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Evaluation of antifungal and antibiofilm activity of commercial mouthwashes sold in Türkiye against oral biofilm-forming yeasts: an *in vitro* microbiological study

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SUMMARY

Introduction: In recent years, the use of mouthwashes has become quite common in various situations, from bad breath to treating minor infections. Although oral care products have a broad spectrum of antibacterial activity, little is known about their antifungal properties, and studies on antifungal and antibiofilm activity on oral-isolated *Candida* isolates are limited. **Objective:** This study aimed to assess the antifungal and antibiofilm activities of the best-selling mouthwashes in Türkiye against yeast strains isolated from the mouth, known for their strong biofilm production. **Methods**: This study investigated the antifungal and antibiofilm activity of twenty-five commercially available types of mouthwash against 19 *Candida* sp. and 1 *Pichia manshurica* strains isolated from the oral cavity of 18- 25-year-olds. **Results**: It was determined that twelve mouthwashes containing cetylpyridinium chloride had antifungal activity. It was determined that MIC values of mouthwashes varied between 0.20 and 1.56 μL/mL. Also, the effect of mouthwashes on mature biofilm and biofilm formation was evaluated according to MIC values. It was determined that mouthwashes are remarkably effective during biofilm formation but have little effect on mature biofilm structure. **Conclusion**: This study demonstrates that mouthwashes may possess not only antibacterial but also antifungal properties. Mouthwashes containing cetylpyridinium chloride have been shown to exhibit antifungal activity against oral *Candida* species, as confirmed by MIC values. Furthermore, these mouthwashes strongly inhibit biofilm formation but have a limited impact on the structure of mature biofilms. These findings suggest that mouthwashes should undergo evaluation for their antifungal effects and may play a significant role in clinical applications, particularly due to their biofilm-inhibiting characteristics.

Keywords: oral hygiene; mouthwash; antifungal activity; antibiofilm activity.

RESUMEN

Evaluación de la actividad antifúngica y antibiofilm de enjuagues bucales comerciales vendidos en Turquía contra levaduras formadoras de biofilm oral: un estudio microbiológico *in vitro*

Introducción: En los últimos años, el uso de enjuagues bucales se ha generalizado en diversas situaciones, desde el mal aliento hasta el tratamiento de infecciones leves. Si bien los productos de higiene bucal

poseen un amplio espectro de actividad antibacteriana, se conoce poco sobre sus propiedades antifúngicas, y los estudios sobre la actividad antifúngica y antibiofilm en cepas de *Candida* aisladas de la cavidad oral son limitados. **Objetivo:** Este estudio tuvo como objetivo evaluar la actividad antifúngica y antibiofilm de los enjuagues bucales más vendidos en Turquía contra cepas de levaduras aisladas de la cavidad oral, conocidas por su alta capacidad de formación de biofilm. Métodos: Se investigó la actividad antifúngica y antibiofilm de veinticinco enjuagues bucales comerciales contra 19 cepas de *Candida* sp. y 1 cepa de *Pichia manshurica* aisladas de la cavidad oral de personas de entre 18 y 25 años. **Resultados:** Se determinó que doce enjuagues bucales que contenían cloruro de cetilpiridinio presentaban actividad antifúngica. Se determinó que los valores de la concentración inhibitoria mínima (CIM) de los enjuagues bucales variaban entre 0,20 y 1,56 μL/mL. Asimismo, se evaluó el efecto de los enjuagues bucales sobre la biopelícula madura y su formación en función de los valores de la CIM. Se determinó que los enjuagues bucales son notablemente eficaces durante la formación de la biopelícula, pero tienen poco efecto sobre la estructura de la biopelícula madura. **Conclusión:** Este estudio demuestra que los enjuagues bucales pueden poseer propiedades tanto antibacterianas como antifúngicas. Se ha demostrado que los enjuagues bucales que contienen cloruro de cetilpiridinio presentan actividad antifúngica contra especies de *Candida* oral, como lo confirman los valores de la CIM. Además, estos enjuagues bucales inhiben fuertemente la formación de biopelícula, pero tienen un impacto limitado en la estructura de las biopelículas maduras. Estos hallazgos sugieren que los enjuagues bucales deben someterse a una evaluación por sus efectos antifúngicos y podrían desempeñar un papel importante en aplicaciones clínicas, en particular debido a sus características inhibidoras de la biopelícula.

Palabras clave: higiene bucal; enjuague bucal; actividad antifúngica; actividad antibiofilm.

RESUMO

Avaliação da atividade antifúngica e antibiofilme de enxaguantes bucais comerciais vendidos na Turquia contra leveduras formadoras de biofilme oral: um estudo microbiológico *in vitro*

Introdução: Nos últimos anos, o uso de enxaguantes bucais tornou-se bastante comum em diversas situações, desde mau hálito até o tratamento de infecções menores. Embora os produtos para higiene bucal apresentem um amplo espectro de atividade antibacteriana, pouco se sabe sobre suas propriedades antifúngicas, e os estudos sobre a atividade antifúngica e antibiofilme em isolados de *Candida* da cavidade oral são limitados. **Objetivo:** Este estudo teve como objetivo avaliar as atividades antifúngica e antibiofilme dos enxaguantes bucais mais vendidos na Turquia contra cepas de leveduras isoladas da cavidade oral, conhecidas por sua forte produção de biofilme. Métodos: Este estudo investigou a atividade antifúngica e antibiofilme de 25 tipos de enxaguantes bucais disponíveis comercialmente contra 19 cepas de *Candida* sp. e 1 cepa de *Pichia manshurica* isoladas da cavidade oral de indivíduos de 18 a 25 anos. **Resultados:** Foi determinado que doze enxaguantes bucais contendo cloreto de cetilpiridínio apresentaram atividade antifúngica. Os valores da CIM (Concentração Inibitória Mínima) dos enxaguantes bucais variaram entre 0,20 e 1,56 μL/mL. Além disso, o efeito dos enxaguantes bucais sobre o biofilme maduro e a formação do biofilme foi avaliado de acordo com os valores da CIM. Constatou-se que os enxaguantes bucais são notavelmente eficazes durante a formação do biofilme, mas têm pouco efeito sobre a estrutura do biofilme maduro. **Conclusão:** Este estudo demonstra que os enxaguantes bucais podem possuir propriedades não apenas antibacterianas, mas também antifúngicas. Os enxaguantes bucais contendo cloreto de cetilpiridínio demonstraram atividade antifúngica contra espécies de *Candida* oral, conforme confirmado pelos valores da CIM. Além disso, esses enxaguantes bucais inibem fortemente a formação do biofilme, mas têm um impacto limitado na estrutura dos biofilmes maduros. Esses achados sugerem que os enxaguantes bucais devem ser avaliados quanto aos seus efeitos antifúngicos e podem desempenhar um papel significativo em aplicações clínicas, principalmente devido às suas características inibidoras de biofilme.

Palavras-chave: higiene bucal; enxaguante bucal; atividade antifúngica; atividade antibiofilme.

1. INTRODUCTION

The oral microbiota is the most complex microbial community in the body, second only to the colon, consisting of approximately 1,000 species of microorganisms residing in various areas such as the teeth, tongue, cheeks, palate, gums, and tonsils [1, 2]. *Candida* species, naturally present in around 75% of healthy individuals as part of the oral microbiota, can become opportunistic pathogens and cause acute or chronic infections. This is particularly likely due to factors like denture use, smoking, a weakened immune system, xerostomia, and broad-spectrum antibiotic treatment. *Candida albicans* is the most prevalent and pathogenic yeast linked to oral candidiasis; however, non-albicans Candida (NCAC) species can also lead to disease [3].

Dental plaque constitutes a structurally and functionally organized multi-species microbial biofilm, playing a significant role in the etiology of oral diseases. The primary factor for maintaining good oral health is the routine management of dental plaque that forms on tooth surfaces and adheres to the gingival margins [4]. Traditional strategies for preventing and controlling dental plaque include mechanical removal and the use of broad-spectrum antibiotics such as chlorhexidine. The effectiveness of mechanical cleaning methods like brushing and flossing relies on personal knowledge, skill, and motivation. Although meticulous mechanical hygiene can mitigate diseases, many individuals struggle to uphold good oral health [5, 6]. Excessive use of chlorhexidine, beneficial for oral health, has been shown to increase the risk of drug resistance among oral bacteria and cause cellular damage [4].

Using toothbrushes, toothpaste, and mouthwash daily is an effective oral hygiene practice [7]. Mouthwash frequently prevent and manage *C. albicans* infections, particularly in dentistry. These mouthwashes include active ingredients such as water, chlorhexidine, ethanol, essential oils, and anti-inflammatory compounds [7, 8]. Mouthwash with antibacterial agents help eliminate bacteria left in the mouth after brushing. Additionally, clinical, and *in vitro* studies have demonstrated that cetylpyridinium chloride (CPC), a cationic quaternary ammonium compound, inactivates oral bacteria and diminishes plaque and gingivitis [7]. However, Ardizzoni *et al.* and Paulone *et al.* indicated that mouthwash containing chlorhexidine digluconate, CPC, and essential oils in their formulations can impact on the hyphal development of *C. albicans* and its biofilm formation and persistence [9, 10].

In recent years, various oral care brands with diverse formulations have claimed that their products enhance oral hygiene, decrease plaque, and reduce gingivitis or tooth decay. Different oral care products contain antimicrobial and antiplaque agents within their formulations. Recently, the use of mouthwash has gained popularity in numerous situations, from combating bad breath to addressing minor infections [6, 11]. Consequently, this study aimed to investigate the antifungal and antibiofilm properties of twenty-five commercial types of mouthwash against 20 yeast isolates sourced from the oral cavities of young individuals.

2. METHODS

2.1. Cultures

Four *Candida albicans* isolates (1, 2, 3, 4), five *Candida dubliniensis* isolates (5, 6, 7, 8, 9), ten *Candida parapsilosis* isolates (11, 12, 13, 14, 15, 16, 17, 18, 19), and one *Pichia manshurica* isolate (20) known to be strong biofilm producer from the researchers' culture collection were used in the study, all isolated from the oral cavity in 18-25-year-olds [12].

2.2. Mouthwashes

Mouthwashes were collected from beauty shops in Çanakkale, Türkiye in 2021. Mouthwashes and their contents (whether they contained alcohol, sodium fluoride, plant extract, or CPC) are detailed in Table 1.

Table 1. Contents of the mouthwashes used in the study.

No	**901**	**902**	**903**	**904**	**905**
Brand	A	A	A	A	A
Product name	Advanced white mild taste	For enhanced protection sensitivity	Total care	Total care mild taste	Fresh brust
Alcohol	-	-	+	-	+
Sodium fluoride	220 ppm	220 ppm	220 ppm	220 ppm	-
Plant extract	-	-	-	-	-
CPC	-	-	-	-	-
Other ingredients	Aqua, Aroma, Caprylyl Glycol, Critic Acid, Eucalyptol, Menthol, Sorbitol, Sodium Saccharin, Sucralose, Sodium Methyl Cocoyl Taurate, Pentasodium Triphosphate, Poloxamer 407, Propylene Glycol, Tetrapotassium Pyrophosphate, Thymol,	Aqua, Aroma, Sorbitol, Sodium Saccharin, Sodium Benzoate, Sucralose, Sodium Lauryl Sulfate, Sodium Methyl Cocoyl Taurate, Phosphoric Acid, Propylene Glycol, 1.4% Dipotassium Oxalate	Aqua, Aroma, Benzoic Acid, Cl 16035, Cl 42090, Eucalyptol, Methyl Salicylate, Menthol, Sorbitol, Sodium Saccharin, Sodium Benzoate, Sucralose, Propylene Glycol, Thymol, Zinc Chloride	Aqua, Aroma, Benzoic Acid, Cl 16035, Cl 42090, Eucalyptol, Methyl Salicylate, Menthol, Sorbitol, Sodium Saccharin, Sodium Benzoate, Sucralose, Sodium Lauryl Sulfate, Poloxamer 407, Propylene Glycol, Thymol, Zinc Chloride	Aqua, Aroma, Benzoic Acid, Cl 42053, Cl 47005, Eucalyptol, Methyl Salicylate, Menthol, Sorbitol, Sodium Saccharin, Sodium Benzoate, Poloxamer 407, Thymol

912	**911**	**910**	**909**	**908**	**907**	**906**
D	C	C	B	A	A	A
Cool mint mouthwash	Promine	Cool mint for sensitive teeth	Gum x Enamel Care	Stay white	Cool mint mild taste	Cool mint
-	-	-	-	+	-	+
*	450 ppm	217 ppm	450 ppm	220 ppm	220 ppm	-
-	-	-	-	-	-	-
+	+	-	+		-	-
Aqua, Critic Acid, Cl 42053, Eucalyptol, Flavor, Menthol, Sodium Saccharin, Sodium Benzoate, Polysorbate 20, Poloxamer 407, Thymol	Aqua, Aroma, Cellulose Gum, Cl 42090, Disodium Phosphate, Glycerin, Methylparaben, Sorbitol, Sodium Saccharin, Sodium Benzoate, Sodium Phosphate, Potassium Nitrate, Peg-60 Hydrogenated Castor Oil, Propylparaben, VP/VA Copolymer, Xanthan Gum	Aqua, Aroma, Cl 42090, Disodium Phosphate, Glycerin, Sorbitol, Sodium Saccharin, Sodium Benzoate, Potassium Nitrate 3% w/w, Potassium Nitrate, Peg-60 Hydrogenated Castor Oil, Poloxamer 407	Aqua, Aroma, Benzoic Acid, Cinnamal, Cl 15985, Cl 42053, Glycerin, Sodium Benzoate, Sucralose, Poloxamer 407, Propylene Glycol	Aqua, Aroma, Benzoic Acid, Cl 42090, Eucalyptol, Methyl Salicylate, Menthol, Sorbitol, Sodium Saccharin, Sodium Benzoate, Sucralose, Sodium Lauryl Sulfate, Poloxamer 407, Thymol, Zinc Chloride	Aqua, Aroma, Benzoic Acid, Cl 42053, Eucalyptol, Methyl Salicylate, Menthol, Sorbitol, Sodium Saccharin, Sodium Benzoate, Sucralose, Sodium Lauryl Sulfate, Poloxamer 407, Propylene Glycol, Thymol	Aqua, Aroma, Benzoic Acid, Cl 42053, Eucalyptol, Methyl Salicylate, Sorbitol, Sodium Saccharin, Sodium Benzoate, Poloxamer 407, Thymol

919	**918**	**917**	**916**	**915**	**914**	**913**
E	E	E	E	D	D	D
Mint refreshment	Plax tea-lemon	Total 12	Plax complete care	Tartar control	Shining white	Mint bamboo charcoal herbal mouthwash
-	-	-	-	+	+	-
225 ppm	225 ppm	225 ppm	225 ppm	-	-	*
-	*Camellia sinensis* Leaf Extract *Citrus limon* Peel	-	-	-	-	*Camellia sinensis* Leaf Extract *Commiphora myrrha*
+	+	+	+	+	+	+
Aqua, Aroma, Cl 42051, Glycerin, Menthol, Sorbitol, Sodium Saccharin, Potassium Sorbate, Poloxamer 407, Propylene Glycol	Aqua, Aroma, Cl 19140, Cl 42051, Glycerin, Menthol, Sorbitol, Sodium Saccharin, Potassium Sorbate, Poloxamer 407, Propylene Glycol	Aqua, Aroma, Cl 17200, Cl 42051, Glycerin, Lactic Acid, Menthol, Sorbitol, Sodium Saccharin, Potassium Sorbate, Poloxamer 407, Propylene Glycol, Zic Lactate	Aqua, Aroma, Cl 42051, Glycerin, Menthol, Sorbitol, Sodium Saccharin, Potassium Sorbate, Poloxamer 407, Propylene Glycol	Aqua, Critic Acid, Cl 42090, Eucalyptol, Flavor, Menthol, Sorbitol, Sodium Saccharin, Sodium Benzoate, Sodium Hydroxide, Polysorbate 20, Poloxamer 407, Thymol, Zinc Chloride	Aqua, Critic Acid, Cl 42090, D-limonene, Eucalyptol, Flavor, Menthol, Sorbitol, Sodium Saccharin, Sodium Benzoate, Sodium Hydroxide, Polysorbate 20, Poloxamer 407, Thymol, Zinc Chloride	Aqua, Critic Acid, Cl 42053, D-limonene, Eugenal, Flavor, Menthol, Sorbitol, Sodium Benzoate, Potassium Sorbate, Polysorbate 20, Poloxamer 407, Propylene Glycol,

925	**924**	**923**	**922**	**921**	**920**
G	G	F	F	F	E
Active oral care flouride protection	Active flouride protection mounthrince green	Bad breath	Klorhex gum care	Full protection	Optic white
-	-	-	-	-	-
225 ppm	225 ppm	-	500 ppm	*	225 ppm
-	-	*Mentha arvensis* plant oil	*Mentha arvensis* plant oil	*Cistus* species Resin Extract *Mentha arven-*	-
+	+	-	+	-	-
Aqua, Aroma, Cl 42051, Glycerin, Menthol, Sodium Saccharin, Polysorbate 20, 2-Bromo-2-Nitropropane-1,3-diol	Aqua, Aroma, Cl 42090, Cl 47005, Flavor, Glycerin, Menthol, Sodium Saccharin, Polysorbate 20, 2-Bromo-2-Nitropropane-1,3-diol	Aqua, Aroma, Citric acid, Cl 42051, Sorbitol, Sodium Benzoate, Sodium Hydroxide, Sucralose, Potassium Sorbate, Peg-40 Hydrogenated Castor Oil, Propylene Glycol, Zinc Citrate	Aqua, Aroma, Ascorbic Acid, Chlorhexidine Digluconate, Cl 42051, Cl 47005, Sorbitol, Sodium Metabisulfite, Sodium Phosphate, Sodium Hydroxide, Sucralose, Peg-40 Hydrogenated Castor Oil, Propylene Glycol	Aqua, Aroma, Citric acid, Cl 16035, Glycerin, Sucralose, Potassium Sorbate, Potassium Sorbate, Peg-40 Hydrogenated Castor Oil, Propylene Glycol,	Aqua, Aroma, Benzyl Alcohol, Cl 42051, Glycerin, Sorbitol, Sodium Saccharin, Potassium Sorbate, Propylene Glycol, PVM/MA Copolymer, Tetrasodium Pyrophosphate, Tetrapotassium Pyrophosphate, Zinc Citrate

"-" = absent, "+" = present, *unknown quantify

2.3. Determination of Antifungal Activity of Mouthwashes

First, the agar well diffusion method was used to determine the antimicrobial activity of mouthwashes. Then, the agar well diffusion method determined the minimum inhibitory concentrations of five types of mouthwashes that were effective. The *in vitro* antifungal activity of mouthwashes was performed by modifying the method outlined in CLSI M44-A [13]. Stock cultures were inoculated on Sabouraud 2% Dextrose Agar (SDA, Neogen), and plates were incubated at 37 °C for 24 hours. After incubation, the isolates were adjusted to 0.5 McFarland density (1-5x10^6 cells/mL) with physiological saline (PS, 0.85% w/v NaCl). The cell suspension

was inoculated on the dried surface of Mueller-Hinton Agar + 2% Glucose, 0.5 μg/mL Methylene Blue Agar (MHA+ GMB) (Himedia, India) plate with the help of a cotton swab. Then, 6 mm diameter wells were opened on the plate, and 20 μL of mouthwash was added. Plates were then incubated at 37 °C for 24 hours. Zone diameters were measured after incubation. The analysis was done in three parallels.

The antifungal activity of mouthwashes was performed *in vitro* as in CLSI M27-A2 [14]. The cell suspension was adjusted to the density of 0.5 McFarland with PS from a fresh culture grown on an SDA medium for 24 hours at 37 °C. Then cell suspension was diluted at 1:100 (1-5x10^3 cell/mL), and the final cell concentration was 0.5-2.5x10^3 cells/mL by inoculating 1:1 into RPMI 1640 medium containing mouthwash.

RPMI 1640 medium (Himedia, India) containing 0.165 M MOPS containing ten different mouthwash concentrations was prepared. Mouthwash concentration was prepared using a two-fold dilution from 1000 μL/mL to 2 μL/mL. 100 μL of the prepared medium was dispensed into the wells of the microdilution plates. 100 μL of the cell suspension was added to the microplates. The final mouthwash concentration was between 1 μL/mL and 500 μL/mL. RPMI 1640 broth medium containing 500 μL/mL mouthwash was used as a negative control, and RPMI 1640 + culture was used as the positive control. Microplates were evaluated at 660 nm in a microplate reader (Thermo Multiscan FC) after 24 hours and 48 hours of incubation at 37 °C. The study was carried out in 3 parallels.

2.4. Effect of Mouthwash on Biofilm Formation

Yeast isolates were resuscitated overnight at 37 °C in 5 mL of Sabouraud 2% Dextrose Broth (SDB) (NCM0147, Neogen, USA/Canada) medium. Revived cultures were adjusted to OD600 = 1.0 (10^7 cells/mL) in an SDB+8% glucose medium containing mouthwash in line with the determined MIC value. Then, 200 μL of inoculated SDB + 8% glucose medium was added to the wells of 96-well flat-bottom microplates. Microplates were incubated at 37°C for 48 hours. After incubation, the microplates were washed three times with sterile PS. After washing, 200 μL of 99% methanol (Merck, Germany) was added for fixation and incubated for 15 minutes. The plates were then emptied and dried at room temperature. Afterwards, 200 μL of 1% (w/v in distilled water) crystal violet (Himedia, India) was added to each well and incubated for 15 minutes. After incubation, the microplates were washed twice with sterile distilled water, and the plates were dried at room temperature. Then, 200 μL of 33% acetic acid (Merck, Germany) was added to the plates and evaluated in a microplate reader (Thermo Multiscan FC) at 570 nm. Only medium + culture was used as the control group, and the results were compared with the control group. The study was conducted in two parallels and three repetitions [12].

2.5. Determination of the Effect of Mouthwash on Mature Biofilm

Evaluation of the effect of mouthwash by MIC values on mature biofilms was done according to Özcan Ateş and Otkun [12]. In the microplate method, we used to evaluate biofilm formation before. After 48 hours of incubation at 37 °C, the plates were emptied, and 200 μL of SDB medium containing mouthwash was added to the wells, then incubated at 37 °C for 24 hours. After incubation, the effect of mouthwash on mature biofilm was evaluated as in the biofilm formation method. The study was carried out in two parallels and three repetitions.

2.6. Statistical analysis

All results are given as mean (M) ± standard deviation (SD) and were evaluated at a 0.05 significance using SPSS Package Program (v23.0, IBM Corp).

3. RESULTS

The agar well diffusion method was initially used to evaluate the antifungal activity of twenty-five commercial mouthwashes against yeasts isolated from the oral cavity. Twelve mouthwashes that contain CPC displayed antifungal activity against these yeasts. The inhibition zone diameters for mouthwashes with antifungal activity are presented in Table 2. However, 13 mouthwashes that did not contain CPC (901, 902, 903, 904, 905, 906, 907, 908, 910, 920, 921, 922, and 923) were found to have no antifungal activity against the tested isolates. Among the tested mouthwashes, only 922 contained chlorhexidine digluconate but did not show antifungal activity against the tested yeasts. It was concluded that, among the mouthwashes identified as having antifungal activity, 909 exhibited the highest antifungal effect against yeasts isolated from the mouth, while 913 exhibited the lowest antifungal effect.

Table 2. Agar well diffusion inhibition zone diameters (in mm) (M ± sd)

Isolate	909	911	912	913	914	915	916	917	918	919	924	925
1	17.15±1.34	11.73±0.65	11.97±0.74	10.97±0.53	11.48±0.56	13.34±0.75	20.44±3.23	17.47±1.56	16.90±1.97	20.12±1.76	15.77±0.99	15.82±1.46
2	18.04±1.13	11.59±0.69	11.52±0.26	10.67±0.58	11.13±0.34	13.20±0.37	18.23±1.97	16.76±1.55	16.24±1.37	17.98±2.11	16.04±0.68	15.66±0.81
3	27.63±4.21	11.74±0.55	12.35±0.88	10.47±0.59	12.36±0.44	16.47±1.58	22.23±1.80	25.76±4.13	19.58±2.58	26.35±3.62	20.31±1.60	21.11±2.64
4	17.88±1.24	11.49±0.75	11.52±0.75	10.38±0.73	11.12±0.92	13.07±1.15	16.92±0.80	17.82±1.57	15.50±0.91	16.20±1.66	15.67±0.70	15.84±1.39
5	19.98±1.94	12.31±0.33	12.65±0.46	12.10±0.82	12.29±0.68	14.57±0.89	18.79±1.16	17.08±2.11	17.90±2.51	20.04±2.28	18.30±1.29	15.78±1.86
6	24.12±3.70	12.36±0.57	13.93±1.87	11.18±0.25	12.56±0.48	16.10±0.83	23.84±3.34	20.71±3.39	17.95±2.03	21.67±2.09	19.94±2.51	21.66±2.89
7	23.74±3.67	13.19±1.43	12.65±0.73	10.73±0.77	12.56±0.55	16.13±1.07	24.23±2.01	22.01±3.90	21.42±1.38	19.23±1.91	21.33±1.75	20.02±2.02

19	18	17	16	15	14	13	12	11	10	9	8
24.53±2.10	27.15±3.62	22.31±1.75	24.42±4.95	24.00±2.49	22.18±2.02	23.77±3.42	29.33±2.76	25.46±2.66	26.69±5.26	23.38±1.96	24.91±2.66
12.12±0.68	11.56±0.54	11.13±0.68	10.25±1.28	12.10±0.54	11.50±1.88	12.32±0.47	10.99±0.81	13.03±0.45	13.48±0.86	10.83±0.72	11.57±0.43
12.60±0.99	12.87±1.45	11.58±0.62	12.18±0.67	12.65±1.02	12.94±0.63	12.06±0.28	11.97±0.71	12.38±0.35	12.95±0.71	9.98±0.89	11.77±0.95
9.72±0.45	8.29±0.39	7.90±0.31	7.91±0.72	9.75±0.52	10.19±0.86	10.29±0.50	9.08±0.97	10.79±0.70	11.80±0.58	8.03±0.83	8.58±0.35
12.27±1.58	10.94±1.40	10.53±0.48	11.02±0.46	11.78±0.34	12.66±0.99	12.23±0.24	12.10±1.23	12.24±1.06	13.35±0.44	10.05±0.73	10.76±0.52
14.95±1.77	15.50±2.76	14.88±1.55	15.51±1.84	15.33±1.59	15.23±1.47	15.29±1.54	15.52±1.52	14.65±0.82	15.47±1.12	14.16±0.83	14.62±0.87
25.55±2.78	23.02±2.96	23.00±3.89	23.11±3.40	21.35±2.62	23.60±4.10	26.11±3.46	20.41±2.28	19.35±4.25	25.00±3.13	20.63±2.21	21.76±1.58
24.57±2.19	22.20±1.93	19.69±3.32	20.79±2.49	20.27±2.84	19.66±2.69	22.75±3.21	24.44±4.51	20.47±3.85	19.31±4.05	22.06±2.31	17.20±0.95
21.45±3.40	18.68±0.26	19.47±1.98	19.54±1.60	17.85±3.00	18.32±1.31	23.54±2.90	20.27±2.16	18.81±3.11	20.71±4.01	20.22±2.34	17.82±1.92
25.89±2.62	19.36±0.90	21.39±2.06	22.06±3.61	18.66±3.20	19.34±2.19	26.37±3.39	23.74±3.91	21.41±5.52	24.14±4.38	20.42±2.12	19.19±1.75
22.30±1.62	16.43±1.63	18.18±1.25	17.42±2.16	18.59±2.30	18.35±1.69	18.49±1.39	18.27±1.45	17.85±1.10	19.25±1.69	17.02±1.27	16.97±1.72
18.88±1.38	17.78±0.88	18.26±0.74	18.01±1.16	18.058±1.30	18.62±1.80	19.16±1.20	20.74±2.59	18.00±2.02	17.65±1.71	16.93±1.33	17.58±2.66

20	35.52±8.37	14.03±0.71	19.79±2.44	16.02±0.81	17.32±1.42	23.96±1.56	31.84±4.57	26.29±6.77	23.36±2.84	32.46±4.16	28.33±3.36	27.76±2.66

The antifungal activity of the mouthwashes was found to be statistically significant, depending on the isolate and the type of mouthwash (P=0.000). Furthermore, both the alcohol and plant extract content of the mouthwashes, along with the amount of sodium fluoride, demonstrated statistically significant antifungal activity (P=0.000). Five mouthwashes exhibiting antifungal activity (909, 911, 915, 919, and 924), each sourced from different manufacturers, were selected, and their MIC values were determined and presented in Table 3.

Table 3. MIC values of mouthwashes (μL/mL)

Isolates	Microorganisms	909	911	915	919	924
1	*C. albicans*	3.90	7.81	15.62	2.00	3.90
2	*C. albicans*	2.00	7.81	15.62	2.00	3.90
3	*C. albicans*	3.90	7.81	7.81	2.00	3.90
4	*C. albicans*	3.90	7.81	15.62	3.90	3.90
5	*C. dublinensis*	2.00	7.81	15.62	2.00	3.90
6	*C. dublinensis*	3.90	7.81	15.62	2.00	3.90
7	*C. dublinensis*	3.90	7.81	15.62	2.00	3.90
8	*C. dublinensis*	3.90	7.81	15.62	3.90	3.90
9	*C. dublinensis*	2.00	7.81	15.62	2.00	3.90
10	*C. parapsilosis*	3.90	7.81	15.62	2.00	3.90
11	*C. parapsilosis*	2.00	7.81	7.81	3.90	3.90
12	*C. parapsilosis*	2.00	7.81	7.81	2.00	3.90
13	*C. parapsilosis*	2.00	7.81	7.81	3.90	7.81
14	*C. parapsilosis*	3.90	7.81	15.62	3.90	3.90
15	*C. parapsilosis*	3.90	7.81	15.62	3.90	3.90
16	*C. parapsilosis*	3.90	7.81	15.62	2.00	7.81
17	*C. parapsilosis*	3.90	7.81	7.81	2.00	3.90
18	*C. parapsilosis*	3.90	15.62	7.81	3.90	3.90
19	*C. parapsilosis*	3.90	7.81	15.62	2.00	3.90
20	*P. manshurica*	2.00	3.90	15.62	2.00	3.90

It was found that the MIC values of 909 and 919 against planktonic yeast isolates with biofilm formation potential ranged from 2.0 to 3.90 μL/mL. The MIC values of 911 and 924 ranged from 3.90 to 7.81 μL/mL. The highest MIC value was 7.81 and 15.62 μL/mL for 915. The MIC values of 909, 915, 919, and 924 were similar across all tested isolates and were not influenced by species differences. However, the MIC value for 911 was influenced by the species. The lowest MIC value (3.90 μL/mL) was observed in the *P. manshurica*. In contrast, the MIC value was 7.81 μL/mL for all tested *Candida* isolates, except for one *C. parapsilosis* isolate. The effects of the mouthwashes on the biofilm formation capacity of yeasts and mature biofilms were also assessed based on the determined MIC values.

The effects of the mouthwashes on biofilm formation are presented in Table 4. In contrast, the impact on mature biofilms is outlined in Table 5. When evaluating the effects of the mouth-

washes on biofilm formation according to the MIC values, it was found that the highest inhibition rate (95.05%) was noted in the 915 against the strong biofilm producer *C. parapsilosis* (16). In comparison, the lowest inhibition rate (1.83%) was seen in 909 against *C. parapsilosis* (18). 911 inhibited biofilm formation in all tested isolates by 2.83% to 91.12%. Conversely, 909 enhanced the biofilm formation potential of six isolates by 11.76% to 53.36%.

Table 4. Effect of mouthwashes on biofilm formation potential of yeasts evaluated at MIC values (in %)

Isolates	Microorganisms	909	911	915	919	924
1	*C.albicans*	37.24	77.49	82.48	72.86	17.62
2	*C. albicans*	**-16.45***	58.69	55.43	32.24	36.71
3	*C. albicans*	73.79	89.57	88.32	65.34	85.14
4	*C. albicans*	11.71	2.83	**-9.92**	1.84	29.19
5	*C. dublinensis*	**-11.76**	19.74	47.39	39.25	**-9.10**
6	*C. dublinensis*	58.10	67.01	74.20	**-5.79**	61.43
7	*C. dublinensis*	**-16.93**	50.50	50.50	61.63	51.16
8	*C. dublinensis*	55.77	89.88	93.65	69.82	89.51
9	*C. dublinensis*	10.80	80.83	90.33	**-12.68**	76.32
10	*C. parapsilosis*	38.45	60.32	65.88	7.53	62.75
11	*C. parapsilosis*	**-16.18**	74.92	72.41	61.87	64.91
12	*C. parapsilosis*	71.71	8.70	**-46.35**	72.38	61.91
13	*C. parapsilosis*	18.15	87.73	88.21	87.14	88.55
14	*C. parapsilosis*	45.44	71.44	82.38	79.20	82.35
15	*C. parapsilosis*	64.13	76.51	76.88	78.26	73.80
16	*C. parapsilosis*	26.85	91.12	95.05	15.37	93.11
17	*C. parapsilosis*	**-53.36**	85.49	89.55	6.94	56.19
18	*C. parapsilosis*	1.83	89.60	85.57	64.25	74.58
19	*C. parapsilosis*	**-24.00**	62.46	70.77	10.88	57.62
20	*P. manshurica*	34.26	72.04	77.92	38.10	66.51

*** ineffective against biofilm formation**

According to the MIC values of the mouthwashes, the highest inhibition rate (52.59%) on the mature biofilm structure formed by the tested yeasts was observed with sample 915, while the lowest inhibition rate (0.49%) was noted with the *C. parapsilosis*, mouthwash of 924. The 909 specifically inhibited the mature biofilm of *P. manshurica* and had no effect on the biofilm structure formed by the tested *Candida* isolates; on the contrary, it increased biofilm formation by up to 217.08%. 911 affected seven isolates, 915 affected eight isolates, 919 affected nine isolates, and 924 affected the mature biofilms of six isolates. Additionally, when the tested mouthwashes were applied at MIC values, they formed a more robust biofilm structure, resulting in considerably increased biofilm production compared to the control group. All mouthwashes impacted the biofilm structure formed by the *P. manshurica* isolate, providing an inhibition rate in the mature biofilm structure of 2.26% and 34.79%.

Table 5. Effect of on mature biofilm structure formed by yeasts evaluated at MIC values (in %).

Isolates	Microorganisms	909	911	915	919	924

1	*C.albicans*	-25.98	**8.13***	-57.70	-71.73	-44.29
2	*C. albicans*	-217.08	-22.25	-57.91	-22.67	-129.54
3	*C. albicans*	-90.55	-22.85	-4.50	-13.70	**5.77**
4	*C. albicans*	-42.06	-40.05	**38.80**	-1.20	-6.31
5	*C. dublinensis*	-80.44	-40.71	-50.44	**51.38**	-21.91
6	*C. dublinensis*	-83.27	**15.61**	**7.33**	**12.82**	-36.17
7	*C. dublinensis*	-33.29	-4.94	**48.68**	**27.01**	**4.90**
8	*C. dublinensis*	-73.64	-19.37	-114.96	-88.92	-105.22
9	*C. dublinensis*	-60.82	-58.95	-65.72	-80.00	-72.81
10	*C. parapsilosis*	-69.48	**6.04**	-8.00	-26.70	-12.80
11	*C. parapsilosis*	-115.68	**4.91**	-40.22	-39.18	-35.35
12	*C. parapsilosis*	-0.76	**31.99**	**50.00**	**11.06**	**0.49**
13	*C. parapsilosis*	-43.86	-12.54	**52.59**	**28.15**	**49.38**
14	*C. parapsilosis*	-30.33	**23.63**	-14.97	30.83	-28.59
15	*C. parapsilosis*	-19.41	**45.12**	**9.42**	**36.07**	-21.52
16	*C. parapsilosis*	-65.51	-50.30	-112.31	-142.93	-37.97
17	*C. parapsilosis*	-156.33	-126.05	-121.84	-118.22	-129.01
18	*C. parapsilosis*	-119.11	-103.12	-57.70	-149.10	-70.48
19	*C. parapsilosis*	-70.16	-4.85	**48.92**	**34.58**	**42.90**
20	*P. manshurica*	**2.29**	**34.79**	**27.54**	**14.77**	**26.77**

*** effective on mature biofilm structure**

When evaluating the effects of mouthwashes on both biofilm formation and mature biofilm, it was found that their effectiveness during biofilm formation was high. In contrast, their impact on the structure of existing biofilm was low.

4. DISCUSSION

The health status of the oral region is closely related to numerous factors, including personal and professional hygiene, regular care, daily dental care, and toothpaste use [15]. Mouthwashes can be mechanical irrigation methods for removing organisms from teeth or supporting integration [16]. Mouthwashes serve various purposes, including teeth whitening, combating bad breath, and treating minor infections. As a result, they contain a range of compounds such as water, antimicrobial agents, and anti-inflammatory substances [8, 17-19]. The antimicrobial agents in mouthwashes specifically help control microorganisms. Using antimicrobial mouthwashes is advisable to reduce the presence of *Streptococcus mutans*, which is known to be the primary cause of dental caries due to its ability to form biofilms on teeth [18, 20]. Literature also indicates that mouthwashes influence microorganisms linked to dental plaque and the oral cavity, exhibiting antimicrobial properties [20-23]. Particularly, mouthwashes that contain alcohol, chlorhexidine, or plant extracts have demonstrated high antimicrobial effectiveness [21-25].

When examining the antifungal effects of mouthwashes, recent studies have shown that those containing CPC are effective against both planktonic and biofilm-embedded fungal cells [7, 26, 27]. CPC is a quaternary ammonium compound categorized within the cationic surfactant group, which interacts with the cell walls of microorganisms, leading to leakage of cytoplasmic material and disruption of their metabolism, ultimately resulting in cell death [18, 20]. CPC possesses plaque and tartar inhibitory properties and has a broad antimicrobial spectrum

that targets gram-positive bacteria and yeasts [18]. You *et al.* found that the MIC values of two different mouthwashes containing CPC were 0.97 and 1.95 μL/mL against the *C. albicans* KCTC 727, while five different mouthwashes without CPC also yielded MIC values ranging from 125 to 250 μL/mL [22]. Korbecka-Paczkowska and Karpiński investigated the anticandidal activity of fifteen mouthwashes available in the European market against ten clinical strains of *C. albicans* obtained from patients diagnosed with candidiasis and two standard cultures (*C. albicans* ATCC 10231 and ATCC 14053). They found that the mouthwashes containing CPC at 0.13% concentration exhibited good activity against *C. albicans* [28]. The study indicated that only mouthwashes containing CPC displayed antifungal activity against yeasts capable of forming biofilms isolated from the mouth, with the MIC values varying between 2.00 and 15.62 μL/mL, consistent with previous literature.

Chlorhexidine is a cationic biguanide with a broad antimicrobial spectrum. It is particularly effective against dental biofilm and gingivitis. Chlorhexidine is highly persistent, capable of binding to tissues in the mouth, which allows it to have a long-term effect after application [20]. Di Lodovico *et al.* found that the MIC value of mouthwashes containing chlorhexidine digluconate at concentrations of 0.05-0.12% against *C. albicans* ranged from 0.02% to 0.09% [29]. Korbecka-Paczkowska and Karpiński reported that the MIC value of mouthwashes containing chlorhexidine digluconate was determined to be 0.12% [28]. While several other studies have also shown that chlorhexidine-containing mouthwashes exhibit antifungal activity against yeast cells [10, 27, 30, 31], only one mouthwash contained chlorhexidine, which was ineffective.

Unlike other yeast species, *Candida* species can exist as either a single cell, a budding cell, or a filamentous yeast form depending on environmental conditions. This phenomenon, known as dimorphic transition, is crucial for adhesion, invasion, tissue damage, dissemination, immune evasion, and virulence related to biofilm formation. Biofilm formation is a significant event in the pathogenesis of many infections, including oral candidiasis [32]. Therefore, the effects of mouthwashes on biofilm formation and mature biofilm were also evaluated in the study. In the antibiofilm activity study, it was determined that CPC-containing mouthwashes affected biofilm formation, while their impact on mature biofilm was seen to be limited. The existing literature on the effects of mouthwashes on biofilms formed by yeasts is quite scarce. Various methods were employed in these limited studies. Using a method like this study, Nikseresht *et al.* evaluated the effects of herbal mouthwashes on the biofilm of *Streptococcus mutans* [33]. Other studies in literature also employed different methods [28, 34, 35]. Therefore, comparing our study results with the available data is quite challenging.

This study has several limitations. It examined mouthwashes sold in beauty salons and markets in Türkiye, but the exact concentrations of the substances in these mouthwashes are not specified in the ingredient list. Because this information is unavailable, comparing and interpreting the study results is challenging. Additionally, due to budget constraints, not all products on the market could be tested. Moreover, these budget limitations restricted the number of cultures studied, making it impossible to increase this number, particularly for antifungal-resistant isolates. These shortcomings hinder the findings of our study.

5. CONCLUSION

Consequently, it was determined that the impact of the five selected mouthwashes on biofilm formation was significant, but their effect on mature biofilm was minimal. Thus, the mouthwashes can be utilized in daily oral hygiene routines. However, the effectiveness of these

mouthwashes against bacterial and fungal microorganisms in both patients and healthy individuals should be further investigated. Their effects on complex biofilms and antifungal resistance isolates must be examined, and the results should be presented.

CONFLICT OF INTERESTS

The authors declared no conflict of interest.

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