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FULL PAPER

Development of indigenous *Streptococcus salivarius* TUCC 1253-fermented soy oral strip using Box-Behnken design for oral health

[1] How, Y.H., [2]Ewe, J.A., [3]Song, K.P., [4] Kuan, C.S., [1]Kuan, C.H. and [1,] *Yeo, S.K.

[1] *School of Biosciences, Taylor's University, 1, Jalan Taylor's, 47500 Subang Jaya, Selangor, Malaysia.*
[2] *Nano and Advanced Materials Institute Limited, Lakeside 1, Units 515-516, 5/F, Lakeside 1, New Territories, Shatin, Science Park Avenue, Science Park, Hong Kong*
[3] *School of Science, Monash University, Jalan Lagoon Selatan, Bandar Sunway, 47500 Subang Jaya, Selangor, Malaysia*
[4] *NeoGenix Laboratoire, Kelana Square, C707, Jalan SS17/26, 47301 Petaling Jaya, Selangor, Malaysia*

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Abstract

The study was to develop *Streptococcus salivarius* TUCC 1253-fermented soy oral strip that exhibits antimicrobial activity against oral pathogens. Indigenous *S. salivarius* TUCC 1253 isolated from healthy human saliva grew well in soy protein isolate after 24 hrs fermentation. Oral strip was developed using hydroxypropyl methylcellulose polymer, propylene glycol plasticizer, *S. salivarius* TUCC 1253-fermented soy, peppermint flavouring and distilled water. The formulation was successfully optimized which gives characteristics such as low moisture uptake, high percent elongation and high tensile strength. The formulation was successfully optimized with a percentage error of not greater than 4.22%. The optimized *S. salivarius* TUCC 1253-fermented soy oral strip (20% v/v inoculum) contains more than 10^8 CFU/g of live cells. The optimized *S. salivarius* TUCC 1253-fermented soy oral strip was able to inhibit all studied oral pathogens (*Enterococcus faecalis*, *Streptococcus pyogenes* and *Staphylococcus aureus*) in both aerobic and anaerobic conditions. Up to our knowledge, this is the first available oral strips containing indigenous oral probiotic-fermented soy which serve as a new alternative for oral health.

1. Introduction

Oral health care has always been a primary concern in our daily routine life. In recent years, protection for oral health has become more prevalence as oral diseases are also directly correlated with other life-threatening diseases. Oral diseases such as dental caries, periodontitis and gingivitis are caused by a complex group of oral pathogens. Hence, it is crucial to balance the oral microflora and reduce oral pathogens in the oral cavity to improve oral health (Leishman *et al.*, 2010).

Indigenous probiotics are probiotics isolated from the host's specific site such as gastrointestinal tract (GIT), oral cavity, respiratory tract, and other sites. Indigenous probiotics used for specific host site have advantages compared to foreign probiotics such as better adaptation; better site colonization; and better antimicrobial activity against specific site pathogens (Kaushik *et al.,* 2009, Maheshwari *et al.,* 2012). However, indigenous oral probiotics are not widely studied and little strain isolates are available especially for the oral site. In addition, most commercial probiotics product for oral health are in the form of tablet/ food product that requires transit through GIT and leaving little viable culture in the oral cavity.

The fermentation process is able to change the qualitative and quantitative composition of soybean components through physical and enzymatic processes. Probiotic-soy fermentation hydrolysed glycoside isoflavones into biological active aglycones. Aglycones provide several health benefits such as antimicrobial, antioxidant, and hypocholesterolemic properties (Yang *et al.,* 2011).

The oral strip is a suitable substance delivery system especially for the oral site due to its ability to bypass hepatic first-pass effect as a substance is absorbed through the oral mucosa. Besides, oral strip dissolves in the oral cavity, hence does not requires water administration and avoid choking (Borges *et al.,* 2015).

*Corresponding author.
Email: *siokkoon.yeo@taylors.edu.my*

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Tropical countries such as Malaysia have high relative humidity up to 86% annually which often caused oral strip's instability upon absorbing moisture (Jamaludin *et al.,* 2015). Besides, tensile strength and percent elongation will also affect the oral strip's stability during transportation and handling (Borges *et al.,* 2015). Hence, it is important to explore the optimized formulation of probiotic-fermented soy oral strip with good characteristics.

In the current market, there are dental care product such as mouthwash and oral probiotic tablet but yet to have probiotic oral strip product that targets to improve oral health. Therefore, the aim of this study was to incorporate indigenous probiotics that are isolated from healthy human saliva for soy fermentation and developed into probiotic-fermented soy oral strip.

2. Materials and methods

2.1 Bacterial culture

S. salivarius TUCC 1253; control strains, *S. salivarius* K12 and *S. salivarius* ATCC 13419; and oral pathogens (*E. faecalis* ATCC 700802; *S. pyogenes* ATCC 19615; *S. aureus* ATCC 25923) (Monash University; Taylor's University, Malaysia) were stored at -80°C and activated in sterile Brain Heart Infusion (BHI) broth (Hi Media, India) for 3 successive times at 37°C for 24 hrs. Activated cultures were washed (Hettich, Germany) with 0.9% (w/v) of sodium chloride solution three times by centrifugation at 3500 x *g* at 4°C for 15 mins.

2.2 Soy fermentation

Activated *Streptococcus* strains were added into soy protein isolate (SPI) for fermentation with 5% (v/v) inoculum (OD_{600} = 0.7) at 37°C for 24 hrs. *Streptococcus* -fermented soy was then freeze-dried for storage.

2.3 Growth of Streptococcus strains in fermented-soy

The viable cell counts of probiotics were conducted via pour plate method. *Streptococcus*-fermented soy (1 mL) was serially-diluted by using sterilized 0.9% (w/v) sodium chloride solution and then plated in BHI agar (Hi Media, India) for 48 h at 37°C. Viable cell counts of *Streptococcus* in SPI were reported as CFU/ mL.

2.4 pH of Streptococcus-fermented soy

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

2.5 Titratable acidity (TA) of Streptococcus-fermented soy

TA was conducted to determine free protons concentration and undissociated acids released by *Streptococcus*-fermented soy according to Fuse *et al.* (1997). After the initial pH of *Streptococcus*-fermented soy was determined, 0.1 N sodium hydroxide (NaOH) solution was gradually added to the fermented soy (5 mL) until the pH reached 8.2 at 25°C. The TA of *Streptococcus*-fermented soy was calculated as shown below.

$$\text{Titratable acidity (\%,v/v)} = \frac{\text{Volume of NaOH (mL)} \times \text{N of NaOH}}{\text{Volume of } \textit{Streptococcus} - \text{fermented soy (mL)}} \times 100$$

2.6 Development of Streptococcus-fermented soy oral strip

S. salivarius TUCC 1253-fermented soy oral strip was prepared according to the method described by Chaudhary *et al.* (2013) with modification. Hydroxypropyl methylcellulose (HPMC) was prepared by dispersing in distilled water with continuous stirring. Freeze-dried *Streptococcus*-fermented soy, plasticizer, and peppermint flavouring were then dissolved using distilled water and mixed thoroughly into HPMC solution. The mixture solutions were poured on a glass with plastic and micrometer film applicator (Microm II, Paul N. Gardner Company Inc., USA) was used to ensure the film's thickness was even. The oral strip was dried at ambient temperature in Biological Safety Cabinet for 24 h and cut into 4 cm x 2 cm area. Different formulations (10 mL) were prepared by incorporating polymers (45-50% w/w), plasticizers (15-20% w/w), *Streptococcus*-fermented soy (100 mg), peppermint flavouring (20 mg) and add up to 10 mL with distilled water using 2-factor 3-level Box-Behnken experimental design (Stat-East Inc., USA) (Table 1). A total of 13 runs of experiment batches were carried out using different levels of independent variables, polymer concentration (X_1) and plasticizer concentration (X_2) (Table 2).

Table 1. Independent variables in Box-Behnken design

Factor	Level used, actual (coded)		
Independent variables	Low (-1)	Medium (0)	High (+1)
X_1 = Concentration of polymer (% w/w)	45	47.5	50
X_2 = Concentration of plasticizer (% w/w)	15	17.5	20

2.7 Characterization tests of Streptococcus-fermented soy oral strip

2.7.1 Tensile strength

Tensile strength was performed according to Irfan *et al.* (2016) with slight modification using texture analyser equipped with a fixture, TA-DGA (Brookfield CT3™

Table 2. Box-Behnken design (2-factor 3-level) with 13 runs

Runs	Batch	Independent variable	
		X_1	X_2
1	OS1	-1	-1
2	OS2	0	0
3	OS3	1	0
4	OS4	0	0
5	OS5	1	-1
6	OS6	0	0
7	OS7	0	1
8	OS8	1	1
9	OS9	0	-1
10	OS10	-1	0
11	OS11	-1	1
12	OS12	0	0
13	OS13	0	0

X_1 = Concentration of polymer (% w/w); X_2 = Concentration of plasticizer (% w/w)

Texture Analyzer, USA) and 50 kg load cell. The test conditions were set at a target distance of 10 mm with 1 s hold time of a 0.25 N trigger load, running at a test speed of 0.50 mm/s. Tensile strength was calculated using the below calculation.

$$\text{Tensile strength } (\text{N/mm}^2) = \frac{\text{Load at failure (N)}}{\text{Strip thickness (mm)} \times \text{Strip width (mm)}}$$

2.7.2 Percent elongation at break

Percent elongation at break was performed according to Irfan *et al.* (2016) using texture analyser (Brookfield CT3^{TM} Texture Analyzer, USA) set at the same test conditions as per tensile strength. Percent elongation at break was calculated using the below calculation.

$$\text{Percent elongation at break (\%)} = \frac{\text{Change in length (mm)}}{\text{Initial length (mm)}} \times 100$$

2.7.3 Moisture uptake

Moisture uptake of *Streptococcus*-fermented soy oral strip was carried out according to Singh *et al.* (2013) with slight modification. The initial weight of oral strip was recorded. The oral strip was cut into a dimension of 2 x 2 cm^2 and placed in a climate chamber (Memmert HPP 110, Germany) with a relative humidity of 86% at 25°C for 7 days. The final weight of the oral strip after 7 days in climate chamber was recorded. Percentage moisture uptake was determined using the below formula.

$$\text{Percentage moisture uptake (\%)} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

2.8 Optimization of Streptococcus-fermented soy oral strip formulation

A 2-factor, 3-level Box-Behnken Design (Design Expert Software 9.0.4., Stat-East Inc., USA) was used for optimization of *Streptococcus*-fermented soy oral strip formulation according to Chaudhary *et al.* (2013) with modification. The 13 runs of experiment batches were evaluated based on the independent variables which were polymer concentration (X_1) and plasticizer concentration (X_2); and dependent variables which were moisture uptake (Y_1); percent elongation (Y_2) and tensile strength (Y_3) of oral strip. The optimized *Streptococcus*-fermented soy oral strip was produced based on the formula given by Design Expert Software.

2.9 Antagonistic effect of Streptococcus spp.-fermented soy against oral pathogens

Antagonistic effect of *Streptococcus*-fermented soy oral strip towards oral pathogens was analysed using agar diffusion well variant method according to Valgas *et al.* (2007) with modification. Oral pathogens (100 mL) (OD = 0.3, 600 nm) were spread on blood agar (blood base No. 2 with sheep blood, Oxoid Ltd., United Kingdom) using a sterile spreader. *Streptococcus*-fermented soy oral strip (20% v/v inoculum) was dissolved using 0.9% sodium chloride and added 20 mL into the agar wells. Plates were incubated at 37°C for 24 h in both aerobic and anaerobic condition using an anaerobic jar. The zone of inhibition was measured in mm and results were interpreted against oral pathogen's respective positive and negative control antibiotic and their concentration as listed in Table 3. Each oral pathogens were interpreted as resistant, intermediate or susceptible to respective control antibiotics according to breakpoints given by CLSI (Table 4) (CLSI, 2012).

Table 3. Positive and negative control antibiotics for respective oral pathogens

Oral pathogen strains	Positive control antibiotic (Susceptible)	Negative control antibiotic (Resistant)
E. faecalis ATCC 700802	Penicillin (10 mg/mL)	Tetracycline (30 mg/mL)
S. pyogenes ATCC 19615	Penicillin (10 mg/mL)	Tetracycline (30 mg/mL)
S. aureus ATCC 25923	Penicillin (10 mg/mL)	Gentamycin (10 mg/mL)

2.10 Statistical analysis

All the data were analysed using SPSS Inc. Software (version 22) (SPSS Inc., Chicago, IL, USA) and presented as means from 2 separate runs. Pair t-test, independent t-test and one-way analysis of variance (ANOVA) with Tukey's test as post-hoc test were used to determine the significant differences between mean; with a significant level of $\alpha = 0.05$.

Table 4. Interpretive criteria for each control antibiotics used on oral pathogens

Strains	Antibiotic	Inhibition zone diameter (mm)		
		Susceptible	Intermediate	Resistant
E. faecalis ATCC 700802	Penicillin	≥15	-	≤14
	Tetracycline	≥19	15-18	≤14
S. pyogenes ATCC 19615	Penicillin	≥24	-	≤23
	Tetracycline	≥23	19-22	≤18
S. aureus ATCC 25923	Penicillin	≥29	-	≤28
	Gentamicin	≥15	13-14	≤12

3. Results and discussion

3.1 Fermentation of soy protein isolate by Streptococcus

Streptococcus spp. is one of the main microorganisms found in the oral cavity (Nicolas and Lavoie, 2011). Figure 1 shows the viability of *Streptococcus* spp. in soy after fermentation. All strains showed a viable count of $>10^8$ CFU/mL in soy protein isolate solution upon fermentation. Viable counts of *S. salivarius* TUCC 1253-fermented soy was 0.8 - 1.1% higher ($p < 0.05$) compared to control *S. salivarius* K12 and ATCC 13419 -fermented soy. *S. salivarius* K12 and ATCC 13419 were used as control because they were previously studied by other studies.

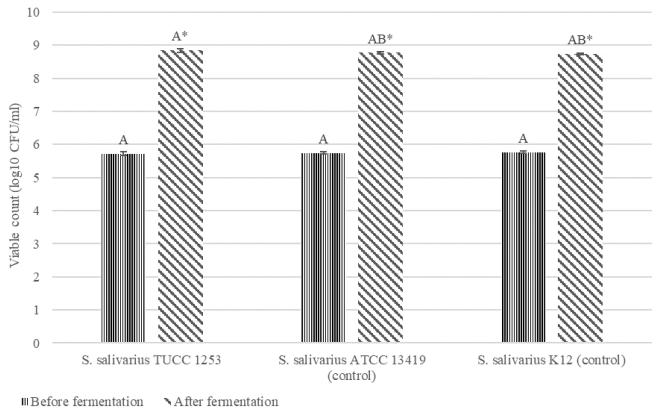

Figure 1. Viability of *Streptococcus*-fermented soy after fermentation at 37°C for 24 hrs (data: means, n = 6). AB means values between *Streptococcus*-fermented soy are significant different ($p < 0.05$) via one-way ANOVA. * means values are significant different ($p < 0.05$) via pair T-test.

As *Streptococcus* spp. originated from the oral cavity, it is desired to be used as an oral probiotic compared to foreign probiotics due to its benefit in adaptation, adhesion ability and exerting antagonistic effect against site-specific oral pathogens (Kaushik *et al.,* 2009; Maheshwari *et al.,* 2012). Although oral *Streptococcus* spp. are not commonly used for soy fermentation, previous studies have demonstrated that these species exert enzymatic activities such as b-glucosidase that allow bioconversion of isoflavones in soy into bioactive compounds which could exert beneficial effects towards host (Michlmayr and Kneifel, 2014).

Lactic acid-forming *Streptococcus* spp. uses nutrients in soy for growth and released by-products such as lactic acid during fermentation (Lu and Wang, 2017). Figure 2 shows that the pH level of all *Streptococcus*-fermented soy decreased ($p < 0.05$) significantly after fermentation. *S. salivarius* TUCC 1253 showed the highest viable count after soy fermentation which is in tandem with the reduction in pH level compared to control strains.

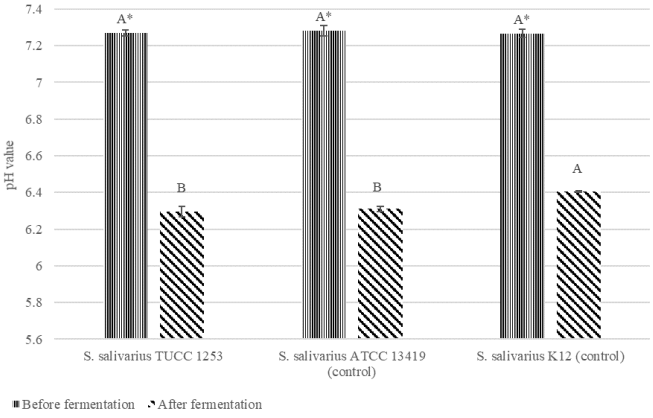

Figure 2. pH of *Streptococcus*-fermented soy at 37°C for 24 hrs (data: means, n = 6). AB means values between *Streptococcus*-fermented soy are significant different ($p < 0.05$) via one-way ANOVA. * means values are significant different ($p < 0.05$) via pair T-test.

The reduction of pH is due to the production of organic acid. This is proven by the results of titratable acidity (Figure 3). TA increased as the amount of organic acid increased. Figure 3 shows that TA of all *Streptococcus*-fermented soy was significantly increased ($p < 0.05$) after 24 hours of fermentation. *S. salivarius* TUCC 1253-fermented soy showed the greatest increase ($p < 0.05$) in TA after 24 hours fermentation. This was in tandem with the results for pH and viability as *S. salivarius* TUCC 1253-fermented soy showed the lowest pH level and highest ($p < 0.05$) viable counts after fermentation compared to control strains. This showed that *Streptococcus* spp. were able to metabolize the nutrients in SPI medium during fermentation.

Oral cavity's pH level is generally maintained

between 6.7 to 7.3 by saliva. Critical low pH (5.5 – 6.0) environment in the oral cavity will increase the risk of cavities, tooth demineralization and tooth decay (Stookey 2008). Considering the pH level of the fermented soy, no neutralisation procedure was needed for *S. salivarius* TUCC 1253-fermented soy before incorporating into the oral strip as pH level was above 6.0.

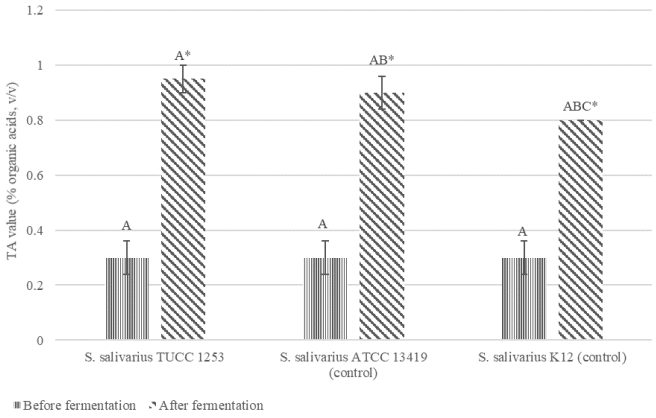

Figure 3. Titratable acidity (TA) of *Streptococcus*-fermented soy after fermentation at 37°C for 24 hrs (data: means, n = 4). ABC means values between *Streptococcus*-fermented soy are significant different ($p < 0.05$) via one-way ANOVA. * means values are significant different ($p < 0.05$) via pair T-test.

3.2 Enumeration of viable count of Streptococcus salivarius TUCC 1253 after freeze drying process and incorporation into oral strip

Viability of probiotic product is expected to sustain throughout the manufacturing process, transportation and storage to ensure sufficient amount of 10^6 to 10^7 CFU/ mL viable count of probiotics during consumption. *S. salivarius* TUCC 1253-fermented soy was freeze-dried in this study prior to incorporating into oral strip and its viability was reported in Table 5. Viability of *S. salivarius* TUCC 1253-fermented soy decreased significantly ($p < 0.05$) by 2.4 $\log_{10}$ CFU after freeze-drying.

Table 5 Viable count of *S. salivarius* TUCC 1253-fermented soy (with 20% v/v inoculum) after freeze drying

	Before freeze drying ($\log_{10}$ CFU/g)	After freeze drying ($\log_{10}$ CFU/g)
S. salivarius TUCC 1253-fermented soy	11.70±0.08*	9.28±0.06

Results are expressed as means ± SD, n = 6. * Significant different between after fermentation and after freeze drying ($p < 0.05$) via pair t-test

Freeze-drying is a process commonly used to preserve nutritional values, colour, taste, smell and texture of the food product. Freeze drying process could reduce viable counts of probiotics due to stress from cold shock (Iaconelli *et al.* 2015). However, Iaconelli *et al.* (2015) showed that freeze-drying is the best preservation method as it preserves bacterial cultivability, enzymatic activity, and revive growth functions after rewetting.

Table 6 shows that viable count of *S. salivarius* TUCC 1253-fermented soy after incorporating into oral strip decreased significantly ($p < 0.05$) by 4.1% compared to before incorporating into the oral strip. However, the final cell count for developed *S. salivarius* TUCC 1253-fermented soy oral strip was more than 8 $\log_{10}$ CFU/g, thus fulfil the requirement as probiotic product.

Table 5. Viable count of *S. salivarius* TUCC 1253-fermented soy (with 20% v/v inoculum) after incorporating into oral strip

	Before freeze drying ($\log_{10}$ CFU/g)	After freeze drying ($\log_{10}$ CFU/g)
S. salivarius TUCC 1253-fermented soy	9.28±0.06*	8.90±0.04

Results are expressed as means ± SD, n = 6. * Significant different between after fermentation and after freeze drying ($p < 0.05$) via pair t-test

Both mixing and air-drying process could contribute to the reduced in probiotic's viable count. Mixing process induced mechanical stress towards probiotic's cell membrane. On the other hand, bacterial cells are made up of 70 - 95% of intracellular water, hence air-drying changes the cells physiology and threatens the survivability of probiotic (Guergoletto 2012). Despite the dropped of viable count, the final viable count of *S. salivarius* TUCC 1253-fermented soy was sufficient to exert beneficial health for human. As the final viable count of *S. salivarius* TUCC 1253-fermented soy was stated in CFU/g, 2 strips (approximately 0.73 g) of *S. salivarius* TUCC 1253-fermented soy oral strip (at least 6 $\log_{10}$ CFU) were recommended to consume daily to achieve beneficial health effect.

3.3 ANOVA statistical analysis for response models

Different formulations of *S. salivarius* TUCC 1253-fermented soy oral strip were evaluated on tensile strength, percent elongation and moisture uptake as shown in Table 7. The responses were subjected to ANOVA analysis. Box-Cox plot was used to assist in transforming the data for response 1 and response 2 into different scales (Osborne, 2010).

ANOVA analysis between 2 factors (HPMC and propylene glycol concentration) against moisture uptake, percent elongation and tensile strength were reported

respectively in Table 8, Table 9 and Table 10. The model for all 3 responses fitted well as all the models' *p*-value were significant ($p < 0.05$). Quality of the fitted model was assessed by further evaluating some model descriptors. All 3 models had adjusted R^2 and predicted R^2 higher than 84.96%, which indicates high in goodness of fit and prediction power. Coefficient of variation (CV) represents the percentage ratio of standard deviation to mean for the overall model (Lim *et al.,* 2018). According to Cui (1989), CV less than 10% is good and less than 20% is acceptable in clinical research. This shows that models of response 1 and 3 showed good reliability (1.26% and 7.89%); while model of response 2 showed acceptable reliability (12.81%). Lastly, adequate precision represents the measure of signal to noise ratio, It is suggested that adequate precision should be more than 4 for better precision, which all models in our study had achieved with more than 34.68 suggesting a good model prediction (Lim *et al.,* 2018).

Table 7. Characterization tests of *S. salivarius* TUCC 1253-fermented soy oral strip with 13 formulation batches

Formulation code	Tensile Strength (N/mm^2)	Percent Elongation at break (%)	Moisture Uptake (%)
OS1	17.429±4.292	2.592±0.211	2.66±0.03
OS2	29.637±3.486	8.921±2.723	4.81±0.04
OS3	35.831±2.102	17.071±2.460	6.17±0.05
OS4	30.238±3.634	12.425±2.839	4.81±0.03
OS5	35.711±2.712	7.154±1.389	5.78±0.05
OS6	29.755±3.543	10.542±4.317	4.84±0.04
OS7	29.213±2.758	21.692±1.219	5.35±0.04
OS8	39.606±5.559	22.792±1.578	6.69±0.05
OS9	29.713±2.575	3.688±0.409	4.38±0.04
OS10	19.635±4.855	3.533±0.403	3.29±0.03
OS11	21.919±1.523	5.921±0.663	3.88±0.03
OS12	30.442±3.217	9.421±4.224	4.83±0.04
OS13	30.414±3.245	7.633±1.500	4.83±0.05

Table 8. ANOVA analysis of response 1 (moisture uptake) of *S. salivarius* TUCC 1253-fermented soy oral strip.

Source	SS[a]	*df*[b]	MS[c]	*F* value	*p* value[d] (prob>F)
Model	534.73	5	106.95	8646.67	< 0.0001
X_1	476.74	1	476.74	38544.6	< 0.001
X_2	57.77	1	57.77	4760.47	<0.001
X_1X_2	0.0214	1	0.0214	1.73	0.1923
X_1^2	0.0277	1	0.0277	2.24	0.139
X_2^2	0.2046	1	0.2046	16.54	0.0001
Residual	0.8782	71	0.0124		
Pure error	0.6163	60	0.0103		
Total	535.62	77			

[a]Sum of squares, [b]Degree of freedom, [c]Mean square, [d]Significant if $p < 0.05$. Adjusted $R^2 = 0.9984$; predicted $R^2 = 0.9980$; percentage of coefficient of variation = 1.26%; adequate precision = 295.3354.

Table 9. ANOVA analysis of response 2 (percent elongation at break) of *S. salivarius* TUCC 1253-fermented soy oral strip.

Source	SS[a]	*df*[b]	MS[c]	*F* value	*p* value[d] (prob>F)
Model	1.09	5	0.2179	96.90	< 0.0001
X_1	0.5429	1	0.5429	241.45	< 0.0001
X_2	0.4722	1	0.4722	210.03	< 0.0001
X_1X_2	0.0025	1	0.0025	1.11	0.2962
X_1^2	0.0351	1	0.0351	15.59	0.0002
X_2^2	0.0112	1	0.0112	4.98	0.0288
Residual	0.1596	71	0.0022		
Pure error	0.1006	60	0.0017		
Total	1.26	77			

[a]Sum of squares, [b]Degree of freedom, [c]Mean square, [d]Significant if $p < 0.05$. Adjusted $R^2 = 0.8632$; predicted $R^2 = 0.8496$; percentage of coefficient of variation = 12.81%; adequate precision = 34.6799.

Table 10. ANOVA analysis of response 3 (tensile strength) of *S. salivarius* TUCC 1253-fermented soy oral strip.

Source	SS[a]	*df*[b]	MS[c]	*F* value	*p* value[d] (prob>F)
Model	2831.27	5	566.25	106.65	< 0.0001
X_1	2721.07	1	2721.07	512.50	< 0.0001
X_2	62.18	1	62.18	11.71	0.0010
X_1X_2	0.5325	1	0.5325	0.1003	0.7524
X_1^2	42.83	1	42.83	8.07	0.0059
X_2^2	0.2489	1	0.2489	0.0469	0.8292
Residual	376.97	71	5.31		
Pure error	231.89	60	3.86		
Total	3695.33	77			

[a]Sum of squares, [b]Degree of freedom, [c]Mean square, [d]Significant if $p < 0.05$. Adjusted $R^2 = 0.8825$; predicted $R^2 = 0.8742$; percentage of coefficient of variation = 7.89%; adequate precision = 36.2380.

3.4 ANOVA analysis of response 1: effect of moisture uptake

A coded model equation was generated to reflect the mathematical relationship between HPMC and propylene glycol concentration on moisture uptake. The model equation of response 1 was transformed into a power scale to meet the normality assumption as stated below.

$$Y_1^{1.38} = 8.78 + 3.64X_1 + 1.27X_2 - 0.03X_1X_2 - 0.04X_1^2 + 0.11X_2^2$$

All model terms with the $p < 0.05$ were considered as significant. The factor that was significant with larger values of sum of squares and *F*-value; and smaller *p*-value in the model had a large impact on the response. Table 8 shows that 3 model terms (X_1, X_2, X_2^2) were significant factors. HPMC concentration (X_1) had the larger positive effect on moisture uptake with 89.0%

contribution. The increased of HPMC concentration caused increased by 2.93% in moisture uptake of the oral strip (Figure 4). This is because HPMC is a water-soluble polymer where it absorbs moisture when exposed to high relative humidity environment (Majumder *et al.,* 2016*)*. On the other hand, propylene glycol concentration caused only slight increase in moisture uptake of the oral strip (Figure 4).

3.5 ANOVA analysis of response 2: Effect of percent elongation at break

The mathematical equation was developed using obtained experimental data where it was transformed to inverse square root. The factors associated to percentage elongation at break of oral strip were described in coded terms below.

$$\frac{1}{\sqrt{Y_2}} = 0.34 - 0.12X_1 - 0.11X_2 + 0.01X_1X_2 + 0.05X_1^2 + 0.03X_2^2$$

Table 9 shows that 4 model terms (X_1, X_2, X_1^2, X_2^2) were significant. HPMC concentration showed a positive effect on percent elongation with 43.09% contribution. The percent elongation at break increased from 4.01% to 16.25% when HPMC concentration increased (Figure 5). This is because HPMC is a water-soluble polymer where it forms bond and trapped water molecules. As water is considered a natural plasticizer, it gives plasticizing effect and improves polymer chain movement making it more elastic and hence higher percent elongation (Lim *et al.,* 2018).

Besides, propylene glycol also had an impact and positive effect on percent elongation at break with 37.48% contribution. The percent elongation at break increased from 4.52% to 17.97% when propylene glycol concentration increased (Figure 5). Propylene glycol is a type of humectant plasticizer where it increased the free space between the polymer chain and allow polymer chain to increased movement and rotation, hence increased in percent elongation (Jantrawut *et al.,* 2017).

3.6 ANOVA analysis of response 3: effect of tensile strength

The mathematical equation was formed using gathered experimental data that explained the relationship between factors and tensile strength in coded terms below.

$$Y_3 = 29.88 + 8.69X_1 + 1.31X_2 - 0.15X_1X_2 - 1.61X_1^2 + 0.12X_2^2$$

There were 3 model terms (X_1, X_2, X_1^2) that were significant as shown in Table 10. HPMC concentration (X_1) was the most critical factor affecting tensile strength, with 73.64% contribution. Figure 6 shows that tensile strength increases by 88.82% as HPMC concentration increase. According to the study by Ismail *et al.* (2017), as the polymer concentration increased, the macro-voids (free space) between the polymer chain

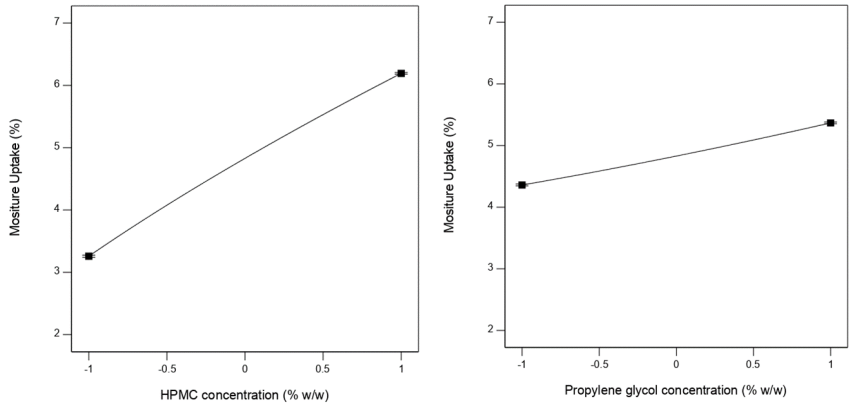

Figure 4. Effect of HPMC concentration (X_1) and propylene glycol concentration (X_2) on response 1 (moisture uptake).

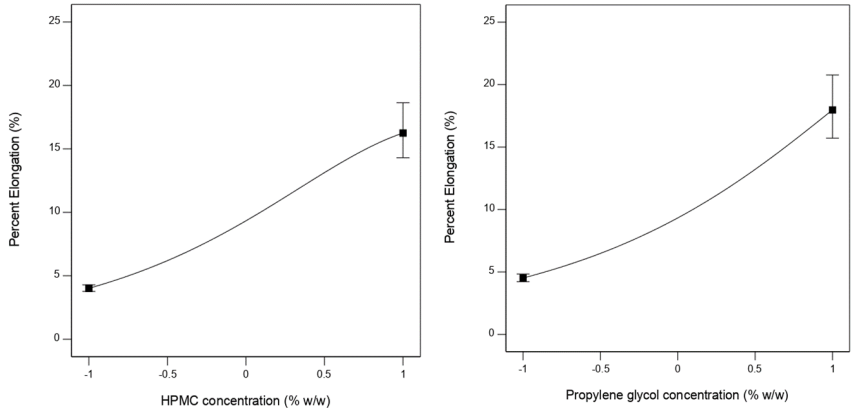

Figure 5. Effect of HPMC concentration (X_1) and propylene glycol concentration (X_2) on response 2 (percent elongation at break).

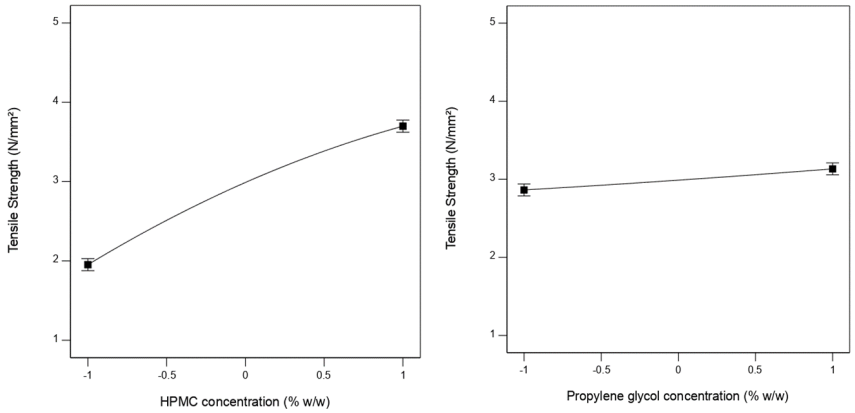

Figure 6. Effect of HPMC concentration (X_1) and propylene glycol concentration (X_2) on response 3 (tensile strength).

reduced, allowing polymer chain to be closer to each other and reduced in movement. Hence, the improved structure of polymer increased in tensile strength. Besides, propylene glycol only had a slight positive effect of 9.17% on tensile strength (Figure 6).

3.7 Optimization of Streptococcus salivarius TUCC 1253 -fermented soy oral strip

The three models generated using Box-Behnken design were used to carry out a multi-response optimization. Derringer's desirability function is an optimization tool used to provide the factors combinations which produced *S. salivarius* TUCC 1253-fermented soy oral strip with desirable responses (Shahabadi and Reyhani, 2014).

The optimization goal of our study is to formulate an oral strip that is physically stable throughout transportation and handling. In our study, moisture uptake goal was set at a minimum level; while tensile strength and percent elongation were set at a maximum level. The desirable coded level of HPMC and propylene glycol concentration given by software was -0.03 (47.43% w/w) and 0.55 (18.88% w/w), respectively.

Optimized *S. salivarius* TUCC 1253-fermented soy oral strip was developed (Figure 7) according to the formulation provided by the software. Experimental data of the 3 responses for optimized strip were collected and compared with the values of the predicted response provided by software (Table 11). The percentage error was not more than 4.04%. This shows that the optimization of *S. salivarius* TUCC 1253-fermented soy oral strip was successful, and the developed response models were accurately explained and predicted.

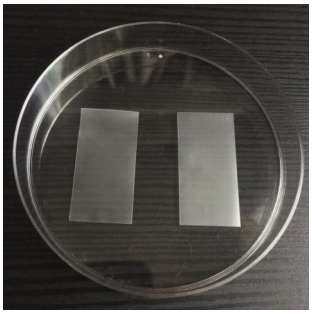

Figure 7. Samples of optimised oral strips containing *S. salivarius* TUCC 1253-fermented soy.

3.8 Antagonistic effect of optimized Streptococcus salivarius TUCC 1253-fermented soy oral strip against oral pathogens

Antagonistic tests were conducted in this study to analyse the effectiveness of *S. salivarius* TUCC 1253-fermented soy oral strip against oral pathogens. *E. faecalis*, *S. pyogenes* and *S. aureus* are facultative anaerobic oral pathogens that are commonly found in the oral cavity that cause oral infections such as caries, periodontitis, and endodontic infections (Pinheiro and Mayer, 2014, Komiyama *et al.,* 2016).

Antagonistic tests of *S. salivarius* TUCC 1253-fermented soy oral strip were carried out under aerobic and anaerobic conditions which mimic oral cavity domain. Inhibitory activities of *S. salivarius* TUCC 1253 -fermented soy oral strip and control antibiotics against oral pathogens were reported in Table 12. The oral strip containing *S. salivarius*-fermented soy showed ($p < 0.05$) inhibitory activity against all studied oral pathogens. *S. salivarius* TUCC 1253-fermented soy oral strip inhibited *E. faecalis*, *S. pyogenes*, and *S. aureus* under aerobic conditions more effectively ($p < 0.05$) than anaerobic conditions by 4.86%, 12.81%, and 11.32%, respectively.

S. salivarius TUCC 1253-fermented soy oral strip showed significantly higher ($p < 0.05$) inhibitory activity towards *E. faecalis*, *S. pyogenes*, and *S. aureus* compared

Table 11. Averaged experimental data and their respective percentage error based on predicted responses

Responses[a]	Predicted[b]	Experimental[c]	Error (%)
Y_1	5.078	5.0433	0.68
Y_2	13.3395	12.8	4.04
Y_3	30.4051	30.0323	1.23

[a]Responses for optimized film: Y_1, moisture uptake (%); Y_2, percent elongation at break (%); Y_3, tensile strength (N/mm^2). [b]Predicted responses by the model. [c]Averaged experimental data based on the calculated optimized solution.

Figure 6. Effect of HPMC concentration (X_1) and propylene glycol concentration (X_2) on response 3 (tensile strength).

	E. faecalis ATCC 700802		*S. pyogenes* ATCC 19615		*S. aureus* ATCC 25923	
	A	AN	A	AN	A	AN
S. salivarius TUCC 1253-fermented soy oral strip	$17.5\pm0.55^{cB*}$	16.67 ± 0.52^{cB}	$22.17\pm0.41^{aB*}$	19.50 ± 0.55^{bB}	$18.67\pm0.52^{bB*}$	16.67 ± 0.52^{cB}
Positive control antibiotic	23.67 ± 1.03^{A}	24.00 ± 1.10^{A}	$27.33\pm0.52^{A*}$	24.83 ± 0.41^{A}	$30.83\pm0.41^{A*}$	29.67 ± 0.52^{A}
Negative control antibiotic	13.50 ± 0.55^{C}	13.5 ± 0.55^{C}	$15.83\pm0.41^{C*}$	13.33 ± 0.52^{C}	$11.33\pm0.52^{C*}$	9.83 ± 0.41^{C}

A: aerobic conditions; AN: anaerobic conditions. Results are expressed as means ± SD, n = 6. ^{abc} means in the same row for *S. salivarius* TUCC 1253-fermented soy oral strip followed by different lowercase letters are significant different ($p < 0.05$) via one-way ANOVA. ^{ABC} means in the same column followed by different uppercase letters are significant different ($p < 0.05$) via one-way ANOVA. * means in the same row between A and AN condition are significant different ($p < 0.05$) via independent T-test. The size of the oral strips is 2 x 4 cm (width x length).

to negative control antibiotic under both conditions. On the other hand, the oral strip showed significantly lower ($p < 0.05$) inhibitory activity towards all studied oral pathogens compared to positive control antibiotic (pathogen showed susceptible) under both conditions.

Bacteriocins such as salivaricin A and salivaricin B were reported to be released by *S. salivarius* to inhibit pathogens such as *S. pyogenes*, *E. faecalis* and *S. aureus* (Barbour *et al.,* 2016). Besides, aglycones (daidzein and genistein) produced by probiotic-fermented soy were reported to exert antimicrobial properties (Verdrengh *et al.,* 2004). Thus, the antimicrobial activity of the oral strips could be a combination effect of fermented soy and probiotics.

Antibiotics were usually prescribed by dentists to treat oral infections. However, antibiotics are reported to cause side effects such as gastrointestinal discomfort and pathogens had developed increasing resistance on over-used antibiotics (Dar-Odeh *et al.,* 2010). In our study, *S. salivarius* TUCC 1253-fermented soy oral strip had shown to exert antagonistic effect against all studied oral pathogens under aerobic and anaerobic environment, which makes a good alternative for the prevention of oral infections. In addition, probiotics oral strips can make a good preventive approach via restoration of oral microbial ecology by increasing good bacteria and reducing pathogens.

4. Conclusion

S. salivarius TUCC 1253-fermented soy oral strip in this study had been successfully optimized and showed potential in improving oral health. To date, this is considered the first study on incorporating *S. salivarius*-fermented soy into oral strip to improve oral health. Future studies could study on the antioxidant and immunomodulatory properties of *S. salivarius*-fermented soy. Besides, in-vivo studies could also be carried out in future studies to explore the effectiveness of *S. salivarius* TUCC 1253-fermented soy oral strip in improving oral infections on human subjects.

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