Interactions between thermal cycled resilient denture lining materials, salivary and serum pellicles and *Candida albicans in vitro*. Part II. Effects on fungal colonization.

Summary

In the present study, the growth of 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) seven commercial soft lining materials were investigated, by adenosine triphosphate (ATP) analysis. In the case of control resilient liners (not thermocycled and uncoated), the fungal colonization appeared to depend upon the type of commercial resilient liner used. Thus, the lowest colonization was observed with fluoric and heat cured silicone materials, cold cure silicone materials, except for one product, and heat cured acrylic resin exhibited the highest colonization capacity, and cold cured acrylic resilient 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, $p<0.01$). These results, taken together, suggest that the aging of the materials and the biological fluids of the host promote yeast colonization on resilient lining 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), 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 resilient lining material 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, 1998a). Since the growth and colonization of *C. albicans* on the resilient lining materials, paricularly on aged materials, is of importance in clinical terms, thus in the present study, fungal colonization on some commercial resilient liners was investigated.

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 resilient lining materials

Samples of acrylics (Bio Resin, Shofu, Kyoto, Japan) and seven commercial resilient lining 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 and resilient liners 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 μl 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 acrylic or resilient liner 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

Effect of resilient liners on fungal colonization

As shown in Fig. 1, the colonization varied depending upon the resilient lining materials and times of thermal cycle on which *Candida* had grown. In the case of nonthermocycled and uncoated resilient liners, the fungal colonization was greatest on resin and EV, least on MB, KD and TSR, whilst SR, SF and MO showed the intermediate.

Effect of thermal cycles of resilient liners on fungal colonization

As shown in Fig. 1, the colonization on MB was significantly promoted by thermocycling (ANOVA, $p<0.01$). In the case of 1000 x thermocycling, the amounts of yeasts colonized on resin and EV were the greatest, and KD, TSR and MB showed the least, whilst SF, MO and SR were intermediate (Table 2). In the case of 10000 x thermocycling, TSR showed the least, with an increase in the order of KD=MB≦MO≦SF≦EV≦SR with the resin the greatest (Table 2).

Effect of proteinaceous pellicles on fungal growth

As shown in Figs. 2 and 3, saliva or serum pellicle essentially promoted the fungal colonization on the resilient liners. The effects were, however, varied depending upon both protein-coats and materials used (ANOVA, $p<0.01$). When the effects of saliva-coats on fungal colonization was analysed, as compared with uncoated control specimens, the fungal colonization on nonthermocycled MB ($p<0.01$), 1000 x thermocycled TSR and MB ($p<0.05$), and 10000 x thermocycled EV, MO, MB, SF, KD ($p<0.01$) and TSR ($p<0.05$) was significantly promoted by saliva-coats (Fig. 2; ANOVA).

When the effects of thermocycling on the fungal colonization on saliva-coated resilient liners were analysed, significant promotion of fungal colonization caused by thermocycling was observed with KD, MB (ANOVA, $p<0.01$) and MO (ANOVA, $p<0.05$)(Fig. 2).

In fungal colonization on saliva-coated nonthermocycled specimens, KD and MB exhibited the least colonization capacity, followed by TSR≦SR, SF, MO≦resin, and EV showed the most (Table 3, ANOVA, $p<0.05$). In the case of saliva-coated 1000 x thermocycled specimens, the amount of fungi colonized on KD, TSR and MB was the lowest, followed by SF and MO≦SR and resin, and that on EV was the most (Table 3; $p<0.01$). In the case of 10000 x thermocycling, the amount of fungi colonized on TSR, MB and KD was the lowest, that on EV most and SR, SF, resin and MO were intermediate (Table 3; $p<0.01$).

When the effects of serum-coats on the colonization of fungi on resilient liners were analysed, as to the nonthermocycled specimens, the fungal colonization on TSR ($p<0.05$) and MB ($p<0.01$) was significantly promoted by serum-coats (Fig. 3). In the case of 1000 x thermocycling, the fungal colonization on TSR, MO, MB, SF and KD was promoted, and the fungal colonization on all resilient materials used was promoted by serum-coats on 10000 x thermocycled specimens (Fig. 3; ANOVA, $p<0.01$).

The least colonization on serum-coated nonthermocycled specimens, was exhibited by KD, MB and TSR, followed by SR≦SF≦resin and EV, and MO showed the most (Table 4, ANOVA, $p<0.05$). In the case of serum-coated 1000 x thermocycled specimens, the amount of fungi colonized on KD, MB and TSR was the lowest, followed by SF, and SR, resin, EV and that on MO was most (Table 4; $p<0.01$). The amount of fungi colonized on TSR, MB and KD after 10000 x thermocycling was significantly lower than that on EV, SR, SF, MO and resin (Table 3; $p<0.01$).

When the effects of thermocycling on the fungal colonization on serum-coated resilient liners were analysed, significant promotion of fungal colonization caused by thermocycling was observed with MB, SF, SR and KD (ANOVA, $p<0.01$)(Fig. 3).

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 have been available on the interactions between resilient liners and fungi. In addition, although the materials are known to show age changes in their physical properties, little attention has been paid on the colonization of *Candida* when they aged.

With nonthermocycled and uncoated resilient liners, the fungal colonization was greatest on resin and EV, least on MB, KD and TSR, and SR, SF and MO showed the intermediate. These results are consistent with the results of the previous part of the study, in which the antifungal effects of resilient liners were observed in relation to the type or ingredient components of the materials (Nikawa et al. 1998b). As in that study, KD and MB, including their ingredient components, exhibited the antifungal effects on both initiation of fungal growth and their growth rate. This could be the main reason why the colonization on KD and MB was the lowest. A similar explanation could be offered on the fungal colonization on SR and SF, because these materials did not exhibited the initial delay in fungal growth, but showed the supression of fungal growth rate. However, the amount of fungi colonized on TSR was relatively low, although this material did not show significant antifungal effects. This phenomenon suggests that the colonization capacity on materials is not only governed by the growth, but also some other factors which may include adherence capacity and surface properties of substrates. Futher study is necessary to clarify the mechanisms.

The thermocycling process essentially promoted the fungal colonization particularly on the pellicle-coated resilient liners, i.e. uncoated MB, saliva-coated MO, MB and KD, and serum-coated MB, SF, SR and KD. A similar explanation, in relation to the antifungal effects of resilient liners, could be offered on this result. In the previous part of the study (Nikawa et al. 1998b), some of resilient materials exhibited a delayed start of rapid decline in pH (KD and MB) which could be attributed to their fungicidal effects agaist initially inoculated fungi and/or, the decrease in the rate of pH reduction accompanied by fungal growth which could be explained to be caused by either the continuous contact of the fungal cells with the antifungal components of the resilient liner (KD and MB) or the leaching out of the some inhibitory components from soft liners to the growth medium (SF and SR), and these types of antifungal effects are significantly reduced by thermocycling (Nikawa et al 1998b). Thus the fact that the thermocycling essentially promoted the fungal colonization could be mainly attributed to

the significant reduction in antifungal effects of the resilient liners during the thermal cycling process.

As compared with uncoated control specimens, the fungal colonization was significantly promoted on nonthermocycled MB ($p<0.01$), 1000 x thermocycled TSR and MB ($p<0.05$), and 10000 x thermocycled EV, MO, MB, SF, KD ($p<0.01$) and TSR ($p<0.05$) by saliva-coats (Fig. 2; ANOVA).When the effects of serum-coats on the colonization of fungi on resilient liners were analysed, as to the nonthermocycled specimens, the fungal colonization on TSR ($p<0.05$) and MB ($p<0.01$) was significantly promoted by the serum-coats (Fig. 3). In case of 1000 x thermocycling, the fungal colonization on TSR, MO, MB, SF and KD was promoted, and the fungal colonization on all resilient materials used was promoted by serum-coats on the 10000 x thermocycled specimens (Fig. 3; ANOVA, $p<0.01$).

Thus, as opposed to the growth correlates, the activity of the yeast colonized on the pellicle coated samples varied considerably, though saliva or serum pellicle essentially promoted the fungal colonization on resilient liners (ANOVA, $p<0.01$). This could be partly attributed to the reduction in antifungal effects of resilient liners by pellicle-coats (Nikawa et al. 1998b).However, in the fungal colonization on pellicle coated resilient liners, not only the effects of liners on fungal growth but also the biological factors should be involved. In fact, in our previous study, biological fluids such as saliva or serum modified the colonization process in a complex manner. We have shown that salivary pellicle retards the fungal colonization in initial phase, however, in the latter stage saliva promoted fungal colonization with cell-cell aggregation and hyphal emergence. In addition to this, a pellicle comprising serum proteins facilitated initial colonization and subsequent development of a candidal biofilm on acrylic. We also demonstrated that a variety of yeast morphological changes, such as blastospores, germ tubes, pseudohyphae and hyphae were involved in the process. Particularly noteworthy was the 'strand-like' adhesins mediating hyphal adhesion to acrylics (Nikawa et al., 1996). In addition to this, we have also reported the hyphal invasion into tissue conditioners induced by pellicle coats (Nikawa et al., 1993; 1997). Further, the quality or property of the surface of the resilient material should be involved in the fungal colonization. Additional studies are required to characterize the relation.

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

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Legends to Figures

Fig. 1 Colonization of *Candida albicans* grown on (a: 0 times, b: 1000 times and c: 10000 times) thermocycled acrylic and resilient lining materials.
Asterisk indicates the significant promotion of fungal colonization caused by thermocycling (** $p<0.01$; * $p<0.05$).

Fig. 2 Colonization of *Candida albicans* grown on saliva-coated (a: 0 times, b: 1000 times and c: 10000 times) thermocycled acrylic and resilient lining materials.
Fungal colonization was significantly promoted by saliva-coats (a; $p<0.01$; $b<0.05$)
Asterisk indicates the significant promotion of fungal colonization caused by thermocycling (** $p<0.01$; * $p<0.05$).

Fig. 3 Colonization of *Candida albicans* grown on serum-coated (a: 0 times, b: 1000 times and c: 10000 times) thermocycled acrylic and resilient lining materials.
Fungal colonization was significantly promoted by serum-coats (a; $p<0.01$; $b<0.05$)
Asterisk indicates the significant promotion of fungal colonization caused by thermocycling (** $p<0.01$; * $p<0.05$).

Table 1 Materials

Type of Materials	Type of curing	Products	Abbr.	Manufacturers
Acrylic Resin	heat curing	Bio Resin	resin	SHOFU Inc. Kyoto, Japan
Soft Lining Materials				
Acrylic	cold curing	Soften	SF	Kamemizu Chem.Ind. Co,. Ltd. Osaka, Japan
		Nissin Soft Reverse	SR	Nissin Dental Products Inc. Kyoto, Japan
Silicone	cold curing	Mollosil	MO	DETAX. Karl Huber. GmbH & Co. KG, Ettlingen, Germany
		Evatouch	EV	Neo Dental Chemical Products Co., LTD.
		Tokuyama Soft Relining	TSR	Tokuyama Corp. Tokyo, Japan
	heat curing	Molloplast B	MB	DETAX. Karl Huber. GmbH & Co. KG, Ettlingen, Germany
Fluoric	heat curing	Kurepeet Dough	KD	Kreha Chemical Industryal Co. Japan

Table 2 Fungal colonization on uncoated control samples

cont 0		cont 1000		cont 10000	
MB	0.347 ± 0.024	KD	0.460 ± 0.055	TSR	0.720 ± 0.181
KD	0.539 ± 0.055	TSR	0.595 ± 0.342	KD	0.790 ± 0.010
TSR	0.709 ± 0.056	MB	0.808 ± 0.121	MB	1.099 ± 0.093
SR	1.472 ± 0.037	SF	1.324 ± 0.009	MO	1.317 ± 0.084
SF	1.501 ± 0.024	MO	1.330 ± 0.109	SF	1.340 ± 0.032
MO	1.552 ± 0.085	SR	1.895 ± 0.075	EV	1.750 ± 0.192
resin	2.111 ± 0.117	EV	1.981 ± 0.073	SR	1.850 ± 0.051
EV	2.243 ± 0.053	resin	2.284 ± 0.034	resin	2.322 ± 0.013

No significant differences were observed between the values connected by bars. (ANOVA, $p>0.05$)

Table 3 Fungal colonization on saliva-coated samples

sal 0		sal 1000		sal 10000	
KD	0.739 ± 0.041	KD	0.946 ± 0.055	TSR	1.362 ± 0.007
MB	1.001 ± 0.034	TSR	1.236 ± 0.019	MB	1.735 ± 0.148
TSR	1.223 ± 0.163	MB	1.370 ± 0.009	KD	1.809 ± 0.153
SR	1.485 ± 0.066	SF	1.655 ± 0.020	SR	1.881 ± 0.079
SF	1.591 ± 0.107	MO	1.662 ± 0.062	SF	1.952 ± 0.043
MO	1.676 ± 0.058	SR	1.824 ± 0.108	resin	2.102 ± 0.050
resin	2.035 ± 0.027	resin	1.933 ± 0.049	MO	2.203 ± 0.049
EV	2.134 ± 0.001	EV	2.222 ± 0.051	EV	2.319 ± 0.018

No significant differences were observed between the values connected by bars. (ANOVA, $p>0.05$)

Table 4 Fungal colonization on serum-coated samples

ser 0		ser 1000		ser 10000	
KD	1.029 ± 0.115	KD	1.360 ± 0.108	TSR	1.627 ± 0.062
MB	1.186 ± 0.170	MB	1.654 ± 0.081	MB	1.842 ± 0.040
TSR	1.360 ± 0.039	TSR	1.716 ± 0.111	KD	1.927 ± 0.027
SR	1.579 ± 0.064	SF	2.149 ± 0.031	EV	2.393 ± 0.014
SF	1.803 ± 0.016	SR	2.256 ± 0.076	SR	2.408 ± 0.009
EV	2.289 ± 0.059	resin	2.326 ± 0.023	SF	2.414 ± 0.021
resin	2.294 ± 0.028	EV	2.359 ± 0.028	MO	2.465 ± 0.064
MO	2.534 ± 0.003	MO	2.505 ± 0.005	resin	2.557 ± 0.008

No significant differences were observed between the values connected by bars. (ANOVA, $p>0.05$)

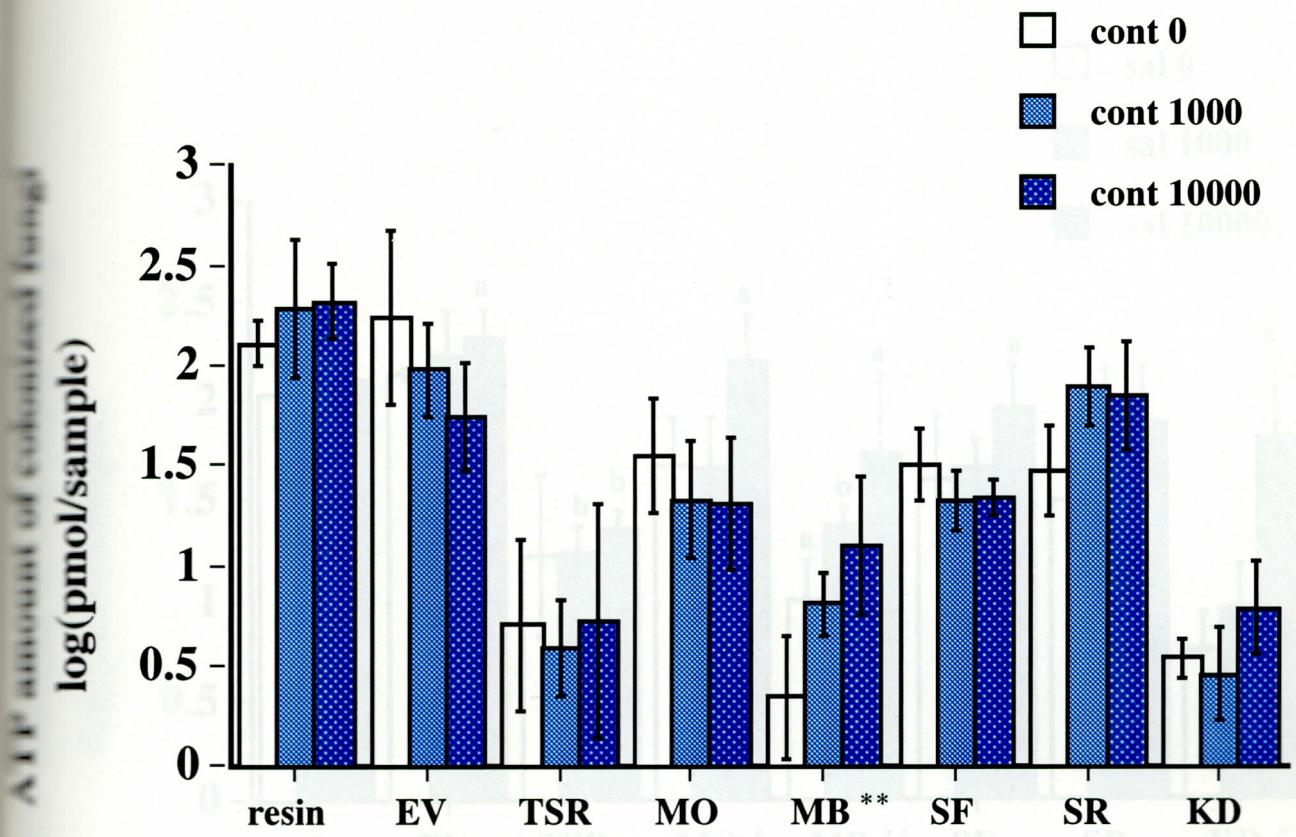

FIG. 1 NIKAWA et al.

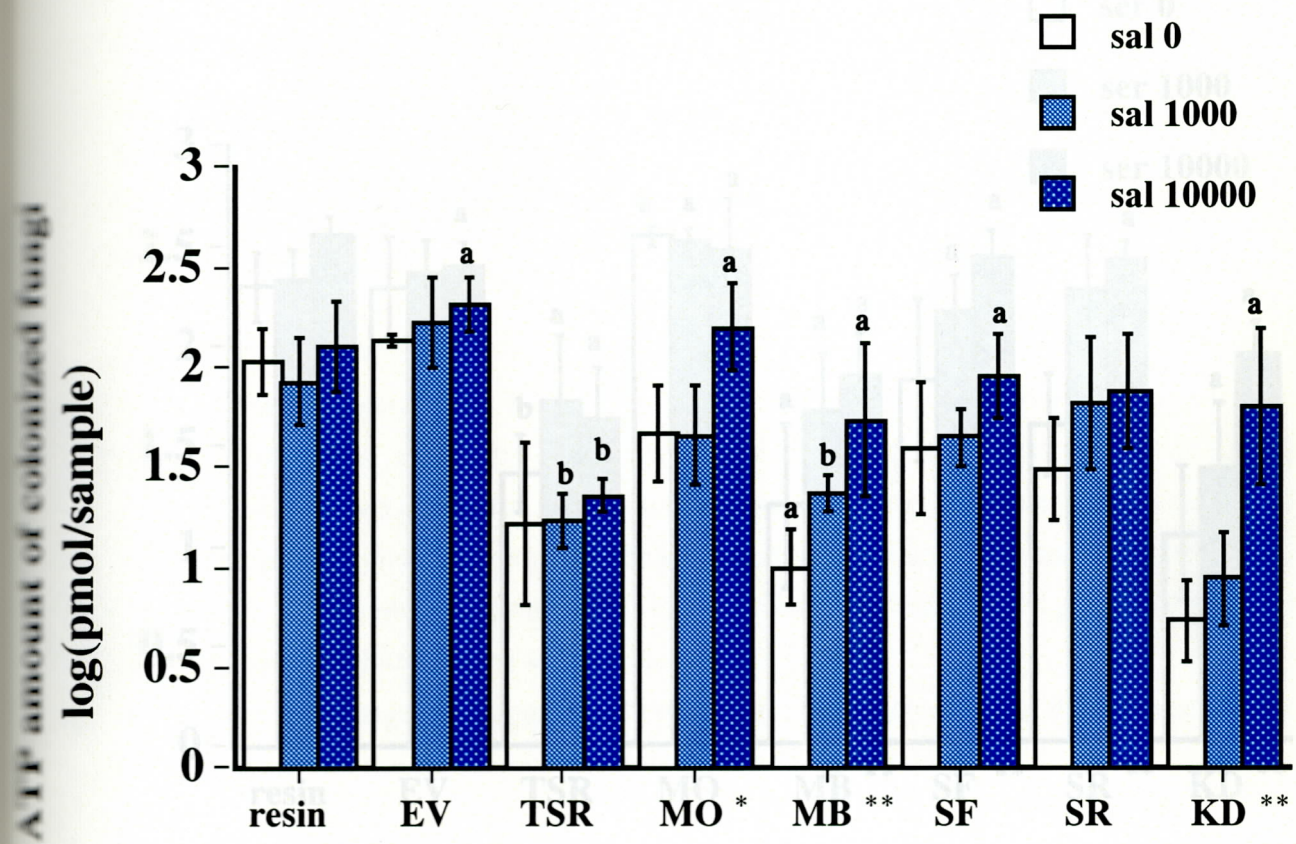

FIG. 2 NIKAWA et al.

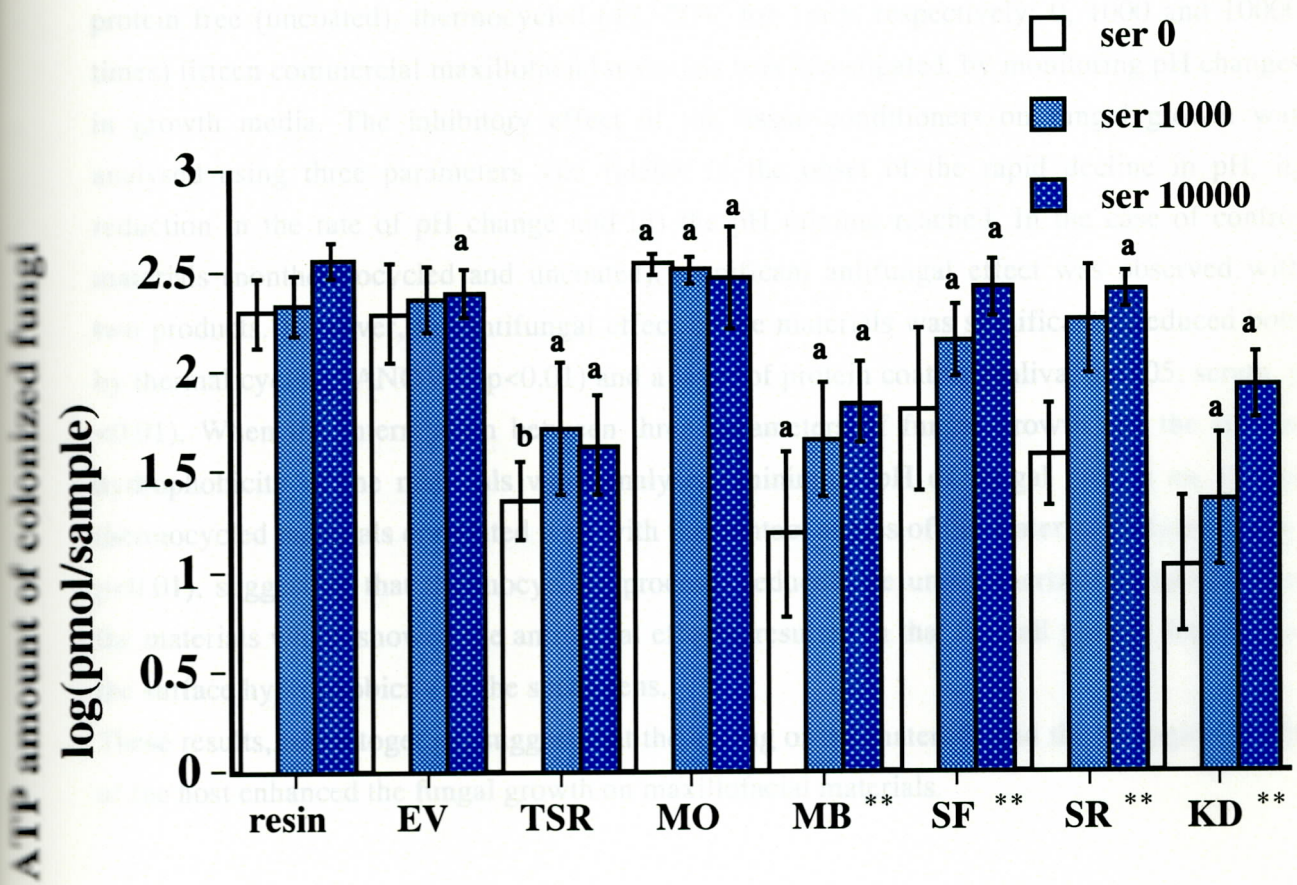

FIG. 3 NIKAWA et al.