# Effect of the high molecular weight chitosan and sodium alginate on *Candida albicans* hydrophobicity and adhesion to cells

**Ana Isabel Azcurra [1], Silvina Ruth Barembaum [1], María Alejandra Bojanich [1], Silvia Edith Calamari [2], Javier Aguilar [3], Luis José Battellino [4], Susana Tereza Dorronsoro [5]**

(1) Jefe de Trabajos Prácticos Cátedra de Introducción a la Química y Física Biológicas, Facultad de Odontología
(2) Profesor Adjunto Cátedra de Introducción a la Química y Física Biológicas, Facultad de Odontología
(3) Jefe de Trabajos Prácticos, Instituto de Virología Varella, Facultad de Ciencias Médicas
(4) Profesor Titular Cátedra de Química Biológica, Facultad de Odontología
(5) Profesor Titular Cátedra de Introducción a la Química y Física Biológicas, Facultad de Odontología, Universidad Nacional de Córdoba, Argentina

*Correspondence:*
*Dra. Ana Isabel Azcurra*
*Departamento de Biología Bucal,*
*Facultad de Odontología, Universidad Nacional de Córdoba.*
*Haya de la Torre S/N. Ciudad Universitaria,*
*Casilla Postal 4, 5000-Córdoba, Argentina.*
*E-mail: azcurra@fcq.unc.edu.ar*

Received: 6-05-2005
Accepted: 4-01-2006

Azcurra AI, Barembaum SR, Bojanich MA, Calamari SE, Aguilar J, Battellino LJ, Dorronsoro ST. Effect of the high molecular weight chitosan and sodium alginate on *Candida albicans* hydrophobicity and adhesion to cells. Med Oral Patol Oral Cir Bucal 2006;11:E120-5.


**Indexed in:**
-Index Medicus / MEDLINE / PubMed
-EMBASE, Excerpta Medica
-Indice Médico Español
-IBECS

Click here to view the article in Spanish

## ABSTRACT

The aim of the present paper is to evaluate the effect of the high molecular weight chitosan (HMWC) and of sodium alginate (NaAL) on surface hydrophobicity of *Candida albicans* and on adhesion of the yeast to epithelial cells and fibroblasts of different proceeding.

For this study, a collection strain and seven isolates of *C. albicans* from saliva (patients with denture stomatitis) were grown in Sabouraud glucose agar supplemented with HMWC or NaAL or in absence of them (control). Hydrophobicity was determined by adhesion to hydrocarbons method using two organic media (xylene and chloroform). For adhesion experiments, aqueous suspensions of yeasts were contacted with solutions of biopolymers and different cells (rat and human fibroblasts and epithelial cells Hep-2). The quantification of adhesion was made by optical microscopy.

*Results:* a decrease in hydrophobicity was observed in the presence of HMWC (44%) and of NaAL (82%) when chloroform was employed as organic medium, meanwhile the decreases were of 30% with HMWC and 19% with NaAL in the presence of xylene. Adhesion of *C. albicans* to epithelial cells and human fibroblasts decreased significantly with both biopolymers. In the case of rat fibroblasts, a decrease was observed only with NaAL. None of experiments showed significant differences associated to fibroblast type.

*Conclusions:* biopolymers showed effectiveness in reducing hydrophobicity and adhesion of *C. albicans* to cells, which are important virulence factors related to colonization of the soft tissues of host or acrylic surfaces present in the oral system.

***Key words:*** *Candida albicans, chitosan, alginate, adhesion, hydrophobicity.*

## RESUMEN

*Objetivo:* Evaluar el efecto del quitosán de alto peso molecular (QAPM) y del alginato de sodio (NaAL) sobre la hidrofobicidad superficial de *Candida albicans* y la adhesión de esta levadura a células epiteliales y fibroblastos de distinto origen.

*Diseño del estudio:* Para el estudio de la hidrofobicidad, las levaduras (n=7) se hicieron crecer en agar glucosado de Sabouraud suplementado con QAPM o NaAL o en ausencia de los mismos (controles). La determinación de la hidrofobicidad se realizó por el método de adhesión a hidrocarburos utilizando dos solventes orgánicos (xileno y cloroformo). En los estudios de adhesión, las levaduras se pusieron en contacto con soluciones de biopolímeros y luego se enfren-

taron a diferentes células (fibroblastos humanos y de rata y células epiteliales Hep-2). La cuantificación se realizó por microscopía óptica.
*Resultados:* Se observó una disminución del 44% de la hidrofobicidad en presencia de QAPM y del 82%, con NaAL, o del 30% con QAPM y 19% con NaAL, cuando los solventes orgánicos empleados fueron cloroformo o xileno, respectivamente. La adhesión de *C. albicans* a células epiteliales y fibroblastos humanos disminuyó significativamente con ambos biopolímeros. En el caso de los fibroblastos de encía de rata, sólo se observó una disminución con NaAL. En ninguno de los experimentos se observaron diferencias significativas en asociación al tipo de fibroblasto empleado.
*Conclusiones:* Los biopolímeros resultaron efectivos en la reducción de la hidrofobicidad y la adhesión de *C. albicans* a células, las cuales son importantes factores de virulencia relacionados con la colonización de los tejidos blandos del hospedador o superficies acrílicas presentes en el sistema estomatognático.

***Palabras clave:*** *Candida albicans, quitosán, alginato, adhesión, hidrofobicidad.*

## INTRODUCTION

The development of new materials in dentistry, as well for prevention as for oral diseases treatment, has increased in recent years. Between them, high molecular chitosan (HMWC), cationic polymer derived from chitin, is used as a vehicle for drug delivery, with osteoconducting properties and favoring fibroblasts proliferation (1-4). Sodium alginate (NaAL), anionic copolymer, is employed for dental impression (5,6). Besides, both biocompatible materials, are used in biomedical applications.

Denture stomatitis, caused mainly by *C. albicans*, can be observed in a high percentage of population wearing dental prosthesis (7,8). The development of the infection depends on many factors, like protein type interaction – protein between *Candida* and host cells, morphology of yeast (blastoconidia or mycelium) and the immunological status of the host (9). The possibility of adhesion to plastic surfaces and soft tissues constitutes a virulence factor that predominates in *C. albicans* respect to other *Candida* species. Many studies on fungal adhesion to plastic materials employed different epithelial cells (10,11). Different interactions participate in this process, being cell surface hydrophobicity (CSH) one of the most important (12). Many methods have been used to determine CSH, but the adhesion to hydrocarbons one is the most employed because of its simplicity (13,14). Factors as temperature, interaction time of both phases, pH values, ionic strength of media, relative concentration of interacting species and acid-base character of the organic solvent employed modify CSH values.

The purpose of this work was to evaluate the effect of HMWC and NaAL on CSH and adhesion of *C. albicans* to epithelial cells and fibroblasts.

## MATERIAL AND METHODS

### Isolation and identification of *Candida*

A collection strain (*C. albicans* ATCC 10231) and isolates from unstimulated saliva of patients with denture stomatitis (n = 7) assisting to Cathedra of Prostodoncia III, Faculty of Dentistry, National University of Cordoba, Argentina (UNC), whom informed consent the participation in the study, were employed in this work. None of patients that consumed antifungal drugs, xerostomyzing medicines or anti-inflammatory therapy by a period of 7 to 10 days before the recollection of the sample was included in the trial. For strain isolation, saliva was seeded in Sabouraud glucose agar (SGA) supplemented with 1% chloramfenicol (Britania, Argentina). All isolations were identified biochemically by carbohydrate assimilation tests (Candifast, International Microbe, France) and identification was completed by micromorphological study on corn meal agar and germinative tube production (15).

### Preparation of the yeast for cell surface hydrophobicity test (CSH)

After identification of isolates, yeast was grown 24-48 h at 37 ºC in SGA without polymer (control) and in the media supplemented with 0.05 g% HMWC (90% deacetylated, 280 cps, MW 300 kDa, Unifarma, Argentina) and 0.2 g% NaAL (420 cps, Kimitsu, Chile). These concentrations correspond to sub-inhibitory ones of polymers determined previously in the described conditions.

### Determination of CSH

CSH was determined by adhesion to hydrocarbons method (13). Briefly, suspensions of yeast in phosphate buffer saline (PBS) were contacted (absorbance at 520 nm = 0.400) to different organic phases, xylene or chloroform, in a volume relation of 5:1; the mixture was mixed 1 min and left to rest for 10 min. CSH was calculated by the absorbance of aqueous phase at 520 nm before and after contacting yeast suspensions to the organic phases (13).

### Growth conditions and preparation of cells

a) Culture cell of human fibroblasts and epithelial cells

Human fibroblasts proceeding from baby foreskin (HF) and an epithelial cell line (Hep–2, ATCC CCl 23) from pharynx carcinoma, donated by the Medical Center of Studies and Clinical Investigations (CEMIC, Buenos Aires, Argentina, and the Virology Institute, Dr. Varella, UNC, respectively), were used. For culture cells, MEM Eagle media (Gibco, USA) with 10% bovine fetal serum and 50 µg/ml gentamicin was used. Culture cells were washed twice with PBS and disintegrated with 0.05% trypsine–EDTA; finally, a suspension of 1 x $10^5$ cells / ml in culture media was prepared. 100 µl of this suspension was left to grow in a 24 well- chamber during 24 h at 37 ºC for adhesion in conditions of 5% CO2 and 100% of humidity (16).

b) Primary culture of gum fibroblasts of rat

Three male Wistar rats (3 to 5 weeks of life, 200 to 300 g) as donors of gum fibroblasts (RF) were used. After the anesthesia, connective tissue of upper and lower gum was extracted, according Freshney´s criteria (16). Samples were washed three times with sterile PBS, and connective tissue was cut in very small portions, cultivated in MEM Eagle media supplemented with 20% bovine fetal serum, and maintained at 37 ºC in the same conditions that human fibroblasts. It was grown from 10 to 14 days or until getting a confluent culture (17). Fibroblasts were harvested with trypsine al 0,05% and resuspended in culture media to a concentration of 1 x $10^5$ cel / ml.

**Preparation of yeasts for adhesion test**

Yeast grown in SGA during 24-48 h at 37 ºC were inoculated in Sabouraud glucose broth and cultured 16-18 h at 37 ºC. Harvested cells were washed by two centrifugation cycles (400 rpm, 5 min) with sterile PBS and a suspension (1 x $10^7$ cells/ ml in PBS) was made. The number of cells was adjusted by recounting in Neubauer's hematological camera.

**Adhesion test**

Epithelial cells and fibroblasts were washed with PBS and contacted with inactivated saliva (30 min 60 ºC) during 1 h at 37 ºC. Cells free of adhered yeast after the contact with saliva was confirmed by microscopic observation. Simultaneously, suspension of *C. albicans* and biopolymers were added (0.25% P/V HMWC in 1% acetic acid and 0.10% P/V NaAL in sterile $H_2O$) (18,19); samples were incubated 1 h at 37 ºC. After this, suspensions were spread on a glass coverslip, air-dried, fixed with methanol and colored with violet crystal. Same procedure without inclusion of biopolymers was made for control samples. The quantification of *C. albicans* adhered to cells was made by optical microscopy (400 X) (n=150) in at least three fields. Images were analyzed by an images analyzer system (Image For Bonus 4.0 Average Cybernetics v. 2002, USA). Fields with conglomerate cells were not quantified (18). Experiments were triply made in three different moments.

**Statistical analysis**

Results were statistically processed by MANOVA (for CSH results), ANOVA and LSD-Fisher test (for adhesion results) with the statistical package SPSS 10.0. A p value ≤ 0.05 was set to determine significant differences.

## RESULTS

**Cell surface hydrophobicity**

The effect of biopolymers on CSH of *C. albicans* is shown in Figure 1. A significant decrease relative to control (p = 0.025) with both organic phases was observed (44% for HMWC and 82% for NaAL in chloroform, and 30% for HMWC and 19% for NaAL, in xylene). CSH values of yeasts grown in the presence of HMWC and NaAL did not show significant differences for both organic solvents. CSH of control yeasts was significantly greater with chloroform than with xylene (p = 0.001). Values obtained in the presence of biopolymers varying the organic phase were not significantly different.

Adhesion to cells

Figure 2 shows that control samples have a greater number of adhered yeasts, independently the type of cell, (Figure 2: A-I, B-I and C-I) than cells treated with NaAL (Figure 2: A-II,B-II,C-II) and HMWC (Figure 2: A-III, B-III, C-III). Adhesion of *C. albicans* to HF and to Hep-2 cells decreased significantly with HMWC and NaAL respect to control (p <0.5). When comparing the number of *C. albicans* adhered to RF, only significant differences were observed between NaAL and control, but not between the polymers. There were no differences in results with both kind of fibroblasts (figure 3).

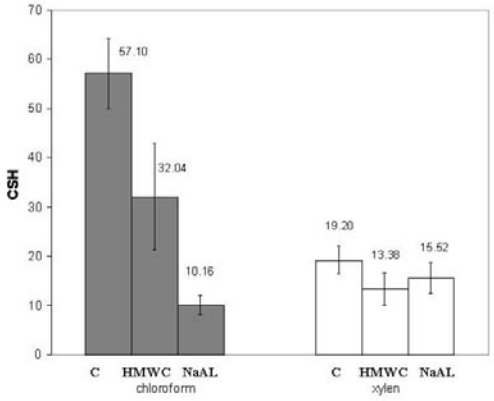


**Fig. 1.** Effect of biopolymers on cellular surface hidrophobicity of *C.albicans* in two different organic media. Media ± standard error. C: control, HMWC: high molecular weight chitosan, NaAL: sodium alginate.

## DISCUSSION

Adhesion of *C. albicans* to host surfaces is considered the initial phase for candidiasis development. This is a complex process that involves chemical and biological factors and forces participating in adhesion; hydrophobic interactions play an important role between them. CSH, virulence factor for the process of fungal adhesion, should not be analyzed purely from a thermodynamic view (12) due diverse microbial structures also contribute to it, such as membrane proteins, lipoproteins, phospholipids and lipopolisaccarides (20). In bibliography, adhesion to hydrocarbons method employing xylene as organic phase is the most utilized one (13,21). This neutral solvent adhere to yeasts only through hydrophobic interactions, while in chloroform it can be evaluate also acid-base interactions type, because of its electronic acceptor character, being microbial surface an electronic donor. This determines greater values of CSH (14) in this organic phase. Our results agree with such differences. When the samples were contacted with HMWC and NaAL, CSH decreased respect to control with both organic solvents.

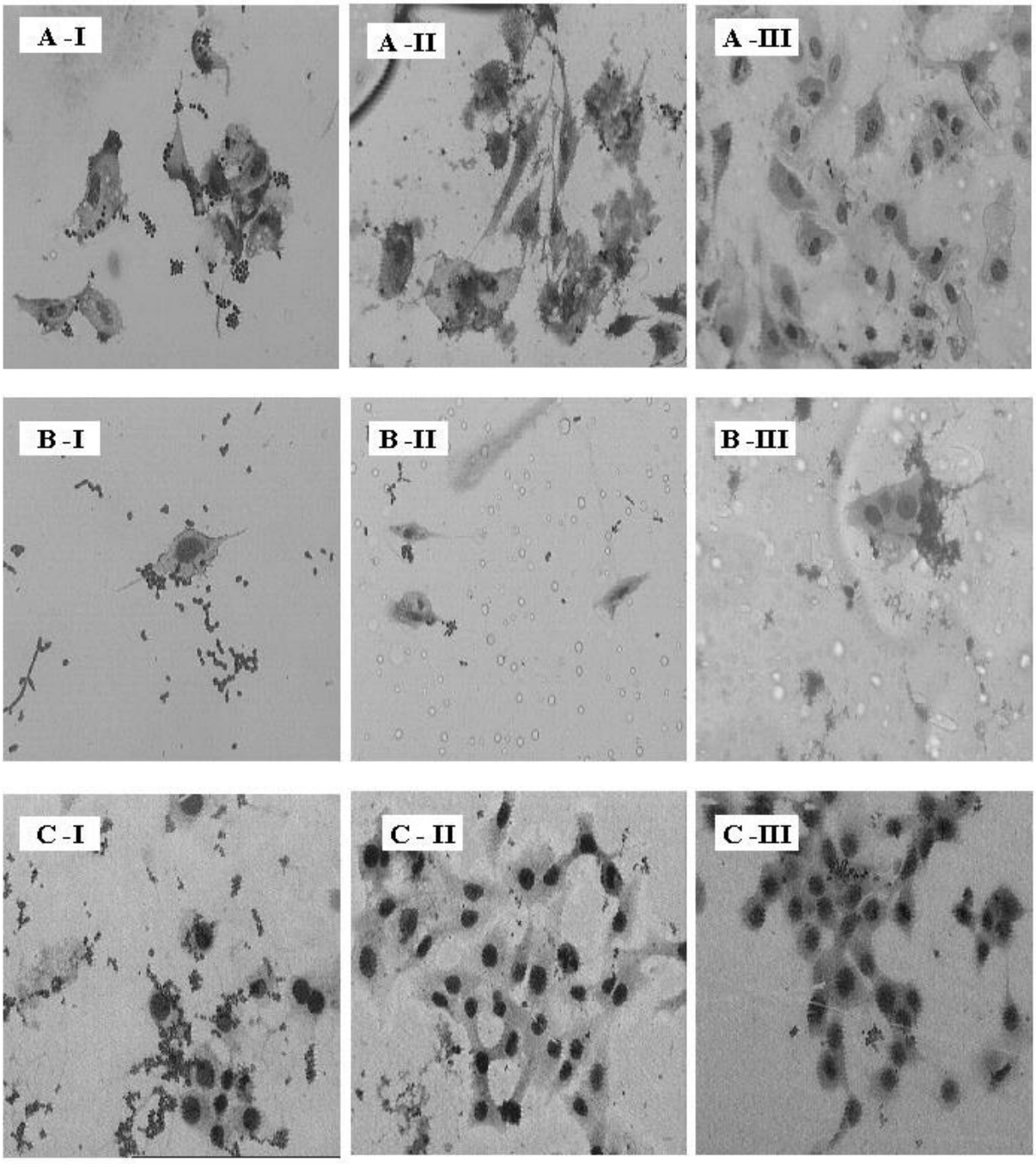


**Fig. 2.** Microphotographs of C. albicans adhered to different types of cells (A: HF; B: RF; C: Hep-2) in absence (I: control) and presence of NaAl (II) and HMW (III). (Magnification: 400 X).

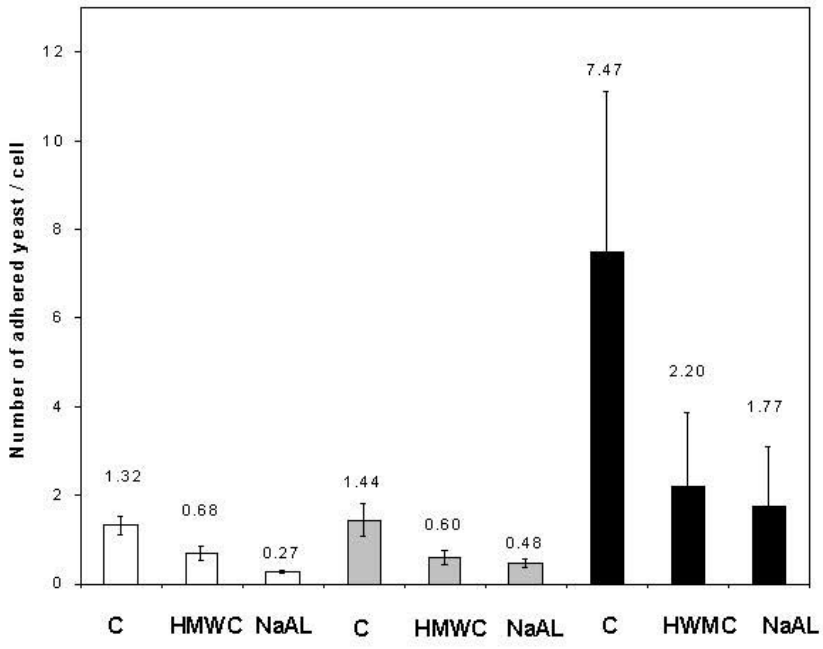


**Fig. 3.** Number of *C. albicans* adhered by cell in the presence and in the absence of biopolymers. Media ± standard error. C: control, HMWC: high molecular weight chitosan, NaAL: sodium alginate

This may be due culture conditions would modify the fungal wall structure, and therefore, the surface charge (21). These finds would suggest that acid-base interactions contribute to antifungal action of these polymers.

Studies referred to fungal adhesion to different surfaces show that *Candida* has numerous adhesins; between them, CR3 receptors, homologous to human integrin, mannoproteins that they can interact to lectins-like molecules of epithelial cells, CR2 receptor, that promotes adhesion to acrylic surfaces (22,23) and receptors for immobilized proteins of salivary pellicle (24). The adhesion is enhanced by traumatisms or occlusion of oral mucosa, as occurs in denture stomatitis (10). In this paper, a greater adhesion to epithelial cells than to fibroblasts was shown, which would be associated to a greater number of receptors in the former ones. The decrease on adhesion of *Candida* produced by biopolymers would be interpreted as a result of modifications of fungal surface charge and of cells (25) because of the physico-chemical characteristics of polymers. Also can be considered a possible blocking effect of these polymers by its viscosity on yeast and cell receptor (19). These results agree with those obtained for CSH, reinforcing the correlation that exists between these virulence factors. The greater effect observed for NaAL than to HMWC may be due to the mechanical properties of the gel, added to modifications that would produce on cellular surfaces.

Oral candidiasis treatment becames more simple in patients with slight immunodeficiencies, in which generally topic antifungal drugs are efficient. These interfere on ergosterol synthesis of cellular membrane of *Candida* or on regulatory enzymes for nucleic acids synthesis, but they also interfere in metabolic pathways of the human cells. Nevertheless, there are chronic oral candidiasis that resist clinical treatment or collateral undesirable effects observed with the use of these antifungal drugs (26). An alternative in treatment protocols is the use of these biopolymers, alone or associated, because they have been employed for adhesive microcapsules preparation with controlled liberation of medicines in oral mucose; also an inhibitory effect on fungal growth and on adhesion of *C. albicans* to epithelial cells by themselves has been reported (27-29). The decrease of CSH and adhesion to biological surfaces in the presence of these products would produce a decrease of pathogenic potential of *C. albicans* (21). Bioadhesive properties of chitosan and alginate, their use as intraoral drugs release (27) as well as inhibition of adhesion to oral cells and non specific antifungal effects of chitosan (29), would permit us to consider these products as an alternative for denture stomatitis treatment, with better acceptance of patients and a decrease of the collateral effects of conventional antifungal protocols.

## REFERENCES

1. Gerentes P, Vachoud L, Doury J, Domard A. Study of chitin based gel as injectable material in periodontal surgery. Biomaterials 2002; 23:1295-02.
2. Liu H, Du Y, Yang J, Zhu H. Structural characterization and antimicrobial activity of chitosan/betaine derivative complex. Carbohydr Polymers 2004; 55: 291–7.
3. Muzzarelli R. Biochemical significance of exogenous chitin and chitosans in animal and patients. Carbohydr Polymers 1993; 20:7-16.
4. Fakhry A, Schneider GB, Zaharias R, Senel S. Chitosan supports the initial attachment and spreading of osteoblasts preferentially over fibroblasts. Biomaterials 2004;25:2075-9.
5. Draget KI, Skjak-Braek, Smirsrod O. Alginate based new materials. Int J Biol Macromol 1997;21:47-55.
6. Flanagan DA, Palenik CJ, Setcos JC, Miller CH. Antimicrobial activities of dental impression materials. Dental Materials 1998;14:399-04.
7. Delgado W, Aguirre JM. Las micosis orales en la era del SIDA. Rev Iberoam Micol 1997;14:14-22.
8. Baena-Monroy T, Moreno-Maldonado V, Franco-Martínez F, Aldape-Barrios B, Quindós G, Sánchez-Vargas LO. Candida albicans, Staphylococcus aureus and Streptococcus mutans colonization in patients wearing dental prosthesis. Med Oral Patol Oral Cir Bucal 2005;10:E27-E39.
9. Cannon RD, Holmes AR, Mason AB, Monk BC. Oral *Candida*: clearance, colonization or candidiasis?. J Dent Res 1995;74:1152-61.
10. Samaranayake LP, Mac Farlane TW. Factors affecting the in vitro adherence of the fungal oral pathogen *Candida albicans* to epithelial cells of human origin. Arch Oral Biol 1982;22:869-73.
11. San Millán R, Elguezabal N, Regúlez P, Moragues MD, Quindós G, Pontón J. Effect of salivary secretory IgA on the adhesion of *Candida albicans* to polystyrene. Microbiology 2000;146:2105-12.
12. Doyle RJ. Contribution of the hydrophobic effect to microbial infection. Microb Infect 2000;2:391-400.
13. Gallardo Moreno AM, Garduño AE, Gonzalez Martin ML, Pérez Giraldo C, Bruque JM, Gómez García AC. Analysis of the hydrophobic behavior of different strains of *Candida parapsilopsis* under two growth temperatures. Colloids Surf B Biointerfaces 2003;28:119-26.
14. Bellon-Fontaine MN, Rault J, van Oss CJ. Microbial adhesion to solvents: a novel method to determine electron-donor/electron-acceptor or Lewis acid-base properties of the microbial cells. Colloids Surf B Biointerfaces 1996;7:147-53.
15. Nota técnica. Medios de cultivo caseros para la identificación de hongos de interés médico. Rev Arg Micol 1994;17:26-33.
16. Freshney RI. Culture of Animal Cells: A Manual of Basic Techniques, New York: Alan R. Liss Inc. Editores; 1987. p. 57-84; p. 107-126.
17. Simain Sato F, Lahmouzi J, Kalykakis GK, Heinen E, Defrensne MP, De Pauw MC et al. Culture of gingival fibroblasts on bioabsorbable regenerative materials in vitro. J Periodontol 1999;70:1234-9.
18. Kimura LH, Pearsall NN. Relationship between germination of *Candida albicans* and increased adherence to human buccal epithelial cells. Infect Immun 1980;28:464-8.
19. Barembaum S, Virga C, Bojanich A, Cornejo S, Calamari S, Pontón J et al. Efecto de QAPM y alginato de sodio sobre la adherencia de *Candida albicans* autóctonas a células epiteliales bucales (*in vitro*). Med Oral 2003;8:188-96.
20. Egusa H, Ellepola An, Nikawa H, Hamada T, Samaranayake LP. Sub- therapeutic exposure to polyene antimycotics elicits a post-antifungal effect and depresses the cell surface hydrophobicity of oral *Candida albicans* isolates. J Oral Pathol Med 2000;29:206-13.
21. Panagoda GJ, Ellepola AN, Samaranayake LP. Adhesion of *Candida parapsilosis* to epithelial and acrylic surfaces correlates with cell surface hydrophobicity. Mycoses 2001;44:29-35.
22. Crocket DN, O'Grady JF, Reade PC. *Candida* species and *Candida albicans* morphotypes in erythematous candidiasis. Oral Surg Oral Med Oral Pathol 1992;73:559-63.
23. Chakir J, Côté L, Coulombe C, Deslauriers N. Differential pattern of infection and immune response during experimental oral candidiasis in BALB/C and DBA/2 (H-$2^d$) mice. Oral Microbiol Immunol 1994;9: 88-94.
24. Sullivan JM, Jenkinson HF, Cannon RD. Adhesion of *Candida albicans* to oral streptococci is promoted by selective adsorption of salivary proteins to the streptococcal cell surface. Microbiol 2000;146:41-8.
25. Singleton DR, Masuoka J, Hazen KC. Surface hydrophobicity changes of two Candida albicans serotype B mnn4delta mutants. Eukaryot Cell 2005;4:639-48.
26. Quindós G, Ribacoba L, Contreras I, Aguirre JM. Tratamiento de las candidiasis orofaríngeas. Rev Iberoam Micol 1996;13: 511-15.
27. Miyazaki S, Nakayama A, Oda M, Takada M, Attwood D. Chitosan and sodium alginate based bioadhesive tablets for intraoral delivery. Biol Pharm Bull 1994;17:745-7.
28. Giunchedi P, Juliano C, Gabbini E, Cossu M, Sorrenti M. Formulation and in vivo evaluation of chlorhexidine buccal tablets prepared using drug-loaded chitosan microspheres. Eur J Pharm and Biopharm 2002;53:233-9.
29. Knapczyk J, Macura AB, Pawlik B. Simple tests demonstrating the antimycotic effect of chitosan. International Journal of Pharmaceutics 1992;80:33-8.

Acknowledgment
Investigation granted by Science and Technology Secretary (SECyT) 05 J/041, National University of Cordoba, Argentina.