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An *in vitro* study on the antifungal and antibiofilm activities of probiotic bacteria against *Candida* species isolated from orthodontic appliances and dental caries

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Abstract

Candida species are opportunistic pathogens that may cause infections in predisposed persons. This work aimed to detect the antifungal and antibiofilm potentials of *Lactobacillus acidophilus* and *Lactobacillus plantarum* supernatants on *Candida* spp. About 60 % and 80 % of *Candida* isolates were recovered from saliva samples of 20 patients with fixed orthodontic appliances, and 20 children having dental caries, respectively. The antifungal susceptibility of *Candida* spp. was investigated using disk diffusion assay. *C. albicans* strains showed low resistance to fluconazole (15 %) and amphotericin B (10 %). Using the agar well diffusion assay, both *L. acidophilus* and *L. plantarum* supernatants inhibited the *in vitro* growth of all tested *Candida* spp. with inhibition zone diameters of (11-19 mm) and (7-16 mm), respectively. On observing the effects of *L. plantarum* and *L. acidophilus* supernatants on *Candida* cells' morphology; the light microscopic examination demonstrated the inhibition in germ tube formation of all tested *C. albicans* with percentages of 68 % and 53 %, and for *C. krusei* with inhibition percentages of 69 % and 59 %, respectively. *L. acidophilus* and *L. plantarum* strains showed high co-aggregation ability with *C. albicans* strains with ranges of 42-49 % and 30-35 %, respectively. The antibiofilm activities of the two *Lactobacillus* supernatants were determined using the tissue culture plate assays. Significant inhibition of biofilms formation by *Candida* spp. was recorded on treatment with *L. plantarum* and *L. acidophilus* supernatants, with reduction percentages of 50-72 % and 74-85 %, respectively.

Keywords: Anti-Candida, Opportunistic fungi, Antibiofilm, Co-aggregation, Germ-tube formation

1. Introduction

Oral microbiota is defined as oral microorganisms such as bacteria, yeasts and viruses that form a contradictory ecosystem in the mouth (Lu *et al*., 2019). A recent study conducted by Razi *et al*., (2020) revealed that the oral cavity provides a nourishing environment for oral microbiota and regulates the bacterial colonization to prevent the invasion of the pathogenic microbes. The oral microbiota plays a key

role in maintaining the oral health. However, in some situations, the invading microbes can cause imbalances in the commensal microbial community of the mouth, leading to dental diseases (Marsh and Zaura, 2017). Caries is an infectious microbial disease of the tooth that results in the local dissolution and destruction of the calcified tissues (Heymann *et al*., 2013). Caries occurs due to complex interactions between dental structures and the oral microbiome; biofilm formation, food residue accumulation, saliva dysfunction and genetic predisposition (Zero, 2006).

In the oral cavity, *Candida* spp. represent commensal yeasts that could contribute to the formation of a complex oral microbial biofilm. The colonization rate of *Candida* spp. is 20- 40 % in healthy individuals and 60 % in immuno-compromised persons, as they become the most prevalent microflora (Signoretto *et al*., 2009). Increase in the intake of sweet diet; poor oral hygiene and the presence of carious lesions in children, support oral colonization by Candida spp. (Shino *et al*., 2016). *C. albicans* is known to be associated with dental caries, but the role of non-*Candida albicans* including *C. krusei* and *C. tropicalis* in the development of dental caries is recently described by Beena, (2020).

Fixed orthodontic appliances are artificial devices in the mouth that greatly affect the health of the mouth and allow the accumulation of plaques and food debris. They can bring a large number of microorganisms with associated infections to the mouth (Atack *et al*., 1996). The previous study of Heintze *et al*., (1999) revealed that during the treatment of fixed orthodontic appliances, festive regions are formed that are suitable for biofilms accumulation. Badiee *et al*., (2011); Castanheira *et al*., (2014) added that increased dental plaque levels are associated with the development of gingivitis. Patients with gingivitis are liable to suffer from periodontal disease. After application of the fixed orthodontic appliances, the plaque pH and the number of microorganisms in the oral cavity change. *C. albicans* and other *Candida* strains aggregate or adhere much more easily to the fixed orthodontic appliances.

The antifungal drugs are very effective however they have many side effects especially the antifungal resistance (Sardi *et al*., 2013). The antimicrobial agents act non-specifically through decreasing the levels of beneficial and harmful oral microorganisms. According to Mishra *et al*., (2016), Chlorhexidine is a broad-spectrum powerful antimicrobial agent but it has side effects such as staining of teeth and unpleasant taste that restrict its utilization as a rinse for a long-term. Probiotics are living bacteria, derived from the genus *Lactobacillus* or *Bifidobacterium* (Doron and Gorbach, 2006). A previous study conducted by Mishra *et al*., (2016) highlighted that probiotics provide a natural protection against the bacteria that are harmful to the teeth and gums; by utilizing the beneficial naturally occurring oral bacteria, thereby maintaining a healthy microbial balance in the oral cavity. The adherence ability of the microorganisms belonging to the same species (auto-aggregation), and ability of the genetically different microorganisms to adhere to each other (co-aggregation); are considered as preliminary screening protocols for detecting the probiotics. Moreover, these abilities are important for the development of oral biofilms, and help in providing protection to the microbiota against shear forces, which occur naturally in the oral cavity (Ledder *et al*., 2008).

According to Pandya, (2016), probiotics help to prevent and treat oral disorders through different mechanisms including; inhibition of pathogen adhesions and biofilm formation, elimination of competitors; affect plaque development and its complex ecosystem by competition with the adherent bacteria, production of chemicals such as organic acids, hydrogen peroxide and bacteriocins that suppress the oral bacteria, regulation of systemic immune function, affecting the local immunity, regulation of mucosal permeability acting as antioxidants, and inhibition of plaque induction by neutralizing the free electrons.

The objectives of this study were to identify Candida spp. that accompany placement of the fixed orthodontic appliances and dental caries, and to

evaluate the effectiveness of using probiotics as antifungal agents; to reduce biofilms formation by Candida spp. in the oral cavity.

2. Material and methods

2.1. Patients and samples

Patients selected for this study were 20 children with dental caries from Pedodontics Department, whereas the other 20 patients had fixed orthodontic appliances, attended for periodical provision in the Outpatient Clinics of Department of Orthodontics, Faculty of Dentistry, Minia University, Minia, Egypt. All patients or children's parents gave their written consent to participate in this study, and were informed them that the use of antimicrobial mouthwashes was banned during the study. Saliva samples were collected from children with caries and from patients using orthodontic appliances; 6 months after starting the orthodontic therapy. The patients were instructed individually to expel saliva into a sterile container until a volume of approximately 3 ml was collected.

2.2. Microorganisms and identification of *Candida* spp.

About 100 µl of each saliva sample was cultured on Sabouraud dextrose agar (SDA) (Lab M, UK) and CHROMagar *Candida* (CHROMagar *Candida*, France) media, and then incubated at 35˚C for 48 h. Generally, CHROM agar *Candida* medium identifies *Candida* spp. by their color and growth pattern (Nadeem *et al*., 2010). For phenotypic differentiation between *Candida* spp., the germ tube test was performed according to Benson, (2002). A small portion of the isolated colonies of the tested Candida spp. was suspended individually in 0.5 ml of human serum in a test tube, and then incubated at 35˚C for 2h. After incubation, a drop of *Candida* serum suspension was placed on a slide; covered with a coverslip, and then checked microscopically for the presence of germ tubes. The probiotic strains of *L. plantarum* ATCC 14917 and *L. acidophilus* ATCC 20552 were provided by the MIRCIN culture collection of the Faculty of Agriculture, Ain Shams University, Egypt.

2.3. Antifungal susceptibility assay

The *in vitro* activities of the different antifungal agents against the isolated *Candida* spp. were measured using the disk diffusion assay, as described by Ng *et al*., (2001). Overnight cultures of Candida spp. were adjusted to 0.5 McFarland turbidity standards, and then 0.5 ml of each culture suspension was spread individually over the surface of Muller-Hinton agar (MHA) (Lab M, India) plates supplemented with 2 % glucose, using a sterile glass spreader. The antifungal disks used were ketoconazole (10 µg), fluconazole (25 µg) and amphotericin B (20 units) (Bio-analyse R, Turkey). These disks were placed on the surface of MHA and then all plates were incubated at 37°C. The inhibition zone diameters were measured using a calibrated ruler after 24h of incubation.

2.4. Detection of antifungal potential of the *Lactobacillus* supernatants against *Candida* spp.

The antifungal potentials of *L. plantarum* ATCC 14917 and *L. acidophilus* ATCC 20552 supernatants against the recovered *Candida* isolates were examined using the agar well diffusion technique, in reference to Toba *et al*., (1991). The two *Lactobacillus* strains were incubated in de Man Rogosa Sharp (MRS) broth at 37°C for 48h. After incubation, the cultures were centrifuged at 10.000 rpm for 10 min.; the supernatants were filtered through a 0.2 µm filter (Aly *et al*., 2018). On the other hand, overnight cultures of the *Candida* isolates were adjusted to 0.5 McFarland turbidity standards using physiological saline. About 100 µl of each Candida suspensions was spread individually over the surface of SDA agar plates using a sterile glass spreader. Wells of 10 mm were made in these seeded SDA plates using a sterile cork borer, and then 100 µl of each *Lactobacillus* supernatant was added individually to each well. The plates were incubated at 35 °C for 48h. Finally, zones of inhibition formed around the wells were measured using a calibrated ruler, and then compared to MRS broth control wells. Three replicates were used for each

Lactobacillus supernatant and the test was repeated thrice.

2.5. Effect of Lactobacilli supernatants on *Candida* spp. germ-tube formation

Tested *Candida* spp. were adjusted to a cell density of 0.5 McFarland in human serum, and then added individually to equal volume of supernatants of the 2 *Lactobacillus* strains, whereas *Candida* cells without *Lactobacillus* supernatants were used as negative control. The tubes were incubated at 37°C for 2 h. After incubation, about 100 *Candida* cells from treated samples and negative control were observed for the presence of germ tubes using a light microscope, according to the method adopted by Liu *et al*., (1994). The percentage of inhibition in *Candida* cells' germination was calculated in three independent assays.

2.6. Surface hydrophobicity of *Lactobacillus* strains

The surface hydrophobicity of cells of the two *Lactobacillus* strains was investigated using the Salt aggregation test (SAT), as described by Andreu *et al*., (1995). Bacterial cells were suspended in phosphate buffered saline (PBS) (pH 6.8) to a final concentration of 5×10^9 cfu/ ml. The bacterial suspensions were mixed individually on a glass slide with equal volumes of different concentrations (0.5, 1.5, 2 and 4 mol/ l) of Ammonium sulfate solution. The lowest concentration of Ammonium sulfate that caused visible aggregation of the *Lactobacillus* cells was defined as the SAT hydrophobicity values. Based on these values, the tested bacterial strains were classified as high hydrophobic (< 0.9 mol/ l), intermediate hydrophobic (0.9–1.5 mol/ l), or hydrophilic (>1.5 mol/ l). For comparison, negative and positive controls were used by mixing equal volumes of the bacterial suspensions with 0.02 M phosphate buffer (pH 6.8) and 4 M Ammonium sulfate solution, respectively. The test was performed in triplicates and repeated twice.

2.7. Auto-aggregation assay of *Lactobacillus* strains and Co-aggregation of *Lactobacillus* strains with *Candida* spp.

The auto-aggregation assay was done according to the method conducted by Schillinger and Lücke, (1989). *L. acidophilus*, *L. plantarum* and *Candida* cultures were adjusted to 5×10^9 cfu/ ml concentration with PBS at pH = 6.8, and the absorbance of each suspension was measured at 600 nm (initial aggregation index). These suspensions were incubated at 37 ºC, and then assessed at intervals of 4, 20 and 24h (final aggregation). The auto-aggregation index was calculated according to Schillinger and Lücke, (1989) as follows:

$$\text{Aggregation \%} = 100 \times (A_{initial}-A_{final})/A_{initial}$$

Where; Aggregation% = Aggregation index, $A_{initial}$ = initial absorbance at 600 nm (at 0 h), A_{final} = final absorbance at 600 nm (at 4 h, 20 h and 24 h according to the assay)

The co-aggregation assay was carried out to detect the ability of Lactobacillus strains to co-aggregate with the tested *Candida* spp., in reference to Reid *et al*., (1990). Lactobacilli and *Candida* spp. were suspended individually in PBS (pH = 6.8), and then adjusted to a final concentration of 5×10^9 cfu/ ml. Equal volumes of Lactobacilli and *Candida* spp. were mixed and then incubated at 37 °C for 24 h. Each assay was performed in triplicates. About 2 ml of each *Lactobacillus* strain and *Candida* sp. were used as controls. Absorbance (A_{600}nm) of the mixtures was measured at 4, 20 and 24 h, and the co-aggregation percentage was calculated according to Ekmekci *et al*., (2009) as:

$$\text{Co-aggregation (\%)} = [((A_{Candida}+A_{Lactobacillus})/2)-A_{mix})]/[(A_{Candida}+ A_{Lactobacillus})/ 2] \times 100$$

Where; $A_{Candida}$, $A_{Lactobacillus}$ and A*mix* represent the absorbance at 600 nm of the control tubes of *Candida* spp., *Lactobacillus* strains and their mixture; respectively, after incubation for the pre-determined time intervals.

2.8. Detection of biofilm formation by *Candida* spp.

Biofilm formation by the *Candida* isolates was detected using the tissue culture plate method, according to Stepanovic *et al*., (2000). The *Candida* isolates were cultured on SDA at 37 °C for 24 h.

Cultures were adjusted with SDB to 10^7 cfu\ ml. An aliquot of 20 µl was distributed individually in each well of a 96-well plate. After that, about 180 µl SDB containing 2.5 % glucose was added, and then the plate was incubated for 48h at 35 °C. After incubation, the wells were washed 3 times using sterile physiological saline, and then fixed with 200 µl of methanol (99 %) for 15 min. At the end of this fixation period, excess methanol was discarded and the wells were left to dry. After dryness, the wells were stained with 200 µl of crystal violet (2 %) for 5 min., washed with dist. water and then dried. The wells were treated with 160 µl of glacial acetic acid (33 %), and then the optical density (OD_{540}) was measured spectrophotometrically. Biofilm formation was evaluated according to the measured OD, where; negative (-) if OD values were $0 \leq OD_{540} \leq 0.120$, weak (+) if $0.121 \geq OD_{540} \leq 0.240$, intermediate (++) if $0.241 \leq OD_{540} \leq 0.500$, and strong biofilm former (+++) if $OD_{540} \geq 0.500$. The assay was carried out in duplicates.

2.9. Inhibitory effects of *Lactobacillus* supernatants on the strong biofilm forming *Candida* spp.

2.9.1. Effect of *Lactobacillus* supernatant on biofilm formation by *Candida* spp.

To test the effect of filtrates of the 2 Lactobacilli strains (*L. plantarum* ATCC 14917 and *L. acidophilus* ATCC 20552) on biofilm formation by the strong biofilm forming *Candida* isolates, an aliquot of 10 µl of *Candida* isolates pre-cultured in SDB (10^7 cfu/ ml) was distributed individually into the wells of 96-well microtiter plate. After that, about 140 µl of SDB containing 2.5 % glucose was added onto each well. A volume of 50 µl of sub-MIC (minimum inhibitory concentration) of the 2 *Lactobacillus* filtrates was added individually to each well, and then the plate was incubated at 35°C for 48 h. *Candida* isolates and SDB were used solely as controls. The degree of biofilm formation was assessed according to the tissue culture plate method described above.

2.9.2. Effect of *Lactobacillus* supernatants on reduction of pre-formed biofilms by *Candida* spp.

For testing for the inhibitory effects of *Lactobacillus* supernatants on the preformed *Candida* biofilms; *Candida* biofilms were formed in the 96-well microtiter plates for 24 h, subsequently 100 µl of the Lactobacilli supernatants was added individually to these preformed biofilms, and then incubated for another 24h. The percent reduction in biofilm formation was calculated according to the following equations, adopted by Kaur *et al*., (2018):

Percentage inhibition = 100 - (OD_{540} of test wells/ OD_{540} of control wells) ×100

2.10. Statistical analysis

Statistical analysis of results was carried out using SPSS version 17.0 (SPSS Inc., Chicago, IL). The antibiofilm activities were analyzed through One-way analysis of variance (ANOVA) and Tukey's multiple-comparison test. Results with a p-value less than 0.05 were considered statistically significant.

3. Results

3.1. Isolation and identification of *Candida* spp.

Out of 20 orthodontic samples, 12 (60%) *Candida* isolates are recovered and identified as; *C. albicans* (8), *C. tropicalis* (2) and *C. krusei* (2). Whereas 16 (80%) *Candida* isolates are recovered from 20 children having dental caries, and are identified as; *C. albicans* (12), *C. tropicalis* (3) and *C. krusei* (1). On CHROMagar, *C. albicans* are observed as green smooth colonies, *C. tropicalis* as blue raised colonies and *C. krusei* as pink fuzzy colonies. However, *C. albicans* is the most prevalent species recovered from both types of samples as shown in Table (1). Results of germ tube test confirmed that both of *C. albicans* and *C. krusei* isolates are germ tube formers.

3.2. Antifungal susceptibility of the *Candida* spp.

The antifungal susceptibility patterns of the 28 *Candida* spp. to ketoconazole, fluconazole and

amphotericin B antibiotics are shown in Table (2). *C. tropicalis* showed higher resistance rate (40 %) to fluconazole, followed by *C. albicans* (15 %) and *C. krusei* (33.3 %), while *C. krusei* demonstrated higher resistance (33.3 %) to amphotericin B. On the other hand, *C. albicans* and *C. tropicalis* presented the highest resistance against ketoconazole (40 %).

Table 1. The prevalence of *Candida* spp. on CHROMagar recovered from orthodontic and caries samples

Orthodontic samples (20)			**Caries samples (20)**		
Candida isolates 12 (60%)			*Candida* isolates 16 (80%)		
C. albicans	*C. tropicalis*	*C. krusei*	*C. albicans*	*C. tropicalis*	*C. krusei*
8 (40 %)*	2 (10 %)	2 (10 %)	12 (60 %)	3 (15 %)	1 (5 %)

*These percentages were attributed to the whole number of samples of each type

Table 2. Antifungal sensitivity of *Candida* spp. recovered from orthodontic and caries samples

Candida spp.	**Antifungal antibiotics**					
	Ketoconazole		Fluconazole		Amphotericin B	
	R	S	R	S	R	S
C. albicans (20)	8 (40 %)*	12 (60 %)	3 (15 %)	17 (85 %)	2 (10 %)	18 (90 %)
C. tropicalis (5)	2 (40 %)	3 (60 %)	2 (40 %)	3 (60 %)	1 (20 %)	4 (80 %)
C. krusei (3)	1 (33.3 %)	2 (66.7 %)	1 (33.3 %)	2 (66.7 %)	1 (33.3 %)	2 (66.7 %)

Where; R: resistant, S: sensitive, * Percentage of inhibition in correlation to the number of each *Candida* sp.

3.3. Antifungal potential of *Lactobacillus* supernatants

The antifungal activity of *Lactobacillus* strains was tested using agar well diffusion assay. Both *L. plantarum* ATCC 14917 and *L. acidophilus* ATCC 20552 supernatants inhibited the growth of all tested *Candida* spp. As shown in Table (3), *L. acidophilus* has higher antifungal efficacy than *L. plantarum* against *C. albicans*; the inhibition zone diameter ranged from 14.2 to 19.7 mm, followed by *C. tropicalis* (11.9-16.6 mm) and *C. krusei* (11-12.1 mm).

3.4. Effect of *Lactobacillus* supernatants on *Candida* spp. germ-tube formation

By examination under the light microscope, it is observed that supernatants of both *Lactobacillus* strains inhibited the germination ability of all the tested *C. albicans* and *C. krusei*. As shown in Fig. (1), the percentage of germ tube formation decreased on using the *L. plantarum* and *L. acidophilus* supernatants after 2 h of incubation by 68 % and 69 % for *C. albicans*, compared to the control cells. On the other hand, Fig. (2) shows that the germination inhibition percentage of *C. krusei* cells on treatment with *L. plantarum* is 53 %, whereas it is 59 % in case of *L. acidophilus*, after 2 h of incubation.

Table 3. Antifungal efficacy of supernatants of the *Lactobacillus* strains against the tested *Candida* spp.

***Candida* isolates**	**Average inhibition zone diameter (mm)**	
	L. plantarum supernatant	*L. acidophilus* supernatant
***C. albicans* 1**	13.2	14.4
2	13.5	14.2
3	16.1	16
4	15.7	17.2
5	16.5	19.7
6	13.5	15.7
7	14.2	17.1
8	16.3	19.5
9	16.5	18.2
10	14.7	17.3
11	15.6	19.4
12	13.8	15.3
13	16.7	18.1
14	15.2	16.9
15	16.2	19.3
16	16.4	19.5
17	16.3	18.6
18	14.2	17
19	14.4	19.1
20	15.5	16.3
***C. tropicalis*1**	9.1	15.2
2	8.3	12-*.4
3	8.6	11.9
4	10.3	16.6
5	11.1	16.2
***C. krusei* 1**	7.3	11
2	10.9	12.1
3	8.3	11.3

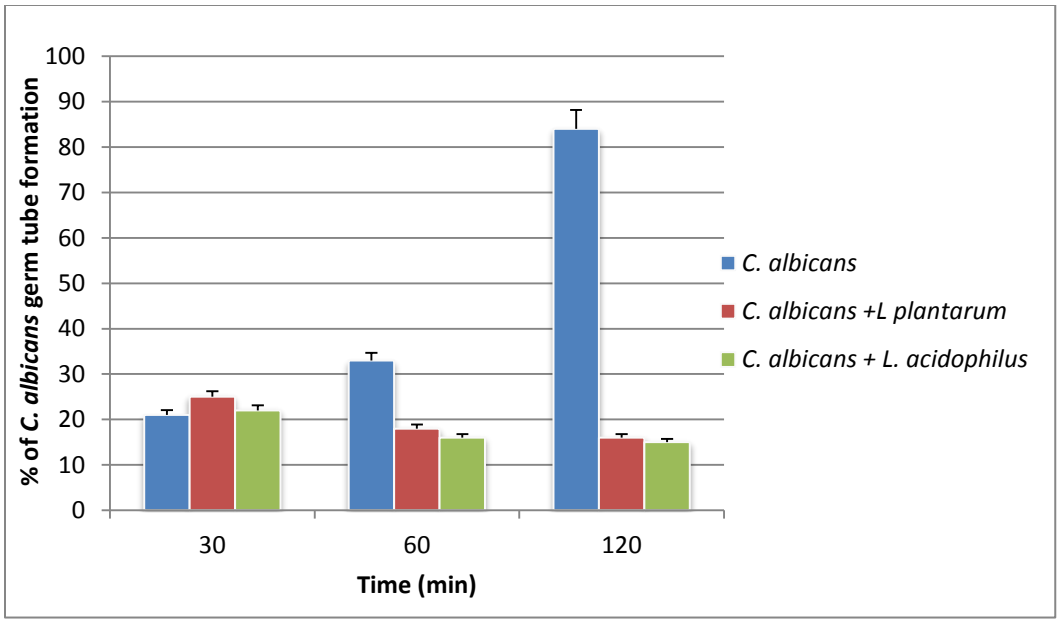

Fig. 1. Effects of supernatants of *Lactobacillus* strains on germ tube formation by *C. albicans*. The Error bars represent the standard deviation

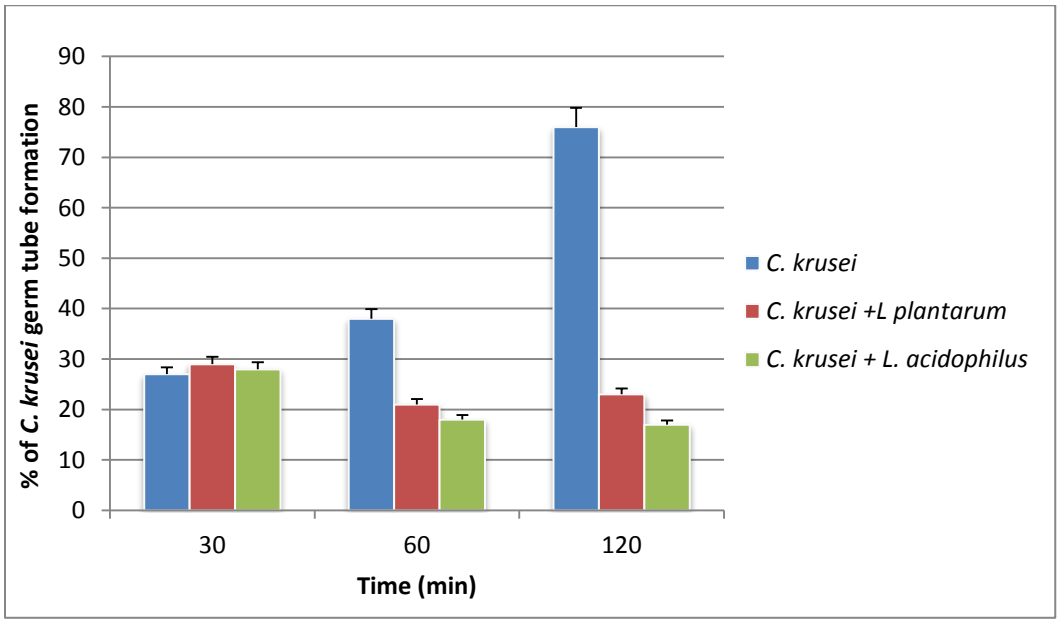

Fig. 2. Effects of supernatants of *Lactobacillus* strains on germ tube formation by *C. krusei*. The Error bars represent the standard deviation

3.5. Surface hydrophobicity, auto-aggregation, and co-aggregation of Lactobacilli with *Candida* spp.

The SAT was used to investigate the hydrophobic/hydrophilic properties of *Lactobacillus* surfaces. *L. plantarum* ATCC 14917 and *L. acidophilus* ATCC 20552 showed hydrophilic properties, as cell aggregates are formed with both tested *Lactobacillus* strains after 1 min. at a salt concentration of 2 mol/ l. Much larger aggregates are observed with *L. acidophilus* than *L. plantarum*.

Regarding auto-aggregation test, the tested Lactobacillus strains showed high ability to auto-aggregate. *L. acidophilus* expressed higher percentage of auto-aggregation after 24 h (71 %) than that recorded by *L. plantarum* (53 %). Auto-aggregation increased with increasing the incubation time, and the highest auto-aggregation is recorded at 24 h, as shown in Table (4).

Also, all the tested *Candida* spp. auto-aggregated and the ranges of auto-aggregation percentage are; 24 – 27 %, 22- 26 % and 23- 29 % after 24 h, for *C. albicans*, *C. tropicalis* and *C. krusei*, respectively. The auto-aggregation percentage of the 2 *Lactobacillus* strains is much higher than the Candida spp. auto-aggregation, tested under the same conditions.

According to results of the Co-aggregation assay, both of *L. acidophilus* and *L. plantarum* showed high ability to co-aggregation with all the tested *Candida* spp. at different percentages; after 4 h, 20 h and 24 h of incubation, as demonstrated in Table 5. The highest co-aggregation percentages of *L. acidophilus* and *L. plantarum* are recorded with *C. albicans* with ranges of 42±5.2- 49±3.2 and 30±3.7- 35±0.7; respectively, as demonstrated in Table (5).

Table 4. Auto-aggregation percentages of the *Lactobacillus* and *Candida* spp.

Strains	**Auto-aggregation (%)***		
	4 h	20 h	24 h
L. acidophilus	27±1.4	53±3.6	71±2.8
L. plantarum	12±3.9	30±1.9	53±2.1
C. albicans (20) **	5±3.2- 7±1.4***	14±2.3- 18±4.2	24±3.7- 27±1.5
C. tropicalis (5)	8±2.2- 10±1.8	18±1.6- 20±1.5	22±2.2- 26±1.8
C. krusei (3)	4±3.2- 6±2.5	13±2.5- 16±4.2	23±4.6- 29±3.2

Where, (*): Results are means of auto-aggregation percentages; (±): SD; (**): Number of isolates; (***): Range of aggregation percentages of the tested strains

Table 5. Co-aggregation percentages for *L. acidophilus* ATCC 20552 and *L. plantarum* ATCC 14917 with oral *Candida* isolates

Strains	Co-aggregation (%)*		
	4 h	20 h	24 h
L. acidophilus with:			
C. albicans (20)**	4±1.1- 6±2.3	25±4.2- 27±5.3	42±5.2- 49±3.2
C. tropicalis (5)	3±2.1- 3±6.8	16±3.1- 17±4.6	27±0.3- 28±1.6
C. krusei (3)	3±1.7- 5±0.2	13±4.5- 15±3.4	26±2.1- 28±0.2
L. plantarum with:			
C. albicans (20)	4±0.1- 6±1.2	19±0.4- 22±1.3	30±3.7- 35±0.7
C. tropicalis (5)	2±3.7- 3±3.6	12±1.9- 14±4.5	23±2.2- 25±3.8
C. krusei (3)	3±3.4- 4±2.7	11±0.8- 13±2.7	21±3.3- 22±4.9

Where; (*) Represent means of Co-aggregation percentages; (±): SD; (**): Number of strains; (***): Range of co-aggregation percentages for the tested isolates

3.6. Ability of biofilm formation by *Candida* spp.

Biofilm formation by *Candida* spp. was detected using the tissue culture plate assay. Out of 28 *Candida* isolates, 66.7% of *C. krusei* showed strong biofilm formation, followed by *C. albicans* and *C. tropicalis* (both 20 %). Conversely, weak or no biofilm formation is observed in 50 %, 40 % and 33.3 % of *C. albicans*, *C. tropicalis* and *C. krusei*; respectively, as shown in Table (6). The strong biofilm forming Candida spp. are *C. albicans* (1, 2, 6, 12), *C. tropicalis* (2) and *C. krusei* (1 and 3).

Table 6. Ability of biofilm formation by different tested *Candida* spp.

Candida spp.	Biofilm formation					
	Strong		Moderate		Non/Weak	
	No.	%	No.	%	No.	%
C. albicans (20)*	4	20	6	30	10	50
C. tropicalis (5)	1	20	2	40	2	40
C. krusei (3)	2	66.7	0	0	1	33.3
Total (28)	7	25	8	28.6	13	46.4

*Total number of isolates, Results are means of two replicates

3.7. Antibiofilm activity of *Lactobacillus* supernatants

Antibiofilm activity of *L. plantarum* and *L. acidophilus* supernatants was tested on both of biofilm formation and mature preformed biofilm by the strong biofilm forming Candida spp. including; *C. albicans* (1, 2, 6, 12), *C. tropicalis* (2) and *C. krusei* (1 and 3). As demonstrated in Fig. (3), significant biofilm inhibition is recorded when *Candida* spp. were treated with *L. plantarum* and *L. acidophilus* supernatants, with percentage inhibition of 50-72 % and 74-85 %, respectively. On studying the effects of the two *Lactobacillus* supernatants on reduction of the preformed biofilms, it is observed that the supernatants effectively eradicated the preformed *Candida* biofilms, but at lower percentages than those recorded during the inhibition of biofilm formation. The tissue culture assay expressed a reduction percentages of 26-35 % and 29-44 % for *L. plantarum* and *L. acidophilus* supernatants; respectively, as presented in Fig. (4).

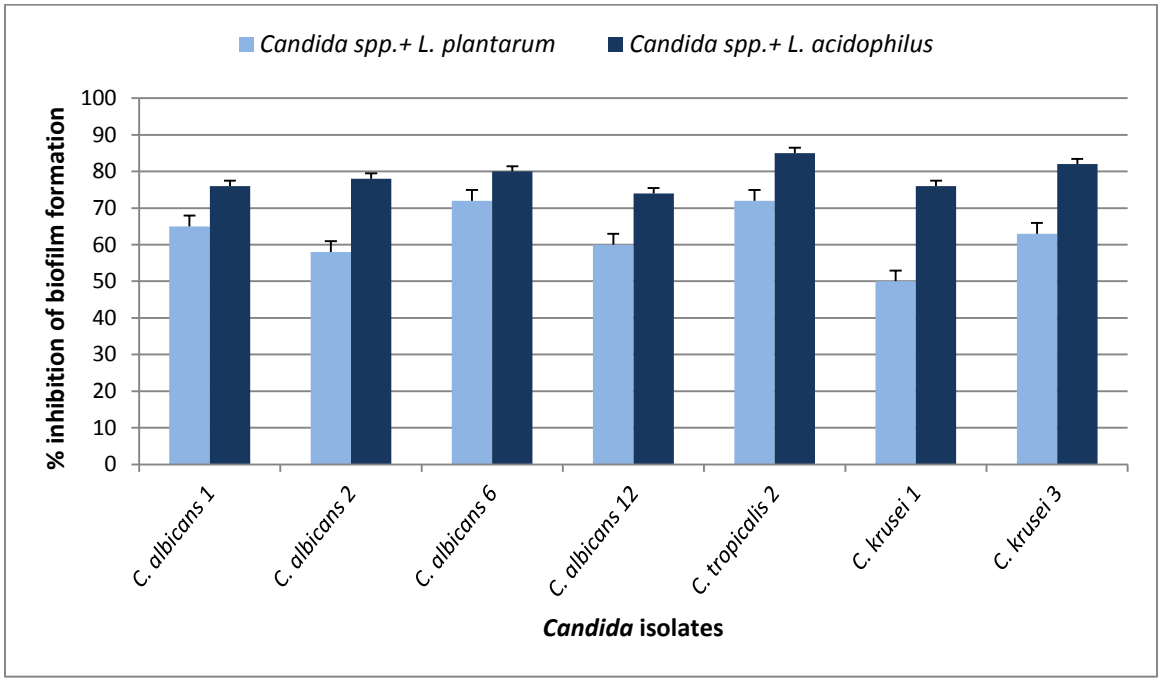

Fig. 3. Antibiofilm effects of *L. plantarum* and *L. acidophilus* supernatants on strong biofilm forming *Candida* spp.

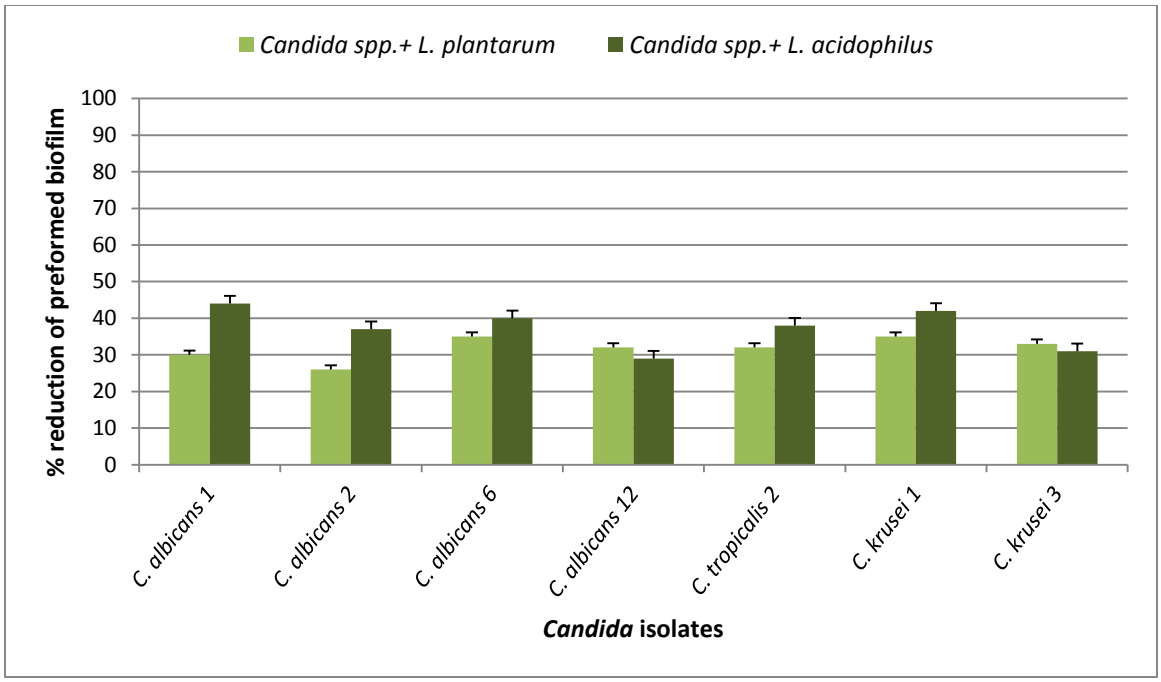

Fig. 4. Effects of supernatants of *L. plantarum* and *L. acidophilus* on reduction of the preformed biofilm by *Candida* spp.

4. Discussion

Treatment with fixed orthodontic devices is associated with significant biofilm accumulation, thus patients are at risk of deteriorating their oral health, as confirmed by Hadj-Hamou *et al*., (2020). In this study, 60 % and 80 % *Candida* spp. were isolated from orthodontic and dental caries samples; respectively, and 3 different species were identified mainly; *C. albicans* (40 % and 60 %), *C. tropicalis* (10 % and 15 %) and *C. krusei* (10 % and 5 %), respectively. In consistence with the current results, Al-Oebady *et al*., (2019) showed higher colonization by *Candida* spp. in presence of fixed orthodontic devices; compared to the normal flora, and *C. albicans* was present at a percentage of 35 %, followed by *C. tropicals* (23.7 %), *C. parapsilosis* (21.1 %) and *C. krusei* (19.5 %). In a similar recent study, Alhasani *et al*., (2020) recorded that *C. albicans* (72.5 %) was the most commonly recovered yeast sp., which colonized the oral cavity after introduction of fixed orthodontic appliances, followed by *C. glabrata* and *C. tropicalis* (both 12.5 %). Moreover and consistent with our results, Mishra *et al*., (2016) found that the prevalence of *C. albicans* was 69.2 % in children with decayed teeth. In addition, Al-hebshi *et al*., (2015) revealed that *Candida* spp. were detected in 63.3 % of the children, where *C. albicans* represented 69 % of the total isolates, followed by *C. tropicalis*, *C. glabrata*, *C. krusei* and unidentified species, which recorded prevalence of 11.8 %, 5.5 %, 2.3 % and 11.4%, respectively.

Recently, Radi and Abdelmonem, (2017) proved that emergence of resistance to the antifungals is a serious health problem. In this research work, the antifungal sensitivity of *Candida* spp. was investigated using the disk diffusion method. We found that *C. tropicalis* and *C. krusei* showed the highest resistance to fluconazole (40 % and 33.3 %) and amphotericin B (20 % and 33.3 %); respectively, while both of *C. albicans* and *C. tropicalis* expressed the highest resistance to ketoconazole (40 %). In contrast to these results; Alhasani *et al*., (2020) demonstrated that all oral isolates of *Candida* spp.

were susceptible to amphotericin B and ketoconazole; however, in accordance with the present study, resistance to fluconazole was recorded in *C. tropicalis* (40 %) and *C. albicans* (13.8 %). Compared to this study, recent study conducted by Al-Oebady *et al*., (2019) revealed higher rate of resistance to amphotericin B in *C. krusei* (60 %), while the ketoconazole resistance rates were 50 %, 75 % and 50 % for *C. albicans*, *C. tropicalis* and *C. krusei*, respectively.

As reported recently by Hadj-Hamou *et al*., (2020), the use of probiotics is proposed to prevent and/or treat oral pathologies such as dental caries and periodontal tissues diseases. In this study, the antifungal activities of *Lactobacillus* supernatants were assessed using agar well diffusion assay. Results showed that all tested *Candida* spp. were inhibited by supernatants of both probiotic bacteria, where *L. acidophilus* had higher antifungal efficacy than *L. plantarum* recording inhibition zone diameters ranging from 11.9-19.7 mm. Current results are consistent with those of the previous study of Radi and Abdelmonem, (2017), who highlighted that *L. acidophilus* had the most effective antifungal efficacy against both *C. albicans* and non-albicans (67.5 %), followed by *L. rhamnosus* (41 %) and *L. casei* (15.5 %); however, *L. plantarum* had the least antifungal potential (9 %). Furthermore, Salari and Almani, (2020) recently investigated the antifungal potencies of cell-free supernatants of various concentrations of *L. acidophilus* and *L. plantarum* on five oral *Candida* spp., and found that *C. albicans* was the most sensitive to the tested Lactobacilli supernatants.

We demonstrated the inhibitory effects of *Lactobacillus* supernatants on germ tube formation, as germination of *Candida* spp. is a virulent determinant. Current results proved that the two *L. plantarum* and *L. acidophilus* supernatants suppressed the formation of germ tubes by the tested *C. albicans* (recording inhibitions of 68 % and 53 %), and *C. krusei* (69 % and 59 %), respectively. In accordance, a previous study conducted by Noverr and Huffnagle, (2004) investigated the effects of supernatants obtained from 2 h probiotic bacterial cultures on the morphology of *C. albicans*. They observed the inhibition of *C. albicans* germ tube formation, while inclusion of 24h cultures completely inhibited this germination. They attributed this inhibitory activity to the presence and accumulation of soluble compounds in the culture supernatants.

In this study, the ability of biofilm formation by the oral *Candida* spp. was detected using the tissue culture plate assay; where *C. krusei*, *C. albicans* and *C. tropicalis*, exhibited strong biofilm formation (66.7 %, 20 % and 20 %, respectively). Similarly, the recent research work conducted by Alhasani *et al*., (2020) tested the ability of the isolated *Candida* spp. to form biofilm using the same assay, and recorded biofilm formation rate of 52.5 % for all the tested of *Candida* spp.; however, biofilm formation occurred more frequently among *C. tropicalis* and *C. glabrata* (both 60 %), than in *C. albicans* (48.3 %).

The current study revealed significant inhibition of biofilm formation by the strong forming *Candida* spp. when treated with *L. plantarum* supernatant, although this inhibition was lower than that recorded by *L. acidophilus*. Similarly, a recent study conducted by Tan *et al*., (2018) proved that *L. acidophilus* cell-free supernatant inhibited *C. albicans* biofilm development and filamentation.

Bacterial surface hydrophobicity is the main mechanism of adhesion in the mouth (Rosenberg *et al*., 1983). In the present study, cells of *L. plantarum* and *L. acidophilus* strains showed hydrophilic properties on testing with the salt aggregation test (SAT). Similarly, the hydrophilic nature of Lactobacilli had also been demonstrated in the previous studies conducted by Deepika *et al*. (2009); Gong *et al*., (2012). However, in contrast to our findings, Piwat *et al*., (2015) stated that most oral Lactobacilli had moderate to high hydrophobicity. This disagreement in results may be attributed to the difference in the methods used to detect the bacterial

hydrophobicity, as reported by Marin *et al*., (1997). A previous study of Van Loosdrecht *et al*., (1987) have proved that for hydrophobic microorganisms, hydrophobicity is the primary factor controlling adhesion, although adhesions in case of hydrophilic microorganisms are dominated by an electrokinetic force.

Our study showed that *L. acidophilus* demonstrated higher percentage of auto-aggregation (71 %) compared to *L. plantarum* strain (53 %) after overnight incubation. The tested *Lactobacillus* strains showed high ability for co-aggregation with all the *Candida* spp. after 24 h of incubation; however, the highest co-aggregation percentages were recorded by *L. acidophilus* and *L. plantarum* with *C. albicans* isolates with range of percentages of 42- 49 % and 30- 35%, respectively.

Similar to the present results, Chervinets *et al*., (2018) showed that tested *Lactobacillus* strains made auto-aggregation and co-aggregation with *Candida* spp., and exhibited high surface hydrophobicity. Sazawal *et al*., (2006); Chervinets *et al*., (2018) reported that auto-aggregation, co-aggregation and surface hydrophobicity are among the most important features of the adaptive potential, which are the basis of biofilm formation. An earlier studies conducted by Al-Ahmad *et al*., (2007); Ledder *et al*., (2008) proved that aggregation aids in the entry of new bacterial species within the biofilms, thus the exchange of genes and metabolic products will be easier and supports the microorganisms' survival in different environments.

Chervinets *et al*., (2018) recently revealed that Lactobacilli incorporated in biofilms can easily synthesize substances that resemble antibiotics, which suppress the growth and proliferation of pathogenic and opportunistic microflora; to ensure survival of Lactobacilli, protection and increase in number. Moreover, a previous study of Vilela *et al*., (2015) highlighted that formation of biofilms is probably reduced by probiotics through the production of inhibitory substances called "bacteriocins". Later, Wannun *et al*., (2016) reported the isolation of a bacteriocin called "fermencin SD11" from *L. fermentum* SD11, which is a human oral *Lactobacillus* that has a powerful inhibitory effect on oral *Candida* cells. The anti-Candida activities can be attributed to several reasons including; co-aggregation, H_2O_2 production and oral pH modification (Jørgensen *et al*., 2017), through releasing large amounts of lactic acid (Denkova *et al*., 2013), and through complete inhibition of fungal biofilms formation (Chew *et al*., 2015).

Conclusion

We concluded that orthodontic treatment with fixed appliances and dental caries caused specific alterations in the oral environment. *Candida* spp. are opportunistic microorganisms that showed resistance to several antifungal drugs and can form biofilms in the oral cavity. Results of this study indicated that attention has to be paid to control the oral Candida infection. Probiotic Lactobacilli have good adaptive properties; antimicrobial and antibiofilm potentials against oral *Candida*. Thus they can be used in prevention and control of these yeast infections. Further investigations should be carried out to assess the characteristics of the probiotic strains before providing them for clinical trials. Screening and characterization of *Lactobacillus* strains are necessary to discover ideal and novel probiotics.

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Conflict of interest

The authors declare no conflict of interests.

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Ethical approval

The protocols used in the study were approved by the Ethics Committee of the Faculty of Dentistry, Minia University, Minia, Egypt.

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