobacillus* sp. strain H, P and X only inhibited the specific strains of *Aggregatibacter actinomycetemcomitans*

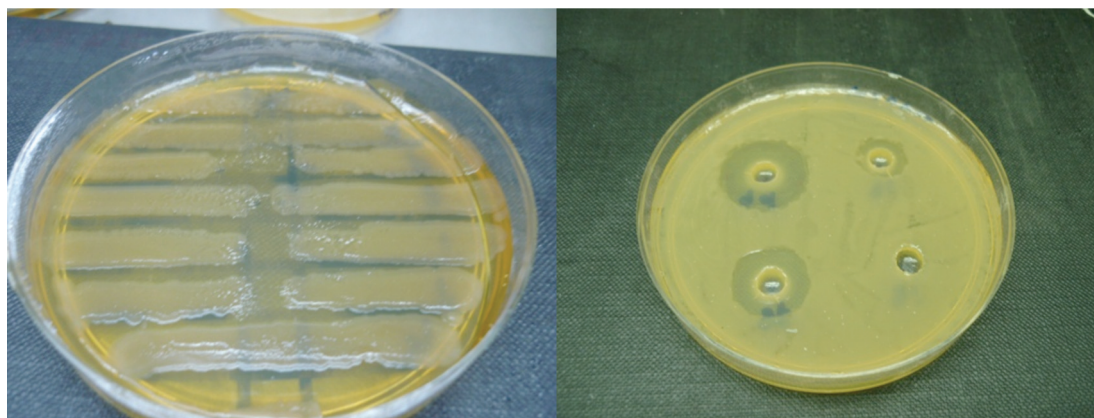
but not black-pigmented strains with mean diameter inhibitory zones (mm \pm SD) of 10.00 ± 2.00 mm (strain 1B), 10.00 ± 2.00 mm (strain 1C) and 12.67 ± 3.21 mm (strain 1A), respectively. These results indicated that only certain strains of *Lactobacilli* from oral cavity have potential to inhibit strains of indicator periodontal pathogens (Table 1, and Figure 1(a)). However, *Lactobacillus* sp. strain W had the capability to inhibit both indicator strains with the mean diameter clear inhibitory zones (mm \pm SD) were significantly positive for *Aggregatibacter actinomycetemcomitans* strain 1A (10.67 ± 0.28 mm) and black-pigmented strain 2A (12.67 ± 1.53 mm) (Table 1). This variation was due to these pathogens (*Aggregatibacter actinomycetemcomitans* and black pigmented bacteria) having the ability to produce its own BLIS to maintain their growth in oral cavity.

Apart from the deferred antagonism test, there were several other methods which can also be used to

determine the production of BLIS. These methods include agar well diffusion method (Gaurav et al. 2010) and modification of the overlay technique (Hillman et al. 1984). In the present study, the eight potential test strains of *Lactobacillus* sp. from deferred antagonism test were further investigated using well diffusion method. Results obtained showed that only three strains inhibited growth of periodontal pathogens. The three potential test strains were *Lactobacillus* sp. strain S, *Lactobacillus* sp. strain V and *Lactobacillus* sp. strain W. Both *Lactobacillus* sp. strain V and *Lactobacillus* sp. strain W were capable of inhibiting indicator black pigmented strain 2A with mean diameter (mm \pm SD) of inhibitory zones of 10.33 ± 0.57 mm and 10.67 ± 2.08 mm, respectively. The mean diameter (mm \pm SD) of inhibitory zone produced by *Lactobacillus* sp. strain V against indicator black pigmented strain 2C was 10.33 ± 1.53 mm (Table 2, Figure 1(b)). The inhibition zones of at least 10 mm

TABLE 1. Mean diameter (mm) \pm standard deviation (SD) zone of inhibition for strains of *Lactobacillus* sp. against periodontal pathogen strains by deferred antagonism test

<i>Lactobacillus</i> sp.	<i>Aggregatibacter actinomycetemcomitans</i>			Black pigmented bacteria		
	1A	1B	1C	2A	2B	2C
H	0	10 \pm 2	0	0	0	0
L	0	0	0	0	0	11.67 \pm 0.58
N	0	0	0	0	0	14.33 \pm 4.04
P	0	0	10 \pm 2	0	0	0
S	0	0	0	16.67 \pm 1.53	10.67 \pm 3.46	0
V	0	1.67 \pm 2.89	9.33 \pm 1.53	7 \pm 3.61	6.33 \pm 4.04	15.33 \pm 4.51
W	10.67 \pm 2.08	0	0	12.67 \pm 1.53	2.65 \pm 0.58	4 \pm 3
X	12.67 \pm 3.21	0	0	0	0	0



(a)

(b)

FIGURE 1. Deferred antagonism test (a) and agar-well diffusion method (b) demonstrating inhibitory activity of *Lactobacillus* sp. against indicator strains

in diameter were significantly positive as suggested by Balakrishnan et al. (2001). BLIS-producing *Lactobacillus* (strain S, V and W) that produced inhibitory activity in both methods represented white to yellowish colonies with smooth margin (Figure 2). The same strain that gave positive results on deferred antagonism test also showed clear inhibitory zones on agar-well method with less than 10 mm and was considered as negative. The present findings showed that the deferred antagonism test was more effective as *Lactobacilli* strains possessed antibacterial activity against more periodontal pathogens, while less active in agar-well diffusion method. These could be attributed to differences in the diffusion rate of bacteriocin in agar medium for both methods (Ahmad et al. 2004). BLIS-producing *Lactobacilli* (strain S, V and W) had the most potential as BLIS producers as both methods showed positive results. TSYCa agar has been used for over 20 years for the detection of BLIS production by streptococci. The BLIS protein secreted by streptococci was relatively small molecules which can easily diffuse through agar (Walls et al. 2003). Thus, *Lactobacillus* sp. strain S, *Lactobacillus* sp. strain V and *Lactobacillus* sp.

strain W were BLIS producers that may be important in periodontal disease prevention.

These potential test strains that showed clear zones of inhibition of at least 10 mm in both methods were further identified with Gram staining. The potential BLIS-producing *Lactobacilli* were identified as Gram-positive coccobacilli and both potential indicator black pigmented strains 2A and 2C were identified as Gram-negative coccobacilli. However, further identification of these three potential test strains and the associate potential indicator strains (black pigmented bacteria strain 2A and 2C) were required for confirmation by using commercial biochemical test kit system API 20A.

CONCLUSION

This study showed that bacteria isolated from the saliva of healthy individuals have potential in production of BLIS. These BLIS-producing bacteria (*Lactobacillus* sp. strain S, *Lactobacillus* sp. strain V and *Lactobacillus* sp. strain W) can be used as a natural alternative antibiotic in combating periodontal pathogens such as black pigmented bacteria.

TABLE 2. Mean diameter (mm) \pm standard deviation (SD) zone of inhibition for strains of *Lactobacillus* sp. against strains of periodontal pathogen by agar-well diffusion method

<i>Lactobacillus</i> sp.	<i>Aggregatibacter actinomycetemcomitans</i>			Black pigmented bacteria		
	1A	1B	1C	2A	2B	2C
H	0	2 \pm 2.65	0	0	0	0
L	0	0	0	0	0	1.33 \pm 1.15
N	0	0	0	0	0	1.33 \pm 1.53
P	0	0	0.67 \pm 1.15	0	0	0
S	0	0	0	10.33 \pm 0.58	1 \pm 1	0
V	0	0	0	0	0	10.33 \pm 1.53
W	2 \pm 1.73	0	0	10.67 \pm 2.08	0	4 \pm 3
X	3 \pm 2	0	0	0	0	0

Mean diameter inhibition zone (mm): 10-20 mm (positive); <10 mm (negative); 0 (no inhibition)



FIGURE 2. Culture morphology of the three strains of BLIS-producing *Lactobacillus* sp.

Therefore, the purified bacteriocin produced by these strains can be used as an anti-periodontal disease agent and has the potential to be incorporated to mouthwash, toothpaste or in lozenges. Replacement therapy using attenuated potential strains also can be used as another alternative in periodontal disease prevention.

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