Biofilm Formation by *Candida* Species on Silicone Surfaces and Latex Pacifier Nipples: An *in vitro* Study

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The present study assessed the growth and development of biofilm formation by isolates of C. albicans, C. glabrata and C. parapsilosis on silicone and latex pacifier nipples. The silicone and latex surfaces were evaluated by scanning electronic microscopy (SEM). The plastic component of the nipple also seems to be an important factor regarding the biofilm formation by Candida spp. The biofilm growth was measured using the MTT reduction reaction. C. albicans was found to have a slightly greater capacity of forming biofilm compared to the other Candida species. Analysis of the pattern of biofilm development by C. albicans, C. glabrata and C. parapsilosis on latex and silicon pacifier shields showed an increased biofilm formation regarding the latter substrate. Silicone was shown to be more resistant to fungal colonization, particularly in the case of C. parapsilosis, despite the lack of any statistically significant differences (P > 0.05). In addition, silicone has a smoother surface compared to latex, whose surface was found to be rugose and irregular.

Keywords: *Candida, latex, silicone, pacifier, biofilm, babies, children*
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INTRODUCTION

Nasal respiration, mastication and deglutition are considered both physiological and functional habits. However, finger-sucking, pacifier-sucking, use of feeding-bottle and the like are described as non-physiological habits, thus being deleterious. Non-nutritive sucking is very common during the early phases of life, remaining as an unwanted habit in approximately 30% of older children. The severity of the problems associated with sucking habits has been well documented and depends on duration, frequency and intensity, although individual predisposition with or without somatic diseases is also involved.[1]

Scherma *et al.*[2] evaluated 33 babies using questionnaires on oral hygiene, use of pacifiers and feeding-bottles and oral health during a 4-month period. They observed that 27.3% of the new-born that used pacifiers and feeding-bottles had more cases of oral candidiasis than those who did not use them. For Zollner,[3] there are differences regarding the presence of *Candida* species in the oral cavity of children who were breast-fed and used no pacifiers or artificial nipples (34.55%) compared to those who were bottle-fed (66.67%). In a study of 300 Turkish children, Kadir[4] *et al.* observed that oral candidiasis was prevalence in 26.35% of the cases, mostly involving *C. albicans* (84.8%) although other species such as *C. parapsilosis, C. krussei, C. kefyr, C, tropicalis* and *C. famata* were also observed.

Candidiasis is the most common oral fungal infection affecting human beings. In fact, *C. albicans* may be considered a normal component of the oral micro-flora since 30-50% of people have this micro-organism. It is important to emphasize that *C. albicans* and other yeast species have virulence factors such as epithelial cell adhesion, dimorphism, blastospore-like structures, pseudohyphae and hyphal forms, enzyme production (proteases and phospholipases) and low- and high-molecular-weight endotoxins, besides their cell wall composition, which facilitates adhesion and penetration

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through the infected tissue.[5]

Although the presence of the yeast is usually benign in a healthy host, the same is not true for immune-suppressed patients. However, there are intrinsic factors which can potentially enhance the ability of *C. albicans* to be pathogenic, such as the presence of adhesins in fungal cells, phenotypic variety and secretion of invasive bio-molecules (e.g. proteases and phospholipases).[6] The ability of the pathogenic *Candida* species to adhere to a variety of surfaces, including inert materials, has received considerable attention. For Klotz,[7] *Candida* species can invade human hosts because of their ability to adhere to plastic surfaces and similar materials. This invasion may occur via adherence to plastic surfaces such as prosthesis, catheters and prosthetic valves, thus disseminating through the vascular system. Studies suggest that *C. albicans* adherence is governed by extra-cellular mannoproteins.[8]

Studies have demonstrated that *C. parapsilosis* is the second most common *Candida* species seen in oral candidiasis. This yeast is frequently associated with diseases affecting premature children in intensive care units.[9] Parenteral nutrition is also related to the risk of candidemia. A glucose-rich milieu promotes the formation of biofilm, particularly *C. parapsilosis*, which increases the capacity of this microorganism to colonize catheters and in turn causes infections in patients receiving intravenous nutrition.[10] *C. parapsilosis* is also associated with nosocomial infections in vascular devices, a fact that can partially explain the biofilm formation in catheters.[11]

The phenomenon of adhesion relies on specialized proteins existing on the cell surface, called adhesins, which in turn attach specifically to amino-acids or sugars existing on the surface of other cells or promote adhesion to abiotic surfaces.[12] The hydrophobic cell surface, in association with attracting forces, contributes to the initial steps of adherence, a finding that seems to be related to adherence to plastic materials. Fungal adhesion is initially affected by a combination of hydrophobicity and electrostatic interaction. The adhesion capacity depends on many factors, such as the hydrophobicity state of the fungal cellular wall, characteristics of the substrate surface and the methodology used. Studies demonstrate that the greater the adhesion, the higher the degree of cell-surface hydrophobicity. This depends on many factors, including the culture medium used and strains chosen.[13]

The plastic component of the nipple also seems to be an important factor regarding the biofilm formation. In 2003, Tamura *et al.*[14] found differences in adherence by comparing urinary catheters made of latex to those made of silicone. Hawser,[15] in 1994, had already compared the biofilm formation of *Candida* species on surfaces of urinary catheters made of silicone, latex, PVC and elastomeric silicone and found different values for each type of material.

Ferey *et al.*.[16] in 2003, analyzed the silicone tubes of hemodialysis machines and found the presence of biofilm inside them in association with a large number of crystals of calcium and magnesium carbonate. These crystals were found to be firmly adhered to the tube surfaces, thus increasing adhesion and biofilm formation.

Comina *et al.*[17] have performed an *in vivo* study on *Candida* biofilm formed on latex and silicone nipples of pacifiers and found differences in the level of contamination, suggesting that the nipples of pacifiers and feeding-bottles can become a potential microbial reservoir.

Therefore, the aim of the present study was to assess the growth and development of biofilm formation by *C. albicans, C. glabrata* and *C. parapsilosis* on silicone and latex pacifier nipples as well as to evaluate the surfaces of these materials microscopically.

MATERIALS AND METHODS

The pacifiers were selected according to the composition of the nipples. Six latex nipples and six silicone nipples of the most commonly available pacifiers, produced by national manufacturers, were used for the study. The nipples were extracted and then cut into discs measuring approximately 0.5 cm in diameter. Each nipple yielded, on average, 10 discs that were stored in flasks and then sterilized with ethylene oxide.

Three *Candida* species were used for biofilm formation, namely, *C. albicans* (ATCC 36.901), *C. parapsilosis* (ATCC 22.019) and *C. glabrata* (ATCC 2001). The yeast species were obtained after growth in YNB medium (Yeast Nitrogen Medium, Difco) with 50 mM of glucose for 48 hours and 37ºC under shaking. Next, they were twice washed with PBS and cellular suspensions of 10^6 yeast/ml were obtained for biofilm formation.

After distributing the discs on 24-well medium plates, 60 ml of previously standardized solution were incubated for 1 hour at 37ºC in order to characterize the period of adhesion. The discs were washed with PBS to remove any non-adhered cells and then incubated with 1ml of cellular suspensions for 24 and 48 hours at 37ºC. Controls, *i.e.* samples without the presence of *Candida*, were incubated in YNB with 50 mM of glucose for the same periods of time (24 and 48 hours).

The biofilm growth on latex and silicone discs was measured using the MTT reduction reaction [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). After biofilm formation, 50 ml of MTT solution (a stock solution containing 5 mg of MTT per ml of PBS, diluted 1:5 in pre-warmed 0.15 M PBS prior to application) was added to each well. After incubation for 5 h at 37ºC the medium plus MTT was removed and the wells were washed three times with 0.15 M PBS (2 ml) to remove all traces of MTT. Dimethyl sulfoxide (1 ml) was then added to solubilize the MTT formazan product. MTT formazan formation was measured at 540 nm by using a spectrophotometer. Control wells contained medium plus MTT to determine background formazan values.

The discs were gently washing with PBS to remove any non-adhered cells and those with biofilm adhering to their surfaces were chemically fixed with 2.5% glutaraldehyde solution containing 3.7% saccharosis for 1 hour at room temperature. Next, the discs were washed again with PBS and then post-fixed with 1% osmium tetra-oxide solution in

Table 1. Quantitative measurement of 24- and 48-hour biofilm formation by *Candida* species on the surfaces of latex discs from three different nipples.Species

	Formazan formation (A 540)					
Species	Nipple A		Nipple B		Nipple C	
	24 h	48 h	24 h	48 h	24 h	48 h
C. albicans	0.090±0.247	[a]	0.194±0.019	0.100±0.008	0.181±0.030	0.065±0.042
C. glabrata	0.128±0.502	0.133±0.060	0.125±0.037	0.138±0.019	0.168±0.006	0.084±0.049
C. parapsilosis	0.080±0.022	0.037±0.011	0.124±0.022	0.016±0.006	0.101±0.001	0.043±0.019

[a]No measurement

Table 2. Quantitative measurement of 24- and 48-hour biofilm formation by Candida species on the surfaces of silicone discs from three different nipples.

	Formazan formation (A 540)					
Species	Nipple A		Nipple B		Nipple C	
	24 h	48 h	24 h	48 h	24 h	48 h
C. albicans	0.131±0.006	0.123±0.102	0.119±0.008	0.101±0.002	0.112±0.018	0.130±0.126
C. glabrata	0.137±0.395	0.093±0.029	0.147±0.013	0.123±0.016	0.117±0.028	0.097±0.043
C. parapsilosis	a	a	0.001±0.001	0.010±0.024	0.0085±0.015	0.021±0.011

[a]No measurement

0.1M sodium cacodylate buffer containing 0.8% potassium ferrocyanate and 5mM calcium chlorate for 30 minutes at room temperature. The samples were gradually dehydrated with ethanol and critically point dried with CO_2. Finally, they were metallized with gold. The images were obtained and analyzed in a JEOL JSM-5800 scanning electronic microscope with a secondary-electron detector.

All the experiments were performed in triplicate and the results expressed through descriptive and inferential statistics by using SPSS 11.0 software.

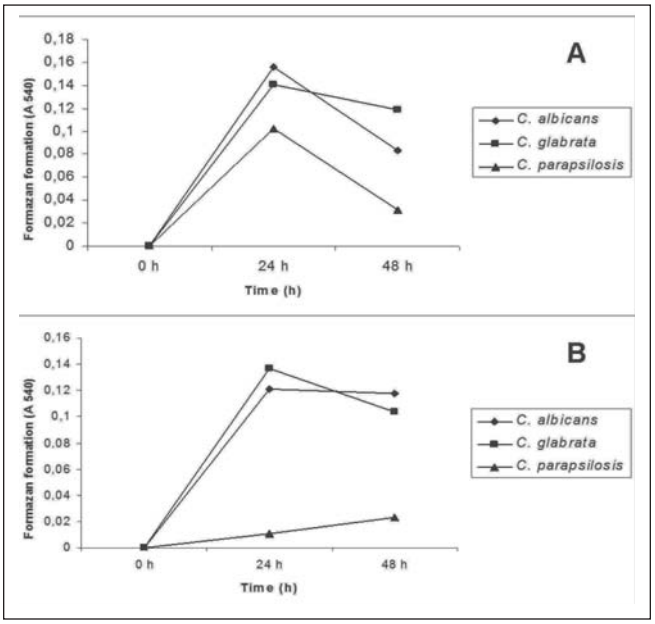
RESULTS

Amongst the different latex pacifiers, no difference in the amount of biofilm was observed after 24 and 48 hours of incubation, regardless of the *Candida* species used (Table 1). The same pattern of biofilm growth was found amongst the silicone samples (Table 2).

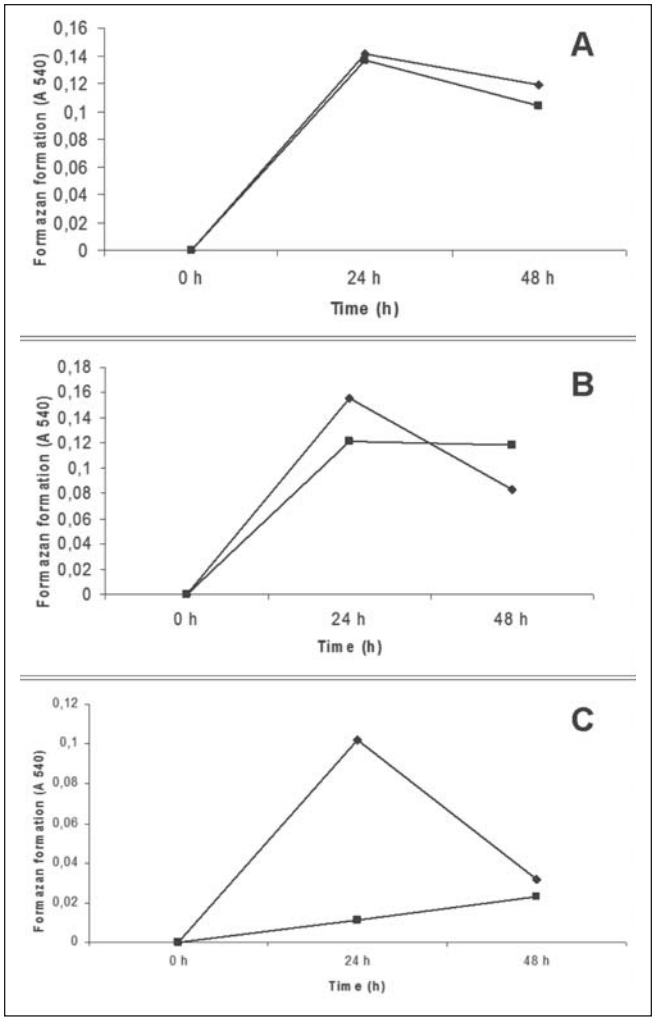
All *Candida* species used in the present study were able to form biofilm on latex and silicone structures. On both substrates, however, *C. albicans* showed a slightly greater ability to form biofilm compared to the other *Candida* species. Graph 1 shows that *C. albicans* is more predominant than *C. glabrata* and *C. parapsilosis* regarding biofilm formation, although no statistical difference was observed between the species. Such a difference was found to be more evident for silicone discs, particularly with regard to the reduction in *C. parapsilosis* biofilm following 24 and 48 hours of incubation.

MTT reduction reactions occurred in all *Candida* species after 24 and 48 hours of incubation, particularly during the former period of time despite the lack of significant difference (Graph 1).

Analysis of the development pattern of biofilm formation regarding *C. albicans, C. glabrata* and *C. parapsilosis* revealed that latex surfaces had more biofilm than the silicone ones (Graph 2). Silicone, therefore, was found to be more resistant to fungal colonization, mainly regarding *C. parapsilosis,* although no statistically significant difference was observed. These findings could be supported by the topographic images of the latex and silicone surfaces obtained by means of the scanning electronic microscopy, which revealed distinctive characteristics between both substrates. Comparatively, latex showed a rugose and irregular surface with clefts and depressions, whereas silicone had a more regular surface with no fissures or clefts (Figure 1A, B).



Graph 1. Biofilm formation by Candida species growing on latex (A) and silicone (B) discs in medium containing 50 mM glucose.



Graph 2. Biofilm formation by *C. albicans* (A), *C. glabrata* (B) and *C. parapsilosis* (C) growing on latex (♦) and silicone (■) discs in medium containing 50 mM glucose.

The biofilm formations produced by *C. albicans* (ATCC 36.901), *C. parapsilosis* (ATCC 22.019), and *C. glabrata* (ATCC 2001) on latex and silicone discs following 48 hours were observed with SEM. Images revealed the presence of different fungal forms such as yeast, hyphae, pseudohyphae and germinative tubes (*C. albicans* only) on both substrates (Figure 1).

DISCUSSION

The choice of silicone and latex pacifier nipples was mostly based on the fact that they are widely used by infants. The use of pacifiers has been increasingly considered a factor contributing to the development of candidiasis in children. Scherma *et al.* (2004)[2] showed that the frequent use of feeding-bottles can irritate the mucosa because of the introduction of a strange body within the oral environment, which may change the oral microbiota. Mattos-Graner[18] and coworkers studied 12-18-month old children and found that 58.3% of the subjects had yeast species in their oral cavity, mostly *C. parapsilosis* and *C. albicans.* Therefore, they concluded that the use of pacifiers is positively associated with the frequency of infection and presence of *Candida* species in the oral cavity, thus suggesting that the pacifier habit is an important factor contributing to colonization and proliferation regarding these micro-organisms.

Candida species have mechanisms permitting them to colonize even inert plastic surfaces. Klotz *et al.*[9] demonstrated that *Candida* adherence to plastic substrates is governed by hydrophobic forces – more appropriately designated as the van der Waals force. In addition, plastic surfaces have several degrees of negativity. The same can be observed in cells having negative surface charges, including yeast species, which adhere to plastic substrates because of simultaneous forces of attraction and repulsion.

The difference in electronegativity between latex and silicone can explain the difference found in the *Candida* biofilm formation on such substrates. Hawser and Douglas 15 obtained similar findings. They found that 100% of the silicone discs studied were more resistant ($P < 0.001$) to bacterial colonization compared to the other materials. The author found that biofilm formation was more extensive on latex discs, whereas 100% of the silicone discs had less biofilm than the other materials studied. These results are in accordance with our findings, showing a greater amount of *Candida* biofilm colonization on latex discs.

Still, according to Hawser,[15] there exists a correlation with *Candida* pathogenicity when different strains are tested in relation to biofilm formation. For instance, *C. glabrata* and *C. parapsilosis* were found to produce less biofilm than *C. albicans,* the most pathogenic form. Findings that *C. albicans* isolates produce consistently greater amounts of *in vitro*-biofilm compared to other *Candida* species have recently been corroborated by Kuhn,[19] which is also in accordance with our results showing that *C. parapsilosis* produced less biofilm.

Scanning electronic microscopy was used by Comina[17] to study pacifier nipples and they described clefts existing on the latex surface which can enhance the bacterial attachment. Clefts are also observed on silicone surfaces, although they are lesser in number and are not so deep, characteristics which do not prevent degradation by *Candida* species. Furthermore, only latex nipples were studied. The author suggests three explanations by citing differences in physical-chemical properties between silicone and latex, differences in the saliva of infants and older children and diet composition.

Amongst the three pacifiers commonly sold in Rio de Janeiro City, no differences were observed regarding biofilm formation. One can suppose, therefore, that today pacifiers are better controlled by the inspection agencies. ABNT (Brazilian Association of Technical Norms) establishes requirements for pacifiers, including their packing, whereas ANVISA (National Health Surveillance Agency - Brazil) rules the manufacturing procedures, sanitary control, and the like. Lima[20] shows that most pacifiers are produced according to such requirements.

Based on these findings, it should be emphasized that good sterilization is important to prevent the pacifier from

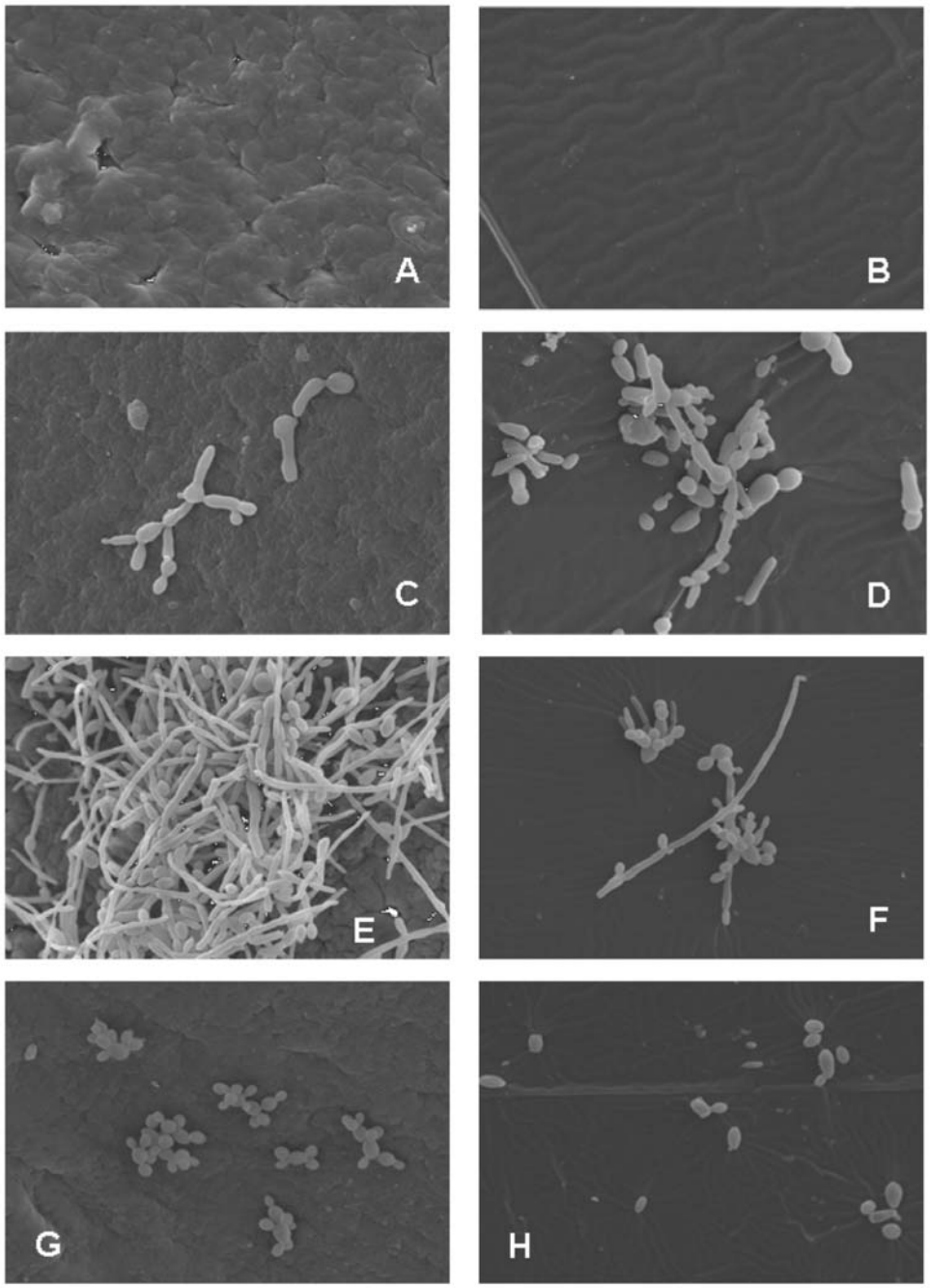


Figure 1. Scanning electron micrographs of biofilm formation by *C. albicans* (ATCC 36.901) (c,d); biofilm formation by *C. parapsilosis* (ATCC 22.019) (e,f), and *C. glabrata* (ATCC 2001) (g,h) on latex and silicone discs, respectively. Mean control group (a,b).

becoming a vehicle of bacterial communication, particularly *Candida* species, which may be related to candidiasis in children.

Based on the results obtained in the present study, one can conclude that silicone nipples are more resistant than the latex ones regarding biofilm formation by *Candida* species. However, it should be emphasized that further studies should be performed in order to better understand the mechanisms involved in the yeast adherence to plastic surfaces. Also it was reported that *C. albicans* has a greater ability to adhere to both latex and silicone substrates compared to the other *Candida* species, a fact that could be explained by its high pathogenicity. In addition, the latex surface was found to be more irregular than the silicone one, thus becoming a potential reservoir not only for *Candida* strains, but also for dental biofilm and consequently a niche for infection. The increasing pressures exerted by inspection agencies requiring better quality control may play an important role in the standardization of materials for manufacturing pacifiers, which might explain the similarity found in the results obtained from silicone and latex nipples.

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