Candida albicans colonization on thermal cycled maxillofacial polymeric materials *in vitro*.

Summary

In the present study, the colonizationof a single isolate of *C. albicans* on saliva-, serum-coated or protein free (uncoated), thermocycled (4°C-70°C for 1min, respectively; 0, 1000 and 10000 times) fifteen commercial maxillofacial materials were investigated, by adenosine triphosphate (ATP) analysis. In the case of control specimens (not thermocycled and uncoated), the fungal colonization appeared to depend upon the type of commercial products used. Thus, the lowest colonization was observed with additional silicone materials, soft acrylic liners with visible light curing, except for one product, whereas visible light curing liners comprising of single paste or single gel exhibited the highest colonization capacity, and cold cured acrylic liners exhibited the intermediate. However, the fungal colonization on the materials was significantly promoted both by thermal cycling (ANOVA; $p<0.01$) and a layer of protein coating (saliva, $p<0.01$; serum, $p<0.01$). When the interrelation between the fungal colonization and the surface hydrophobicity of the materials were analysed, fungal colonization on 1000- and 10000-thermocycled materials correlated well with the contact angles of the materials (Student t-test, $p<0.01$), being consistent with the thermodynamic theory.

These results, taken together, suggest that the aging of the materials and the biological fluids of the host promote yeast colonization on maxillofacial materials.

Introduction

Denture stomatitis is an erythematous pathogenic condition of the denture bearing mucosa and caused mainly by microbial factors, especially *Candida albicans*. It has been shown that the main reservoir of *C. albicans* and related *Candida* species is the fitting surface of upper denture (Davenport 1970) and that soft lining materials are easily colonized and deeply infected by these organisms (Allison & Douglas 1973; Douglas 1979). In the pathogenesis of denture stomatitis, the growth of large numbers of *Candida* on the fitting surface of the denture and the following acid production by grown yeasts are known as one of the most important factors (Odds 1988) through the direct cytotoxicity, activatation of acid proteinase and phospholipase produced by these yeasts and promotion of *Candida* adherence (Samaranayake & MacFarlane 1985, 1990; Samaranayake et al., 1984).

Despite the realization that successful candidal adherence on a denture surface is an important step in the pathogenesis of this condition (Rotrosen et al., 1986), the role played by saliva or serum pellicles during the colonization process and subsequent multilayer biofilm formation is poorly understood. Indeed components of saliva or serum proteins, such as mucins, fibrinogen and complements specifically bind to *Candida* blastospores and germ tubes possibly modifying the process (Bouali et al., 1986; Bull and Turner, 1984; Page and Odds, 1988; Tronchin et al., 1987). In recent investigations we have demonstrated the existence of specific interactions between mannoprotein adhesin of *C. albicans* and sugar-moiety of salivary proteins, including mucins, during candidal adherence to protein adsorbed surfaces (Nikawa and Hamada, 1990; Nikawa et al., 1992-a), and that salivary and serum pellicles promote fungal colonization on tissue conditioning materials (Nikawa et al. 1993). In contrast, some researchers have demonstrated that pretreatment of acrylic strips and/or yeast cells with whole saliva decreases the initial adherence of *C. albicans* to denture acrylic, whereas a serum pellicle promotes adherence (McCourtie and Douglas, 1981; Samaranayake et al., 1980). It is therefore evident that the relationship between the salivary or serum pellicle on denture material surfaces and candidal colonization is a complex subject, particularly taking the surface aging of materials into account.

Clinically, it has recently been pointed out that the continuous swallowing or aspiration of microorganisms from denture plaque exposes patients, particularly the immunocompromised host or medicated elderly, to the risks of unexpected infections (Nikawa et al, 1998). Since the colonization of *C. albicans* on the materials frequently used for maxillofacial protheses, is of importance in clinical terms, thus in the present study, fungal colonization on some commercial products was investigated (Nikawa et al., 1996).

Materials and Methods

Microorganisms and growth condition

Candida albicans IFO 1385, purchased from the Institute for Fermentation, Osaka, was used and cultured as previously described (Nikawa et al., 1989, 1993, 1994; Nikawa & Hamada 1990). Briefly, the yeasts were grown at 37°C with reciprocal shaking (150 rev/min), in yeast nitrogen base medium (Difco, Detroit, USA) containing 250mM glucose. Batches of medium were inoculated with overnight cultures of the yeast, the yeast was harvested in the late exponential growth phase, washed twice with distilled water and suspended to final concentrations (10^7 cells/ml) (Nikawa et al., 1997a,b)

Acrylic resin and lining materials

Samples of acrylics (Bio Resin, Shofu, Kyoto, Japan) and fifteen commercial maxillofacial materials summarized in Table 1 were processed according to manufacturer's directions, and each of them was prepared to a uniform size (10mm × 10mm × 0.7mm thickness) with smooth surfaces by placing glass slides over them as previously described (Nikawa et al., 1994, 1995,1996).

Specimens of each of the materials were thermocycled between 4°C and 70°C with an immersion time of 60 seconds in each bath, and the growth assay were taken after thermal cycling 0, 1000 and 10000 times.

Saliva and serum

Pooled unstimulated whole saliva was collected from five healthy candidates and clarified, according to the method of Cannon et al. (1995) with modification, by centrifugation at 12,000× g for 15min at 4℃. Human serum was purchased from Sigma Chemical Co. (St Louis, MO, USA). Whole saliva and serum were stored at -25℃ before use (Nikawa et al. 1990, 1996).

Assay procedures

The colonization assay was conducted as follows (Nikawa et al. 1993, 1996, 1997b). The specimens of acrylic resin and maxillofacial materials were coated with saliva or serum by placing them in wells of Multiwell tissue culture plates (Nunclon^R Delta, Nunc, Kamstrup, Denmark), into which were dispensed 500 μ l of the protein solution per well, and incubating for 1 hour at 37℃. Saliva or serum was substituted with an equal volume of sterile distilled water in the control wells. After incubation the protein solution was aspirated, 50 microliters

of yeast suspension (1×10^7 cells/ml) was inoculated on the surface of each specimen and the whole assembly was incubated at 37℃ for 2 hrs to promote yeast adherence and colonization. Subsequently, 2.0 ml of Sabouraud broth was carefully dispensed into each well, incubated at 37℃ for 120hrs.

Afterwards each specimen was carefully removed, to determine the amount of colonization including, cavitation or invasion, washed vigorously and ultrasonically for a total of 15min with distilled water to remove the biofilm yeasts other than firmly attached, cavitated or invased organisms (Nikawa et al., 1997b). Then, the washed samples were immersed in 1.0 ml of the reagent containing benzalkonium which extracts intracellular ATP (Siro, Romer & Lovgren, 1982) and allowed to react for 15 min at room temperature (Nikawa et al. 1996). The resultant reagent solution was then subjected to an ATP-measuring system (ATP-AF 100, TOA Electronics Ltd, Tokyo, Japan) to determine the the amount of fungi colonized (Berlutti et al., 1993).

The assays were carried out on two independent occasions, with quadruplicated samples on each occasion and the values obtained were averaged to give the final data with standard deviations. All the numerical data obtained were analyzed by analysis of variance (ANOVA) and Tukey's multiple range test at 5% level.

Results

Surface hydrophobicity of the materials

The surface hydrophobicity of each maxillofacial material were shown as Young's contact angle against water, in Table 2, silicone materials essentially showed the highest hydrophobicity, reduced in the order of VLC acrylic materials or cold curing acrylic, and heat curing acrylic exhibited the la\east (ANOVA, $p<0.05$; Table 2).

Effect of maxillofacial materials on fungal colonization

As shown in Fig. 1, the colonization varied depending upon the materials and times of thermal cycle on which *Candida* had grown. In the case of unthermocycled and uncoated samples, the fungal colonization was greatest on PER form, decreased in the order of Lightdon-U≧ Resiline, Light Liner Hard, Rebaron LC≧ Astron LCH≧ Rebaron, CosmesilT001, Ever Soft≧ Light Liner Soft, Ideal, and resin Cosmesil HC, Astron Soft, Silkskin and Episil showed the least adherent capacity (ANOVA $p<0.05$; Table 3).

Effect of thermal cycles of maxillofacial materials on fungal colonization

As shown in Fig. 1, the fungal colonization was significantly promoted by thermocycling on Astron Soft, Ever Soft and Light Liner Soft (ANOVA, $p<0.01$). In the case of 1000 thermocycling, the amounts of yeasts colonized on resin and Light Liner Soft, Lightdon-U, Rebaron, Astron LCH and Resiline were the greatest, and CosmesilT001, Episil, Ideal, Cosmesil HC and Silkskin were the least, whilst resin, Astron Soft, Rebaron LC, PER form, Ever Soft and Light Liner Hard were intermediate (ANOVA $p<0.01$; Table 3). In the case of 10000 x thermocycling, the differences between the materials were significantly reduced (ANOVA, $p<0.01$).

Effect of proteinaceous pellicles on fungal growth

As shown in Fig. 1, biological fluids, particularly the serum, essentially promoted the fungal colonization on the materials. The effects were, however, varied depending upon both protein-coats and materials used (ANOVA, $p<0.01$;Tables 4 & 5). When the effects of saliva-coats on fungal colonization was analysed, saliva-coats essentially reduced the differences in the amount of colonization on the materials (ANOVA; $p>0.05$; Table 4).

In contrast, the fungal colonization on serum-coated samples seemed to depend on the components of the materials. For example, serum-coated silicone materials tends to show the lower colonization capacity, whilst VLC materials comprised of single paste or single gel, cold-cured acrylic materials or heat-cured acrylic resin (control) tends to show the greater adherence capacity (ANOVA, $p<0.01$; Table 5).

Surface hydrophobicity of the materials and fungal colonization

When the interrelation between the fungal colonization and the surface hydrophobicity of the materials were analysed, the exponential of the amount of fungal colonization on uncoated 1000-thermocycled or 10000-thermocycled materials apeared to negatively correlate well with the contact angles of the materials (Fig. 2; Student t-test, $r=0.698$ and $r=0.680$; $p< 0.01$), altough the higher correlation coefficient were obtained when the curves of second order (Fig. 2; Student t-test, $r=0.894$ and $r=0.761$; $p<0.01$).

Discussion

In the successful colonization, subsequent plaque formation and development of pathogenesis, the adherence of *Candida* to solid surfaces such as acrylics or denture lining materials has been thought to be the first step (Rotrosen et al., 1986). The following colonization, then included the growth of adherent cells or the coadhesion of floating or

growing cells to adherent ones. Only limited data has been available on the interactions between maxillofacial materials and fungi. In addition, although the materials are known to show age changes in their physical properties, little attention has been paid on the growth of *Candida* when they changed.

With nonthermocycled and uncoated liners, the fungal colonization was greatest on PER form, decreased in the order of Lightdon-U≧Resiline, Light Liner Hard, Rebaron LC≧ Astron LCH≧ Rebaron, CosmesilT001, Ever Soft≧ Light Liner Soft, Ideal, and resin Cosmesil HC, Astron Soft, Silkskin and Episil showed the least adherent capacity (ANOVA $p < 0.05$; Table 3). These results are consistent with the results of the previous part of the study, in which the antifungal effects of materials were observed in relation to the type or ingredient components of the products. In the previous study, Light Liner Soft and Astron Soft exhibited the antifungal effects on both initiation of fungal growth and growth rate. Besides this, the finding that all five products of silicone liners also exhibited the lowest colonization capacity should be explained by the hydrophobic interactions between fungi and the surface of materials based on thermodynamic theory (Minagi et al., 1985; Nikawa et al. 1989).

When the finding that saliva-coats essentially reduced the differences in the amount of colonization on the products, is consistent with our previous study, in which the saliva-coats minimumized the effects of the surface hydrophobicity of tissue conditioners on their adherence (Nikawa et al, 1992-b).

In contrast, the finding that the fungal colonization on serum-coated samples seemed to depend on the components of the materials should be of interest in clinical terms, because these materials often contact the inflamatory tissues. However, the mechanisms of this phenomenon should be complex, because interactions between liners, proteins comprising serum, and the yeasts should be involved (Nikawa et al, 1996). Further analysis is requied to clarify this phenomenon.

The findings that the exponential of the fungal colonization on uncoated 1000-thermocycled or 10000-thermocycled materials correlated well with the contact angles of the materials (Fig. 2; Student t-test, $p<0.01$) should be of interest, because the thermocycling process essentially reduced the residual unpolymerized components which showed the antifungal activity in the previous study, resulted in the thermodynamic (hydrophobic) properties of the materials predominantly affected the fungal colonization, since fungi colonize on uncoated materials in monolayer (Nikawa et al, 1993). In addition, Minagi et al. (1985) have reported the number of adherent cells negatively correlated well with the hydrophobicity of the denture materials, whereas we observed the similar correlation between the exponentiol of the colonized fungi and the surface hydrophobicity of the materials. This

may explained by the colonization parameter theory of Caldwell (1984), who exhibited the adherent cell exponentially increase during the colonization process.

On the other hand, when the curves of second orders were applied, higher correlation were observed (Fig. 2), and the peaks of the colonization were observed at 76.8 and 73.3 in degrees of contact angles of the materials. The findings may imply that the colonization of *C. albicans* prefer to occur on the surface of materials of contact angles of approximately 75 in degrees. It is generally accepted that the aggregation or adherence between two materilas or particles prefer to occur when the thermodynamic properties of one material is similar to that of another. Further analysis is required to clarify the relationship between surface properties of cells, that of substrates and colonization.

Finally, our findings, that either the thermocycling or the proteinaceous pellicles, essentially promoted the fungal colonization on resilient liners through various mechanisms, suggested that appropriate control for denture plaque is essential to the long-term usage of the maxillofacial materials.

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Legends to Figures

Fig. 1 Colonization of *Candida albicans* grown on thermocycled acrylic and lining materials. Asterisk indicates the significant promotion of fungal colonization caused by thermocycling (* $p<0.05$).

Fig. 2 Correlation between fungal colonization on 1000-times and 10000-times thermocycling lining materials, and the surface hydrophobicity of the materials.

Table 1 maxillofacial materials

	manufacturer	type
Astron LC Hard [a]	Astron Dental Corp, Wheeling, IL, USA	VLC*
Astron LC Soft [a]	Astron Dental Corp, Wheeling, IL, USA	VLC
COSMESIL HC [b]	Principality Medical Ltd, Newport, UK	condensation curing
COSMESIL T001 [b]	Principality Medical Ltd, Newport, UK	addition curing
EPISIL [b]	Dreve-Dentamid GmbH, Unna, Germany	addition curing
Ever Soft [c]	Myerson Austenal Ltd, Middlesex, UK	cold curing
IDEAL [b]	Orthomax, Bradford, UK	addition curing
Resiline [a]	Dentsply/York Div, York, PA, UK	VLC
Lihgt Liner Hard [a]	H.J. Bosworth, Skokie, IL, USA	VLC
Light Liner Soft [a]	H.J. Bosworth, Skokie, IL, USA	VLC
Lightdon-U [a]	Dreve-Dentamid GmbH, Unna, Germany	VLC
PER form [a]	Whaledent Dentalprodukte GmbH, Fieldberg, Germany	VLC
Rebaron LC [a]	GC Inc. Tokyo, Japan	VLC
Rebaron [c]	GC Inc. Tokyo, Japan	cold curing
SILSKIN 2000 [b]	DePuy Healthcare, Leeds, UK	addition curing
Bio Resin (resin)	Shofu Inc. Kyoto, Japan	heat curing

*VLC: visible light curing

[a]VLC acrylic materials cured in the TRIAD 2000curing unit.

[b]Silicone materials cured at room temperature 23±2oC and 50±5 % relative humidity.

[c]Acrylic materials cured at room temperature 23±2oC and 50±5 % relative humidity.

Table 2 Contact angles of maxillofacial materials

	contact angle*
Astron LCH	70.42 ±4.76
Astron Soft	86.07 ±2.07
COSMESIL HC	113.08 ±0.91
COSMEIL T001	109.43 ±1.81
EPISIL	105.12±1.38
Ever Soft	99.58±2.29
IDEAL	110.93±2.03
Resiline	60.02 ±3.63
Lihgt Liner Hard	87.68 ±5.18
Light Liner Soft	79.07 ±4.69
Lightdon-U	86.68±2.98
PER form	99.82±2.06
Rebaron LC	84.70 ±3.85
Rebaron	81.70 ±0.88
SILSKIN 2000	108.10 ±5.37
Bio Resin (resin)	58.80 ±1.20

*Mean contact angle ±SD calculated from six specimens by the sessile drop method using distilled water as sensing liquid.

Table 3 Multiple range test on the data of fungal colonization on uncoated thermocycled denture liners*

cont 0	cont 1000	cont 10000
Episil	Cosmesil T001	Episil
Silkskin	Episil	Silkskin
Astron Soft	Ideal	PER form
Cosmesil HC	Cosmesil HC	Rebaron
resin	Silkskin	Cosmesil HC
Ideal	*resin*	Ideal
Light Liner Soft	Astron Soft	Cosmesil T001
Ever Soft	Rebaron LC	Rebaron LC
Cosmesil T001	PER form	*resin*
Rebaron	Ever Soft	Astron Soft
Astron LCH	Lihgt Liner Hard	Ever Soft
Rebaron LC	Resiline	Lihgt Liner Hard
Lihgt Liner Hard	Astron LCH	Light Liner Soft
Resiline	Rebaron	Astron LCH
Lightdon-U	Lightdon-U	Resiline
PER form	Light Liner Soft	Lightdon-U

* No significant differences were observed with the sample connected by bars.

Table 4 Multiple range test on the data of fungal colonization on saliva-coated thermocycled denture liners.

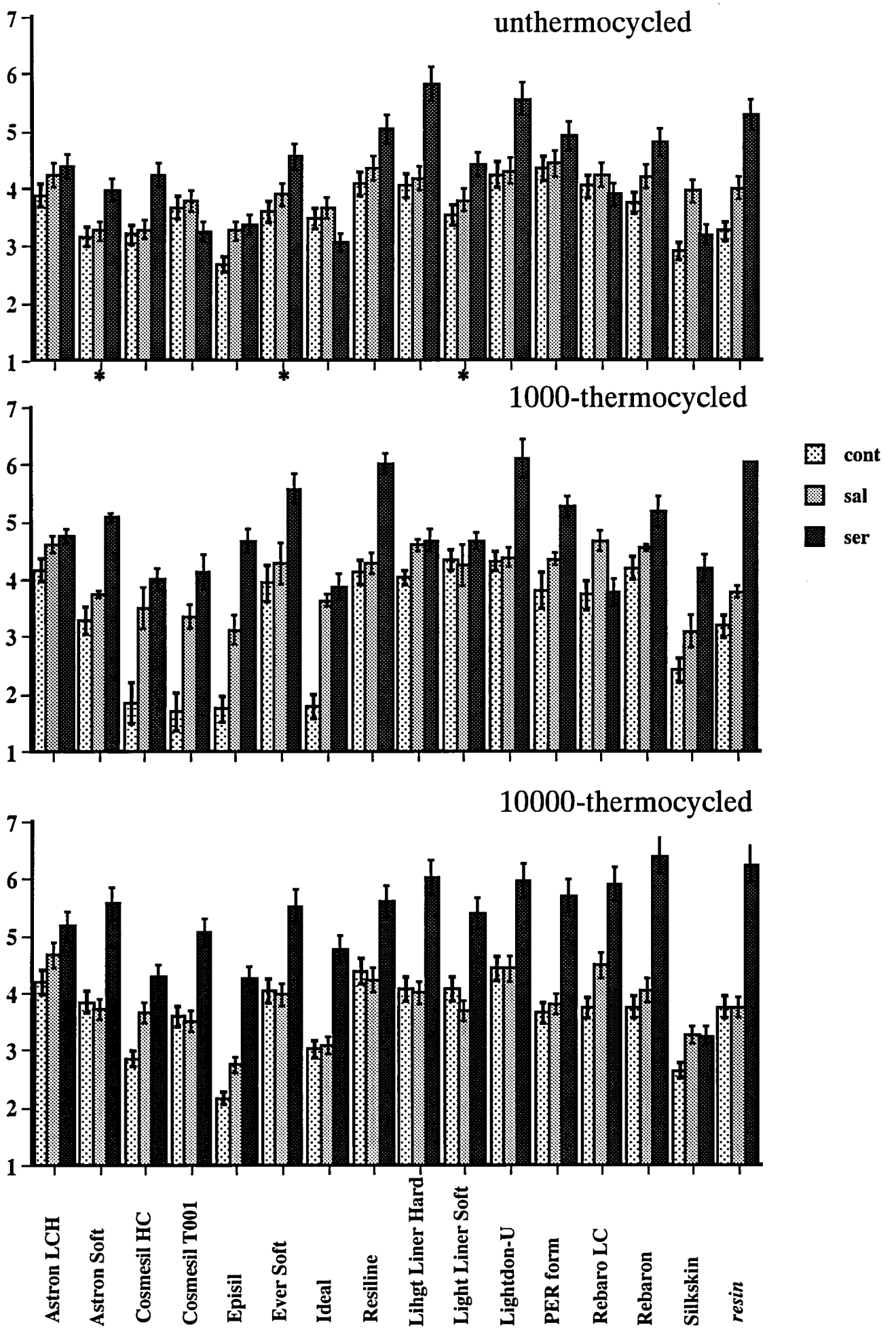
sal 0	sal 1000	sal 10000
Episil	Silkskin	Episil
Astron Soft	Episil	Ever Soft
Resiline	Cosmesil T001	Rebaron
Cosmesil HC	Cosmesil HC	Ideal
Ideal	Ideal	Silkskin
Cosmesil T001	Astron Soft	Cosmesil T001
Light Liner Soft	resin	Cosmesil HC
Ever Soft	Light Liner Soft	Light Liner Soft
Silkskin	Ever Soft	Astron Soft
resin	Resiline	resin
Lihgt Liner Hard	PER form	PER form
Rebaron	Lightdon-U	Lihgt Liner Hard
Astron LCH	Rebaron	Resiline
Rebaron LC	Lihgt Liner Hard	Lightdon-U
Lightdon-U	Astron LCH	Rebaron LC
PER form	Rebaron LC	Astron LCH

* No significant differences were observed with the sample connected by bars.

Table 5 Multiple range test on the data of fungal colonization on serum-coated thermocycled denture liners*

ser 0	ser 1000	ser 10000
Ideal	Rebaron LC	Silkskin
Silkskin	Ideal	Episil
Cosmesil T001	Cosmesil HC	Cosmesil HC
Episil	Cosmesil T001	Ideal
Rebaron LC	Silkskin	Cosmesil T001
Astron Soft	Light Liner Soft	Astron LCH
Cosmesil HC	Episil	Light Liner Soft
Astron LCH	Lihgt Liner Hard	Ever Soft
Light Liner Soft	Astron LCH	Astron Soft
Ever Soft	Astron Soft	Resiline
Rebaron	Rebaron	PER form
PER form	PER form	Rebaron LC
Resiline	Ever Soft	Lightdon-U
resin	Resiline	Lihgt Liner Hard
Lightdon-U	resin	resin
Lihgt Liner Hard	Lightdon-U	Rebaron

* No significant differences were observed with the sample connected by bars.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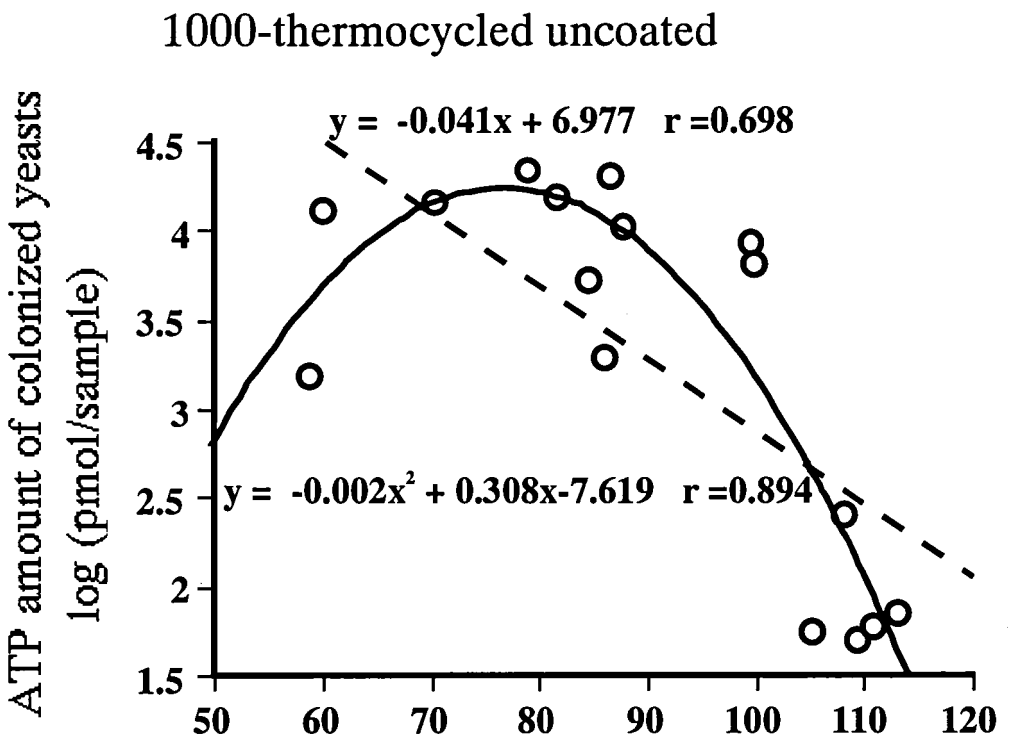
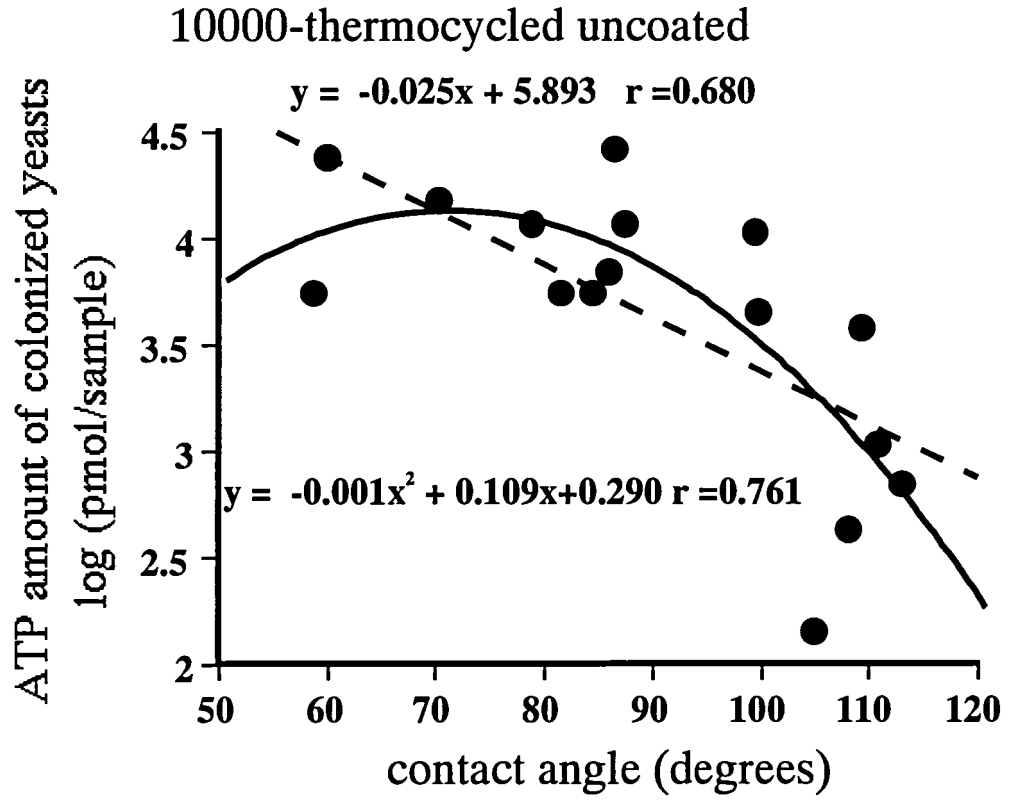

1000-thermocycled uncoated
y = -0.041x + 6.977 r =0.698
y = -0.002x² + 0.308x-7.619 r =0.894
ATP amount of colonized yeasts
log (pmol/sample)
4.5
4
3.5
3
2.5
2
1.5
50
60
70
80
90
100
110
120


10000-thermocycled uncoated
y = -0.025x + 5.893 r =0.680
y = -0.001x² + 0.109x+0.290 r =0.761
ATP amount of colonized yeasts
log (pmol/sample)
4.5
4
3.5
3
2.5
2
50
60
70
80
90
100
110
120
contact angle (degrees)