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Nurul Alia Risma Rismayuddin, Syarifah Nurhikmah Izzati Syed Nasarudin, Nurul Ruziantee Ibrahim, et al.

Synbiotic *Smallanthus Sonchifolius* (Yacon) and *Streptococcus Salivarius* Inhibit *Candida Albicans* Biofilm Formation

Nurul Alia Risma Rismayuddin[1, 2, a)], Syarifah Nurhikmah Izzati Syed Nasarudin[1, 3, b)], Nurul Ruziantee Ibrahim[1, 4, c)], Ali Alnuaimi[5, d)], Mohd Hafiz Arzmi[1, 2, e)]

[1]*Cluster of Cancer Research Initiative IIUM (COCRII), International Islamic University Malaysia, Kuantan Campus, 25200 Kuantan, Pahang, Malaysia.*
[2]*Department of Fundamental Dental and Medical Sciences, Kulliyyah of Dentistry, International Islamic University Malaysia, Kuantan Campus, 25200 Kuantan, Pahang, Malaysia.*
[3]*Department of Biomedical Sciences, Kulliyyah of Allied health Sciences, International Islamic University Malaysia, Kuantan Campus, 25200 Kuantan, Pahang, Malaysia.*
[4]*Department of Oral Maxillofacial Surgery and Oral Diagnosis, Kulliyyah of Dentistry, International Islamic University Malaysia, Kuantan Campus, 25200 Kuantan, Pahang, Malaysia.*
[5]*La Trobe Rural Health School, La Trobe University, 3086 Victoria, Australia.*

[a)]*aliarismayuddin@gmail.com*
[b)]*syarifahnurhikmah951@gmail.com*
[c)]*drnurulruziantee@iium.edu.my*
[d)]*doctorali74@gmail.com*
[e)]*Corresponding author: hafizarzmi@iium.edu.my*

Abstract. Oral biofilms are comprised of extracellular polysaccharides and polymicrobial microorganisms. The objective of this study was to determine the effect of synbiotic *Smallanthus sonchifolius* (yacon) with *Streptococcus salivarius* K12 (K12) on polymicrobial biofilm formation of *Candida albicans* with the hypothesis that polymicrobial biofilm biomass of *C. albicans* is inhibited by synbiotic *S. sonchifolius* with K12. Initially, disk diffusion and well diffusion assay were conducted to determine the susceptibility of *C. albicans* towards *S. sonchifolius* and K12. Following that, *C. albicans* was mixed with *S. salivarius* in nutrient broth (NB) or RPMI-1640 to determine the effect of probiotic on the polymicrobial biofilm. To determine the effect of synbiotic, similar protocol was repeated by adding 800 mg mL-1 of *S. sonchifolius* aqueous extract extract into the same followed by a 72 h incubation. Finally, biofilm biomass was measured using a crystal violet assay. *C. albicans* ATCC MYA-4901, ALC2 and ALC3 were found to be susceptible to *S. sonchifolius* extract and *S. salivarius* K12. However, the biofilm of all of *C. albicans* strains ATCC MYA-4901, ALC2 and ALC3 were found to reduce ranged in between 20% to 39.4% when co-cultured with synbiotic compared to prebiotic culture in NB. In conclusion, synbiotic *S. sonchifolius* with K12 inhibit polymicrobial biofilm. This indicates the potential use of synbiotic in dental application for the prevention *C. albicans* infection.

INTRODUCTION

Probiotics are living microorganisms that, when given in an adequate amount, bring a health benefit to the host. Probiotics have been reported to promote oral health by preventing periodontal illnesses, oral cavities, halitosis, and oral candidiasis [1]. *Streptococcus salivarius* is a normal inhabitant of the human oral cavity and gut, and its effect on oral health was previously discussed [2–4]. The murine experimental oral candidiasis also showed that *S. salivarius*

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K12 was dose-dependent in protecting against severe fungal infection. In addition, prebiotics is oligosaccharides, non-digestible carbohydrates commonly used to improve and stimulate a balanced microbiome [5].

Smallanthus sonchifolius (yacon) is an underutilised plant consumed as a traditional root-based fruit in South America and is mainly known as an abundant source of fructooligosaccharides (FOS) [6]. FOS are considered prebiotics, and *S. sonchifolius* FOS prebiotic effects have been demonstrated in vitro, showing that they were selectively fermented by *Bifidobacteria* and *Lactobacilli*.

The growth and activity of probiotics are enhanced by non-digestible oligosaccharides, namely, prebiotics [7-8], which are unable to be digested by the host but do enhance the beneficial effects of probiotics by selectively stimulating the growth and activities of the probiotics [9]. Thus, the combination of these two is known as synbiotic. A previous study has also demonstrated synbiotics' action in suppressing oral pathogens without interfering with a safe oral environment where it neutralises the growth of oral pathogenic microorganisms [7].

The microbiome in the oral cavity is a group of microorganisms that play an important role in the normal oral physiological system [10]. However, oral pathogenesis can occur under conditions where the oral surroundings are imbalanced (also known as dysbiosis) [11]. About 700 kinds of microorganisms inhabit the human oral cavity. *C. albicans* is part of a normal microbiome and does not cause any harm [12]. However, when the host defences are compromised, they can become pathogenic and cause serious problems [13]. *C. albicans* can cause oral candidiasis due to an overgrowth of the pathogenic fungus in immunocompromised individuals under several situations, for instance, in HIV-infected patients [14]. Therefore, maintaining an optimum environment in the oral cavity is essential for a healthy oral microbiome.

This study aimed to determine the effect of synbiotic *S. sonchifolius* with *S. salivarius* K12 on polymicrobial biofilm formation of *C. albicans* with the hypothesis that the synbiotic *S. sonchifolius* and *S. salivarius* K12 inhibit polymicrobial biofilm.

MATERIALS AND METHODS

Growth of Microorganisms

C. albicans American Type Cell Culture (ATCC) MYA-4901, genotype B isolated from HIV patient (ALC2), and oral cancer isolate (ALC3) were used in this study (Fig. 1). *C. albicans* strains were revived in yeast peptone dextrose (YPD) broth (Difco, USA) and incubated at 37°C aerobically for 24 h. To grow bacteria, stock cultures of *S. salivarius* K12 were revived by sub-culturing onto brain heart infusion (BHI) broth (Difco, USA). The agar plates were incubated at 37°C for 48 h.

Well Diffusion Assay

Well diffusion assay was carried out to determine the susceptibility of *C. albicans* towards *S. salivarius* K12 and *S. sonchifolius* [15]. In brief, a sterile swab was used to transfer *C. albicans* colonies onto the Mueller Hinton agar plates illustrated in (Fig.1). Following that, a five-millimetre diameter hollow tube was used to prepare the wells on the agar. Later, 100 µL of supernatant of probiotic *S. salivarius* was added into the wells. All the samples were tested in three biological replicates. Finally, the zone of inhibition of *C. albicans* was measured after 18 h incubation at 37˚C. A similar protocol was repeated by replacing *S. salivarius* with 100 µL of 800 mg mL-1 of *S. sonchifolius* water-based extract [16].

Static Biofilm Formation

Biofilm formation was analysed under static conditions by using a quantitative assay according to a previously published protocol [17]. *C. albicans* and *S. salivarius* K12 were grown on YPD and BHI agar, respectively, for 24 h at 37°C (Fig.1). Several single colonies of *C. albicans* and *S. salivarius* K12 were resuspended in nutrient broth (NB) and RPMI-1640 to an absorbance of 0.5 at 620 nm wavelength (OD_{620nm}) to standardised to a final density of 10^5 cells mL^{-1} and 10^7 cells mL^{-1}, respectively. To determine the effect of *S. salivarius* K12 on polymicrobial biofilm of *C. albicans*, the bacterium and the yeast were mixed thoroughly using a vortex mixer for 30 sec. To determine the effect of synbiotic to *C. albicans* polymicrobial biofilm, a similar protocol was repeated by adding 800 mg mL-1 of *S. sonchifolius* extract into each well of a sterile 96-well plate. The plates were incubated for 72 h at 37°C to mimic the

dynamic of the oral environment. The medium was replenished aseptically every 24 h. Mono-culture *S. salivarius* biofilm was also developed as the negative control.

Crystal Violet Assay

Crystal violet (CV) assay was performed according to the protocol outlined by the previously established study [18]. Initially, the biofilm in each well of the 96-well plate was washed twice with sterile PBS to remove non-adherent cells (Fig. 1). 200 µL of methanol was added to each well for fixation and incubated for 15 min at 25°C. The supernatant was then discarded, and the plate was air-dried for 45 min. 200 µL of 0.1% (w/v) CV solution was added into each well and incubated for a further 20 min at 25°C. The plate was washed gently twice using running distilled water, and 200 µL of 33% (v/v) acetic acid was added to de-stain the biofilm. The plate was incubated for five minutes at room temperature. A 100 µL aliquot of this solution was transferred to a new sterile 96-well plate, and the absorbance was measured at $OD_{620\ nm}$ using a microtiter plate reader (TECAN Sapphire, M200 Pro).

Statistical Analysis

All experiments were conducted in three biological and three technical replicates (N=9). Using SPSS software version 25.0, all data were statistically analysed using One-way analysis of variance (ANOVA) associated with post hoc Dunnet and Tukey's test (Fig. 1). This test was used to compare biofilm biomass between *C. albicans* strains in prebiotic *S. sonchifolius* and synbiotic *S. sonchifolius* with K12. Independent T-test was conducted to compare biofilm biomass between prebiotic and synbiotic. Data were considered significant when $P < 0.05$.

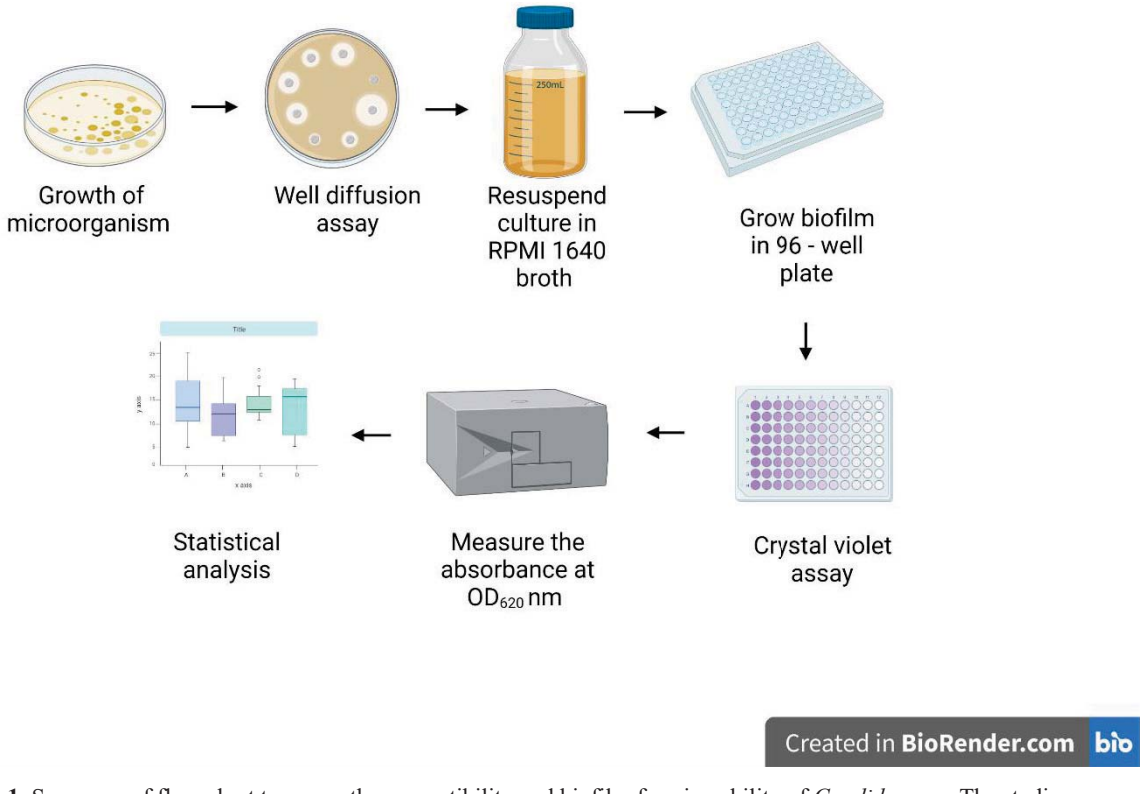

FIGURE 1. Summary of flow chart to assess the susceptibility and biofilm forming ability of *Candida* spp. The studies were conducted in three biological and three technical replicates.

RESULTS AND DISCUSSION

Antifungal Activity of Prebiotic *S. Sonchifolius* and Probiotic *S. Salivarius* K12

S. sonchifolius aqueous extract showed no antifungal activity against all *C. albicans* strains when tested using the well diffusion assay. In addition, American Type Cell Culture (ATCC) MYA-4901, genotype B isolated from HIV patient (ALC2), and oral cancer isolate (ALC3) *C. albicans* were also resistant towards probiotic *S. salivarius* K12 supernatant when tested using the well diffusion assay. This data indicated that *C. albicans* is resistant towards prebiotic *S. sonchifolius* and probiotic *S. salivarius* K12. These findings are similar to a previous study in which *C. albicans* was found to be resistant to *S. sonchifolius* [19].

Effect of Prebiotic *S. Sonchifolius* and *Synbiotic S. Sonchifolius* with *S. Salivarius* K12 on *C. Albicans* Biofilms Biomass

The effect of prebiotic and synbiotic on the polymicrobial biofilm formation of *C. albicans* was assessed using crystal violet (CV) assay (Table 1, Fig. 2). The biofilm of NB-grown *C. albicans* ATCC MYA-4901, ALC2, and ALC3 culture with *S. sonchifolius* showed total biomass of 0.289 ± 0.053, 0.454 ± 0.275 and 0.833 ± 0.389, respectively. In comparison, NB-grown *C. albicans*, when treated with *S. sonchifolius* and *S. salivarius*, had total biofilm biomass of 0.230 ± 0.064, 0.485 ± 0.028 and 0.666 ± 0.476 for *C. albicans* ATCC MYA-4901, ALC2 and ALC3, respectively. Higher biomass was observed in clinical strains compared to the lab strain, with ALC3 was significantly higher than *C. albicans* ATCC MYA-4901 in prebiotic. In comparison, ALC2 was found to be significantly more biofilm biomass compared to the lab strain when co-cultured in synbiotic ($P < 0.05$).

The biofilm biomass of RPMI-grown *C. albicans* ATCC MYA-4901, ALC2 and ALC3 varied with biofilm biomass of 0.087 ± 0.015, 0.182 ± 0.128 and 0.374 ± 0.399, respectively when co-cultured with prebiotic. Meanwhile, *C. albicans* that were co-cultured with synbiotic *S. sonchifolius* and K12 exhibited biofilm biomass of 0.286 ± 0.127 (ATCC-MYA-4901), 0.330 ± 0.095 (ALC2) and 0.861 ± 1.074 (ALC3). These results showed that C. albicans grown with prebiotic in NB exhibited higher biofilm biomass compared to C. albicans grown in RPMI ($P < 0.05$).

TABLE 1. The effect of prebiotic and synbiotic on the biofilm biomass *Candida* species.

Media	Strains	Means biofilm biomass	
		Prebiotic	Synbiotic
NB	ATCC MYA-4901	0.289*#	0.230#
		(0.053)	(0.064)
	ALC2	0.454*	0.275#
		(0.485)	(0.028)
	ALC3	0.833#	0.666
		(0.389)	(0.476)
RPMI	ATCC MYA-4901	0.087*	0.286
		(0.015)	(0.127)
	ALC2	0.182*	0.330
		(0.128)	(0.095)
	ALC3	0.374	0.861
		(0.399)	(1.074)

Data were absorbance measured at OD_{620nm}. Data were means from three biological ant three technical replicates. SD are given in parenthesis. Mono-culture *S. salivarius* K12 exhibited biofilm biomass of 0.180 ± 0.030 and 0.404 ± 0.177, respectively. Prebiotic represents *C. albicans* grown in with *S. sonchifolius* extract while synbiotic represents *C. albicans* cultured with *S. sonchifolius* extract and *S. salivarius* K12. NB: Nutrient broth and RPMI: RPMI-1640. ATCC: *C. albicans* ATCC MYA-4901,

ALC2: *C. albicans* HIV isolates, and ALC3: *C. albicans* oral cancer isolates. The results indicate the mean values in reduction of biomass in percentage compared to the control group. Significant differences were observed between media (*) and *C. albicans* strains (#) ($P < 0.05$). Data were analysed using one-way analysis of variance (ANOVA) associated with *post hoc* Tukey and Dunnet's test and considered significantly different when $P < 0.05$.

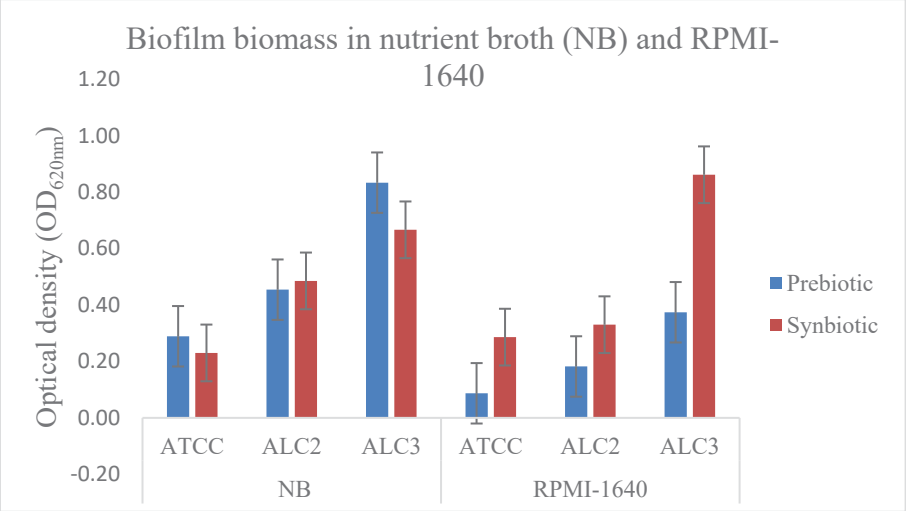

FIGURE 2. Data were absorbance measured at OD_{620nm}. Prebiotic represents *C. albicans* that was grown in with *S. sonchifolius* extract while synbiotic represents *C. albicans* cultured with *S. sonchifolius* extract and *S. salivarius* K12. NB: Nutrient broth and RPMI: RPMI-1640. ATCC: *C. albicans* ATCC MYA-4901, ALC2: *C. albicans* HIV isolates, and ALC3: *C. albicans* oral cancer isolates. The results indicate the mean values in reduction of biomass in percentage compared to the control group. Significant differences were observed between media grown *C. albicans* and between *C. albicans* strains ($P<0.05$). Data were analysed using One-way analysis of variance (ANOVA) associated with *post hoc* Tukey and Dunnet's test and considered as significantly different when $P < 0.05$.

S. salivarius was found to produce urease enzymes that contribute to the stability of oral communities [20]. Another study showed that exo-beta-D-fructosidase (FruA) produced by *S. salivarius* plays an essential role in developing oral biofilm formation by commensal bacteria and may regulate microbial pathogenicity in the oral cavity [21]. *S. sonchifolius* is well known to have a prebiotic effect. Its ability to ferment fructooligosaccharides has been proven by different probiotic strains such as *Lactobacillus acidophilus* NRRL-1910, *Lactobacillus plantarum* NRRL B-4496 and *Bifidobacterium bifidum* ATCC 1569 [22]. Thus, the increased biofilm in synbiotic is suggested due to the increase of *S. salivarius* in biofilm. In addition, NB has been shown to produce yeast form, while RPMI-1640 can produce a hyphal form of *C. albicans*. In a healthy oral cavity, yeast form is more important in initiating *C. albicans* adhesion to the oral surface, both hard and soft tissues. Thus, our study indicated that the synbiotic is more efficient in preventing *C. albicans* biofilm in the oral cavity. Nevertheless, further study is required to identify the distribution of microorganisms in this consortium.

CONCLUSION

Inhibition of synbiotic *S. sonchifolius* and *S. salivarius* K12 on *C. albicans* biofilm is media dependent. Thus, it has the potential as a natural anticandidal or antibiofilm agent that can be used in the coating of dental materials.

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