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Soy fermentation by indigenous oral probiotic *Streptococcus* spp. and its antimicrobial activity against oral pathogens

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Abstract

The present work aimed to determine the antagonistic effect of probiotic-fermented soy against oral pathogens. Indigenous oral probiotics (*Streptococcus salivarius* Taylor's University Collection Centre (TUCC) 1251, *S. salivarius* TUCC 1253, *S. salivarius* TUCC 1254, *S. salivarius* TUCC 1255, and *S. orisratti* TUCC 1253) were incorporated into soy fermentation at 37°C for 24 h. Growth characteristics, β-glucosidase activity, and total isoflavones content of *Streptococcus* strains following soy fermentation were analysed. Antimicrobial test of *Streptococcus*-fermented soy was carried out against oral pathogens *Enterococcus faecalis* American Type Culture Collection (ATCC) 700802, *Streptococcus pyogenes* ATCC 19615, and *Staphylococcus aureus* ATCC 25923. Streptococcus strains showed a significant increase in growth following soy fermentation. *S. salivarius* TUCC 1253-fermented soy showed significantly higher extracellular β-glucosidase activity and amount of aglycones. *S. salivarius* TUCC 1253-fermented soy showed antimicrobial effect against all oral tested pathogens in both aerobic and anaerobic conditions. These results showed that *S. salivarius* TUCC 1253-fermented soy could potentially be used as a preventive action or alternative treatment for oral infections.

Introduction

Oral health is important as dental plaque that is formed by complex communities of oral pathogens will lead to periodontal diseases such as periodontitis and gingivitis. Besides, oral health also associates with cardiovascular diseases such as stroke, atherosclerosis, and other artery diseases (Leishman *et al.,* 2010). Thus, it is significant to improve oral health by balancing the oral microflora and preventing the growth of oral pathogens in the oral cavity.

Indigenous probiotics are probiotics that are isolated from the original site of the host. Researchers suggested that using indigenous probiotics for specific host site may provide benefits such as better survivability as compared to foreign site-probiotics as foreign site-probiotics may require different nutrients for growth (Maheshwari *et al.,* 2012). There are specific and non-specific binding for bacterial adhesion, where specific binding showed high cell surface hydrophobicity and strong bacterial adhesion. Indigenous probiotics have been reported to have high cell surface hydrophobicity thus better site adhesion and colonisation ability as compared to foreign site-probiotics (Duary *et al.,* 2011). Besides, studies also showed that indigenous probiotics exert higher antibacterial properties such as higher production of organic acids against specific site pathogens as compared to foreign site-probiotics (Kaushik *et al.,* 2009).

Bacteriocins produced by the probiotics are harmless towards the human body, but antagonistic against pathogens (Yang *et al.,* 2014). Bioactive compounds such as soy aglycones and peptides from fermented soy are also claimed to exert antimicrobial properties against pathogenic biofilms. Examples of antimicrobial activities are producing bacteriocins or organic acids, modifying the pH of the oral cavity, and balancing the oral microflora by competing for nutrients and site adhesion with oral pathogens (Dhayakaran and Priyadharshini, 2014). A study carried out by Chaleshtori *et al.* (2017) showed that 100 mg/mL of soy isoflavones was able to inhibit *Staphylococcus aureus*. The study by Laodheerasiri and Horana Pathirage (2017) also reported that total isoflavones extracted from soybean flour, roasted soybean, and raw soybean by the ethanol-hexane method were all able

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to inhibit *Escherichia coli* and *Sta. aureus* at the concentrations of 0.031, 0.125, and 0.250 mg/mL, respectively.

To date, much research have been conducted on probiotics for dental care, and there are various oral probiotic products in the market; however, there is yet to be a probiotic-fermented soy product in the market or research that targets to improve oral health.

Materials and methods

Bacterial cultures

Oral probiotics (*Streptococcus salivarius* TUCC 1251, *S. salivarius* TUCC 1253, *S. salivarius* TUCC 1254, *S. salivarius* TUCC 1255, and *S. orisratti* TUCC 1253) isolated from healthy human saliva, and *S. salivarius* K12 isolated from tablet (BLIS K12™, Blis Technologies, Dunedin, New Zealand) in a previous study (Choo *et al.,* 2020) were used in the present work. Oral probiotics and oral pathogens (*Enterococcus faecalis* ATCC 700802, S. pyogenes ATCC 19615, and *Sta. aureus* ATCC 25923) (Taylor's University, Selangor, Malaysia; Monash University, Selangor, Malaysia, respectively) were kept at -80°C and propagated in sterile brain heart infusion broth (HiMedia, Mumbai, India) using 10% (v/v) inoculum for three successive times and incubated for 24 h at 37°C. The activated cells were centrifuged with 0.9% (w/v) sodium chloride at 3,500 *g* at 4°C for 15 min. *S. salivarius* K12 and *S. salivarius* ATCC 13419 were used as control and activated as above.

Soy fermentation

Soy protein isolate (SPI) powder (V.I.S. Foodtech, Kuala Lumpur, Malaysia) was diluted (40 g/L) using ultra-pure distilled water and autoclaved for 15 min at 121°C. Activated probiotic strains were incorporated into soy protein isolate (SPI) medium for fermentation by 5% (v/v) inoculum (optical density = 0.7, 600 nm) at 37°C for 24 h.

Growth of indigenous Streptococcus-fermented-soy

The viability of indigenous oral probiotics before and after 24 h soy fermentation was determined. One millilitre of probiotic-fermented soy before and after fermentation was serially diluted by 10-fold using 0.9% (w/v) sodium chloride solution. Then, 1 mL of probiotic-fermented soy was plated on brain heart infusion agar using the pour plate method. Plates were then incubated for 48 h at 37°C. Colony-forming units (CFU) of *Streptococcus* strains were calculated using Eq. 1:

CFU/mL = number of colonies formed × dilution factor of sample / 1 mL of sample (Eq. 1)

Determination of pH

The pH of *Streptococcus*-fermented soy before and after fermentation was determined using a pH meter (pH 700, Eutech Instruments, Singapore).

Titratable acidity (TA) of Streptococcus-fermented soy

Free proton concentration and undissociated acids produced by *Streptococcus* strains in soy fermentation were determined by TA according to Phromthep and Leenanon (2017). The initial pH of *Streptococcus*-fermented soy was measured using a calibrated pH meter. Then, 0.1 N sodium hydroxide (NaOH) solution was progressively added until the pH reached the point of 8.2 at 25°C. The TA of *Streptococcus*-fermented soy was calculated using Eq. 2:

TA (%, v/v) = (volume of NaOH (mL) × N of NaOH / volume of sample) × 100 (Eq. 2)

Intra- and extracellular β-glucosidase activities in Streptococcus strains

The intracellular β-glucosidase activity of *Streptococcus* strains was determined following the method of Ewe *et al.* (2011). Activated probiotics (10%, v/v) were extracted and determined using the rate of hydrolysis of *p*-nitrophenyl β-D-glucopyranoside (*p*NPG), where the amount of *p*-nitrophenol released was measured at 420 nm using a spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that released one μmol of *p*-nitrophenol from *p*NPG per mL per min under assay condition. The protein concentration of the crude enzyme extract was determined following the Bradford method (Bradford, 1976) using bovine serum albumin as standard. The specific activity of the microorganism was expressed as mU of β-glucosidase activity per mg of protein.

The extracellular β-glucosidase activity of *Streptococcus*-fermented soy was determined by measuring the rate of hydrolysis of *p*NPG (Sigma-Aldrich, St. Louis, United States) as described by Ewe *et al.* (2011). The amount of *p*-nitrophenol released was measured as mentioned in the determination of intracellular β-glucosidase activity.

Isoflavone contents in Streptococcus-fermented soy

The isoflavone extraction from probiotic-fermented soy was performed and analysed following the method of Ewe *et al.* (2011). The extracted isoflavones were subjected to High-Performance Liquid Chromatography (HPLC) analysis.

The HPLC system with a UV detector

(Shimadzu Corporation, Kyoto, Japan) set at 259 nm and was fitted with an Inertsil ODS-3 column (150 × 3 mm, 5 mm; GL Sciences, Tokyo, Japan) and operated at a flow rate of 1 mL/min. The mobile phase consisted of solvent A (water:phosphoric acid, 1000:1; v/v) and solvent B (water:acetonitrile:phosphoric acid, 200:800:1; v/v/v). Gradient elution was used to isolate isoflavones and set as solvent A 100% (2 min) → 65% (29 min) → 50% (31 min) → 100% (40 min) → 100% (43 min). Stock solutions of HPLC-grade daidzin, daidzein, glycitin, glycitein, genistin, and genistein (Sigma-Aldrich, St. Louis, USA) were used as standards.

Antimicrobial effect of Streptococcus-fermented soy against oral pathogens

Antimicrobial activity of *Streptococcus*-fermented soy towards oral pathogens was analysed using agar diffusion well variant method by Valgas *et al.* (2007) with modification. One hundred microliters of the activated oral pathogens (optical density = 0.3, 600 nm) were uniformly spread on blood agar (blood base No. 2 with sheep blood; Oxoid Ltd., Cheshire, UK). Agar wells were cut using sterile cork borer (5 mm diameter). Twenty microliters of *Streptococcus*-fermented soy (20% (v/v) inoculum) was added into the agar wells. All the plates were incubated at 37°C for 24 h in both aerobic, and anaerobic conditions (using an anaerobic jar). The diameter of the zone of inhibition was measured in mm using a calibrated ruler, and results were interpreted against oral pathogen's respective positive control antibiotic (5 µL) and negative control antibiotic (5 µL). Penicillin (10 µg/mL) was used as positive control antibiotic for all three oral pathogens. Tetracycline (30 µg/mL) was used as negative control antibiotic for *Ent. faecalis* and *S. pyogenes*; while gentamicin (10 µg/mL) was used as negative control antibiotic for *Sta. aureus*. The antibiotic concentrations followed the recommendations by the Clinical and Laboratory Standards Institute (CLSI) guidelines. Each oral pathogen was interpreted as resistant, intermediate, or susceptible to respective control antibiotics following the breakpoints described in CLSI guidelines (CLSI, 2012).

Statistical analysis

All the data were statistically analysed (SPSS version 22; Chicago, Illinois, USA) and presented as means from two separate runs. The significant difference between means was determined using pair T-test in section 2.3. - 2.5.; independent *T*-test in section 2.8.; and one-way analysis of variance (ANOVA) and Tukey's test as a *post-hoc* test in section 2.3. - 2.8., with a significant level of $\alpha = 0.05$.

Results

Viability of Streptococcus-fermented soy

The viability of *Streptococcus* strains in fermented soy was determined to ensure that a minimum amount of 10^6 - 10^7 CFU/mL would be present in the *Streptococcus*-fermented soy, as illustrated in Figure 1. The viability of *Streptococcus* strains significantly increased ($p < 0.05$) to 10^8 CFU/mL (increase of 42.0 - 54.4%) following fermentation, which showed that SPI was a suitable carrier that provided favourable environment and nutrients for *Streptococcus* strains. The viability of *S. salivarius* TUCC 1253-fermented soy was significantly higher ($p < 0.05$) as compared to the other *Streptococcus*-fermented soy.

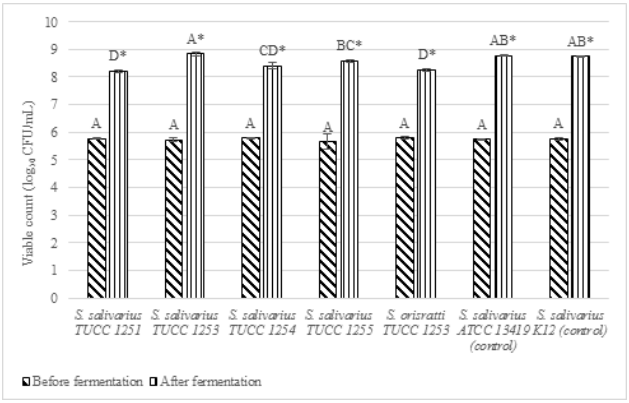

Figure 1. Viability of *Streptococcus*-fermented soy following 24 h fermentation at 37°C. Data are means ± standard deviation, $n = 6$. Capital letters indicate significant difference ($p < 0.05$) via one-way ANOVA. Asterisk (*) indicate significant difference ($p < 0.05$) via pair *T*-test.

pH changes in Streptococcus-fermented soy

The pH of the oral cavity is generally maintained between 6.7 - 7.3 by saliva. As *Streptococcus*-fermented soy was to be used as the active ingredient to improve oral health, its pH level must be ascertained.

The pH level of all *Streptococcus*-fermented soy decreased ($p < 0.05$) significantly by 11.1 - 13.4% following fermentation (Figure 2). The significant drop ($p < 0.05$) in pH level and increase ($p < 0.05$) in the growth of *Streptococcus* strains following soy fermentation showed that nutrients in the SPI medium were utilised for growth. *S. salivarius* TUCC 1253-fermented soy showed the highest viability and most significant decrease ($p < 0.05$) in pH level following fermentation; while *S. salivarius* TUCC 1251 and *S. orisratti* TUCC 1253 with the lowest viability showed the least significant decrease ($p < 0.05$) in pH level following soy fermentation. This showed that there was a relationship between the increase in *Streptococcus* strains growth and the decrease in pH level following soy fermentation.

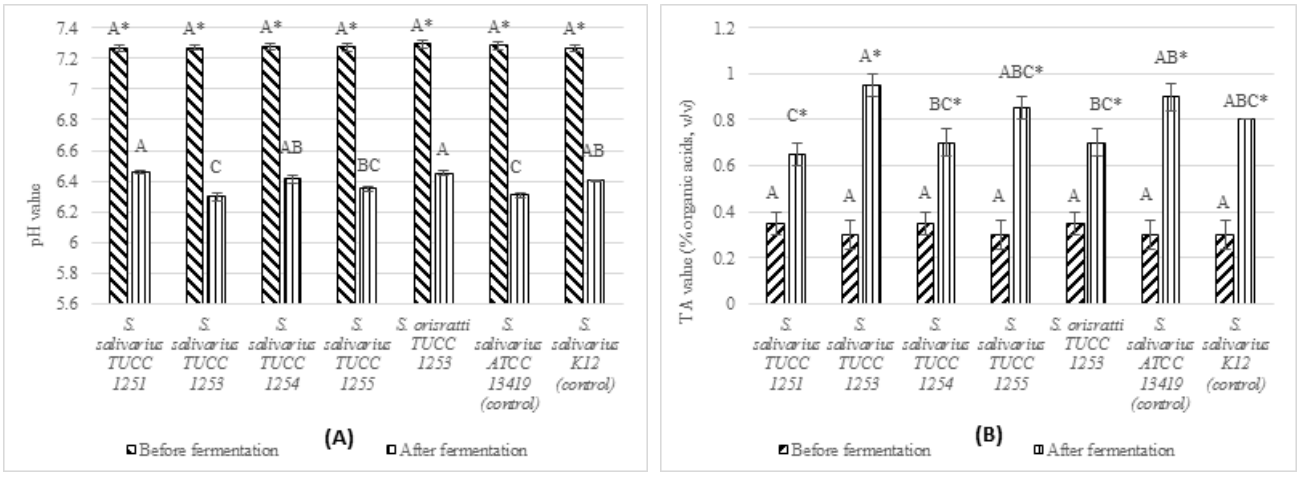

Figure 2. (A) pH and (B) titratable acidity (TA) of *Streptococcus*-fermented soy following 24 h fermentation at 37°C. Data are expressed as means ± standard deviation, $n = 6$. Capital letters indicate significant difference ($p < 0.05$) via one-way ANOVA. Asterisk (*) indicate significant difference ($p < 0.05$) via pair *T*-test.

Titratable acidity (TA) of Streptococcus-fermented soy

TA is commonly used to evaluate the amount of organic acids present in the medium by titration with an alkali. The TA of *Streptococcus*-fermented soy before and after fermentation are shown in Figure 2. All *Streptococcus*-fermented soy showed significant increase ($p < 0.05$) in TA by 85.7 - 216.0% following fermentation. *S. salivarius* TUCC 1253-fermented soy showed the greatest increase ($p < 0.05$) in TA, the greatest decrease ($p < 0.05$) in pH level, and the highest increase ($p < 0.05$) in viability after fermentation. These showed that the amount of organic acids produced correlated with the growth of *Streptococcus* strains and pH level following fermentation.

The intracellular β-glucosidase activity of Streptococcus strains

The intracellular β-glucosidase activity was conducted to determine the presence of β-glucosidase in *Streptococcus* strains (Table 1). All *Streptococcus* strains were demonstrated to have β-glucosidase enzyme. Both *S. salivarius* TUCC 1251 and TUCC 1255 showed significantly higher ($p < 0.05$) total specific activity, followed by *S. salivarius* TUCC 1253 and TUCC 1254; *S. orisratti* TUCC 1253, *S. salivarius* ATCC 13419 and K12 showed lesser ($p < 0.05$) total specific activity of intracellular β-glucosidase activity among all the tested strains.

The extracellular β-glucosidase activity of Streptococcus-fermented soy

Table 1 shows that all *Streptococcus* strains demonstrated extracellular β-glucosidase activity following fermentation. *S. salivarius* TUCC 1253 showed significantly the highest ($p < 0.05$) total specific activity, followed by *S. salivarius* ATCC 13419, TUCC 1255, *S. orisratti* TUCC 1253, *S. salivarius* TUCC 1254, and TUCC 1251; *S. salivarius* K12 showed lower ($p < 0.05$) in the total specific activity of extracellular β-glucosidase activity.

Total isoflavones in Streptococcus-fermented soy

Table 2 shows the amounts of glycosides

Table 1. The intra- and extracellular β-glucosidase activities of *Streptococcus* strains.

***Streptococcus* strains**	**Intracellular β-glucosidase activity**			**Extracellular β-glucosidase activity**		
	Total volume activity (U/mL)	**Total protein concentration (mg/mL)**	**Specific activity (U/mg protein)**	**Total volume activity (U/mL)**	**Total protein concentration (mg/mL)**	**Specific activity (U/mg protein)**
S. salivarius TUCC 1251	0.226 ± 0.011^{A}	0.534 ± 0.020^{A}	0.424 ± 0.370^{A}	0.224 ± 0.113^{E}	0.825 ± 0.024^{A}	0.271 ± 0.010^{EF}
S. salivarius TUCC 1253	0.165 ± 0.021^{B}	0.455 ± 0.021^{B}	0.364 ± 0.056^{AB}	0.424 ± 0.014^{A}	0.621 ± 0.016^{D}	0.683 ± 0.021^{A}
S. salivarius TUCC 1254	0.122 ± 0.011^{C}	0.340 ± 0.008^{C}	0.358 ± 0.031^{AB}	0.242 ± 0.008^{DE}	0.853 ± 0.020^{A}	0.284 ± 0.012^{DE}
S. salivarius TUCC 1255	0.199 ± 0.023^{A}	0.473 ± 0.033^{B}	0.420 ± 0.029^{A}	0.270 ± 0.008^{C}	0.697 ± 0.018^{B}	0.388 ± 0.014^{C}
S. orisratti TUCC 1253	0.097 ± 0.011^{CD}	0.287 ± 0.010^{D}	0.338 ± 0.048^{B}	0.251 ± 0.010^{D}	0.830 ± 0.016^{A}	0.302 ± 0.014^{D}
S. salivarius ATCC 13419 (control)	0.090 ± 0.011^{D}	0.272 ± 0.017^{D}	0.331 ± 0.049^{B}	0.315 ± 0.010^{B}	0.682 ± 0.018^{BC}	0.462 ± 0.024^{B}
S. salivarius K12 (control)	0.156 ± 0.015^{B}	0.460 ± 0.023^{B}	0.341 ± 0.054^{B}	0.165 ± 0.010^{F}	0.654 ± 0.018^{CD}	0.252 ± 0.013^{F}

Data are means ± standard deviation, $n = 6$. Means within the same column followed by different uppercase letters are significantly different ($p < 0.05$) via one-way ANOVA.

Table 2. The concentrations of isoflavones in *Streptococcus*-fermented soy.

Streptococcus strains	Concentration of isoflavones (μg/mL)							
	Daidzin (glycoside)	Glycitin (glycoside)	Genistin (glycoside)	Total Glycosides	Daidzein (aglycone)	Glycitein (aglycone)	Genistein (aglycone)	Total aglycones
S. salivarius TUCC 1251	1.700 ± 0.012^{C}	0.422 ± 0.085^{A}	1.628 ± 0.017^{C}	3.750 ± 0.099^{B}	0.875 ± 0.012^{E}	0.097 ± 0.007^{B}	1.772 ± 0.087^{C}	2.744 ± 0.086^{D}
S. salivarius TUCC 1253	ND^{F}	ND^{B}	ND^{F}	ND^{E}	2.040 ± 0.023^{A}	0.231 ± 0.018^{A}	2.873 ± 0.223^{A}	5.144 ± 0.254^{A}
S. salivarius TUCC 1254	1.875 ± 0.012^{A}	0.465 ± 0.085^{A}	1.931 ± 0.022^{B}	4.271 ± 0.093^{A}	0.956 ± 0.009^{D}	0.092 ± 0.007^{B}	1.809 ± 0.166^{C}	2.857 ± 0.163^{D}
S. salivarius TUCC 1255	0.417 ± 0.013^{E}	0.153 ± 0.070^{B}	0.117 ± 0.003^{E}	0.687 ± 0.057^{D}	1.643 ± 0.023^{B}	0.199 ± 0.013^{A}	2.627 ± 0.158^{AB}	4.469 ± 0.163^{B}
S. orisratti TUCC 1253	1.118 ± 0.015^{D}	0.329 ± 0.080^{A}	0.759 ± 0.013^{D}	2.206 ± 0.067^{C}	1.251 ± 0.011^{C}	0.134 ± 0.009^{B}	2.318 ± 0.127^{B}	3.703 ± 0.128^{C}
S. salivarius ATCC 13419 (control)	ND^{F}	0.108 ± 0.065^{B}	ND^{F}	0.108 ± 0.065^{E}	2.040 ± 0.015^{A}	0.200 ± 0.043^{A}	2.761 ± 0.261^{A}	5.001 ± 0.268^{A}
S. salivarius K12 (control)	1.819 ± 0.020^{B}	0.452 ± 0.056^{A}	2.159 ± 0.023^{A}	4.430 ± 0.068^{A}	0.912 ± 0.008^{E}	0.091 ± 0.012^{B}	1.573 ± 0.242^{C}	2.576 ± 0.250^{D}

Data are means ± standard deviation, $n = 6$. Means within the same column followed by different uppercase letters are significantly different ($p < 0.05$) via one-way ANOVA. Isoflavones concentration of unfermented soy protein isolate (control) were 2.002 ± 0.031 μg/mL of daidzin, 0.468 ± 0.063 μg/mL of glycitin, 3.796 ± 0.044 μg/mL of genistin, 6.266 ± 0.124 μg/mL of total glycosides, 0.620 ± 0.015 μg/mL of daidzein, 0.076 ± 0.016 μg/mL of glycitein, 0.544 ± 0.749 μg/mL of genistein, and 1.240 ± 0.095 μg/mL of total aglycones. ND = not detected.

(daidzin, glycitin, genistin) and their respective aglycones (daidzein, glycitein, genistein) present in different *Streptococcus*-fermented soy. Total glycosides of unfermented SPI were higher than all *Streptococcus*-fermented soy, while total aglycones of all *Streptococcus*-fermented soy were higher than unfermented SPI. This shows that β-glucosidase activity had taken place following probiotic fermentation and bio-converted isoflavones from glycosides to aglycones. All *Streptococcus*-fermented soy had a decrease in glycosides by 29.3 - 100.0%; and an increase in aglycones by 107.7 - 314.8%, as compared to unfermented SPI.

No glycosides were detected in *S. salivarius* TUCC 1253-fermented soy, which was in line with the extracellular β-glucosidase activity. *S. salivarius* TUCC 1253-fermented soy had shown higher ($p < 0.05$) extracellular β-glucosidase activity where the enzyme hydrolysed all glycosides in SPI, thus resulting in lower ($p < 0.05$) glycosides concentration as compared to *S. salivarius* ATCC 13419-fermented soy.

Antimicrobial effect of S. salivarius TUCC 1253-fermented soy against oral pathogens

The effectiveness of *S. salivarius* TUCC 1253-fermented soy in reducing the oral pathogens under different conditions is shown in Table 3. *S. salivarius* TUCC 1253 was chosen to be tested for antimicrobial effects due to its better growth in SPI and higher aglycone content when compared with the other *Streptococcus*-fermented soy. *Ent. faecalis*, *S. pyogenes*, and *Sta. aureus* are facultative anaerobic oral pathogens frequently found in the oral cavity. *Ent. faecalis* had been found in subgingival samples of periodontitis patients and caused endodontic infection (Pinheiro and Mayer, 2014). Besides, *Ent. faecalis* was also reported by Kouidhi *et al.* (2011) to have a high carriage rate of 46.9% in dental caries-active Tunisian children. On the other hand, McCormack *et al.* (2015) showed that *Sta. aureus* was frequently found through oral rinse, mouth, and tongue swab. The study also reported that significant isolates of *Sta. aureus* was isolated from patients with oral complications and infections such as angular cheilitis, suspected candidal infection, erythema, and pain in the oral cavity. Besides, Kouidhi *et al.* (2010) also reported that *Sta. aureus* was found in 90 caries-active Tunisian children. A study carried out by Fox *et al.* (2006) reported that *S. pyogenes* was detected in the oral cavity of patients diagnosed with streptococcal pharyngitis. Besides, a case reported by Inagaki *et al.* (2017) also showed that *S. pyogenes*

Table 3. Zone of inhibition (mm) of *S. salivarius* TUCC 1253-fermented soy against oral pathogens.

	Enterococcus faecalis ATCC 700802		*Streptococcus pyogenes* ATCC 19615		*Staphylococcus aureus* ATCC 25923	
	A	AN	A	AN	A	AN
S. salivarius TUCC 1253-fermented soy	20.50 ± 0.55^{cdB}*	19.50 ± 0.55^{eB}	26.33 ± 0.52^{aB}*	24.33 ± 0.52^{bB}	21.33 ± 0.52^{cB}*	19.67 ± 0.52^{deB}
Positive control antibiotic	23.67 ± 1.03^{A}	24.00 ± 1.10^{A}	27.33 ± 0.52^{A}*	24.83 ± 0.41^{A}	30.83 ± 0.41^{A}*	29.67 ± 0.52^{A}
Negative control antibiotic	13.50 ± 0.55^{C}	13.5 ± 0.55^{C}	15.83 ± 0.41^{C}*	13.33 ± 0.52^{C}	11.33 ± 0.52^{C}*	9.83 ± 0.41^{C}

A = aerobic condition; AN = anaerobic condition. Data are means ± standard deviation, $n = 6$. Means within the same row for *S. salivarius* TUCC 1253-fermented soy followed by different lowercase letters are significantly different ($p < 0.05$) via one-way ANOVA. Means within the same column followed by different uppercase letters are significantly different ($p < 0.05$) via one-way ANOVA. *means within the same row between A and AN conditions are significantly different ($p < 0.05$) via independent *T*-test.

had caused an oral infection in an edentulous patient. The oral cavity is the major gateway into the human body, hence by reducing pathogens that are frequently found in the oral cavity, it could reduce the risk of cross-infection at other body sites.

Antimicrobial tests of *S. salivarius* TUCC 1253-fermented soy were conducted under aerobic and anaerobic conditions that resemble the oral cavity environment. *S. salivarius* TUCC 1253-fermented soy showed antimicrobial effect towards all oral pathogens tested. *S. salivarius* TUCC 1253-fermented soy inhibited *S. pyogenes* under aerobic conditions more effectively ($p < 0.05$) as compared to other two oral pathogens in both conditions. *S. salivarius* TUCC 1253-fermented soy showed significantly higher ($p < 0.05$) inhibitory activity towards *Ent. faecalis*, *S. pyogenes*, and *Sta. aureus* under aerobic conditions as compared to anaerobic conditions by 5.0, 7.9, and 8.1%, respectively. Probiotics tested in the study conducted by Annuk *et al.* (2003) showed higher inhibitory activities against pathogens with the presence of oxygen due to poor growth of probiotics under anaerobic conditions.

S. salivarius TUCC 1253-fermented soy inhibited *Ent. faecalis*, *S. pyogenes*, and *Sta. aureus* more effectively ($p < 0.05$) under both conditions when compared with the negative control antibiotic, which showed resistance. On the other hand, *S. salivarius* TUCC 1253-fermented soy inhibited *Ent. faecalis*, *S. pyogenes*, and *Sta. aureus* less effectively ($p < 0.05$) under both conditions when compared with positive control antibiotic, which showed susceptibility.

Discussion

Streptococcus strain colonises 20% of the total bacteria in the oral cavity (Nicolas and Lavoie, 2011). *S. salivarius* K12 and M18 were commonly used as oral probiotics to prevent various oral diseases such as reducing dental plaque, anti-caries, and reducing streptococcal pharyngotonsillitis (Burton *et al.,* 2006). Indigenous *Streptococcus* strain is more preferable to be used as an oral probiotic due to its advantage in survivability, colonising ability, and antimicrobial properties against site-specific oral pathogens as compared to foreign site-probiotics (Kaushik *et al.,* 2009; Duary *et al.,* 2011; Maheshwari *et al.,* 2012). *Streptococcus* strains are not commonly used for probiotic-soy fermentation. However, the β-glucosidase enzyme in *Streptococcus* strains could bio-convert isoflavones in SPI into bioactive compounds which could exert beneficial effects towards host (Michlmayr and Kneifel, 2014). Besides, SPI could also provide nutrients for probiotic growth.

The dominant sugar found in soy is sucrose which is utilised by probiotic as a nutrient for growth (Božanić *et al.,* 2011). A study conducted by Garro *et al.* (1998) showed that *S. salivarius* growth increased to 10^8 CFU/mL upon fermentation after metabolising 60 - 75% of sucrose in soymilk. Soy also acts as a probiotic delivery carrier where it stabilises the viability of probiotics throughout storage and upon absorption in the host, as shown in an *in vitro* study conducted by Sagheddu *et al.* (2018).

Streptococcus strains are lactic acid-formers that utilise the carbon sources and nutrients in soy medium during fermentation and produce by-products such as lactic acid, which causes a decrease in pH level (Lee *et al.,* 2017). Teeth demineralisation is a chemical reaction where minerals such as calcium and phosphate are removed from hard tissues such as cementum, dentin, and enamel when the oral cavity's pH is reduced to critical pH (5.5 - 6) (Stookey, 2008). However, the pH level of *Streptococcus*-fermented soy in the present work was above 6.0, thus, safe to be used in the oral cavity without the risk of cavities and tooth demineralisation.

The production of organic acids is favourable as organic acids are claimed to be able to exert antimicrobial properties (El Baaboua *et al.,* 2018). The TA of *Streptococcus*-fermented soy following fermentation in the present work ranged from 0.65 - 0.95%, and are comparable with other studies which reported the range of 0.31 - 1.82% (Jimoh and Kolapo, 2007; Obadina *et al.,* 2013).

Soy isoflavones initially exist in glycoside form, where they go through biotransformation into bioactive form, isoflavone aglycones, with the assistance of β-glucosidase enzyme through probiotic fermentation (Yang *et al.,* 2011). Intracellular β-glucosidase activity is β-glucosidase that is present in probiotic strain cells, and mostly found in LAB (Yuksekdag *et al.,* 2017). There are no other studies conducted on the analysis of β-glucosidase activity for *S. salivarius* and *S. orisratti* to date.

The extracellular β-glucosidase activity represents the amount of β-glucosidase enzyme released from cells into the medium upon fermentation. Extracellular β-glucosidase activity in *Streptococcus*-fermented soy showed different trends from intracellular β-glucosidase activity in their respective *Streptococcus* strains. This could be due to the cell membrane permeability where the amount of β-glucosidase enzyme present in the cells and released from the cells are different. Besides, different *Streptococcus*-fermented soy also showed different extracellular

β-glucosidase activities which could be affected by different pH levels in Streptococcus-fermented soy, as enzyme activity is also affected by pH due to change in protein structure of the enzyme (Duarte *et al.,* 2013).

Soy isoflavones initially exist in glycosides form (genistin, daidzin, glycitin), where they go through biotransformation into bioactive form, isoflavone aglycones (genistein, daidzein, glycitein) with the assistance of β-glucosidase enzyme (Izumi *et al.,* 2000). Bioactive isoflavones, which are aglycones, are more easily absorbed by the human body into peripheral circulation as compared to its glycoside form (Izumi *et al.,* 2000). Besides, aglycones have been claimed by many studies to have various health benefits, such as antioxidant properties (Tamang *et al.,* 2016), antimicrobial properties (Abd El-Gawad *et al.,* 2014) and immunomodulatory properties. A study conducted by Verdrengh *et al.* (2004) also concluded that both daidzein and genistein showed antimicrobial activity against *Staphylococcus* strains. Another study by Ulanowska *et al.* (2006) also concluded that soy genistein was able to exhibit antimicrobial properties against *E. coli.*

Oral pathogen invades host by colonising the oral epithelial cells, then exhibiting virulence to avoid the human immune system, then travel to various organs and cause infections (Clark and Bavoil, 1994). Chemical treatments such as prescribing antibiotics and mechanical treatments such as restorative work are carried out by dentists to reduce the oral pathogens (Dar-Odeh *et al.,* 2010). However, mechanical treatments could be expensive for certain countries or rural areas (Cruz Martínez *et al.,* 2017) and increase antibiotic resistance due to over-prescription. Antibiotics are also associated with side effects such as gastrointestinal disturbances or anaphylactic shock (Dar-Odeh *et al.,* 2010). Hence, preventive action and alternative treatment could be used to reduce oral pathogens in the oral cavity.

Probiotic-fermented soy can act as both preventive action and alternative treatment to improve oral health. *S. salivarius* exerts antimicrobial effect by producing salivaricin A and salivaricin B that inhibits oral pathogens such as *S. pyogenes*, *Ent. faecalis*, and *Sta. aureus* (Wescombe *et al.,* 2006; Barbour *et al.,* 2016; Therdtatha *et al.,* 2016). Studies conducted by Verdrengh *et al.* (2004) and Dhayakaran and Priyadharshini (2014) also showed that both glycosides and aglycones were able to inhibit pathogens such as *Listeria monocytogenes*, *E. coli*, and *Sta. aureus*. However, there is no guideline for isoflavones on oral health, but it could be an added antimicrobial properties source for *Streptococcus*-fermented soy to improve oral health.

Conclusion

SPI served as a good probiotic carrier for indigenous *Streptococcus* strains as showed by the increase ($p < 0.05$) in viability after fermentation. The extracellular β-glucosidase activity was comparable with total aglycones content in *Streptococcus*-fermented soy, where *S. salivarius* TUCC 1253-fermented soy showed the highest ($p < 0.05$) for extracellular β-glucosidase activity and total aglycones content. *S. salivarius* TUCC 1253-fermented soy also showed an antimicrobial effect on oral pathogens *Ent. faecalis*, *S. pyogenes*, and *Sta. aureus* under both aerobic and anaerobic conditions. The present work demonstrated that *S. salivarius* TUCC 1253-fermented soy had the potential to improve oral health, and may be useful for future novel oral health applications.

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