Chapter

Characterization and Virulence of *Candida* Isolated from Children with Dental Caries and Its Susceptibility to Various Antimicrobial Agents

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Abstract

Candida is known to be associated with early colonization of cariogenic microorganisms leading to dental caries and there is a need to determine the effectiveness of various chemotherapeutic agents against it. The study is aimed to isolate, characterize *Candida* from the dental plaque of children with dental caries, to study its virulence factors and the antimicrobial activity of coconut oil, probiotics, 0.2% chlorhexidine and ketoconazole on *C. albicans*. Samples were collected using sterile cotton swabs from children with dental caries and streaked on Sabouraud's dextrose agar plates and incubated at 37°C for 24 h. Candidal colonies were isolated, species identified, and virulence factors tested, and its susceptibility to 0.2% chlorhexidine, probiotics, coconut oil, and ketoconazole was determined using disc diffusion method. *C. albicans* was the predominant species isolated, and virulence factors such as phospholipase, hemolysin, germ tube, and hyphal formation were seen. The mean zone of inhibition for chlorhexidine was found to be 21.8 mm, for coconut oil it was 16.8 mm, for probiotics it was 13.5 mm, and for ketoconazole it was 22.3 mm. The difference between the groups was not statistically significant. Thus chlorhexidine and coconut oil were found to exhibit significant antimicrobial activity which is comparable with ketoconazole.

Keywords: *Candida albicans*, virulence, children with dental caries, antifungal susceptibility, hemolysis, phospholipase

1. Introduction

Dental caries is defined as an infectious microbiological disease of the teeth that results in localized dissolution and destruction of the calcified tissue [1]. Dental plaque (biofilm) is defined as a soft thin film of food debris, mucin, and epithelial cells that adheres to the tooth surface, providing the medium for the growth of various bacterial species [1]. The term "cariogenic bacteria" refers to certain pathogenic microorganisms, which have the ability to ferment the carbohydrates and produce acids as a by-product [1]. Microflora responsible for caries development usually belongs to the normal physiologic flora with a low cariogenic potential (low virulence). But changes in the oral environment leading to a shift in the balance

between the cariogenic microflora, host defenses such as resistance and acid susceptibility of the tooth, plaque and saliva increase the cariogenic potential of the microflora (increase its virulence) and initiate caries.

Streptococcus mutans have been implicated as the most important bacteria for caries initiation and its progression. They exhibit a number of virulent characteristics that makes the plaque or biofilm cariogenic. They produce various acids, especially lactic acid, which demineralizes the tooth enamel. They also produce extracellular polysaccharides that allow for further plaque growth. In addition to S. *mutans*, *Lactobacilli* and the yeasts are important in the pathogenesis of dental caries.

Candida is a normal commensal in the oral cavity and participates in the formation of complex microbial oral biofilm. The percentage of *Candida* species colonization ranges from 20 to 40% in healthy individuals to about 60% in immuno-compromised people where it becomes the predominant flora [2]. Poor oral hygiene, increase in the intake of sugary foods and presence of carious lesions in children, favors candidal colonization [3]. The microbiology of dental plaque resulting in dental caries has been researched extensively. *Candida* seems to play an important role in microbial adherence to dental surfaces in coaggregation with *S. mutans* [4]. The synergistic action of *Candida* along with mutans streptococci enhances its cariogenicity and its adherence to the oral biofilm and carious tooth substance [4–7]. *C. albicans* is found to ferment glucose and maltose, producing both acid and gas and its contribution to overall microbial acid production seems to be important . Other factors attributing to the cariogenic ability of Candida are its adherence to saliva proteins, ability to penetrate into dentinal canals, and its enzymatic activity to degrade collagen [5]. *C. albicans* is known to be associated with dental caries, but more recently the role of nonalbicans candida (NAC) including *C. glabrata, C. guilliermondii*, *C. krusei,* C. *kyfer,* and *C. tropicalis* in the development of dental caries has been reported [3–9].

Research on the chemotherapeutic approaches to reduce the levels of *C. albicans* resulting in dental caries has been limited, and there is a need to determine the effectiveness of various chemotherapeutic agents against it. Ketoconazole is an antifungal imidazole compound which has been found to be very active against both superficial and systemic fungal infections. The inhibitory effect of ketoconazole on *C. albicans*, as determined by incomplete respiration or impairment of respiratory function, occurred at the lowest concentration observed among the imidazole compounds [10]. So it is taken as a standard drug against which others are compared.

Chlorhexidine is a biocide that is widely prescribed in dentistry both as an antiseptic mouthwash and a denture disinfectant. It has a broad spectrum of antimicrobial activity against a variety of organisms, including *C. albicans*. It acts as a fungicide leading to the coagulation of nucleoproteins and changes in cell walls allowing the escape of cytoplasmic components through the plasmalemma. It is also capable of inhibiting candidal adhesion to biological and inert surfaces [11]. Coconut (*Cocos nucifera*), the unique source of various natural products is consumed as a part of the staple diet in many countries and useful for the development of medicines against various diseases. The parts of its fruit like coconut kernel and tender coconut water are of a great medicinal value because of its antimicrobial and antioxidant property [12]. Lauric acid, a medium chain fatty acid (MCF), which is predominant in coconut oil, has proved to have antimicrobial, antiviral, and anti-inflammatory action. Probiotics can be defined as living microbes, or as food ingredients containing living microbes, that beneficially influence the health of the host when used in adequate numbers [13]. They have been used to modify microfloral ecosystems and have shown some success as a therapeutic for oral diseases.

The study aims to isolate, characterize *Candida* from the dental plaque attached to the tooth surfaces of children with dental caries, to study its virulence factors,

and to test the susceptibility of *C. albicans* to ketoconazole, 0.2% chlorhexidine, probiotics, and coconut oil, and to compare their antimicrobial efficacy.

2. Materials and methods

Subjects for the study were selected from the children who consulted the outpatient Department of Pediatric and Preventive Dentistry, Kannur Dental College, Anjarakandy. Based on the caries experience (dmfs index) that was recorded using visible light, mouth mirror, and CPI probe, 50 children with dental caries were selected for the study. Informed written consent was obtained from the parent/guardian of the children. Exclusion criteria included the children who were on topical or systemic antibiotics or antifungal medication. This study was reviewed and approved by the Institutional Ethical Committee of Kannur Medical College.

2.1 Armamentarium

1. Mouth mirror
2. Explorer, Tweezer
3. Sterile Cotton swabs
4. Magnifying glass
5. Culture media-Sabouraud's Dextrose Agar, Corn Meal Agar, Hichrome Agar (HI MEDIA), Mueller Hinton Agar, Blood Agar
6. Serum
7. Culture plates
8. Stock vials
9. Glass slide
10. Cover slips
11. Incubator
12. Light microscope
13. Saline solution
14. Filter paper discs
15. 2% ketoconazole (KevonR)
16. 0.2% chlorhexidine (Hexidine mouthwash)
17. Probiotics (VizylacR, lactic acid *Bacillus* 120×10^6)
18. Coconut oil

Samples were collected using sterile cotton swabs. Swabbing was done over the buccal, lingual, proximal, and cervical portion of the tooth and immediately transferred to the lab for microbiological analysis. The samples were inoculated for culture on Sabouraud's Dextrose Agar (SDA) plates supplemented with 1% chloramphenicol with pH 6.6 to prevent bacterial overgrowth. The plates were incubated at 37°C for 24–72 h. Isolates were identified by colony morphology on SDA plates. Growth appears in 1 to 2 days as creamy, smooth, convex pasty colonies with a moldy odor. Culture is said to be negative if there is no growth even after 72 h of incubation. The positive cultures were stocked in SDA stock vials (**Figure 1**).

Isolates were speciated based on the conventional methods of germ tube test and Corn Meal Agar (Dalmau Plate Culture Technique) and by Hichrom Agar-Candida Differential Media. For germ tube test, a small portion of an isolated colony of the yeast to be tested was inoculated into the 0.5 ml human serum and incubated at

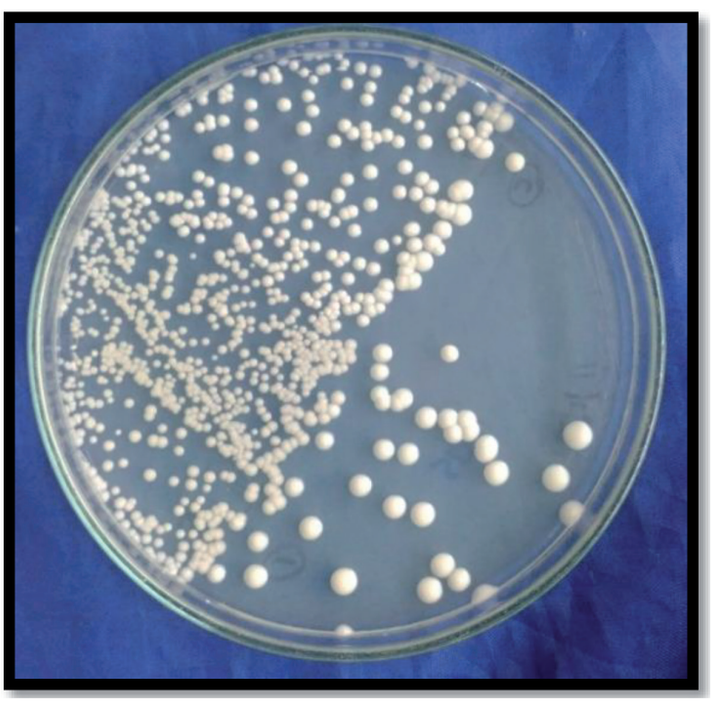

Figure 1.
Growth of Candida *on SDA.*

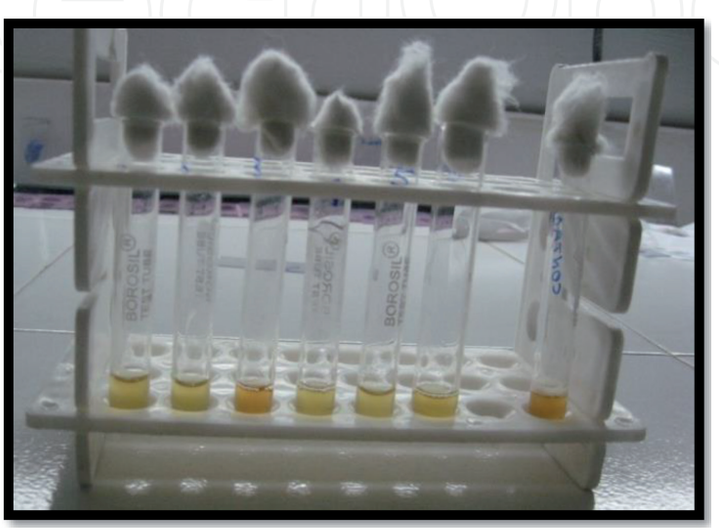

Figure 2.
Inoculation of Candida *in human serum for germ tube testing.*

37°C for 2 h (**Figure 2**). After 2 h of incubation, a drop of the yeast serum suspension was placed on a glass slide, overlaid with a cover slip and examined microscopically for the presence of germ tube under low power microscope. Test is said to be positive if tube like extensions from the yeast cell is seen within 2 h of inoculation and the isolate was considered as *C. albicans.* For Corn Meal Agar (Dalmau Plate Culture Technique), an isolated colony from the primary culture media was picked using a straight wire and inoculated into cornmeal agar plate at 45° angles to the culture media. A sterile cover slip was placed over the surface of the agar, covering a portion of the inoculated streaks (**Figure 3**).

The plates were incubated at 28°C for 48 h. The areas where the agar was streaked were examined under microscope. Isolates with large, highly refractile thick walled cell, single or multiple, terminal or intercalary chlamydospores were identified as *C. albicans* (**Figure 4**). Species identification was also done by streaking the samples on HiCrome Agar media (Himedia, India) and incubated at 37°C for 24 h. Colonies were identified depending on their color and pattern of growth.

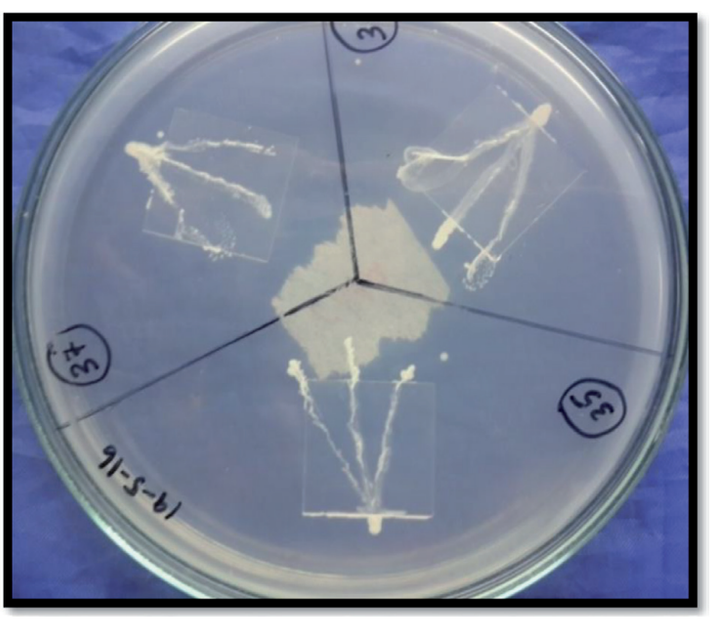

Figure 3.
Dalmau plate culture on Corn Meal Agar.

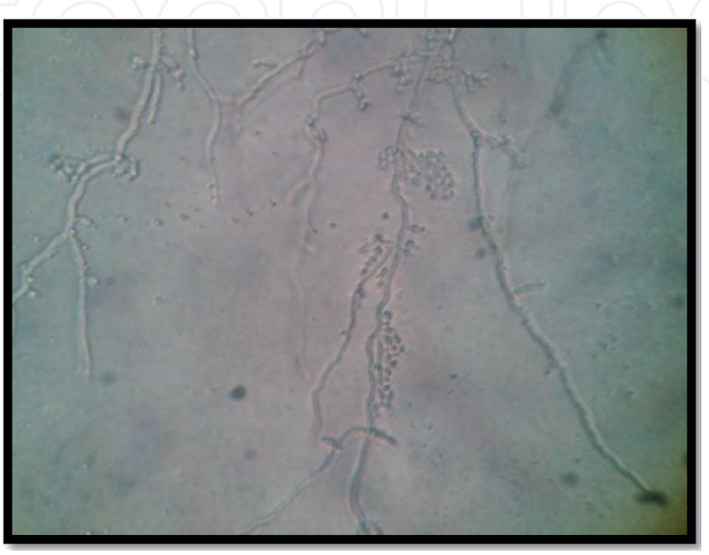

Figure 4.
Chlamydospore formation of C. albicans*-microscopic view (high power).*

The virulence markers like hemolysis and phospholipase were tested on the *Candidal* isolates. For hemolysis test, the Candidal isolates were seeded onto blood agar enriched with 1% glucose and incubated at 37°C for 48 h in a 5% CO_2 atmosphere. Hemolytic activity was defined as the formation of a translucent halo around the colonies. To determine phospholipase activity, test medium containing 65 g SDA, 58.4 g NaCl, and 5.5 g $CaCl_2$ was dissolved in 980 ml distilled water and sterilized at 121°C for 12 min [9]. Egg yolk was centrifuged at 5000*g* for 30 min. The supernatant was removed and added to cooled medium (45–50°C) (2%), mixed, and dispensed in plates. An aliquot (10 μl) of the yeasts suspension was inoculated onto test medium and incubated at 37°C for 4 days. Colony diameter and colony diameter plus precipitation zone were measured for each isolate and the zone of phospholipase activity was calculated [14] (**Figure 5**).

$$Pz = \frac{\text{Colony diameter}}{\text{Colony diameter} + \text{Zone of Precipitation}} \quad (1)$$

Five classes were described for phospholipase activity including;
Pz value = 1 means that the test strain is negative for phospholipase,
Pz <0.90–0.99 = weak phospholipase activity (+),
Pz = 0.80–0.89 = poor phospholipase activity (++);
Pz = 0.70–0.79 = moderate phospholipase activity (+++) and
Pz < 0.70 = large phospholipase activity (++++).

Kirby Bauer's Disc Diffusion method is used to test the antifungal activity of 2% ketoconazole (KevonR), 0.2% chlorhexidine (Hexidine mouthwash), probiotics (VizylacR, lactic acid *Bacillus* 120 × 10^6), and coconut oil against *C. albicans.* Suspensions of *C. albicans* were prepared in saline solution adjusted to the turbidity of 0.5 McFarland and streaked onto Mueller-Hinton agar supplemented with 1% glucose evenly. 0.2% chlorhexidine, coconut oil and probiotics (Vizylac, lactic acid *Bacillus*), and 2% ketoconazole were applied on filter paper discs of 6 mm separately (4.0 μL/disc) and allowed to dry. Then the discs of chlorhexidine, coconut oil, probiotics, and ketoconazole are placed on its surface at equal distance and

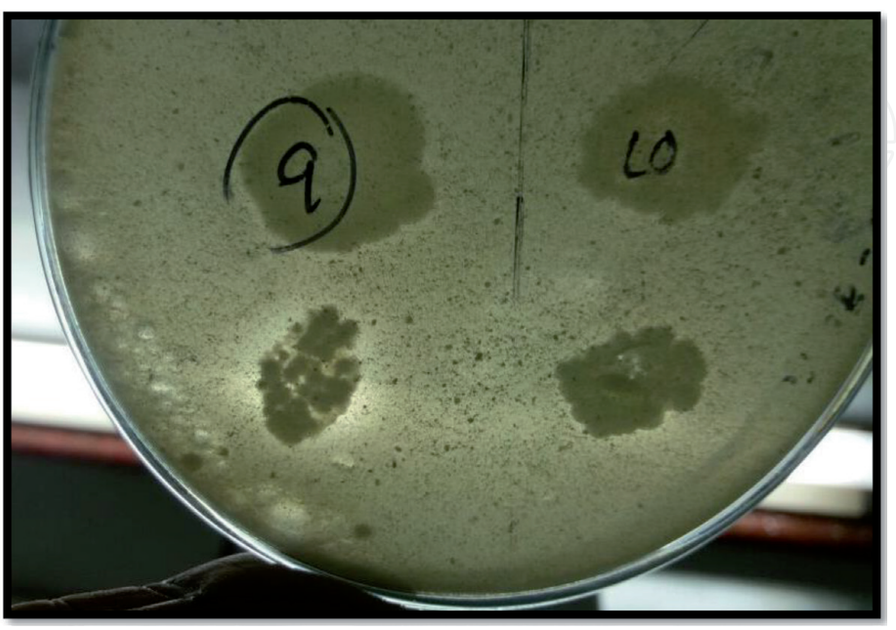

Figure 5.
Phospholipase test.

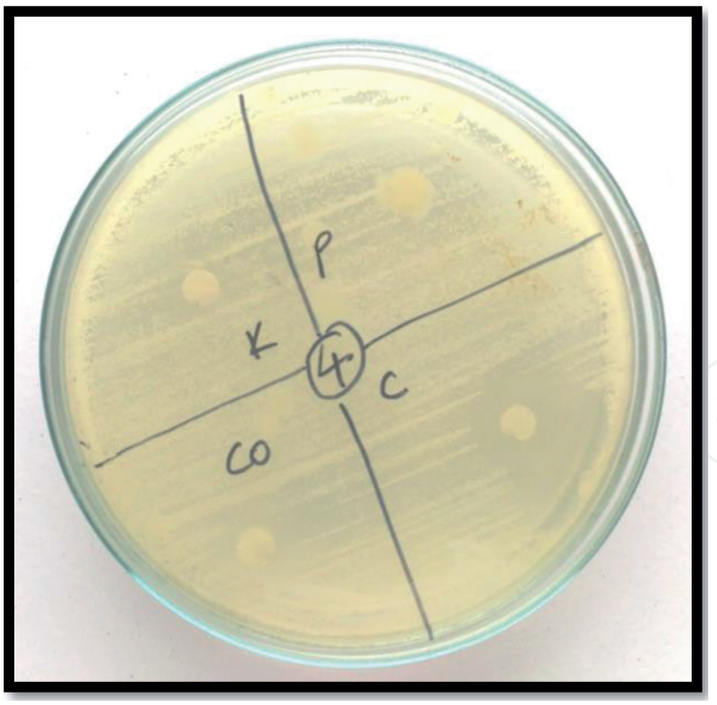

Figure 6.
Zone of inhibition observed around the discs.

incubated at 37°C for 24 h. Twenty isolates of *C. albicans* were tested in this manner. The zone of inhibition around the discs was observed (**Figure 6**), which will be measured and compared.

The phenotypes and the susceptibility of the isolates to the antifungals were compared against one another by the nonparametric Kruskal-Wallis, for multiple independent groups, or Mann-Whitney, for two independent groups, tests. The results were considered statistically significant at $P \leq 0.05$.

3. Results

Candida was identified by its morphological features of cream, smooth, pasty convex colonies with a moldy odor on SDA. Candidal carriage among the children was found to be 84% (42 children-positive), and *C. albicans* was found to be the predominant species identified. The presence of *C. albicans* was confirmed by observing the Germ tube formation and the formation of chlamydospore. On HiCrome agar, *C. albicans* were seen as light green-colored smooth colonies, *C. tropicalis* as metallic blue-colored raised colonies, *C. glabrata* as cream smooth colonies, and *C. krusei* appeared as purple fuzzy colonies (**Figure 7**). The distribution of various species of *Candida* identified is given in **Table 1** and **Figure 8**.

Virulence factors such as hemolysin, phospholipase, and germ tube formation were expressed by the *Candidal* isolates in the study. Phospholipase was tested positive in 92.8% of the isolates, hemolysis in 4.76%, and germ tube and hyphal formation in 5.76% as seen in **Table 2** and **Figure 9**. When various species were analyzed for their virulence factors, it was seen that 8% *C. albicans* showed hemolysis, 96% of them were positive for phospholipase test and all of them showed germ tube and hyphal formation. Hemolysis and germ tube formation was not detected in the rest of the species of Candida. For phospholipase, all the isolates of *C. tropicalis*, C. *guilliermondii*, *C. glabrata*, and C. *kyfer* and 60% of isolates of *C. krusei* showed phospholipase production (**Table 3**).

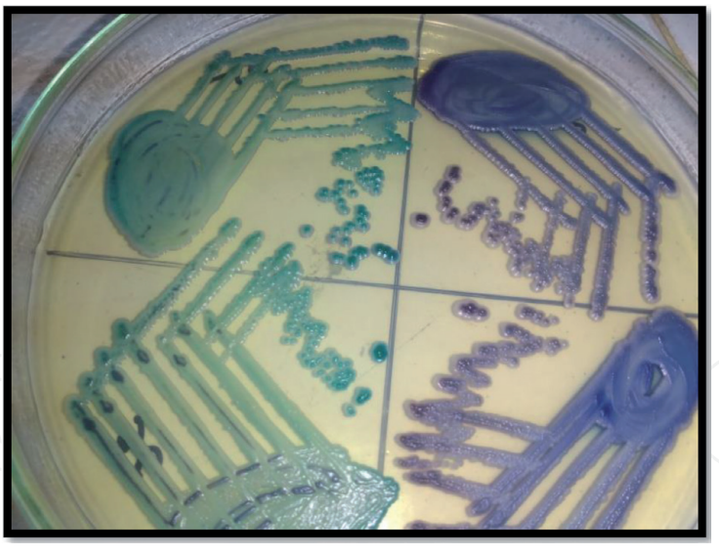

Figure 7.
Candidal colonies on HiChrome Agar.

Species	Frequency (%)
Candida albicans	25 (59.5)
Candida glabrata	3 (7.1)
Candida guilliermondii	1 (2.4)
Candida krusei	5 (11.9)
Candida kyfer	1 (2.4)
Candida tropicalis	7 (16.7)
Total	42(100)

Table 1.
Comparison of species distribution of Candida *among the children with dental caries.*

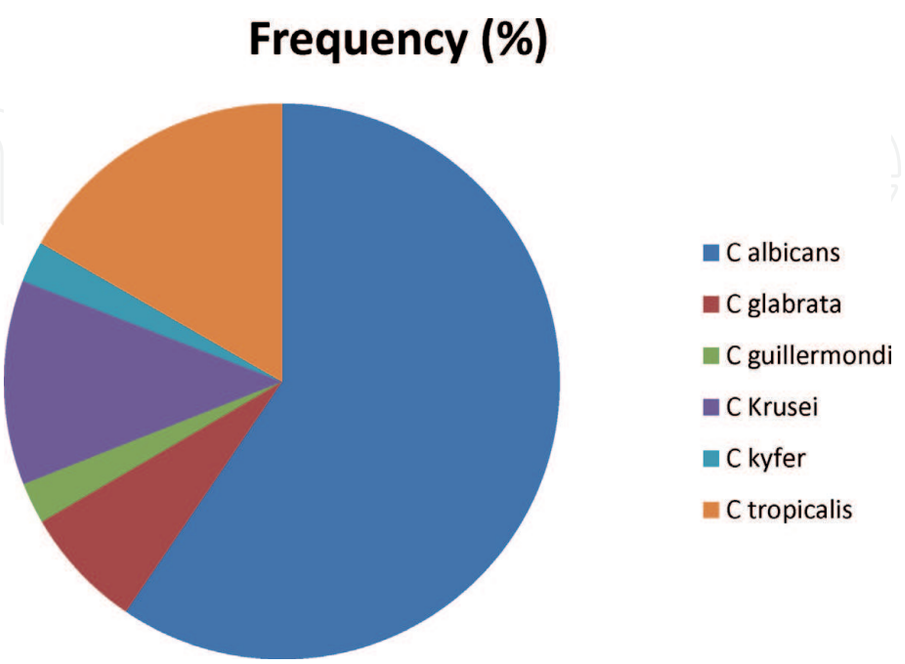

Figure 8.
Species distribution of Candida.

Virulence factors	Number (%)
Phospholipase test	
Positive	39 (92.8)
Negative	3 (7.14)
Hemolysis test	
Positive	2 (4.76)
Negative	40 (95.2)
Germ tube and hyphal formation	
Positive	25(59.52)
Negative	17 (40.47)

Table 2.
Virulence factors exhibited by Candida *isolates.*

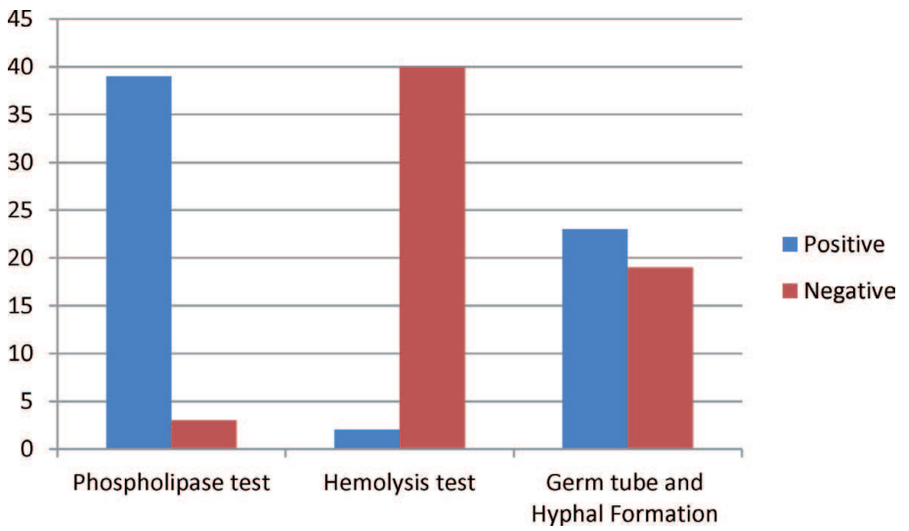

Figure 9.
Virulence factors of Candida.

Species of Candida	N	Hemolysis test		Phospholipase		Germtube and hyphal formation	
		P(%)	N(%)	P(%)	N(%)	P(%)	N(%)
C. albicans	25	2(8)	23(92)	24(96)	1(4)	25(100)	0 (0)
C. tropicalis	7	0 (0)	7(100)	7(100)	0 (0)	0 (0)	7(100)
C. guilliermondii	1	0 (0)	1(100)	1(100)	0 (0)	0 (0)	1(100)
C. krusei	5	0 (0)	5(100)	3(60)	2(40)	0 (0)	5(100)
C. glabrata	3	0 (0)	3(100)	3(100)	0 (0)	0 (0)	3(100)
C. kyfer	1	0 (0)	1(100)	1(100)	0 (0)	0 (0)	1(100)
Total	42	2(4.76)	40(95.2)	39 (92.8)	3(7.14)	25 (59.52)	19(40.47)

Table 3.
Virulence factors exhibited by individual species of Candida P-Positive , N-Negative.

The antifungal susceptibility test showed that *C. albicans* was susceptible to ketoconazole, chlorhexidine, coconut oil, and probiotics by having a clear zone of inhibition. **Table 4** shows comparison of zone of inhibition between different

Antimicrobial agents	N	Mean (mm)	Std. deviation	Chi-square	P value
Ketoconazole	20	22.30	15.076	7.429	0.059NS
Chlorhexidine	20	21.80	8.458		
Coconut oil	20	16.80	12.846		
Probiotics	20	13.50	13.656		
Total	80	18.60	13.033		

Table 4.
Comparison of zone of inhibition of antimicrobial agents against Candida albicans *NS: not significant Kruskal-Walla ANOVA.*

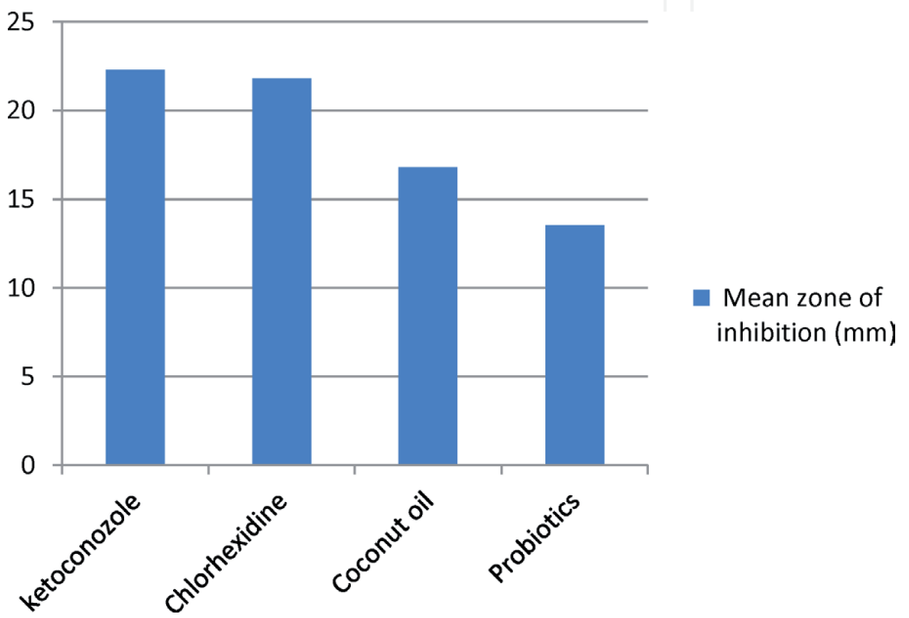

Figure 10.
Mean zone of inhibition of the antimicrobial agents against Candida albicans.

Antimicrobial agents	N	Mean	Std. deviation	Mean difference	Z-value	P value*
Ketoconazole	20	22.30	15.076	−0.5	−0.611	0.542
Chlorhexidine	20	21.80	8.458			

**Mann-Whitney U test.*

Table 5.
Comparison of zone of inhibition between ketoconazole and chlorhexidine.

Antimicrobial agents	N	Mean	Std. deviation	Mean difference	Z-value	P value*
Ketoconazole	20	22.30	15.076	−5.5	−1.761	0.078
Coconut oil	20	16.80	12.846			

**Mann-Whitney U test.*

Table 6.
Comparison of zone of inhibition between ketoconazole and coconut oil.

Antimicrobial agents	N	Mean	Std. deviation	Mean difference	Z-value	P value*
Ketoconazole	20	22.30	15.076	−8.8	−2.272	0.023
Probiotics	20	13.50	13.656			

**Mann-Whitney U test.*

Table 7.
Comparison of zone of inhibition between ketoconazole and probiotics.

groups. It was found that the mean zone of inhibition for ketoconazole was 22.3 mm, while it was 21.8 mm for chlorhexidine, 16.8 mm for coconut oil, and 13.5 mm for probiotics (**Figure 10**). The difference between the groups was not statistically significant (Chi-square value 7.42, *P* value 0.06).

The comparison of the zone of inhibition between ketoconazole and chlorhexidine showed that the mean zone of inhibition for ketoconazole was 22.3 mm, whereas for chlorhexidine, it was 21.8 mm. The difference was not statistically significant (*P* value 0.54) (**Table 5**). The comparison of the zone of inhibition between ketoconazole and coconut oil also showed no statistically significant difference (*P* value 0.07) (**Table 6**). However, in the comparison of the zone of inhibition of ketoconazole and probiotics, there was found to be a statistically significant difference between the groups (*P* value 0.02) (**Table 7**).

4. Discussion

Candida species colonizes the oral cavity of infants. Transmissions by mother during childbirth, pacifier use, and feeding habits are factors related to *Candida* oral colonization [15]. A study by Xiao revealed that mothers of the children affected by Early Childhood Caries (ECC) also have high *C. albicans* carriage, and most of the children were carrying the same *C. albicans* strains as their mothers [16].

The age of infection plays an important role in the disease process and the optimal period to intervene with preventive strategies [17].

Candida is a common commensal of the normal oral microbiota [18]. It is an opportunistic pathogen and has the ability to cause a variety of infections in immuno-compromised hosts like oral candidiasis which manifests as oral thrush in infants and chronic atrophic candidiasis in adults. Its ability to exist both in yeast and pseudohyphal/hyphal form plays an important role in its virulence [19]. While yeast form is a normal commensal of the oral cavity, pseudohyphal (budding shape) is associated with a fungal (saprophytic) condition, and the presence of hyphal forms is seen to be associated with active symptomatic infections. It displays many pathogenic forms due to which it is capable of adhering to various surfaces of host organisms, interfering with their immunological system, and producing several catabolytes [20].

Candida spp. is acidogenic and has the ability to ferment carbohydrates. Klinke et al. [4] have shown that in an environment with a pH below 5.5, which is relevant for ECC formation, acidification by *S. mutans* decreases considerably and ceases around pH 4.2, whereas *Candida* can still secrete acid at pH 4.0. It also produces several organic acids including pyruvic acid and acetate [21]. Abundant H + ATPase on the plasma membrane of yeasts pumping out proteins from the cell is induced by glucose and makes a contribution to the acidification [22]. Furthermore, it was

shown that *Candida* was capable of dissolving the hydroxyapatite at an approximately 20-fold rate higher than *S. mutans*, despite a lower number of yeast cells in the culture [4, 5, 7–20]. Acidification causing demineralization of dental tissues plays a major role in the progression of dental caries. The potential of *C. albicans* to adhere to saliva proteins and *S. mutans*, its acid producing capability, its ability to penetrate into dentinal canals, and its enzymatic activity to degrade collagen indicates its cariogenic ability and possible role in the progression of dental caries.

Of the different species of *Candida*, the most prevalent one recovered from the oral cavity is *C. albicans.* Identification of infecting strains of *Candida* is important because isolates of *Candida* species differ widely in their ability to cause infection as well as in their susceptibility to antifungal agents [23]. Hence, the present study is undertaken to identify and to characterize *Candida* species, to study its virulence factors and the antimicrobial activity of coconut oil, probiotics, 0.2% chlorhexidine, and ketoconazole on *C. albicans*.

Candidal growth was observed to be 84% among the ECC children, whereas it was only 24% among the caries free group. The results show that there is significant association between the Candidal carriage and the presence of ECC. This is in correlation with the previous studies by Hossain et al. [24], de Carvalho [20], Tony Jose [25], Ann Thomas [26], Fragkou et al. [5], and others. However in the studies by Maijala et al. [27], Peretz et al. [28], and Ratson et al. [29], no significant association between *Candida* and dental caries was found. This could be attributed to factors like difference in saliva rate, composition, buffering capacity etc. that influence the carious process. And it was observed that these authors employed other technical methods for detection of yeasts in the carious tooth samples and did not make cultures to identify them, which is considered to be a gold standard method for detection of yeast. But in the present study, a correlation between the caries experience of the children and *Candida* in terms of isolation frequency and numbers was observed, this being in line with most previous findings. And based on the fact that *Candida* is able to colonize the tooth surface, invade the dentinal tubules [30], produce a large amount of acids provoking demineralization of the dental enamel [21], and dissolution of hydroxyapatite [7], it has been hypothesized that *C. albicans* is a relevant pathogen involved in the progression of caries [27]. *C. albicans* also actively participates in cariogenic biofilms, through synergistic interaction with S. *mutans.* Evidence of enhanced exopolymeric matrix production, facilitated by the increased surface area associated with hyphal networks, supports mixed biofilm growth of dense communities cemented to tooth enamel, thus causing progression of dental caries [31].

The Candidal carriage in the present study (84%) is lesser than that of studies conducted by Merchant et al. [5] and Ann Thomas [26], where the prevalence of *Candida* species in ECC children was found to be 89 and 100% respectively. It was observed that the present study showed higher rate of Candidal carriage when compared to the studies by Hodson and Craig [32], Hossain et al. [24], de Carvalho [20], and Tony Jose [25], where the *Candida* carriage in ECC was found to be in the range of 56–67%. The differences in living environment, ecological environment of the oral cavity, and geographical variation and food habits of individuals might have influenced this variation among the rate of Candidal carriage among different studies [20].

The *Candida* species growth among the caries free children was observed to be 24% in this study. This is in correlation with the previous studies by Merchant et al. [5], Rozkiewicz [33], Jose [25], Thomas [26], Hossain [24], where the *Candida* frequency among those without caries was found to be in the range of 2–38%. The frequency of yeast carriage also varies due to differences in age, body fluids, mucosal surface, and natural barriers against yeast colonization [20].

5. Distribution of candida species

Even though *C. albicans* is recognized as the most prevalent species, many other species of *Candida* are identified with a potential clinical importance as they differ in the expression of virulence factors and antifungal susceptibility. Non *albicans Candida* species (NAC) are on the rise due to increasing immuno-compromised states [34]. Different species of *Candida* differ in their adherence to the oral tissues and hence their virulence.

Both conventional methods like germ tube test and chlamydospore formation on Dalmau plate culture and more advanced methods like CHROM AGAR *Candida* differential media (Hi Media) were used in the present study, for differentiation between different species of *Candida*. *C. albicans* was identified by the formation of germ tube and chlamydospores. CHROM AGAR is a relatively rapid method to differentiate between different *Candida* species. It facilitates the detection and identification of *Candida* species from mixed culture and provides result in 24–48 h. Previous studies shows that the sensitivity and specificity of CHROM agar for *Candida albicans* were 100 and 96%, *C. tropicalis* were 100 and 100%, *C. krusei* were 100 and 100%, and *C. glabrata* 75 and 100%, respectively [34].

The overall distribution of *Candida* species among the isolates was observed as follows: *C. albicans* (61.1%), *C. glabrata* (5.6%), C. *guilliermondii* (3.7%), *C. krusei* (11.1%), C. *kyfer* (3.7%), and *C. tropicalis* (14.8%). No isolates of *C. dubliniensis* was observed in the present study, which is similar to the studies by Moreira et al. [35], Martins et al. [36], de Carvalho [20], Al Hebshi et al. [37, 38], and Cortelli et al. [39]. But in the studies by Jabra Rizk et al. [40], Al Ahmed et al. [41], and Moraga et al. [42], C. *dubliniensis* was found in statistically significant proportions in caries active children. C. *dubliniensis* that shares a similar morphology with *C. albicans* is not frequently detected in various studies due to difficulty in differentiating between these yeast species, and it is more commonly isolated from immuno-compromised – HIV positive subjects [43]. However Kniest et al. [38] reported a case of isolating C. *dubliniensis* from plaque and carious dentine of a healthy 5 year old boy.

In the present study, *C. tropicalis* was found to be the most predominant among the Non *albicans Candida* species (14.8%), which is in correlation with the studies by de Carvalho [20], Cortelli et al. [39], Martins et al. [36], and Al Hebshi et al. [37]. However in the study by Al Hebshi et al. [38], *C. krusei* was most prevalent (6.9%) with the lowest counts for *C. tropicalis* (3.1%), and in a study done by Jabra Rizk et al. [40], *C. glabrata* (23%) was predominant. *C. kusei* is found in significant proportions in HIV and leprosy patients, and it is intrinsically resistant to the widely used triazole antifungal fluconazole and poses therapeutic problems [44]. An extensive diversity in the nonalbicans species was observed among different studies. Intrinsic differences in the pediatric population like differences in dietary intake, malnutrition, vitamin deficiency etc. may favor the presence of different yeast species. Interactions among *Candida* species exists that favor coexistence of two or more species (synergistic) or render presence of particular species unlikely (antagonistic). The carriage of *C. tropicalis* and that of *C. glabrata* appears mutually exclusive, while carriage of *C. albicans* favors the presence of *C. glabrata* [37]. More studies are needed to explore this further.

Among the Candidal isolates, *C. albicans* has shown the highest prevalence. This may be attributed to its capacity to form germ tubes, facilitating adhesion [7]. Other factors such as molecular adhesion and invasion into host cells, the secretion of hydrolases, the yeast-to-hypha transition, contact sensing and thigmotropism, biofilm formation, and phenotypic switching contribute to its pathogenic potential [45]. The adhesion of *C. albicans* to intact and denatured type I collagen was found to be significantly greater than those of other species and suggested that *C. albicans*

possessed the ability to adhere specifically to extracellular matrix as compared to other *Candida* species [46].

Virulence of *Candida* species is a significant factor that contributes to its colonization, pathogenicity, and infection of tissues [45]. In the present study, *Candida* expressed virulence factors such as formation of germ tubes, hyphae, hydrolytic enzymes such as phospholipases and hemolysin. Phospholipase acts by degrading the cell membrane of tissues and epithelial cells. *Candida* acquires iron from host tissue for its metabolism, growth, and invasion during host infection by the enzyme called hemolysin. There are reports of a higher production of virulence factors such as phospholipase among NAC than *C. albicans* [47]. Similar results are found in our study as all the isolates (100%) of *C. tropicalis*, C. *guilliermondii*, *C. glabrata*, and C. *kyfer* showed phospholipase production whereas only 96% of *C. albicans* were positive for phospholipase. However in the present study, the factors such as hemolysin production, germ tube, and hyphae were seen exclusively in *C. albicans.*

6. Virulence markers of candida species

C. albicans is an opportunistic pathogenic microorganism that has developed several virulence factors facilitating the invasion of host tissues [48]. It has the ability to persist on mucosal surfaces of healthy individuals [49]. In the oral cavity, it resists the mechanical washing action of a relatively constant flow of saliva towards the esophagus which contributes to its colonization and pathogenicity [50]. Its adhesion to host epithelial cells and biomaterials, formation of germ tubes and hyphae, the production of hydrolytic enzymes such as proteinases and phospholipases, and hemolytic capacity contribute to its colonization, pathogenicity, and infection of tissues [51].

The production of virulence factors is associated with the ability of *Candida* to cause infections [52]. Hemolytic capacity is an important virulence factor, which allows fungi of the genus *Candida* to acquire iron from host tissues, which then is used by the fungus for metabolism, growth, and invasion during host infection [53]. Phospholipase enzyme digests the host cell membrane phospholipid, causing cell lysis and changes in the surface features that enhance adherence and consequent infection. The ability to switch between the yeast form and the filamentous form is also an important virulence factor seen in *C. albicans*.

In the present study, germ tube and hyphal formation, an important virulence factor was seen among all the isolates of *C. albicans*. Phospholipase production was seen among 92.8% of the isolates which is higher than the values obtained from the previous studies by Deepa et al. [54] on Candidal isolates from Oral Candidiasis patients (52.6%) and Udaylaxmi et al. [16] on children on age 5–10 years with dental caries (47.6%). However, the results of the present study are lesser than that of Ali Zarei Mahmoudabadi et al. [55] on *C. albicans* isolated from vagina and urine samples, where phospholipase production was seen to be 100%. The phospholipase acts by degrading the cell membrane of tissues and epithelial cells, and it is an important virulence factor in progression of dental caries.

Hemolysis was shown by 2% among the Candidal isolates in the present study, which is lesser than the previous studies conducted by Rossinni et al. [56], Deepa et al. [54], and Udaylaxmi et al. [9], where the hemolysin activity was seen among 92, 63.1, and 100% of the isolates, respectively. There could be varied reasons for this variation. *Candida* strains in HIV-infected individuals have increased expression of virulence attributes as suggested by the strongly positive hemolytic activity among HIV individuals [56]. There are various factors that influence the morphology of yeast and its virulence, such as environmental changes like glucose

starvation, growth temperature, carbohydrate rich diet, and the presence of streptococci [57].

Ketoconazole is an antifungal imidazole compound that exhibits a significant activity against a broad range of superficial and systemic infections caused by pathogenic yeasts, dermatophytes, and filamentous fungi, including *C. albicans*. It inhibits respiration by inhibiting the activity of NADH oxidase at the mitochondrial level which is its primary site of action. It is known to stimulate phagocytosis and inhibit ergosterol biosynthesis which is a characteristic constituent of yeast cell membranes thus inhibiting the filamentous growth of *C. albicans* [10]. Hence, ketoconazole is taken as the standard antifungal agent in the present study and other antimicrobial agents were compared with it.

0.2% Chlorhexidine digluconate is commonly used as an antiseptic mouth rinse because of its wide spectrum of antimicrobial activity. It is capable of inhibiting candidal adhesion to biological and inert surfaces resulting in biofilm [11]. It acts as a fungicide by coagulating the nucleoproteins of the cell walls causing the escape of cytoplasmic components through the plasmalemma [3]. A significant antimicrobial activity was shown by chlorhexidine, in the present study, with a mean zone of inhibition of 21.8 mm, and the difference with that of ketoconazole was not statistically significant (*P* value 0.54).

Coconut oil is known to exhibit antimicrobial activity against *S. mutans* and *C. albicans*. It has a unique role in the diet as an important physically functional food and is composed of medium chain fatty acids (MCFs) like lauric acid, caprylic acid, myristic acid, capric acid, linoleic acid, oleic acid, stearic acid, and palmitic acid. Lauric acid constitutes majority of MCFs in coconut oil and have similar beneficial effects as MCFs in mother's milk [58]. Monolaurin and other medium chain monoglycerides are shown to have the capacity to alter microbial cell walls, penetrate and disrupt cell membranes, and inhibit enzymes involved in energy production and nutrient transfer, leading to the death of the bacteria [59]. In the present study, coconut oil has shown antifungal activity that is comparable to that of ketoconazole. Previous studies have shown *C. albicans* to be highly susceptible to coconut oil [60], especially to the lauric acid of coconut oil [61].

Probiotics are live micro-organisms which, in adequate amounts, confer a health benefit to the host. Use of probiotics to replace cariogenic bacteria with noncariogenic beneficial microflora has shown promising results. Taking probiotics in cheese is found to reduce the prevalence of *C. albicans* [62]. In the present study, *C. albicans* was found to be susceptible to probiotics.

7. Conclusion

In the present study, the Candidal carriage among the children with dental caries was found to be 84%. In addition to *C. albicans*, non albicans *Candida* such as *C. tropicalis*, C. *guilliermondii*, *C. krusei*, *C. glabrata*, and *C. kyfer* were isolated from the teeth in children with dental caries which indicate its role in the production of dental caries. Various virulence factors such as phospholipase, hemolysin, and germ tube formation seem to affect its pathogenicity. This study scientifically proves the antifungal activity of chlorhexidine, coconut oil, and probiotics. The antifungal activity of coconut oil is found to be higher than that of probiotics against *C. albicans*.

However, further studies emphasizing the various other virulence factors such as proteinase production and phenotypic switching responsible for the virulence of the non albicans *Candida* need to be researched. Further studies must be carried out to determine the antimicrobial efficacy, the MIC, and MFC of these agents and more clinical studies have to be conducted to validate the same.

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Conflict of interest

I declare there is no conflict of Interest.

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References

[1] Heymann HO, Swift EJ, Ritter AV, Sturdevant CM. Sturdevant's art and science of operative dentistry. In: Dental Caries: Etiology, Clinical Characteristics, Risk Assessment, and Management. 6th ed. St. Louis, Mo: Elsevier/Mosby; 2013. pp. 41-88

[2] Signoretto C, Burlacchini G, Faccioni F, Zanderigo M, Bozzola N, Canepari P. Support for the role of Candida spp. in extensive caries lesions of children. New Microbiologica. 2009;**32**:101-107

[3] Shino B, Peedikayil FC, Jaiprakash SR, Bijapur GA, Kottayi S, Jose D. Comparison of antimicrobial activity of Chlorhexidine, coconut oil, probiotics, and ketoconazole on *Candida albicans* isolated in children with early childhood caries: An in vitro study. Scientifica. 2016;**2016**:7061587. DOI: 10.1155/2016/7061587

[4] Klinke T, Kneist S, de Soet JJ, Kuhlisch E, Mauersberger S, Forster A, et al. Acid production by oral strains of Candida albicans and lactobacilli. Caries Research. 2009;**43**:83-91

[5] Fragkou S, Balasouli C, Tsuzukibashi O, Argyropoulou A, Menexes G, Kotsanos N, et al. Streptococcus mutans, Streptococcus sobrinus and Candida albicans in oral samples from caries-free and caries-active children. European Archives of Paediatric Dentistry. 2016;**17**:367-375

[6] Beena MS, Peedikayil FC, GufranAfmed MB, Chandru TP, Soni K, Dhanesh N. Comparison of Candida species isolated from children with and without early childhood caries: A descriptive cross-sectional study. Journal of the Indian Society of Pedodontics and Preventive Dentistry. 2017;**35**:296-300

[7] Nikawa H, Yamashiro H, Makihira S, Nishimura M, Egusa H, Furukawa M, et al. Invitro cariogenic potential of Candida albicans. Mycoses. 2003;**46**:471-478

[8] Barberi DDSAV, Vicente VA. Analysis of the in vitro adherence of Streptococcus mutans and Candida albicans. Brazilian Journal of Microbiology. 2007;**38**:624-631

[9] Udayalaxmi J, Shenoy N. Comparison between biofilm production, phospholipase and haemolytic activity of different species of Candida isolated from dental caries lesions in children. Journal of Clinical and Diagnostic Research: JCDR. 2016;**10**(4):DC21

[10] Uno J, Shigematsu ML, Arai T. Primary site of action of ketoconazole on Candida albicans. Antimicrobial Agents and Chemotherapy. 1982;**21**(6):912-918. DOI: 10.1128/aac.21.6.912

[11] Ellepola ANB, Samaranayake LP. Adjunctive use of chlorhexidine in oral candidosis: A review. Oral Diseases. 2001;**7**:11-17

[12] DebMandal M, Mandal S. Coconut (*Cocos nucifera L.: Arecaceae*): In health promotion and disease prevention. Asian Pacific Journal of Tropical Medicine. 2011;**4**(3):241-247

[13] Ashwell M. Concepts of functional foods Brussels: International Life Sciences Institute Europe. 2002

[14] Price MF, Wilkinson ID, Gentry LO. Plate method for detection of phospholipase activity in Candida albicans. Sabouraudia. 1982;**20**(1):7-14

[15] De Carvalho FG, Parisotto TM, Hebling J, Spolidorio LC, Spolidorio DM. Presence of Candida spp. in infants oral cavity and its association with early childhood caries.

Brazilian Journal of Oral Sciences. 2007;**1**:1249-1253

[16] Xiao J, Moon Y, Li L, Rustchenko E, Wakabayashi H, Zhao X, et al. Candida albicans carriage in children with severe early childhood caries (S-ECC) and maternal relatedness. PLoS One. 2016;**11**(10):e0164242

[17] Tinanoff N, O'sullivan DM. Early childhood caries: Overview and recent findings. Pediatric Dentistry. 1997;**19**:12-16

[18] Cannon RD, Chaffin WL. Oral colonization by Candida albicans. Critical Reviews in Oral Biology and Medicine. 1999;**10**(3):359-383

[19] Featherstone JD. The science and practice of caries prevention. Journal of the American Dental Association. 2000;**131**(7):887-899

[20] de Carvalho FG, Silva DS, Hebling J, Spolidorio LC, Spolidorio DM. Presence of mutans streptococci and Candida spp. in dental plaque/dentine of carious teeth and early childhood caries. Archives of Oral Biology. 2006;**51**(11):1024-1028

[21] Samaranayake LP, Hughes A, Weetman DA, MacFarlane TW. Growth and acid production of Candida species in human saliva supplemented with glucose. Journal of Oral Pathology & Medicine. 1986;**15**(5):251-254

[22] Manavathu EK, Dimmock JR, Vashishtha SC, Chandrasekar PH. Proton-pumping-ATPase-targeted antifungal activity of a novel conjugated styryl ketone. Antimicrobial Agents and Chemotherapy. 1999;**43**(12):2950-2959

[23] Allen CM, Saffer A, Meister RK, Beck FM, Bradway S. Comparison of a lesion inducing isolate and a non lesional isolate of Candida albicans in an immunosuppressed rat model of oral candidiasis. Journal of Oral Pathology & Medicine. 1994;**23**(3):133-139

[24] Hossain H, Ansari F, Schulz-Weidner N, Wetzel WE, Chakraborty T, Domann E. Clonal identity of Candida albicans in the oral cavity and the gastrointestinal tract of pre-school children. Oral Microbiology and Immunology. 2003;**18**(5):302-308

[25] Jose T, Thomas A, Pidamale R, Mhambrey S, Shetty SB. Research article. Correlation between C. albicans, S. mutans, S. sanguinis and lactobacillus in ECC, S-ECC and caries free children. International Journal of Recent Scientific Research. 2014;**5**(2):352-356

[26] Thomas A, Mhambrey S, Chokshi K, Chokshi A, Jana S, Thakur S, et al. Association of Oral Candida albicans with severe early childhood caries-a pilot study. Journal of Clinical and Diagnostic Research: JCDR. 2016;**10**(8):ZC109

[27] Maijala M, Rautemaa R, Järvensivu A, Richardson M, Salo T, Tjäderhane L. Candida albicans does not invade carious human dentine. Oral Diseases. 2007;**13**(3):279-284

[28] Peretz B, Mazor Y, Dagon N, Bar-Ness GR. Candida, mutans streptococci, oral hygiene and caries in children. The Journal of Clinical Pediatric Dentistry. 2011;**36**(2):185-188

[29] Ratson T, Greenstein RB, Mazor Y, Peretz B. Salivary Candida, caries and Candida in toothbrushes. The Journal of Clinical Pediatric Dentistry. 2012;**37**(2):167-170

[30] Şen BH, Safavi KE, Spångberg LS. Colonization of Candida albicans on cleaned human dental hard tissues. Archives of Oral Biology. 1997;**42**(7):513-520

[31] O'Donnell LE, Millhouse E, Sherry L, Kean R, Malcolm J, Nile CJ,

et al. Polymicrobial Candida biofilms: Friends and foe in the oral cavity. FEMS Yeast Research. 2015;**15**(7):fov077

[32] Hodson JJ, Craig GT. The incidence of Candida albicans in the plaques of teeth of children. Journal of Dentistry. 1972;**22**(8):296-301

[33] Rozkiewicz D, Daniluk T, Zaremba ML, Cylwik-Rokicka D, Stokowska W, Pawińska M, et al. Oral Candida albicans carriage in healthy preschool and school children. Advances in Medical Sciences. 2005;**51**:187-190

[34] Devi LS, Maheshwari M. Speciation of Candida species isolated from clinical specimens by using Chrom agar and conventional methods. International Journal of Scientific and Research Publications. 2014;**4**:1-5

[35] Moreira D, Spolidório DM, Rodrigues JA, Boriollo MF, Pereira CV, Rosa EA, et al. Candida spp. biotypes in the oral cavity of school children from different socioeconomic categories in Piracicaba-SP, Brazil. Pesquisa Odontológica Brasileira. 2001;**15**(3):187-195

[36] Martins AC, Maluf ML, Svidzinski TI. Prevalence of yeast species in the oral cavity and its relationship to dental caries. Acta Scientiarum: Health Sciences. 2011;**33**(1):107-112

[37] Al-hebshi NN, Abdulhaq A, Quadri MF, Tobaigy FM. Salivary carriage of Candida species in relation to dental caries in a population of Saudi Arabian primary school children. The Saudi Journal for Dental Research. 2015;**6**(1):54-59

[38] Al-Hebshi NN, Al-Maswary EA, Al-Hammadi ZO, Ghoname N. Salivary Candida species carriage patterns and their relation to caries experience among yemeni children. Oral Health & Preventive Dentistry. 2015;**13**:41-49

[39] Cortelli SC, Junqueira JC, da Silva FI, Ito CY, Cortelli JR. Correlation between Candida spp. and DMFT index in a rural population. Brazilian Journal of Oral Sciences. 2015;**5**(17):1007-1011

[40] Jabra-Rizk MA, Torres S, Rambob I, Meiller T, Grossman L, Minah G. Prevalence of oral Candida species in a North American pediatric population. The Journal of Clinical Pediatric Dentistry. 2007;**31**(4):260-263

[41] Al-Ahmad A, Auschill TM, Dakhel R, Wittmer A, Pelz K, Heumann C, et al. Prevalence of Candida albicans and Candida dubliniensis in caries-free and caries-active children in relation to the oral microbiota—A clinical study. Clinical Oral Investigations. 2015;**23**:1-9

[42] Lozano Moraga CP, Rodríguez Martínez GA, Lefimil Puente CA, Morales Bozo IC, Urzúa Orellana BR. Prevalence of Candida albicans and carriage of Candida non-albicans in the saliva of preschool children, according to their caries status. Acta Odontologica Scandinavica. 2016;**27**:1-6

[43] Erköse G, Erturan Z. Oral Candida colonization of human immunodeficiency virus infected subjects in Turkey and its relation with viral load and CD4+ T-lymphocyte count. Mycoses. 2007;**50**(6):485-490

[44] Samaranayake L. Commensal oral Candida in Asian cohorts. International Journal of Oral Science. 2009;**1**(1):2

[45] Mayer FL, Wilson D, Hube B. Candida albicans pathogenicity mechanisms. Virulence. 2013;**4**:119-128

[46] Makihira S, Nikawa H, Tamagami M, Hamada T, Nishimura H,

Ishida K, et al. Bacterial and Candida adhesion to intact and denatured collagen in vitro. Mycoses. 2002;**45**:389-392

[47] Butola R, Agwan V, Thakuria B, Madan M. A comparative study of virulence factors in clinical isolates of Candida species. International Journal of Current Microbiology and Applied Sciences. 2015;**4**:716-722

[48] Schaller M, Borelli C, Korting HC, Hube B. Hydrolytic enzymes as virulence factors of Candida albicans. Mycoses. 2005;**48**(6):365-377

[49] Bhat V. Prevalence of Candida associated denture stomatitis (CADS) and speciation of Candida among complete denture wearers of south west coastal region of Karnataka. Microbiology. 2013;**7**:8

[50] Noumi E, Snoussi M, Hentati H, Mahdouani K, Del Castillo L, Valentin E, et al. Adhesive properties and hydrolytic enzymes of oral Candida albicans strains. Mycopathologia. 2010;**169**(4):269-278

[51] Sitheeque MA, Samaranayake LP. Chronic hyperplastic candidosis/ candidiasis (Candidal leukoplakia). Critical Reviews in Oral Biology and Medicine. 2003;**14**(4):253-267

[52] Aher CS. Species distribution, virulence factors and antifungal susceptibility profile of Candida isolated from Oropharyngeal lesions of HIV infected patients. International Journal of Current Microbiology and Applied Sciences. 2014;**3**(1):453-460

[53] Almeida RS, Wilson D, Hube B. Candida albicans iron acquisition within the host. FEMS Yeast Research. 2009;**9**(7):1000-1012

[54] Deepa K, Jeevitha T, Michael A. In vitro evaluation of virulence factors of Candida species isolated from oral cavity. Journal of Microbiology and Antimicrobials. 2015;**7**(3):28-32

[55] Mahmoudabadi AZ, Zarrin M, Miry S. Phospholipase activity of Candida albicans isolated from vagina and urine samples. Jundishapur Journal of Microbiology. 2010;**3**(4):169-173

[56] Rossoni RD, Barbosa JO, Vilela SF, Jorge AO, Junqueira JC. Comparison of the hemolytic activity between C. albicans and non-albicans Candida species. Brazilian Oral Research. 2013;**27**(6):484-489

[57] Holmes AR, Cannon RD, Jenkinson HF. Interactions of Candida albicans with bacteria and salivary molecules in oral biofilms. Journal of Industrial Microbiology. 1995;**15**(3):208-213

[58] Peedikayil FC. Is coconut oil good for oral health? A review. Journal of Health Research and Review. 2019;**6**:1-4

[59] Verallo-Rowell VM, Dillague KM, Syah, Tjundawan BS. Novel antibacterial and emollient effects of coconut and virgin olive oils in adult atopic dermatitis. Dermatitis. 2008;**19**(6):308-315

[60] Ogbolu DO, Oni AA, Daini OA, Oloko AP. In vitro antimicrobial properties of coconut oil on Candida species in Ibadan, Nigeria. Journal of Medicinal Food. 2007;**10**(2):384-387

[61] Devan K, Peedikayil FC, Kottayi S, Dhanesh N, Sursh KR. Antimicrobial efficacy of mdium chain fatty acids as root canal irrigants:An invitro study. Journal of the Indian Society of Pedodontics and Preventive Dentistry. 2019;**37**:258-264

[62] Hatakka K, Ahola AJ, Yli-Knuuttila H, et al. Probiotics reduce the prevalence of oral candida in the elderly—A randomized controlled trial. Journal of Dental Research. 2007;**86**(2):125-130