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FULL PAPER

Candida albicans survival, growth and biofilm formation are differently affected by mouthwashes: an *in vitro* study

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SUMMARY

Candida albicans is the most common cause of oral mycoses. The aim of the present study was to investigate *in vitro* the susceptibility of *C. albicans* to mouthwashes, in terms of growth, survival and biofilm formation.

Candida albicans, laboratory strain SC5314, and 7 commercial mouthwashes were employed: 3 with 0.2% chlorhexidine digluconate; 1 with 0.06% chlorhexidine digluconate and 250 ppm F^- sodium fluoride; 3 with fluorine-containing molecules. None of the mouthwashes contained ethanol in their formulations. The anti-Candida effects of the mouthwashes were assessed by disk diffusion, crystal violet and XTT assays. By using five protocols combining different dilutions and contact times the mouthwashes were tested against:

1) *C. albicans* growth;
2) biofilm formation;
3) survival of fungal cells in early, developing and mature Candida biofilm.

Chlorhexidine digluconate-containing mouthwashes consistently exhibited the highest anti-Candida activity, irrespective of the protocols employed. Fungal growth, biofilm formation and survival of Candida cells within biofilm were impaired, the effects strictly depending on both the dilution employed and the time of contact.

These *in vitro* studies provide evidence that mouthwashes exert anti-Candida activity against both planktonic and biofilm fungal structures, but to a different extent depending on their composition. This suggests special caution in the choice of mouthwashes for oral hygiene, whether aimed at prevention or treatment of oral candidiasis.

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INTRODUCTION

The human oral cavity is the most important microbial gateway to the body (Dewhirst *et al.,* 2010). The oral cavity of healthy individuals harbours a wide variety of environmental microorganisms, mostly bacteria, but also fungi, protozoa and viruses (Scannapieco, 2013), the so-called "resident microbiota". The physiological role of such microorganisms and their relevance as aetiological causes of oral infectious diseases are being investigated and increasingly elucidated (O'Donnell *et al.*, 2015). *Candida albicans* is a well-known example of a microbial agent, frequently harboured as a commensal of human healthy mucosae, which can behave as an opportunistic pathogen both locally and distally. Indeed, candidiasis is the most common opportunistic fungal infection affecting the oral mucosa (Garcia-Cuesta *et al.*, 2014). Local lesions, most commonly caused by *C. albicans* (Dağistan *et al.*, 2009; Muadcheingka and Tantivitayakul, 2015), occur especially in people with poor oral hygiene, hyposalivation (secondary to drugs, disease, or radiation therapy), nutritional deficiencies, dental prosthesis, tissue alteration, immunodeficiency and chemotherapeutic or systemic steroid use (Garcia-Cuesta *et al.*, 2014; Lalla *et al.*, 2013).

C. albicans is characterized by numerous virulence factors such as adherence, secretion of proteinases, dimorphic transition from yeast-to-hypal form and production of biofilm (Ramage *et al.*, 2005; İnci *et al.*, 2012; Mohan das and Ballal, 2008; Vinitha and Ballal, 2007). In particular, the development of biofilm occurs by a series of sequential phases over a period of 24-48 hours. At the beginning, yeast cells adhere to a biotic or abiotic surface. Then, hyphal and/or pseudohyphal extensions appear and begin to proliferate. Gradually, an extracellular polymeric matrix is produced, accumulated and packed, embedding tightly adherent fungal cells (Finkel and Mitchell, 2011). Over time, non-adherent newly formed yeast cells can detach from mature biofilm and disperse, thus allowing the fungus to colonize distant sites and expand the infection. By organizing in such a sessile structure, *C. albicans* enhances its pathogenic potential, showing increased resistance to immune-mediated defences (Mayer *et al.*, 2013) and reduced susceptibility to antifungal drugs, such as fluconazole and amphotericin B, and disinfectants (i.e. chlohexidine) (Tobudic *et al.*, 2012; Desai and Mitchell, 2015; Rahmani-Badi *et al.*, 2015; Uygun-Can *et al.*, 2016).

Good oral hygiene consists of effective tooth-brushing with toothpaste, that allows mechanical removal of the

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plaque. Brushing efficacy can be enhanced by additional treatment with mouthwashes (MoWs) (Haps *et al.*, 2008; Teles and Teles, 2009; Kuo *et al.*, 2013) that allow further mechanical removal of microorganisms and sometimes provide antimicrobial activity delaying plaque formation. The numerous commercially available MoWs can be divided into two groups according to their formulation: the first contains chemical substances with antibacterial activity (i.e. chlohexidine digluconate); the second consists of MoWs whose formulation does not include any antibacterial substance. Initial evidence describes the efficacy of MoWs in terms of antifungal activity (Fu *et al.*, 2014).
The aim of this *in vitro* study was to examine the effects of MoWs on *C. albicans* at different morphological stages; specifically, yeast cell growth, hyphal formation, biofilm production and/or survival of Candida cells within biofilm (as assessed at different development stages) were investigated.

MATERIALS AND METHODS

Candida albicans strain

C. albicans, reference strain SC5314, was employed. Fungal cultures were maintained by biweekly passages onto Sabouraud dextrose agar (SDA) plates (OXOID, Milan, Italy). The day before each experiment, fresh cultures were seeded onto SDA plates and incubated at 37°C. After overnight incubation, fungal cells were harvested by a sterile inoculating loop, suspended in phosphate-buffered saline (PBS, EuroClone, Whethereby, UK), washed twice by centrifugation at 3500 rpm for 10 minutes, counted by Burker's chamber and resuspended at $1x10^6$ yeast cells/ml in RPMI 1640 (Gibco, Grand Island, NY, USA), supplemented with 10% heat inactivated foetal bovine serum (Defined Hyclone, Logan, Utah, USA), 50 µg/ml gentamicin (EuroClone, Whethereby, UK) and 2 mM L-glutamine (EuroClone, Whetherby, UK), hereafter referred to as cRPMI. This fungal cell suspension, composed of yeast-like forms, was used throughout the study to set up all the experiments.
For long-term storage, *C. albicans* was maintained as frozen stocks at -80°C, in glycerol solution 20% (v/v).

Mouthwashes

Seven commercial MoWs, operationally indicated with Arabic numbers from 1 to 7, were employed. MoW 1 (CURASEPT 0.20, Curadent Healthcare S.p.A., Saronno (VA), Italy), MoW 2 (DENTOSAN COLLUTORIO, Recordati S.p.A., Milan, Italy) and MoW 3 (MERIDOL COLLUTORIO, Gaba-Colgate-Palmolive, Swidnica, Poland) contained 0.2% chlorhexidine digluconate; MoW 7 (PARODONTAX, GlaxoSmithKline, Brentford, UK) contained 0.06% chlorhexidine digluconate and 250 ppm F^- sodium fluoride; MoW 4 (ELMEX SENSITIVE PROFESSIONAL, Gaba, Therwil, Switzerland), MoW 5 (LISTERINE TOTAL CARE ZERO, Johnson & Johnson, Maidenhead, UK) and MoW 6 (MERIDOL COLLUTORIO, Gaba Vebas S.r.l., Rome, Italy) included fluorine-containing molecules in their formulation.

Disk-diffusion test

The disk-diffusion assay was performed according to the Clinical and Laboratory Standards Institute guideline CLSI M-44A (Sheehan & National Committee for Clinical Laboratory Standards, 2004), with minor modifications. Briefly, a sterile cotton swab was dipped in *C. albicans* yeast stock suspension ($1\text{-}5x10^6$ cells/mL, turbidity corresponding to the 0.5 McFarland standard) and inoculated on Mueller Hinton agar (MHA) supplemented with 2% glucose and 0.5 mg/mL methylene blue. The plates were allowed to dry for 3 to 5 minutes. Then, neutral filter paper disks (6 mm) were placed onto the MHA surface and embedded with MoWs (13 µl/disk); the positive control (C+) disk contained amphotericin B (AmB; 250 µg/ml); the negative control (C-) disk contained PBS. Plates were then incubated at 37°C, examined after 24 or 48 h and the growth inhibition haloes around each disk were measured. The results were expressed as halo diameters in mm.

Biofilm production

C. albicans biofilm production was performed on flat-bottom 96-well plates (Corning Incorporated, NY, USA), as described elsewhere (Pierce *et al.*, 2008) with minor modifications. Briefly, yeast cell suspensions ($1x10^6$ yeast cells/ml in cRPMI prepared as detailed above) were added to each well (100 µl/well). The plates were incubated for 24 or 48 h at 37°C and 5% CO_2. Then, Crystal Violet (CV) assay and 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT, Sigma, St Louis, MO, USA) reduction assay were performed to quantify biofilm mass and fungal cells viability, respectively, as detailed elsewhere (Pierce *et al.*, 2008; Mazaheritehrani *et al.*, 2014). The absorbance was measured spectrophotometrically by the SunRise Microplate Reader (Sunrise, Tecan, Salzburg, Austria). For biofilm, the absorbance was measured at 540 nm and the colour intensity was proportional to biofilm biomass; for XTT assay, the absorbance was measured at 450 nm and 620 nm.

Protocol for assessment of mouthwash effects on Candida yeast cell biofilm formation

Yeast cell suspensions (500 µl/tube) prepared in cRPMI as detailed above were exposed to each MoW (500 µl/tube), at 5 different dilutions (1:2, 1:4, 1:16, 1:32, 1:64). After different times of incubation (1', 5', 10', 30') at 37°C in the presence of 5% CO_2, fungal cells were washed twice in PBS by centrifugation at 4500 rpm for 8 min. The pellets were then suspended with 1 ml of cRPMI, dispensed into flat-bottom 96-well plates and incubated for 48 h at 37°C in the presence of 5% CO_2. Then, CV assay was performed. Each experimental group consisted of 3 replicates.

Protocol for assessment of mouthwash effects on survival of Candida cells embedded in early, developing and mature biofilm

Antifungal effects of the 7 MoWs were investigated by 3 different protocols, on early (Protocol A), developing (Protocol B) and mature (Protocol C) biofilms as below.
Protocol A = early biofilm (24 h-old biofilm + MoWs): 24 h-old biofilms were exposed to the MoWs; specifically, 5 different MoW dilutions (1:2, 1:4, 1:16, 1:32, 1:64) were added to each well (100 µl/well) and allowed to incubate for 1, 5, 15 and 30 minutes, at 37°C. Then, XTT assay was performed.
Protocol B = developing biofilm (24 h-old biofilm + MoWs +24 h): 24 h-old biofilms were exposed to the MoWs (1:2, 1:4, 1:16, 1:32, 1:64 dilutions) for 1, 5, 15 and 30 minutes at 37°C. Then, after washing with 200 µl of PBS, cRPMI was added to each well (200 µl/well) and the plates were incubated for additional 24 h, at 37°C. Then, XTT assay was performed.

Protocol C = mature biofilm (48 h-old biofilm + MoWs): 48 h-old biofilms were exposed to the MoWs (1:2, 1:4, 1:16, 1:32, 1:64 dilutions) for 1, 5, 15 and 30 minutes, at 37°C. Then, XTT assay was performed.
Each experimental group consisted of 3 replicates.

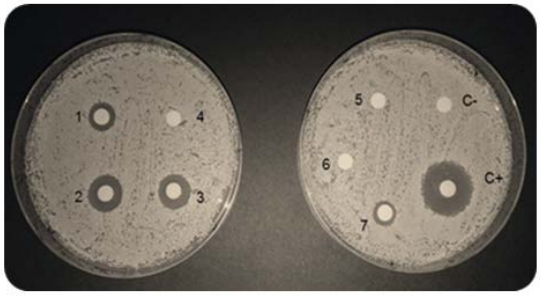
Statistical analyses
All the data presented in the Tables are expressed as mean optical densities (OD) of 3 to 5 independent experiments. Each condition was assessed in triplicate. Analysis of variance (ANOVA) and Bonferroni post-hoc test were carried out by IBM SPSS Statistics 22 to assess overall difference in OD readings obtained between different groups in relation to control groups. Statistical significance was set at $p \leq 0.05$, while $p < 0.001$ was indicative of highly statistically significant differences.

RESULTS

Effects of mouthwashes on Candida growth as assessed by disk diffusion test
Initially, to assess the effects of MoWs on *C. albicans* growth, the disk diffusion test was employed. *Figure 1A* shows a representative experiment. MoWs 1, 2, 3 and 7 produced evident inhibition areas, whereas MoWs 4, 5 and 6 did not have any effect. Negative (PBS) and positive control (AmB) disks provided no and large haloes, respectively. As assessed by 3 independent experiments, the measurement of the disk diffusion zone diameters returned values ranging from 14 mm (MoW 2) to 9 mm (MoW 7), as detailed in *Figure 1B*.

Effects of mouthwash pretreatment on biofilm formation by Candida yeast cells
In order to assess the effects of MoWs on the ability of *C. albicans* to form a biofilm, yeast cells were treated with each MoW at 5 different dilutions and for 4 different contact times and then incubated in cRPMI for 48 h in order to produce biofilm. The impact of the MoWs was evaluated by microscopy and CV assay. Negative control samples, not treated with MoWs, showed a densely packed biofilm (*Figure 2A*). On the contrary, by MoWs treatment, biofilm formation was affected differently according to the MoW employed, to its working-strength dilution and to the contact time. *Figure 2B* shows a sample treated with MoW 2 at 1:4 dilution for 15' contact time; under these conditions, biofilm formation did not occur and only few and damaged yeast cells were detectable. Quantitative analysis was then performed by CV assay. As detailed in *Table 1*, MoWs 2 and 3 produced the highest antifungal effects, as

A

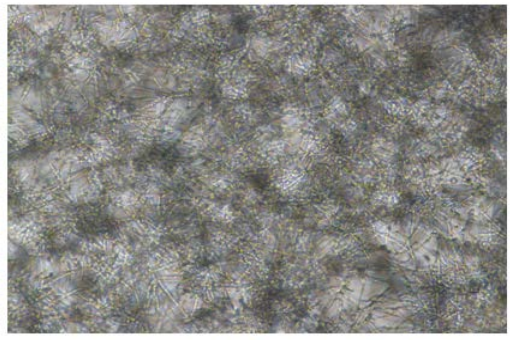


B

MOUTHWASH	HALO DIAMETER (mm)
1	11 ± 0
2	14 ± 0
3	13 ± 0
4	NO EFFECT
5	NO EFFECT
6	NO EFFECT
7	9 ± 0
C +	20 ± 0
C -	NO EFFECT

Figure 1 - *Assessment of antifungal activity by disk diffusion assay. A) C. albicans yeast cells (1 x 10^6 cells) were inoculated on plates of Muller-Hinton agar with 2% Glucose and 0.5 µg/mL Methylene Blue Dye Medium. Neutral filter paper disks were placed onto the plates surface and embedded with MoWs (13 µl/disk). Each plate was examined after 24 h of incubation at 37°C. The disk diffusion haloes were measured and the diameters (mm) were reported. B) Values represent mean ±SD of the haloes observed in three independent experiments. C+ indicates the positive control disk containing AmB (250 µg/ml). C- indicates negative control disk containing PBS.*

A



B

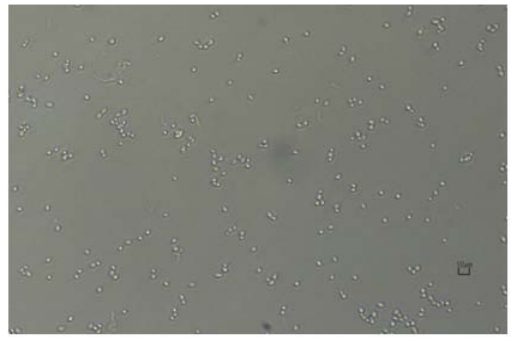


Figure 2 - *Morphology of C. albicans exposed or not to MoW 2. C. albicans yeast cells (1x10^6 cells) were exposed or not to MoW 2 (1:4 dilution), for 15', at 37°C in the presence of 5% CO_2. Then, Candida cells were washed twice in saline, suspended in cRPMI and dispensed into flat-bottom 96-well plates and incubated for additional 48 h at 37°C in the presence of 5% CO_2. A) Negative control sample, consisting of untreated cells: a densely packed biofilm was evident. B) Sample treated with MoW 2: biofilm was not formed and only scant yeast cells could be detected. Optical microscope images were taken at magnification of 20X. Bar: 10 µm.*

established by the drastic decrease in OD levels. MoW 2, in particular, was effective in impairing Candida biofilm formation up to 1:16 dilution, at all the contact times and up to 1:32 dilution, at 30 minutes contact time. MoWs 5, 6, and 7 showed a time- and dilution-dependent decreasing efficacy, whereas MoWs 1 and 4 had no antifungal effects, regardless of both the contact time and dilution employed (*Table 1*).

Effects of mouthwashes on survival of Candida cells embedded in early, developing and mature biofilm

The MoW effects on *C. albicans* early, developing and mature biofilm were assessed by protocols A, B and C, as detailed above. Biofilm formation and survival of Candida cells embedded in biofilm had been assessed by CV and XTT assays, respectively.

As shown by Protocol A (24 h-old + MoWs), *C. albicans* in early biofilm was susceptible to all the MoWs, although to a different extent. MoW 4 had the lowest effects, being effective only at long contact times and low dilutions (*Table 2*).

Protocol B (24 h-old + MoWs + 24 h) demonstrated that MoWs 2 and 3 retained antifungal activity against *C. albicans* developing biofilm up to 1:16 dilution (*Table 3*). In particular, MoW 2 was effective at all the contact times when diluted 1:2 and 1:4, while a minimum of 5 minutes were necessary when diluted 1:16. MoWs 1, 6 and 7 had effects but only when employed for long contact times (≥15 minutes) and only at 1:2 or 1:4 dilutions. MoWs 4 and 5 did not display any effect on developing biofilm regardless of dilution or the contact time employed (*Table 3*).

Finally, in Protocol C (48 h-old + MoWs), MoWs 1, 2, 3, 5 and 7 showed significant effects against *C. albicans* mature

Table 1 - *Effects of mouthwash pre-treatment on Candida biofilm formation by Crystal violet assay.*

Mouthwash	Contact times	Mouthwash dilutions (Od values)				
		1:2	1:4	1:16	1:32	1:64
1	1'	3.98	3.56	3.58	3.81	3.79
	5'	4.00	1.96	2.00	2.86	3.06
	15'	3.96	2.66	2.97	3.62	3.63
	30'	3.99	1.61	1.80	1.85	3.26
2	1'	0.19**	0.45**	1.01**	3.77	3.87
	5'	0.21**	0.71**	0.74**	2.39	2.58
	15'	0.18**	0.11**	0.11**	2.37	3.65
	30'	0.18**	0.14**	0.28**	0.39**	2.11
3	1'	0.20**	0.09**	0.86**	3.97	3.95
	5'	0.18**	0.60**	0.76**	1.73	2.41
	15'	0.18**	0.09**	0.78**	1.83	3.71
	30'	0.17**	0.27**	0.32**	0.72**	2.95
4	1'	4.00	3.12	3.73	3.84	3.94
	5'	4.00	2.74	2.77	2.78	2.72
	15'	4.00	3.23	3.46	3.54	3.67
	30'	4.00	3.21	3.39	3.34	3.21
5	1'	0.20**	3.32	3.49	3.64	3.66
	5'	0.16**	2.07	2.64	2.67	2.99
	15'	0.17**	2.58	3.55	3.57	3.58
	30'	0.18**	2.14	3.02	3.29	2.92
6	1'	0.17**	0.22**	3.07	3.56	3.76
	5'	0.19**	0.32**	1.67	2.56	2.57
	15'	0.20**	0.21**	2.51	3.39	3.61
	30'	0.21**	0.11**	0.11**	3.13	3.29
7	1'	0.19**	0.22**	3.52	3.71	3.84
	5'	0.19**	0.11**	2.30	2.82	2.83
	15'	0.21**	0.67**	2.71	4.00	3.39
	30'	0.17**	0.32**	1.24*	2.48	3.33

C. albicans yeast cells (1x10^6/ml) were exposed or not to MoWs (at the indicated dilutions), for 1', 5', 15' and 30', at 37°C in the presence of 5% CO_2. Then, Candida cells were washed twice in saline, suspended in cRPMI and dispensed into flat-bottom 96-well plates (1x10^5/well) and incubated for additional 48 h at 37°C in the presence of 5% CO_2. The Crystal violet assay was then performed and the OD recorded. The numbers reported in the table are the mean values of 3 independent experiments. The values highlighted in grey, less than 1.5, were arbitrarily considered as conditions of no biofilm formation. Asterisks indicate statistically significant (p≤0.05,) or highly significant (p<0.001,**) differences from the corresponding negative controls, that returned the following* values: 3.91 for 1' contact time; 3.76 for 5' contact time; 3.78 for 15' contact time; 4.00 for 30' contact time.

Table 2 - *Effects of mouthwashes on early biofilm, by XTT assay.*

Mouthwash	Contact times	Mouthwash dilutions (Od values)				
		1:2	1:4	1:16	1:32	1:64
1	1'	0.18**	2.03	2.62	2.84	2.85
	5'	0.33**	0.67**	2.04	2.81	2.89
	15'	0.13**	0.24**	0.79**	2.92	2.83
	30'	0.11**	0.13**	0.22**	2.75	2.80
2	1'	0.05**	0.09**	0.26**	2.42	2.44
	5'	0.05**	0.05**	0.12**	2.39	2.58
	15'	0.04*	0.05*	0.08*	2.09	2.84
	30'	0.04**	0.06**	0.04**	0.79**	2.65
3	1'	0.08**	0.10**	0.36**	2.87	2.86
	5'	0.06**	0.05**	0.23**	2.53	2.89
	15'	0.05**	0.07**	0.08**	2.12	2.91
	30'	0.05**	0.07**	0.04**	1.47**	2.78
4	1'	1.85	2.93	2.94	2.84	2.84
	5'	1.94	2.49	2.84	2.87	2.86
	15'	0.28**	1.01**	2.60	2.81	2.80
	30'	0.17**	0.30**	1.72	2.87	2.73
5	1'	0.11**	2.49	2.86	2.82	2.87
	5'	0.09**	0.31**	2.88	2.81	2.85
	15'	0.08**	0.10**	2.90	2.85	2.81
	30'	0.07**	0.07**	2.90	2.75	2.73
6	1'	0.17*	1.88	2.84	2.86	2.91
	5'	0.26**	0.39**	2.24	2.94	2.91
	15'	0.12**	0.18**	1.33	2.92	2.86
	30'	0.10**	0.15**	0.58**	2.93	2.80
7	1'	0.10**	0.35**	2.75	2.89	2.89
	5'	0.07**	0.16**	0.45**	2.87	2.90
	15'	0.05**	0.07**	0.23**	2.61	2.43
	30'	0.05**	0.07**	0.16**	2.88	2.77

C. albicans yeast cells, suspended in cRPMI, were dispensed into flat-bottom 96-well plates (1x10^5/well) and incubated for 24 h at 37°C in the presence of 5% CO_2. Then, Candida cells were exposed or not to MoWs (at the indicated dilutions), for 1', 5', 15' and 30' at 37°C in the presence of 5% CO_2. Subsequently, the XTT assay was performed and the OD recorded. The numbers reported in the table are the mean values of 3 independent experiments. The values highlighted in grey, less than 0.5, were arbitrarily interpreted as an indication of no fungal cells viability. Asterisks indicate statistically significant (p≤0.05,) or highly significant (p<0.001,**) differences from the corresponding negative controls, that returned the following values: 2.97 for 1' contact time; 2.98 for 5' contact time; 2.99 for 15' contact time; 2.99 for 30' contact time.*

biofilm (*Table 4*). In particular, MoWs 2 and 3 had an effect up to 1:16 dilution for all the contact times. Conversely, MoWs 4 and 6 had similar scant effects, being effective only at contact times ≥15 minutes and at the minimal 1:2 dilution (*Table 4*).
When the CV assay was performed in parallel cultures, we consistently found no significant differences among MoW-treated vs untreated biofilm (data not shown).

DISCUSSION

Here, we show that mouthwashes exert anti-Candida activity, and that such phenomenon strictly depends on their composition as well as on fungal morphological/functional status. Indeed, yeast cell growth, hyphal formation, biofilm production and survival of Candida cells embedded in biofilm are differentially affected.

The health status of the oral cavity strictly depends on several factors, including regular care by professional and personal hygiene, the latter with the daily use of toothbrush and toothpaste (Axelsson and Lindhe, 1981). Removal of tooth- or mucosa-associated microorganisms is facilitated by mechanical washing with MoWs, that represent useful adjuncts for this purpose (Haerian-Ardakani *et al.*, 2015). MoWs are made up of water and active components exerting anti-microbial and anti-inflammatory effects. Their use is strongly advised in a variety of situations ranging from halitosis to the treatment of minor infections (Haps *et al.*, 2008; Teles and Teles, 2009; Kuo *et al.*, 2013; Watanabe *et al.*, 2015). Some MoWs have been found to remove effectively dental plaque (Haerian-Ardakani *et al.*, 2015) and oral cavity-associated microorganisms (Silverman and Wilder, 2006). Several studies report data concerning the antimicrobial activity

Table 3 - *Effects of mouthwashes on developing biofilm, by XTT assay.*

Mouthwash	*Contact times*	*Mouthwash dilutions (Od values)*				
		1:2	*1:4*	*1:16*	*1:32*	*1:64*
1	1'	2.76	2.64	2.47	2.42	2.53
	5'	2.71	2.73	2.62	2.48	2.63
	15'	2.65	1.82	2.39	2.31	2.31
	30'	0.07**	2.26	2.66	2.51	2.60
2	1'	0.04**	0.04**	2.30	2.41	2.47
	5'	0.04**	0.01**	0.22**	2.57	2.66
	15'	0.03**	0.05**	0.05**	2.55	2.58
	30'	0.03**	0.04**	0.03**	2.62	2.43
3	1'	0.04**	0.43**	2.45	2.53	2.59
	5'	0.03**	0.04**	1.55*	2.48	2.63
	15'	0.03**	0.04**	0.03**	2.62	2.59
	30'	0.04**	0.04**	0.06**	2.60	2.63
4	1'	2.83	2.68	2.52	2.56	2.58
	5'	2.89	2.71	2.65	2.56	2.58
	15'	2.81	2.88	2.76	2.57	2.54
	30'	2.49	2.56	2.81	2.60	2.62
5	1'	2.85	2.77	2.52	2.52	2.55
	5'	2.87	2.71	2.51	2.53	2.52
	15'	2.77	3.07	2.52	2.52	2.55
	30'	2.73	2.76	2.53	2.42	2.50
6	1'	2.83	2.70	2.48	2.45	2.42
	5'	2.45	2.78	2.84	2.62	2.67
	15'	2.17	1.77	2.58	2.31	2.42
	30'	0.06**	2.06	2.50	2.63	2.64
7	1'	2.63	2.74	2.48	2.58	2.56
	5'	1.10*	2.78	2.59	2.53	2.61
	15'	0.03**	2.13	2.68	2.22	2.41
	30'	0.05**	0.15**	2.64	2.49	2.57

C. albicans yeast cells, suspended in cRPMI, were dispensed into flat-bottom 96-well plates (1x10^5/well) and incubated for 24 h at 37°C in the presence of 5% CO_2. Then, Candida cells were exposed or not to MoWs (at the indicated dilutions), for 1', 5', 15' and 30' at 37°C in the presence of 5% CO_2. Subsequently, Candida cells were washed with saline, resuspended in cRPMI and incubated for further 24h. Finally, the XTT assay was performed and the OD recorded The numbers reported in the table are the mean values of 3 independent experiments. The values highlighted in grey, less than 0.5, were arbitrarily interpreted as indication of no fungal cells viability. Asterisks indicate statistically significant (p≤0.05,) or highly significant (p<0.001,**) differences from the corresponding negative controls, that returned the following values: 3.06 for 1' contact time; 3.04 for 5' contact time; 3.25 for 15' contact time; 3.03 for 30' contact time.*

Table 4 - *Effects of mouthwashes on mature biofilm, by XTT assay.*

Mouthwash	*Contact times*	*Mouthwash dilutions (Od values)*				
		1:2	*1:4*	*1:16*	*1:32*	*1:64*
1	1'	0.31**	0.73**	1.92	2.27	2.53
	5'	0.19**	0.49**	1.72	2.77	2.74
	15'	0.11**	0.34**	0.66**	2.74	2.67
	30'	0.11**	0.17**	0.28**	2.05	2.67
2	1'	0.08**	0.04**	0.35**	2.42	2.51
	5'	0.05**	0.03**	0.19**	2.06	2.80
	15'	0.04**	0.02**	0.06**	1.85	2.80
	30'	0.05**	0.02**	0.01**	0.86	2.66
3	1'	0.09**	0.05**	0.46**	2.40	2.55
	5'	0.05**	0.07**	0.17**	2.13	2.54
	15'	0.05**	0.03**	0.12**	2.22	2.69
	30'	0.06**	0.03**	0.09**	1.27	2.67
4	1'	2.16	1.87	2.58	2.62	2.68
	5'	2.43	1.73	2.23	2.48	2.67
	15'	0.50**	1.43	2.54	2.69	2.63
	30'	0.27**	0.77**	2.31	2.65	2.70
5	1'	0.35**	2.41	2.69	2.65	2.72
	5'	0.14**	1.27	2.43	2.41	2.72
	15'	0.09**	0.49**	2.76	2.56	2.62
	30'	0.09**	0.21**	2.73	2.52	2.63
6	1'	1.02**	2.35	2.64	2.69	2.63
	5'	0.66**	1.41	1.93	2.41	2.65
	15'	0.19**	1.21**	2.50	2.66	2.61
	30'	0.14**	0.67**	2.18	2.68	2.76
7	1'	0.21**	1.71	2.72	2.59	2.78
	5'	0.10**	0.27**	1.45	2.38	2.51
	15'	0.06**	0.21**	0.86**	2.57	2.52
	30'	0.06**	0.06**	0.41**	2.59	2.75

C. albicans yeast cells, suspended in cRPMI, were dispensed into flat-bottom 96-well plates (1x10^5/well) and incubated for 48 h at 37°C in the presence of 5% CO_2. Then, Candida cells were exposed or not to MoWs (at the indicated dilutions), for 1', 5', 15' and 30' at 37°C in the presence of 5% CO_2. Subsequently, the XTT assay was performed and the OD recorded. The numbers reported in the table are the mean values of 3 independent experiments. The values highlighted in grey, less than 0.5, were arbitrarily interpreted as indication of no fungal cells viability. Asterisks indicate statistically significant (p≤0.05,) or highly significant (p<0.001,**) differences from the corresponding negative controls, that returned the following values: 3.02 for 1' contact time; 3.07 for 5' contact time; 3.08 for 15' contact time; 3.07 for 30' contact time.*

of commercially available MoWs. In particular, MoWs containing alcohol and/or chlorhexidine have the highest antibacterial effects (Watanabe *et al.*, 2015; Herrera *et al.*, 2003; Ghapanchi *et al.*, 2015) and are the most effective in reducing bacterial counts in the oral cavity (Haerian-Ardakani *et al.*, 2015). As for their antifungal effects, it has been recently demonstrated that cetylpiridinium chloride-containing MoWs are effective against planktonic and biofilm-embedded fungal cells (Fu *et al.*, 2014). Nevertheless, chlorhexidine-containing MoWs remain the most effective against both planktonic (Giuliana *et al.*, 1997; Meiller *et al.*, 2001) and sessile fungal cells (Fu *et al.*, 2014). Such studies have employed *Candida spp.* as the most common fungal colonizers of oral cavity (Muadcheingka and Tantivitayakul, 2015).

In this study, we describe the differential effects of 7 commercial MoWs with different composition, whose antifungal efficacy has not been assessed before. Antifungal activity was initially established by disk diffusion test. According to the inhibition halo sizes, we interpreted large (MoWs 2 and 3), intermediate (MoWs 1 and 7) or no (MoWs 4, 5 and 6) halo as high, low and absent antifungal effects exerted by the different MoWs on *C. albicans* yeast cells growth.

Notoriously, the assay suffers from intrinsic limitations, especially when comparing the effectiveness of different compounds and/or formulations, since the results may also depend on their ability to diffuse through the solid medium. Nevertheless, this widely employed and easy-to-perform assay was used to screen the differential efficacy of the different MoWs.

Furthermore, it should be noted that only MoWs without ethanol were investigated to avoid possible ethanol-mediated side-effects and because of the possible toxic effects of ethanol on host cells (Haerian-Ardakani *et al.*, 2015; Antunes *et al.*, 2014). In fact, initial evidence suggests a relationship between frequent use of MoWs with a high ethanol content and the increased risk for upper aero-digestive tract tumours (Ahrens *et al.*, 2014).

Unlike other fungal species, *C. albicans* can grow either as a unicellular yeast budding cell or as a filamentous fungal form, strictly depending upon environmental conditions. Such phenomenon, known as dimorphic transition, is important for virulence, affecting adhesion, invasion, tissue damage, dissemination, immune evasion and also biofilm formation (Jacobsen *et al.*, 2012). Biofilm formation is a crucial event in the pathogenesis of many infections, including oral candidiasis, and the phenomenon evolves through distinct and predictable steps (initial presence of planktonic yeast cells, production of early, developing and lately mature sessile structures). In the present study, we set up four different protocols with the aim of addressing MoW effects on different stages of Candida growth, biofilm production, maturation and survival of biofilm-embedded fungal cells. Each MoW was used at different dilutions and incubation times. In particular, the 1-30 minute contact time range was chosen in an attempt to mimic as close as possible real life situations where, according to the instructions, MoWs have to be used for 30 to 60 seconds, followed by 30 minutes of no drinking and/or eating. As recently recommended (Ramage, 2006), two different assays were employed, namely CV and XTT assays, to evaluate both total biomass and residual metabolic activity, upon fungal exposure to MoWs.

In all the protocols here employed, MoWs 2 and 3 were the most potent. In particular, they were effective in preventing biofilm formation and also in impairing *C. albicans* early and mature biofilm (up to 1:16 dilution and regardless of the contact time). Yet, on developing biofilm, slight differences occurred, MoW 2 still being effective at 1:16 dilution for 5', whereas MoW 3 required 15' to achieve similar effects. Notwithstanding such difference, MoWs 2 and 3 had similar effects in all the other conditions.

Likely, their high performance is due to their chlorhexidine digluconate content in, which is the same for both (0.20%). This conclusion is in line with previous studies describing the ability of chlorhexidine to exert antifungal activity (Fu *et al.*, 2014; Giuliana *et al.*, 1997; Okuda *et al.*, 1998; MacNeill *et al.*, 1997; Rahmani-Badi *et al.*, 2015; Uygun-Can *et al.*, 2016).

MoWs 1 and 7 were characterized by an overall intermediate efficiency, the latter being slightly better. In particular, only MoW 7 was effective against biofilm formation at 1:4 dilution and at all the contact times, whereas MoW 1 effects were negligible. In addition, MoW 7 was slightly more effective than MoW 1 against developing biofilm (15' vs 30' contact times, at 1:2 dilution). Furthermore, it should be noted that MoW 1 efficacy was inferior to that ascribed to MoWs 2 and 3. Interestingly, MoW 1 has the same chlorhexidine digluconate content of MoWs 2 and 3 (i.e., 0.20%); yet, it includes also a particular system (antidiscoloration system, ADS) aimed at reducing the formation of brown teeth pigmentation by chlorhexidine. Our results provide the first evidence on the possibility that this ADS system may reduce the effect of chlorhexidine and thus the efficacy of the MoW, at least in terms of antifungal effect. In line with this speculation, although MoW 7 contains a lower concentration of chlorhexidine digluconate (0.06%), it exhibited an overall better antifungal performance than MoW 1.

MoWs 4, 5 and 6 do not contain chlorhexidine digluconate. Notwithstanding the lack of this molecule, MoWs 5 and 6 showed a good performance in several protocols. Despite the lack of inhibition haloes in the disk diffusion test, they both impaired biofilm production (MoW 6, at 1:4 dilution; MoW 5, at 1:2 dilution). In addition, they both exerted anti-fungal effects on early and mature biofilm, whereas only MoW 6 affected the developing biofilm (at 1:2 dilution for 30' contact time). In our opinion, the appreciably lower efficacy of MoW 6 may be partially explained considering its physico-chemical features. By microscope observation, MoW 6 appears as a persistent precipitate, likely preventing its diffusion within biofilm, as well as within the agar during the disk diffusion assay. Among the three MoWs without chlorhexidine digluconate, MoW 4 appears as the least effective one. It had little effect on early and mature biofilm, and only at low dilutions and long contact times.

It should be mentioned that MoW 4 is characterized by a jelly consistence. This feature was deliberately introduced by the manufacturer to allow a long-lasting protective barrier in the surface of the oral cavity, which may facilitate prolonged occlusion of dentinal tubules; in turn, this would reduce sensitivity, providing effective pain relief. It is possible that this physico-chemical property somehow delayed or impaired the interaction between Candida cells and the MoW active ingredients in our *in vitro* model, thus strongly limiting the occur-

rence of antifungal effects. Finally, it is known that the formulation of MoW 5 includes several extremely volatile essential oils, such as menthol, eucalyptol and thymol, which are known to exert anti-*Candida* effects (Alili *et al.*, 2014). It is possible that, in our model, the incubations carried out at 37°C, both for the disk diffusion test and the CV/XTT assays, may have facilitated an evaporation of such substances, thus losing any possible effects on Candida cells and biofilm.

In our hands, the XTT but not CV assay highlighted differences in MoW efficacy against preformed biofilm: as is well known, the CV assay quantifies the total amount of biofilm mass, whereas XTT assay yields information on the actual metabolic activity of biofilm-embedded Candida cells.

By combining the results provided using such two assays, it emerges that MoW anti-Candida activity is accomplished via impairment of fungal metabolism and/or viability while total biofilm biomass remains substantially unaffected. By a similar approach, Fu and coworkers (2014) demonstrated the efficacy of several MoWs on Candida developing biofilm. Nevertheless, it is worth noting that the contact times with the MoWs on their protocol ranged from 24 to 46 hours, whereas we employed short contact times (1 to 30 minutes) in an attempt to mimic real life practical application times of MoWs as closely as possible.

To our knowledge, this is the first report describing the effectiveness of 5 out of 7 MoWs in preventing biofilm formation by Candida yeast cells, depending upon MoW dilution and contact time. Moreover, 7 out of 7 MoWs were effective, although to a different extent, on preformed early and late biofilm. Conversely, developing biofilm was affected only by 2 out of 7 MoWs (i.e., MoW 2 and MoW 3): probably, an increased plasticity of Candida cells during biofilm development phase may account for their reduced susceptibility to MoWs. Although further in-depth studies are warranted to assess the degree of fungal viability in each different condition by a validated metabolic assay (XTT), we provide the first indication on Candida metabolism impairment by MoWs depending upon biofilm development stage and MoW composition.

In conclusion, this pilot study was performed using a reference strain, well-known for its geno-phenotypic peculiarities, including strong biofilm production. If the above-described phenomena are replicated on clinical isolates from oral candidiasis, practical guidelines might be drawn on how to prescribe MoWs in clinical practice, whether prevention or rather treatment of oral mycoses has to be achieved.

Conflict of interest

We declare we have no conflict of interest with any of the companies, which manufacture the mouthwashes employed in this study.

Abbreviations

MoW/MoWs: Mouthwash/Mouthwashes.
SDA: Sabouraud dextrose agar.
cRPMI: RPMI 1640 supplemented with foetal bovine serum, L-Glutamine and gentamicin.
AmB: amphotericin B.
CV: Crystal Violet assay.
XTT: 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide assay.

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