Synbiotic *Musa acuminata* skin extract and *Streptococcus salivarius* K12 inhibits *Candida* species biofilm formation

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The study aimed to determine the effect of synbiotic *Musa acuminata* skin extract (MASE) and *Streptococcus salivarius* K12 (K12) on *Candida* species biofilm formation. Liquid chromatography quadrupole time-of-flight (LC-Q-TOF-MS) was conducted to characterize MASE. To determine the effect of synbiotic on *Candida* biofilm, 200 µL of RPMI-1640 containing *Candida*, K12, and MASE were pipetted into the same well and incubated at 37 °C for 72 h. A similar protocol was repeated with K12 or MASE to determine the probiotic and prebiotic effects, respectively. Dimorphism, biofilm biomass, and *Candida* total cell count (TCC) were determined. A total of 60 compounds were detected in MASE. *C. albicans* (ALT5) and *Candida lusitaniae* exhibited the highest reduction in biofilm biomass when co-cultured with prebiotic (77.70 ± 7.67%) and synbiotic (97.73 ± 0.28%), respectively. All *Candida* spp. had decreased TCC and hyphae when co-cultured with synbiotic. In conclusion, MASE and K12 inhibit *Candida* biofilm formation.

Keywords: *Musa acuminata*, *Streptococcus salivarius*, *Candida* species, synbiotic, prebiotic, biofilm

Introduction

Oral diseases are caused by various factors, including poor oral hygiene, heavy alcohol consumption, tobacco smoking, unbalanced diet, immunodeficiency, and microbial infection such as *Candida* (Khajuria and Metgud 2015; Grossmann et al. 2020). Among the important clinical manifestations of oral candidiasis are denture-associated stomatitis (Tobouti et al. 2016), rhomboid glossitis (Kaur et al. 2017), leukoplakia (Millsop and Fazel 2016), angular cheilitis (Shetti et al., 2011), and chronic mucocutaneous candidiasis (Kopacova and Spacek 2005). Even though *Candida albicans* is the most isolated yeast associated with human infections; however, non-*albicans Candida* spp. (NAC) such as *Candida tropicalis*, *Candida parapsilosis*, and *Candida glabrata* have also been reported to be associated with 35% to 65% of all systemic *Candida* infections (Krcmery and Barnes 2002).

The formation of biofilms is considered one of the most important virulence factors of *Candida* species (Wall et al. 2019). The biofilms comprise a well-structured microbial consortium encapsulated within an extracellular polysaccharide (EPS) matrix. Compared to its planktonic counterparts, *Candida* which exist as biofilm exhibits increased resistance toward various antifungals, including fluconazole (Seneviratne et al. 2008). In the oral cavity, *Candida* spp. commonly found in polymicrobial biofilms. A previous study has shown that *C. albicans* developed polymicrobial biofilms with *Streptococcus mutans*, a key pathogen in caries development (Bowen et al. 2018).

Probiotics are living microorganisms that confer a health benefit to the host when given in sufficient quantities. The microorganisms have also been reported to possess inhibitory properties against oral carcinogenesis (Morelli and Capurso, 2012; Kamaluddin et al. 2020). The underlying mechanisms for their anti-cancer properties include the suppression of microbial growth involved in mutagenesis, alteration of carcinogenic metabolism, oxide preservation of DNA, and immune system regulation (Abedin-Do et al. 2015).

Streptococcus salivarius K12 is a Gram-positive oral probiotic bacterium commonly isolated in healthy human oral cavities. The bacterium has been reported to suppress various potentially deleterious bacteria such as *Staphylococcus aureus* and *Corynebacterium* in the upper respiratory tract (Kaci et al. 2014; Bidossi et al. 2018). The benefit of *S. salivarius* K12 on oral health has also been previously discussed (Ishijima et al. 2012; Moon et al. 2016; Zupancic et al. 2017).

Prebiotics are functional food with health-promoting properties (Powthong et al., 2020). Non-digestible oligosaccharides lactulose, β-galactooligosaccharides (GOSs), inulin, and fructooligosaccharides, are among the prebiotics known to promote a healthy gut (Singh and Bhatia 2011; Figueroa-Gonzalez et al. 2019). A study has shown that prebiotics can enhance the growth of gut probiotics such as *Lactobacilli*, *Bacillus*, and *Bifidobacteria*, which is beneficial for local and systemic health (Slomka et al. 2018). Nevertheless, the benefit of prebiotics on oral health remains unclear.

Synbiotic is a dietary supplement that is comprised of probiotics and prebiotics (Hemarajata and Versalovic 2013). Synbiotics have been described to suppress the growth of oral pathogens (Kojima et al. 2016). However, the potential of synbiotics in preventing the colonization of *Candida* spp. has not been widely studied.

Fruits are rich in prebiotics. *Musa acuminata*, commonly known as bananas, have been shown to possess some prebiotic compounds (Powthong et al. 2020). Therefore, the objective of this study was to determine the effect of synbiotic *Musa acuminata* skin extract (MASE) and *Streptococcus salivarius* K12 (K12) on *Candida* biofilm formation with the hypothesis that synbiotic MASE and K12 inhibit biofilm formation of *Candida* spp.

Materials and methods

Growth of microorganisms

C. albicans ATCC MYA-4901 (ALT5), *C. albicans* HIV isolates (ALC2) and *C. albicans* oral cancer isolates (ALC3), *Candida dublieniesis* ATCC MYA-2975 (CD), *Candida glabrata* ATCC 90030, *Candida krusei* 14243 (CK), *Candida lusitaniae* ATCC 34449 (CL), *Candida parapsilosis* ATCC 22019 (CP) and *Candida tropicalis* ATCC 13803 (CT) were used in this study. To grow *Candida* spp., stock cultures of the yeasts were revived in yeast peptone dextrose (YPD) broth (Difco, USA) and incubated at 37 °C overnight. Following that, the yeasts were subcultured on YPD agar and incubated at 37 °C aerobically for 24 h. To grow *S. salivarius* K12 (K12), the bacterial stock was revived in brain heart infusion (BHI) broth (Difco, USA) and incubated at 37 °C overnight. Subsequently, the bacterium was subcultured on BHI agar and incubated at 37 °C aerobically for 24 h.

Musa acuminata sampling

M. acuminata was obtained from FELDA Bukit Goh, Kuantan, Pahang, Malaysia. The cultivation was conducted according to the standards of the Department of Agriculture, Malaysia. To reduce the variability, the sample was collected from a single banana plantation once in the morning before transferring to the laboratory for extraction.

Musa acuminata skin extraction

Musa acuminata skin was extracted according to the protocol outlined by Tin et al. (2015). Briefly, *M. acuminata* skin was separated from the flesh and washed under running water. The skin was cut into small pieces, oven-dried at 50°C for three days, and ground to a fine powder using a mechanical blender (Philips, Malaysia). The powdered *M. acuminata* was weighed and stored in sterile tubes at room temperature before extraction. Following that, 50

g of the powder was soaked in one liter of sterile distilled water for three days at room temperature. The concoction was then filtered using Whatman filter paper No. 1. The filtrate was evaporated using a freeze-dryer (Martin Christ, Germany). The percentage extraction yield was calculated according to the following equation (Truong et al., 2019):

Extraction yield = [Weight of the extract after evaporating solvent and freeze-drying (g) / dry weight of the sample (g)] × 100%

The extract was kept in a dry cabinet at 30% relative humidity until further used. Filter sterilization method was also conducted to sterilize the extract using a PES 0.22 µM sterile syringe filter membrane (Tricell Bioscience Resources, Malaysia) before microbiological testing (Harjanti et al., 2020).

LC-QTOF-MS analysis

Initially, 20% (w/v) of MASE diluted in distilled water flowed through in ACQUITY UPLC-BEH C18 column (Waters, USA) at a 0.5 ml min^{-1} flow rate. The mobile phase was prepared using a binary solvent manager; solvent A (0.1% formic acid) and solvent B (acetonitrile) (Sigma Aldrich, Germany). The gradients elution was 99% of solvent A and 1% of solvent B (0 min and 0 to 5 min), 65% of solvent A and 35% of solvent B (5 to 16 min), 0% of solvent A and 100% of solvent B (16 to 18 min), and 99% of solvent A and 1% of solvent B (18 to 20 min), at a 0.6 mL min^{-1} flow rate. The seal wash time and the highest-pressure limit were set at 5 min and 1800 psi, respectively. Positive and negative ionization modes were performed at 2.50 kV capillary voltage and 1.2 bar nebulizer pressure. The drying gas was set at 8 L min^{-1} at 200 °C, and the mass range was 50 to 1000 m/z. The sample was assessed using LC-QTOF-MS (Vion Ion Mobility QTOF MS, Waters, USA), and the data were analyzed using Profile Analysis 2.1 (Bruker, Germany). The presence of secondary metabolites in MASE was assigned using LC-QTOF-MS analysis. A comparison of mass spectrometry (MS)

fragmentation patterns with Waters VR UNIFY library was conducted to identify the compounds based on the spectral match.

Static biofilm formation

The effect of synbiotic, probiotic, and prebiotic on *Candida* spp. biofilms were determined using a semi-quantitative static biofilm assay (Arzmi et al. 2016; Mokhtar et al. 2021). To determine the effect of synbiotic on *Candida* spp. biofilms, 200 µL of RPMI-1640 containing 40 mg of MASE (200 mg mL^{-1}), 3 x 10^6 cells of K12, and 3 x 10^4 cells of *Candida* spp. were pipetted into the same well of a 96-well plate. A similar procedure was repeated for polymicrobial biofilms to determine the effect of the probiotic K12 by inoculating 3 x 10^4 cells of *Candida* spp. suspension with 3 x 10^6 cells of K12.

To determine the effect of the prebiotic on the biofilms, 200 µL of RPMI-1640 containing 40 mg of the extract (200 mg mL^{-1}) and 3 x 10^4 cells of *Candida* spp. were inoculated in the same well. Wells containing *Candida* spp. or K12 only, served as the growth control. Finally, the 96-well plate was incubated for 72 h at 37 °C aerobically, and the medium was replenished aseptically every 24 h. The experiment was performed in three biological replicates to ensure reproducibility. The concentration of MASE used in the biofilm study was optimized to 200 mg mL^{-1}, which has been shown to allow the probiotic to remain viable.

Crystal violet assay

A crystal violet (CV) assay was performed according to the protocol by Alnuaimi et al. (2013) to quantify the biofilm biomass. Initially, the wells containing biofilms were washed twice with sterile PBS to remove the non-adherent cells. Later, the biofilms in the wells were fixed by adding 200 µL of methanol and incubating for 15 min at 25 °C. The supernatant was discarded, and the plate was air-dried for 45 min. Later, 200 µL of 0.1% (w/v) CV solution was

added to each well and incubated for 20 min at 25 °C. The plate was washed gently twice using sterile distilled water to remove the unbound stain. Subsequently, the biofilms were de-stained with 200 µL of 33% (v/v) acetic acid for five minutes at room temperature. Finally, 100 µL of the acetic acid solution was transferred to a new sterile 96-well plate, and the absorbance was measured at OD_{620nm} using a microtiter plate reader (Tecan NanoQuant Infinite M200, CA).

Quantification of Candida cell count

Following 72 h incubation of static biofilm, each well of a 96-well plate was washed twice with 200 µL of sterile PBS to remove the non-adherent cells. Subsequently, 200 µL of PBS was added to the well, and the biofilm was mechanically disrupted using a sterile pipette tip. Finally, *Candida* spp. total cell count was measured using a hemocytometer, an established protocol to quantify *Candida* cell count by Taff et al. (2012) and Alnuaimi et al. (2013). This analysis was performed in three technical and three biological replicates to ensure reproducibility.

Dimorphism assay

Gram staining was performed to determine *Candida* morphology following biofilm growth in RPMI-1640. *C. albicans* ATCC MYA-4901 (ALT5), *C. albicans* HIV isolates (ALC2), and *C. albicans* oral cancer isolates (ALC3) were selected for the assay. Similar protocol as in the static biofilm formation was repeated except that the biofilm was developed in a 6-well plate. Following incubation, the supernatant was discarded, and each well was washed carefully with PBS twice to remove the non-adherent cells. Finally, Gram staining was performed, and the sample was observed under a light microscope at 1000x magnification (Olympus, CH Series, Japan).

Statistical analysis

The results were analyzed using SPSS Statistic version 26.0. One-way analysis of variance (ANOVA) associated with *post hoc* Dunnet and Tukey's test was used to compare biofilm biomass and cell count between *Candida* spp. in the control group, prebiotic MASE, and synbiotic MASE with K12. The data are considered statistically significant where $P<0.05$.

Results

Total yield and chemical composition of Musa acuminata skin extract

The total yield of freeze-dried *M. acuminata* skin extract was 25.2%. The chemical compounds identified in *Musa acuminata* skin extract (MASE) using LC-QTOF-MS and their functions were summarised in Table 1. The base peaks for base peak intensity (BPI) plot and assigned compounds in positive ion modes were also reported (Figure 1). LC-QTOF-MS analysis showed that 60 phenolic compounds were tentatively assigned in positive and negative ion modes. Based on the peak response of electrospray ionization (ESI) and BPI analysis, eight predominant compounds were identified, which include four in positive ion mode (Mahuannin, Glabrol, 7-Hydroxy-3-(4'- hydroxybenzylidene)- chroman-4-one and (2S)-3',4'-Methylenedioxy-5,7- dimethoxyflavane) and four in the negative ion mode (Moscatilin, Didemethoxylcurcumin, Torachrysone-8-O-β-D-glucopyranoside, and Yakuchinone A).

Effect of Musa acuminata extract and Streptococcus salivarius K12 on Candida morphology

The images of *C. albicans* and NAC biofilms Gram-stained were illustrated in Figure 2 and Figure 3, respectively. *C. albicans* ALC3 developed more hyphae than ALT5 and ALC2 in monoculture biofilm after 72 h incubation at 37°C (Figure 2). Only ALC3 remained in hyphal form when grown with prebiotic. Our study also showed that *C. tropicalis* formed hyphae in monoculture biofilm after 72 h incubation at 37°C in RPMI-1640 (Figure 3). In

addition, the incubation of NAC with probiotic, prebiotic, and synbiotic was found to inhibit the hyphal formation of the yeasts.

Effect of Musa acuminata extract and Streptococcus salivarius K12 on Candida biofilm biomass

The effect of probiotics, prebiotics, and synbiotics on biofilm biomass was assessed using a CV assay (Table 2). Co-culture of probiotic, prebiotic, and synbiotic with *Candida* spp. exhibited reduction of biofilm biomass ranging from 59.64 ± 65.00% to 81.51 ± 3.80%, 63.77 ± 16.19% to 77.70 ± 7.67% and 96.37 ± 1.08% to 97.73 ± 0.28%, respectively ($P<0.05$, Table 3). Probiotic K12 had reductions of above 50% against *Candida* spp. biofilm with CG had the highest antibiofilm activity (81.51 ± 3.80%), followed by ALC2 (78.03 ± 2.34%) and CP (59.64 ± 65.00%). Except for CL, there was an increase of biofilm when co-cultured together. The greatest reduction in biofilm biomass was observed in ALT5 when co-cultured with prebiotic MASE (77.70 ± 7.67%), followed by ALC2 (69.41 ± 8.99%), and CK (63.77 ± 16.19%). The biofilm biomass of synbiotic-treated *Candida* spp. biofilm was significantly decreased compared to the respective prebiotic-treated biofilm ($P<0.05$). The highest reduction was noted in CL (97.73 ± 0.28%) followed by ALC3 (96.76 ± 0.91%), and CK (96.37 ± 1.08%).

Effect of Musa acuminata extract and Streptococcus salivarius K12 on Candida species cell count

C. albicans total cell count decreased significantly, ranging from $(0.17 \pm 0.29) \times 10^5$ cells mL^{-1} to $(2.50 \pm 0.50) \times 10^5$ cells mL^{-1} when co-cultured with probiotic K12 compared with monoculture *C. albicans* biofilm ($p<0.05$). In polymicrobial biofilms, CD exhibited the highest total cell count with $(2.50 \pm 0.50) \times 10^5$ cells mL^{-1} followed by ALC2 with $(1.00 \pm 0.87) \times 10^5$ cells mL^{-1} and ALC3 with $(0.17 \pm 0.29) \times 10^5$ cells mL^{-1}.

Prebiotic-treated *Candida* spp. biofilm exhibited a total cell count ranging from (0.67 ± 0.29) x 10^5 cells mL^{-1} to (2.17 ± 0.29) x 10^5 cells mL^{-1}. The biofilm of prebiotic-treated ALC3 had the highest total cell count with (2.17 ± 0.29) x 10^5 cells mL^{-1} followed by CD with (1.17 ± 1.26) x 10^5 cells $mL^{-1}$and CG with (0.67 ± 0.58) x 10^5 cells mL^{-1} (Table 4).

The total cell count of *Candida* spp. in the biofilm was also found to be significantly decreased between (0.17 ± 0.29) x 10^5 cells mL^{-1} to (1.00 ± 0.87) x 10^5 cells mL^{-1} when co-cultured with synbiotic compared to prebiotic (P<0.05) (Table 3). ALC2 and ALC3 exhibited the highest total cell count in synbiotic culture with (1.00 ± 0.02) x 10^5 cells mL^{-1} and (1.00 ± 0.87) x 10^5 cells mL^{-1}, respectively. Meanwhile, CG, CK, and ALT5 exhibited the lowest total cell count with (0.17 ± 0.29) x 10^5 cells mL^{-1}. Finally, the comparison between *Candida* spp. biofilm showed that probiotic, prebiotic, and synbiotic inhibitory effect was species-dependent.

Discussion

The prevention of oral diseases, based on the restoration of the ecological balance of microorganisms rather than eliminating the disease-associated species, has been proposed recently (Allaker and Stephen 2017). One extensively experimented approach in establishing ecological balance in a microbial community is probiotics and prebiotics either as the sole agent or as a mixture known as synbiotic. Until now, the role of synbiotics in oral *Candida* colonization remains unclear. Hence, this study is the first to evaluate the effect of synbiotics based on K12 and MASE on seven *Candida* spp. including *C. albicans, C. dublieniesis, C. glabrata, C. krusei, C. lusitaniae, C. parapsilosis*, and *C. tropicalis.*

The present study demonstrated the inhibitory effect of prebiotic and synbiotic on *Candida* spp. biofilm and hyphal formation (Table 2, Table 3, Table 4, Figure 2, and Figure 3). The anti-biofilm activity of prebiotic and synbiotic is suggested to be due to the presence of

potential antifungal compounds in MASE and the anti-biofilm of K12. Based on LC-QTOF-MS analysis, 60 chemical components were identified, including various flavonols, phenolics, and other metabolites (Table 1). This finding agrees with previous studies showing the antimicrobial and antioxidant properties of phenolic compounds isolated from banana peels against *Candida* spp. (Fadhilah and Mohamad 2014; Sirajudin et al. 2014; Kapadia et al. 2015; Pereira and Maraschin 2015; Harlina and Azizah 2019). In addition, our previous study has also shown that K12 can inhibit the biofilm formation of *C. albicans* (Mokhtar et al. 2021).

Based on LC-QTOF-MS analysis, glabrol has been shown to be one of the predominant compounds in MASE. Glabrol is a member of the flavonoid group, a well-known group of antimicrobial agent compounds that target microbial cell membranes by binding to peptidoglycan, phosphatidylglycerol, and cardiolipin, dissipating proton move force, and increasing membrane permeability (Shuai-cheng et al. 2019), resulting in the reduction of cell size and leakage of intracellular components (Lee et al. 2018). The impact of flavonoids in inhibiting biofilm formation has also been demonstrated previously (Lee, Woo, and Lee 2018). In addition, previous studies have shown the ability of glabrol to deform *C. albicans* cell wall and subsequently inhibit the growth and biofilm formation of the yeast (Hibbett et al. 2007). Therefore, the presence of glabrol in prebiotic and synbiotic may have a role in the inhibition of *Candida* spp. biofilms.

The exposure to the synbiotic reduced the biofilm biomass of the tested *Candida* spp. Comparing total cell counts between the prebiotic and synbiotic treated biofilms confirmed that the synbiotic had more inhibitory effect on *Candida* spp. total cell count in biofilms and the hyphal formation compared to prebiotic, thus supporting the hypothesis of the present study. These findings also indicated that the presence of probiotics can improve the anti-biofilm and anti-hyphae activity of the prebiotic, suggesting the synergistic effect between the probiotic and prebiotic. Probiotics were reported to elicit anti-biofilm properties by competing with

human pathogens for specific host binding sites during infection (Song et al. 2015). In addition, the metabolites of probiotics have also been shown to inhibit the adhesion of pathogenic bacteria to the substratum (Monteagudo-Mera et al. 2019). Furthermore, a study on the oral candidiasis mouse model also showed that K12 inhibited *C. albicans* infection dose-dependently (Ishijima et al. 2012). This inhibition is reported due to the presence of salivaricin A2 (SalA2) and salivaricin B (SboB), which are produced by K12 (Hogan et al. 2004; Nickerson 2006; Morales and Hogan 2010; Barbour et al. 2016; Figueroa-Gonzalez et al. 2019).

In addition, the present study demonstrated that inhibition of *Candida* by prebiotic and synbiotic was species and strain-dependent. Such variations may be associated with the differential expression of proteins such as secreted aspartyl proteinases (Sap) and hyphal cell wall protein (Hwp) that are important in the colonization of *Candida* spp. in the oral cavity (Lie Tobouti et al. 2016; Castilho et al. 2018). Our present study showed that monospecies ALC3 and *C. tropicalis* formed hyphae when grown in RPMI-1640 (Figure 2 and Figure 3). However, no hyphal was observed when grown with prebiotic and synbiotic. *Candida* spp. were reported to form yeast morphology, germ tubes, pseudo-hyphae, and/or hyphae that play a key role in the infection at a different rate depending on the species (Kunyeit et al. 2019). Thus, these attributes could justify the different levels of biofilm formation and hyphal inhibition between *Candida* species in the present study.

Conclusion

Synbiotic of *M. acuminata* skin extract and *S. salivarius* K12 inhibits *Candida* biofilm and hyphae formation. A future study focusing on the *in vivo* assessment is proposed to identify the potential use of the synbiotic in the prevention and management of *Candida* spp. infections in the oral cavity would likely reveal alternative therapeutic modalities for oral candidiasis.

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Declaration of interest

The authors declare no conflict of interest.

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Table 1. Chemical compounds identified in *Musa acuminata* skin extract using LC-QTOF-MS and their functions.

Table 2. Biofilm biomass scores of *Candida* species and *Streptococcus salivarius* K12.

Table 3. The effect of probiotic, prebiotic, and synbiotic on *Candida* biofilm biomass.

Table 4. The effect of probiotic, prebiotic, and synbiotic on *Candida* species total cell count in biofilm.

Figure 1. LC-Q-TOF-MS chromatogram in positive ion mode for BPI plot (A1) and tentative assigned compounds (A2), and negative ion mode for BPI plot (B1) and tentative assigned compounds (B2). The predominant compounds of *M. acuminata* skin extract (MASE) in positive ion mode were Mahuannin F (Rt = 0.53 min) at m/z = 543.1321, Glabrol (Rt = 6.12 min) at m/z = 393.2090, 7-Hydroxy-3-(4'-hydroxybenzylidene)-chroman-4-one (Rt = 12.14 min) at m/z=291.0654, and (2S)-3',4'-Methylenedioxy-5,7- dimethoxyflavane (Rt=16.70 min) at m/z = 337.1041. While the predominant compounds of MASE in negative ion mode were: Moscatilin (Rt=5.34 min) at m/z = 304.1285, Didemethoxylcurcumin (Rt=5.47 min) at m/z = 308.1024, Torachrysone-8-O-β-D- glucopyranoside (Rt=10.72 min) at m/z = 408.1424, and Yakuchinone A (Rt=17.91 min) at m/z= 312.1756.

Figure 2. The gram-stained biofilms were visualized using a light microscope with 1000 × magnification. The probiotic group represents *Candida albicans* grown in *S. salivarius* K12, the prebiotic group represents *C. albicans* grown in MASE only, while the synbiotic group represents *C. albicans* that were grown in the presence of MASE and *S. salivarius* K12. Light microscope images showing Gram-stained biofilms of ALT5: *C. albicans* ATCC MYA-4901, ALC2: *C. albicans* HIV isolates, and ALC3: *C. albicans* oral cancer isolates. Biofilms were grown for 72 h at 37ºC aerobically. Biofilms were developed as monoculture *C. albicans* or by inoculating the yeast with *S. salivarius* K12 (K12) alone, *M. acuminata* skin extract (MASE) alone, or synbiotic K12 and MASE. Shown are representative sections for each strain and treatment group.

Figure 3. The gram-stained biofilms were visualized using a light microscope with 1000 × magnification. The probiotic group represents *Candida* species grown in *S. salivarius* K12, the prebiotic group represents *Candida* species grown in MASE only, while the synbiotic group represents *Candida* spp. that were grown in the presence of MASE and *S. salivarius* K12. Light

microscope images showing Gram-stained biofilms of non-albicans *Candida* CD: *C. dubliniensis* CG: *C. glabrata*, CK: *C. krusei*, CL: *C. lusitaniae*, CP: *C. parasilopsis* and CT: *C. tropicalis*. Biofilms were grown for 72 h at 37ºC aerobically. Biofilms were developed as monoculture *C. albicans* or by inoculating the yeast with *S. salivarius* K12 (K12) alone, *M. acuminata* skin extract (MASE) alone, or synbiotic K12 and MASE. Shown are representative sections for each strain and treatment group.