ANTI-BIOFILM ACTIVITY OF FERMENTED SOYBEAN TEMPEH EXTRACTS AND FRACTIONS AGAINST ORAL PRIMARY COLONIZER BACTERIA

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ABSTRACT

In early dental plaque formation, oral primary colonizers, *Actinomyces viscosus* is initially attached to the pellicle-coated tooth surface to form a biofilm. Tempeh is a Southeast Asian traditional food produced from fermented soybean. This study, for the first time, investigated the *in vitro* inhibitory effects of tempeh extracts and fractions in removing oral primary biofilm on a polystyrene 96-well microtiter plate. Initial cell attachment was evaluated in this study after treatment with tempeh extracts and fractions at various concentrations (0 to 3 mg/mL). The growth and development of the biofilm was assessed using the crystal violet assay. Anti-biofilm activity of tempeh extract and fractions were measured as the percentage of the biofilm inhibition after the treatment in comparison with the untreated control. Following fractionation of chloroform extract, the resulting hexane fraction was found to be active against *A. viscosus*. Data from colorimetric assay were confirmed by scanning electron microscopy (SEM) analysis. SEM analysis revealed significant reduction in biomass, as well as decrease of total cell volume and thickness when intact *A. viscosus* biofilms were treated with chloroform extract and hexane fraction. These results suggest that soybean tempeh is a promising source of bioactive compounds that can be used as natural ingredients for future development of oral care products targeting biofilm formation.

Keywords: *Actinomyces viscosus*, anti-biofilm activity, tempeh, fermented soybean, oral bacteria

Introduction

Dental plaque biofilms exist via the attachment, detachment and proliferation of oral bacteria on the tooth surface. This is primarily associated with adhesion of bacteria to pellicle coating the oral surface (Steinberg D. 2000). Biofilms are well known as an initiator for primary dental diseases (Takahashi N. et al., 2011) by safeguarding the bacteria against the action of antibiotics and antimicrobial agents through the matrix barrier (Costerton W. et al., 2003). Taking into account that adherence is essential for colony formation; preventing bacterial adhesion would appear to be a logical approach for reducing biofilm formation (Kostakioti et al., 2013). If this adhesion could be disrupted by food products, the bacteria would not be able to colonize nor cause infection (Nataro and Kaper, 1998; Nagy and Fekete, 2005). The efficient tooth colonizer, *A. viscosus* is a Gram positive bacterium primarily adhering to dental surface contributing to oral biofilm development at initial stages (Socransky et al. 1977; Theilade et al. 1982; Nyvad and Kilian 1987; Gibbons 1989; Marsh and Martin 1992). Therefore, the reduction of initial *A. viscosus* biofilm development is an effective way to prevent plaque related periodontal diseases.

Forasmuch as, biofilm forming bacteria are more resistant to antimicrobial agents (Stewart and Costerton, 2001), developing new strategies to control this variegated mode of bacterial life is greatly challenging (Trentin et al., 2013). In recent years, plant products which may be bioactive against cariogenic and periodontopathogen have been of great interest to the researchers in the field of oral biofilm control, mainly due to their few after-effects. Plant phenolic compounds which already revealed their antimicrobial and anti-biofilm activities (Slobodníková, Livia et al., 2016) have brought hope.

It has become a hot spot for researchers to delve into traditional fermented food products that can control biofilm formation. A shift of interest has been recently experienced with anti-biofilm activity from fermented foods of plant origin such as red wine against oral *Streptococcus mutans* biofilm (Maria Daglia et al., 2010), *Lactobacilli* extracts from traditional Tunisian fermented food against *Listeria monocytogenes* biofilm (Ben Slama et al., 2013), *Lactobacillus* strains isolated from Sorghum-based traditional fermented food active against *Pseudomonas aeruginosa* and *Klebsiella pneumonia* biofilm (Rao et al., 2015), lactic acid bacteria isolated from barley, traditional dried meat and fermented olive were characterized for their anti-biofilm activities against oral bacteria (Fadia Ben Taheur et al., 2016), Kimchi - a traditional Korean fermented food showed potential for invasion protection against *Pseudomonas aeruginosa* (Khan and Kang, 2016) and loads more.

With this background, we began anti-biofilm studies of tempeh, fermented food from soybeans by fungus, *Rhizopus oligosporus*. Tempeh is an emblematic traditional food from Indonesia and is a commonly available in Malaysia. During fermentation, the soybeans are bound together by the mycelium of the mould into a compact cake and macronutrients are enzymatically degraded. Interest in the potential of soybeans as an attractive functional food product has been on the rise for daily consumption due to its increased nutritive value during fermentation (Murata K et al., 1967).

In regard to tempeh, it has already been documented to exert various microbial activities (Murakami et al., 1984; Sutardi and Buckle 1985; Keuth and Bispington, 1993; Kiers et al., 2002; Tsangalis et al., 2002; Roubos et al., 2008; Haron et al., 2010; Haron et al., 2011). Tempe also has been shown to inhibit ETEC infection in rabbits (Karyadi et al., 1990; Karmini et al., 1997). Kiers et al. (2002) focused on ETEC adhesion to piglet brush border cells, as affected by tempeh. Moreover, Roubos van den Hill et al. (2009) confirmed the interference of tempeh toward Enterotoxigenic *Escherichia coli* adhesion for intestinal epithelial cells to intestinal epithelial cells of pig and human origin, Caco-2 cells. A study by Mo et al. (2011) comparing the anti-adhesive activity of tempeh and tofu from soybean origins disclosed that both have an anti-adhesive activity against *Escherichia coli* *in vitro*. In addition to it, recent study by Kuligowski M. et al. (2013) has successfully evaluated the antibacterial properties of soybean tempeh that has influence on human intestine bacteria with the use of an *in vitro* digestion model. This result strengthened previous observation of the anti-adhesion properties of soybean tempeh.

It is noteworthy that the crude tempeh does exert its anti-adhesion effect against wide range bacteria species. This study was therefore undertaken to investigate for the first time the *in vitro* anti-biofilm potential of tempeh against *A. viscosus* biofilms with the use of microtiter model.

Materials and Methods

Preparation of tempeh sample

Commercial tempeh samples were purchased from a cottage industry producer in Sepang, Selangor DE, Malaysia. The tempeh blocks were lyophilised to obtain a constant mass and the percentage of water and solid content were calculated. Then, the tempeh was ground to a fine powder using a mechanical blender. The dry weight was determined, and the powder was stored in an air-tight container at -20 °C until further use.

Bacterial strains and culture conditions

The oral microorganism used in this study is *Actinomyces viscosus* (ATCC 43146). This strain was cultured in brain heart infusion (BHI; Merck, Germany) broth under anaerobic condition in a pack-rectangular anaerobic jar (Mitsubishi Gas Chemical Co.Inc, Japan), 37 °C for 16-24 h and sub-culturing was done twice weekly. For growth curve analysis, *A. viscosus* were grown as described above, was diluted in BHI to obtain a final bacterial optical density measurement at 600 nm (OD_{600 nm}) of 0.1 McFarland Standard. The cultures were then incubated at 37 °C overnight.

Sequential solvent extraction and fractionation

The tempeh powder was sequentially extracted with a series of solvents differing in polarity. First, it was extracted using hexane (1: 10 w/v) at room temperature using incubator shaker at the speed of 200 rpm for 24 h. After overnight extraction, the extract was vacuum filtered. The residue from the filtration was air-dried and used in subsequent extraction with dichloromethane, chloroform, ethyl acetate, methanol and sterile water, respectively. The extracts obtained were concentrated in a rotary evaporator (Büchi, Switzerland) at 40°C followed by oven-drying to yield hexane (HE), dichloromethane (DcmE), chloroform (ChE), ethyl acetate (EaE) and methanol (ME) extracts. Water extract (WE), was lyophilized until a constant mass was achieved.

By using a separatory funnel, ChE suspended in 100 mL of distilled water was sequentially partitioned with hexane, diethyl ether and ethyl acetate. This produced the following fractions: hexane (HF), diethyl ether (DeeF), ethyl acetate (EaF) and water (WF). All extracts and fractions were dissolved in 100% dimethyl sulfoxide (DMSO) except WE and WF were dissolved in distilled water. For bioassays, DMSO percentage of the samples was adjusted to a final concentration of 10% (v/v).

Biofilm inhibition assay

The effect of tempeh extracts and fractions on *A. viscosus* biofilm formation was measured by the method modified from O'Toole, G. A. (2011). Overnight culture of *A. viscosus* was diluted in BHI broth to obtain an OD₆₀₀ of 0.1. The wells of a sterile 96-well flat bottom tissue culture plate, each contained 100 µL of final concentrations of extracts/ fraction (0–3 mg/ml) from stock solution of 100 mg/ml, with 100 µL of an overnight culture of bacteria, were incubated for a biofilm initiation. One hundred microlitres of water and vancomycin (100 µg/ml) in sterile BHI broth were added as negative and positive controls, respectively. For quantitative assays, we typically use triplicate wells for each treatment. Un-inoculated BHI broth was incorporated as a negative control. Following incubation, the crystal violet staining assay was performed to assess biofilm biomass.

Crystal violet staining assay

Cell attachment was indirectly assessed using the crystal violet (CV) staining. Following incubation, the plates were washed three times with sterile distilled water to remove unbound cells. The plates were air-dried. Then the generated biofilm was fixed with 10% formaldehyde, and washed again. Following drying, the wells were stained with 100 μ l of 0.1% crystal violet and incubated at room temperature for 15 min after which the plates were washed three times with sterile distilled water to remove unabsorbed stain. Following 1 h of incubation, the CV was washed twice with distilled water to remove the unbound crystal violet and the stained biofilms were dried at room temperature. Next, 200 μ L of isopropanol was added to each well to dissolve the dye on the biofilm. The plate was rocked at room temperature for 10 min, and the absorbance was read at 600 nm using microplate reader (FLUOstar Omega, BMG LabTech).

The results expressed as percentage inhibition (Eqn 1) against the extract concentrations

Percentage inhibition [1]

$$= \frac{OD_{\text{Negative control}} - OD_{\text{Experimental}}}{OD_{\text{Negative control}}} \times 100$$

The mean absorbance (OD_{600 nm}) of the samples was determined, and percentage inhibition obtained (Eqn 1).

Statistical analysis

All experiments were conducted in triplicates. The differences in mean values among different groups were tested and the values are expressed as mean $\pm$ standard errors (SE). Statistical analysis was carried out using Microsoft Excel. Data obtained analyzed using Student's t-test. The significance of results was set at $p < 0.05$ (*) and $p < 0.01$ (**).

Scanning electron microscopy (SEM) analysis

The structure of *A. viscosus* biofilms formed after treatment with ChE and HF, as well the untreated control, respectively, was observed by SEM modified from Yutao et al., 2016. Briefly, 100 μ L of overnight-grown *A. viscosus* suspension diluted in fresh BHI at an initial OD₆₀₀ of 0.1 and 100 μ L BHI broth with HF (final concentration 3 mg/ml) were added. Glass coverslips (4 mm in diameter) were prefixed in each well. After incubation for 24 h, the biofilm-coated glass coverslips were immersed in 2.5% glutaraldehyde at 4°C overnight, washed three times with distilled water and dehydrated using ascending graded series of ethanol (30%, 50%, 70%, 80%, 90%, and 100%). The samples were then oven dried. Subsequently, the dried samples were adhered onto double-sided adhesive conductive carbon tape (which was mounted on a copper stage). The samples were then coated with platinum (JEOL JFC-1600 Auto Fine Coater) before viewing by SEM (JSM-6701F, JEOL, Japan).

Results and Discussion

Extraction yield of soybean tempeh

The solvent extraction of bioactive compounds from plants has been widely investigated. Different types of solvents produce different extraction yields. The difference in polarities of the extraction solvents may influence the solubility of the chemical constituents in a sample and thus the extraction yield (Lapormic B et al., 2005; Turkmen N et al., 2006). The yield of crude extracts as shown in Table 1 was calculated based on the 60g of dried and ground tempeh powder initially used for solvent extraction. Yield was calculated using the formula in Eqn 2.

$$\text{Yield (\%)} = \frac{\text{Dry weight of solvent extract (g)}}{\text{Dry weight of tempeh powder used (g)}} \times 100 \%$$

Polar solvents extract polar compounds while non-polar solvents extract non-polar compounds (Handa SS et al., 2008). The yield of HE (18.8 %) was the highest while EaE gave the lowest (1.78 %). This suggests that soybean tempeh contained more non-polar than polar compounds. Higher yield achieved by hexane, dichloromethane and chloroform could be explained by their ability to dissolve more non-polar lipophilic compounds (Cosa P et al., 2006). According to De Reu et al., 1994, the concentration of free fatty acids increased in the final product of soybean fermentation (De Reu et al., 1994). Thus, it can be deduced that 27% out of total recovery yield was non-polar nature due to the higher presence of fatty acids in tempeh sample.

The yield of solvent fractions derived from ChE was calculated based on 4g of crude ChE (Table 2). HF was the most abundant extract with 86% yield which was 7 fold higher than the yield of DeeF. No recovery of samples was found for EaF and WF. This suggests that ChE mostly consisted of non-polar compounds. The repeated hexane extraction step on concentrated ChE was crucial to acquire a mixture of volatile oils, fats and waxes (Houghton and Raman, 1998). According to Oufnac et al., 2007, in partitioning process alkaloids, aglycones and volatile oils will be forced into the hexane layer while the water layer embodies water-soluble carbohydrate polymers, glycosides and amino acid. Thus, the HF expected to have mixture of volatile oils, and lipid soluble compounds.

Anti-biofilm activity of tempeh extracts and fractions on A. viscosus

Biofilms are notoriously difficult to eradicate because of host defence and inherent resistance to antibiotics and biocides. Furthermore, there are several mechanisms used to explain the resistance of biofilms to antimicrobials (Sillankory S et al., 2003), which makes it difficult to predict the behavior of biofilm cells. In the present study, the initial cell attachment with and without tempeh treatment was evaluated after 24 h and the percentage of *A. viscosus* biofilm inhibition was plotted. By comparing the values obtained, it can be seen that tempeh concentrations significantly affected the removal and/or inactivation of biofilm matrix ($p < 0.05$). To the best of our knowledge, the effect of tempeh extract on the *A. viscosus* oral biofilm has not yet been documented.

In regards to *A. viscosus*, only the ChE showed anti-biofilm effect at the four tested concentrations (0.375, 0.75, 1.5 and 3 mg/ml) while the HE and DcmE showed moderate anti-biofilm activity as illustrated in Figure 1, unlike the EaE, ME and WE which exhibited no such activity even at the

highest concentration of 3 mg/ml (Data not shown). Thus, ChE was chosen candidate to be further partitioning. Figure 2 shows that the biofilm of *A. viscosus* was inhibited by HF at the two highest concentrations used (1.5 and 3 mg/ml). In this study, we identified that the biofilm of *A. viscosus* was greatly inhibited by ChE and HF, and this effect was dosage dependent. The success of ChE and HF in inhibiting cell attachment as shown in this study is a promising tool for reducing bacterial colonization and plaque development subsequently leads to periodontal diseases.

It is well known that any kind of agent earmark bacterial viability can be expected to urge selective pressure on the development of antimicrobial resistance. In contrast, repression by natural compounds of bacterial virulence factors that do not affect bacterial growth has advantages such as preserving the host indigenous microflora with less selective pressure on the development of bacterial resistance (Clatworthy A.E et al., 2007; Rasko D.A et al., 2010; Yamasaki S et al., 2011). In the present study, none of the tempeh extracts/ fractions inhibited the growth of *A. viscosus*. We therefore conclude that the inhibition of adhesion by ChE and HF cannot have been caused by antibacterial activity on *A. viscosus*, but of an interaction between the extracts and the bacteria, resulting in a loss of adhesion capability.

Incubation of *A. viscosus* with ChE and HF reduced adhesion strongly. Washing of the bacteria, which removed non-bound extracts did not restore the adhesion, which suggested a strong interaction between *A. viscosus* and component(s) in the tempeh extracts, which was not lost by washing steps. According to Cerca et al., 2005, it is easier for plant extract to interfere initial cell attachment than inhibiting the growth of preformed biofilm. On the other hand, a notable progress in biofilm exopolymer observed with EaE, ME and WE in this assay may be because of the presence of certain compounds within the extracts that provided a conditioning film promoting microbial adhesion. The enhancement effect observed in this study complements the study reported elsewhere that some natural compounds promote the growth of bacteria (Ofek et al.2003; Sandasi et al. 2008).

SEM analysis on monobiofilm morphology and cellular thickness

The morphology alteration and cellular thickness of *A. viscosus* biofilm was observed by SEM. As is shown in Figure 3, the biofilm matrix was recorded relatively thick in control while in the ChE and HF treated sample, thickness was reduced up to 67% and 90%, respectively. As in figure 4, the biofilm integrity and structure in ChE and HF treated sample is gradually being disrupted while homogeneous biofilm was formed in untreated control. Treated *A. viscosus* biofilm became very sparse and could not cover the surface of the glass slips, and the bacteria cluster became much smaller compared with the control group.

Results of the present study clearly demonstrated that ChE and HF could inhibit the initiation of *A. viscosus* biofilm exopolymer. The possible explanation could be given is that the active extract may form a coating film on the bacterial surface, or it occupy the specific fimbriae available binding sites, so that they cannot adhere anymore to the tooth pellicle, but instead aggregate into clumps of bacterial cells. Congregate of bacteria were indeed observed via microscopically. This experiment demonstrated that higher concentrations of samples are required to significantly inhibit biofilm cells. This is an expected result since bacteria in the biofilm are strongly protected and less susceptible to antimicrobial agents than in planktonic form.

Therefore, we suppose it can logically reduce oral biofilm incidence rate for susceptible population. If this speculation is correct, tempeh extracts may be used as an integrated drug in oral hygiene care to

prevent and cure the common oral diseases, including dental caries, gingivitis, periodontal disease, and oral ulcer. To confirm our speculation, more clinical studies of soybean tempeh need to be conducted.

Conclusion

It is the first study to identify the inhibitory effects of tempeh on *A. viscosus* oral biofilm formation. The results of this study show potential anti-biofilm activity for tempeh extracts as they are able to inhibit the initial stage of biofilm formation and subsequent growth. The SEM images obtained revealed significant morphological changes and cellular thickness to the bacterial biofilm. However, further studies should be considered, for example, the effects on other cariogenic bacteria, and the clinical study on dosage, efficacy, and cytotoxicity effects of soybean tempeh.

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Appendix

Table 1. The weight and percentage yield of crude extracts from tempeh.

Crude extract	Weight (g)	Yield (%)
HE	11.25	18.8
DcmE	3.05	5.08
ChE	2.15	3.58
EaE	1.07	1.78
ME	3.35	5.58
WE	2.40	4.00

Table 2. The weight and percentage yield of fractions partitioned from ChE.

Fraction	Weight (g)	Yield (%)
HF	3.44	86.0
DeeF	0.50	12.5
EaF	0.0	-
WF	0.0	-

Figure 1: Percent inhibition of biofilm formation exhibited by dichloromethane (DcmE), hexane (HE) and chloroform (ChE) extracts of tempeh against *A. viscosus* biofilm. Average data (n=3) are presented with SE. A value of $p < 0.05$ (*) or $p < 0.01$ (**) was performed by Student's t-test and compared to control group without treatment.

Figure 2: Percent inhibition of biofilm formation exhibited by diethyl ether (DeE) and hexane (HF) fractions against *A. viscosus* biofilm. Average data (n=3) are presented with SE. A value of $p < 0.05$ (*) or $p < 0.01$ (**) was performed by Student's t-test and compared to control group without treatment.

Figure 3: Effect of ChE and HF on monobiofilm thickness profile (μm). *A. viscosus* was incubated for 24 h in BHI broth, with ChE (B) and HF(C) in a concentration of 3 mg/ml. The untreated control (A) group was also conducted via vertical view SEM imaging, under magnification X5000.

Figure 4: Effect of HF on biofilm morphology. *A. viscosus* was incubated for 24 h in BHI broth, with ChE (B) and HF (C) in 3 mg/ml. The untreated control (A) was also conducted under X500, X2000 and X5000.