Support for the role of *Candida* spp. in extensive caries lesions of children

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SUMMARY

Candida spp. are frequently detected in the mouths of children with extensive caries lesions compared with caries-free subjects. In this study we evaluated the presence of *Candida* spp. in association with mutans streptococci and lactobacilli in the saliva of children with dental decay, before and after anti-caries treatment.
Samples of saliva from 14 children with caries lesions and from 13 caries-free subjects were evaluated for the presence of mutans streptococci, lactobacilli and *Candida* spp. by culture. Eleven of 14 carious subjects hosted *Candida* spp. in their saliva as against only 2 out of 13 subjects without caries lesions. Carious subjects were treated by adopting a conventional protocol for caries disease (rinses with a mouthwash containing 0.2% chlorhexidine and fluorine). After treatment, the salivary bacterial counts decreased for mutans streptococci and in some cases for lactobacilli, but large numbers of *Candida* spp. remained in the saliva of several children. The latter were treated with the antifungal drug nystatin (oral rinses) and evaluation of the level of yeasts in the saliva showed disappearance of the microorganism in several cases. The results indicate that antiseptic treatment alone for dental decay is not sufficient for the eradication of microorganisms potentially responsible for caries lesions, in particular when yeasts are present. We hypothesize that the oral cavity of children could act as a reservoir of fungi, and eradication could be needed to prevent both exacerbation of caries lesions, and colonization by *Candida* spp. of other host sites.

KEY WORDS: Oral microbiology, *Candida* spp., Dental caries, Feeding-bottle syndrome

Received June 27, 2008 *Accepted* July 31, 2008

INTRODUCTION

Although mutans streptococci and lactobacilli are considered the main aetiological agents of human dental caries, fungal oral microflora has been found to be involved by numerous studies of dental decay, however its actual role as a risk factor has yet to be completely clarified. A certain body of clinical and microbiological evidence has suggested a correlation between high prevalence of yeast *Candida* spp. in dental plaque and saliva and the development of active carious lesions (Russel *et al.*, 1991; Sedgley *et al.*,1997; Jacob *et al.*, 1998; Marchant *et al.*, 2001; Moalic *et al.*, 2001; Nikawa *et al.,* 2003; Ersin *et al.*, 2006, Galbiatti de Carvalho *et al.*, 2006).
Yeasts are microorganisms normally present in the oral cavity of healthy individuals, and according to several studies, the percentage of *Candida* spp. colonization ranges from 20% to 40% of healthy individuals (Odds, 1998) and become predominant flora in more than 60% of immunocompromised subjects (Moore *et al.*, 1993). *Candida* spp. are able to colonize several surfaces of the oral cavity including the tongue, palate, cheek and hard surfaces of teeth so much as to be

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included among the components of dental plaque, and, finally, are present in saliva as a consequence of oral surface colonization (Russel *et al*., 1991; Coulter *et al*.,1993; Cannon and Chaffin,1999; Radford *et al*., 2000; Moalic *et al*., 2001; Wall-Manning *et al*., 2002).

More than one hundred species of *Candida* spp. have been identified and characterized to date, but the pathogenetic effects of only 22 of them have been recognized in humans and include *Candida albicans*, *Candida tropicalis, Candida krusei, Candida parakrusei, Candida parapsilosis, Candida pseudotropicalis, Candida zeylanoides, Candida glabrata* and *Candida guilliermondii* (Hazen 1995; de Hooq *el al*., 2000).

C. albicans is the fungal species most frequently isolated in humans; it is included among the normal inhabitants of the human mucosa, but, under particular predisposing physiological or pathological conditions, it is capable of provoking pathologies via an endogenous infectious mechanism. It is endowed with dimorphism, i.e. is able to exist both as a yeast and in pseudohyphal and hyphal form, and this property is referred to as the major virulence determinant (Berman and Sudbery, 2002). The presence of hyphal forms has often been associated with active symptomatic infections while the budding shape has been associated with a saprophytic condition. *C. albicans* displays many pathogenetic factors, in that it is capable of adhering to various surfaces, interfering with the immunological system of the host organism as a dimorphic yeast, and producing several catabolytes (Yang , 2003). Based on the fact that this yeast is able to colonize hard tooth surfaces, invade the dentinal tubules (Sen e*t al*., 1997), produce a large amount of acids provoking demineralization of the dental enamel (Samarayake *et al*., 1986) and dissolution of hydroxyapatite (Nikawa *et al*. 2003), it has been hypothesized that *C. albicans* is a relevant pathogen involved in the progression of caries (Maijala *et al*., 2007). Akdeniz and co-workers (2002) have shown that the prevalence of *Candida* spp. was 69.2% in children with more than 4 decayed teeth and 5% in caries-free subjects. In addition, as far as carious destructive vestibular lesions in children are concerned - the so-called "feeding-bottle syndrome" - the incidence of *Candida* spp. is again elevated (Hodson and Craig, 1972; Boyd and Gregg, 1995).

In this study we examined the presence of *Candida* spp. in association with mutans streptococci and lactobacilli in the saliva of children with extensive caries lesions, before and after anti-caries treatment.

We hypothesize that the oral cavity of these children could act as a reservoir of these fungi and eradication could be needed for preventing both exacerbation of caries lesion and colonization of other human sites by *Candida* spp.

MATERIALS AND METHODS

Patient selection

Twenty-seven young patients (13 males and 14 females, aged 3-10 years) were enrolled among those attending the Pedodontics Outpatient Unit of the Dental Clinic of the University of Verona. Exclusion criteria were antibiotic and/or antimycotic treatment in the previous 3 months at least and the presence of chronic diseases such as allergies and diabetes. The parents of all enrolled children were aware of the experimental design and gave their written informed consent.

The young subjects were divided into two groups: Group 1 was composed of 14 subjects with caries lesions, 8 of them with feeding-bottle syndrome and 6 with multiple caries lesions. Group 2 was composed of 13 caries-free subjects.

Experimental design

The study was divided into three steps. In the first step all the patients selected were enrolled (group 1 and group 2), while in the second step only patients with caries lesions participated (group 1). In the third step only selected patients belonging to group 1 (caries lesions) with high levels of *Candida* spp. after anti-caries treatment were enrolled.

In the first step patients underwent clinical examination and collection of saliva; in the second step the patients in group 1 were treated according to the protocol for caries disease consisting in twice-daily rinses with a mouthwash containing 0.2% chlorhexidine and fluorine for 2 weeks. In the third step, the patients that maintained *Candida* spp. in their saliva after anti-caries treatment were treated with an antifungal drug (nystatin) consisting in thrice-daily administration by oral rinse for 10 days.

Collection of saliva
At least 2 ml of stimulated saliva were collected from each subject in a sterile container, stored in melting ice, and immediately transferred to the microbiology laboratory for microbiological evaluation. The maximum time between sample collection and laboratory processing was 1 hour.

Microbiological procedures
Samples of saliva, prior to microbiological procedures, were thoroughly shaken for 30 s in a Vortex mixer and exposed for 30 s to an ultrasonic bath (Branson mod. 1210). This treatment was the minimum needed to obtain the highest disgregation and dispersion of microorganisms in the sample without interference with their culturability (data not shown).
Suitable sample dilutions (10-fold) of saliva were plated on Gold agar [Mitis-Salivarius agar (Difco) containing 15% sucrose and 0.2 U/l bacitracine] to evaluate mutans streptococci (Schaeken *et al*., 1986), on Rogosa agar (Difco) to evaluate the presence of lactobacilli and on Sabouroud agar to evaluate the presence of yeasts (Difco).
Plates of Rogosa agar were incubated at 37°C for 72 hours in an anaerobic atmosphere in an MK3 anaerobic work station and incubator (du Scientific), plates of Gold agar were incubated in a 5% CO_2 enriched atmosphere at 37°C for 72 hours in a Haereus incubator and plates of Sabouroud agar were incubated at 37°C for 72 hours in an aerobic incubator. The colonies which appeared were counted and numbers referred to one ml of saliva.

RESULTS

Evaluation of microbial counts in saliva
Tables 1 and 2 show pH values and microbial counts in the saliva of each subject with caries lesions and of each caries-free subject, respectively. Table 3 summarizes the mean values of saliva pH and microbial counts of all the subjects enrolled and divided into caries and caries-free.
As far as the salivary pH is concerned, significant differences appeared between the two groups, with lower values in subjects affected by carious pathology in comparison with caries-free subjects (mean values 6.2 vs. 7.3, respectively, $P<0.001$). Subjects with caries lesions (Table 1) displayed higher counts of all three microbial species evaluated when compared with the caries-free subjects (Table 2). In detail, all the samples of saliva from subjects in group 1 had elevated counts of *S. mutans* (mean value $2.1x10^4$ CFU/ml), high levels of lactobacilli, except for sample E (mean value $1.4x10^4$ CFU/ml), and only three samples (namely A, E and F) were negative for the presence of *Candida* spp. In this case the mean value was $3.35x10^3$ CFU/ml. Table 2, on the contrary, shows that caries-free subjects had lower levels of *S. mutans* counts (mean value $2.4x10^3$ UFC/ml) than carious subjects, and low levels of lacto-

TABLE 1 - *pH and counts of specific microorganisms in the saliva of 14 subjects with caries lesions.*

Subject	*pH*	*CFU/ml of saliva*		
		S. mutans	*Lactobacilli*	*Candida spp.*
A	6.5	$2.8x10^2$	$5.0x10^2$	0
B	6.0	$1.6x10^4$	$3.0x10^4$	$6.0x10^3$
C	6.5	$3.2x10^4$	$5.0x10^4$	$1.1x10^3$
D	6.0	$5.1x10^4$	$4.0x10^3$	$7.0x10^1$
E	7.0	$6.3x10^3$	0	0
F	7.0	$4.1x10^4$	$1.0x10^2$	0
G	5.5	$1.6x10^4$	$8.2x10^4$	$8.8x10^3$
H	6.0	$3.0x10^4$	$4.7x10^3$	$9.1x10^1$
I	6.0	$6.0x10^4$	$1.0x10^3$	$2.3x10^1$
L	6.0	$1.0x10^4$	$1.0x10^3$	$2.0x10^3$
M	6.0	$7.1x10^3$	$2.1x10^3$	$2.0x10^3$
N	6.0	$4.2x10^3$	$1.0x10^3$	$8.0x10^3$
O	5.5	$1.6x10^3$	$1.0x10^4$	$8.0x10^3$
P	6.5	$2.4x10^4$	$1.4x10^4$	$3.9x10^3$

TABLE 2 - *pH and counts of specific microorganisms in the saliva of 13 caries-free subjects.*

Subject	*pH*	*CFU/ml of saliva*		
		S. mutans	*Lactobacilli*	*Candida spp.*
1	7.5	$3.1x10^2$	0	0
2	8.0	$5.1x10^3$	$2.4x10^2$	0
3	7.5	$3.5x10^2$	$2.5x10^3$	0
4	7.5	$4.2x10^3$	0	0
5	7.5	$4.0x10^3$	$8.0x10^2$	0
6	7.5	$6.1x10^3$	0	0
7	7.5	$1.0x10^3$	$1.5x10^2$	0
8	6.5	$7.0x10^3$	$1.2x10^2$	0
9	6.5	$2.1x10^3$	$3.7x10^2$	0
10	7.5	$8.0x10^2$	$3.0x10^2$	$1.0x10^2$
11	7.0	$6.1x10^1$	$8.0x10^2$	$1.5x10^2$
12	7.5	$4.2x10^1$	$6.5x10^2$	0
13	6.5	$2.0x10^2$	0	0

bacilli counts, whereas only two samples were positive for *Candida* spp.

Evaluation of microbial counts in saliva after anti-caries treatment

Table 4 shows saliva pH values and microbial saliva counts of each subject with caries lesions (group 1) after anti-caries treatment. Comparing data from Table 1 (before anti-caries treatment) and those of Table 4 (after anti-caries treatment), we observed that the level of *S. mutans* decreased in all samples of saliva and that, in many of them (50%) the value was reduced to zero. The lactobacilli count also decreased for almost all the samples, whereas the level of *Candida* spp. was slightly reduced in about 50% of the samples, while a substantial decrease was observed in 4 subjects (namely C, D, G and H). pH values changed and increased in the majority of samples.

TABLE 3 - *Mean values (±SD) of both pH and counts of specific microorganisms in saliva of the subjects with and without caries lesions.*

Parameter	*Mean values (±SD) in subjects*	
	With caries	*Caries-free*
pH	6.2±0.4	7.3±0.4 (P<0.001)
S. mutans	$2.1x10^4±2.53x10^3$	$2.4x10^3±1.9x10^3$ (P=0.00149)
Lactobacilli	$1.4x10^4±2.4x10^4$	$4.6x10^2±6.8x10^2$ (P=0.04962)
Candida spp.	$3.35x10^3±3.4x10^3$	$2.2x10^1±4.7x10^1$ (P=0.00208)

Data are from Tables 1 and 2. Microorganism counts are expressed as CFU/ml of saliva. P values in brackets were determined by comparing caries-free subjects with those affected by caries lesions.

Evaluation of Candida spp. counts in saliva after antimycotic treatment

The young subjects in group 1, who were still positive for the presence of *Candida* spp. in the saliva after anti-caries treatment (10 patients corresponding to 71% of the total) were locally treated with nystatin. Data reported in Table 5 show that only three patients (30%), were negative for *Candida* spp. after the antifungal treatment, although the *Candida* spp. load greatly decreased in many samples of saliva. Only after a second treatment did *Candida* spp. counts decrease in all the samples of saliva and in many of them were reduced to zero. Finally, in order to evaluate the efficacy of the antimycotic therapy over time, we re-evaluated saliva load of *Candida* spp. in those patients with initial higher loads. Results have

TABLE 4 - *pH and counts of specific microorganisms in the saliva of subjects with caries lesions after anticaries treatment consisting in twice-daily rinses with a mouthwash containing 0.2% chlorhexidine and fluorine for 2 weeks.*

Subject	*pH*[a]	*CFU/ml of saliva*		
		S. mutans	*Lactobacilli*	*Candida spp.*
A	6.5	0	50	0
B	7.5	0	$1.0x10^4$	$3.0x10^3$
C	7.5	0	$3.1x10^2$	70
D	6.5	50	$1.0x10^3$	0
E	7.5	26	0	0
F	7.0	$1.0x10^2$	0	0
G	6.5	$1.0x10^2$	$3.2x10^2$	15
H	6.0	$1.0x10^2$	$4.7x10^3$	10
I	6.5	0	30	$2.0x10^2$
L	6.5	0	$8.0x10^3$	$2.4x10^3$
M	6.5	40	$8.0x10^2$	$1.4x10^3$
N	6.5	$3.2x10^2$	$1.0x10^3$	$6.3x10^2$
O	6.0	0	$1.0x10^4$	$1.6x10^3$
P	7.5	0	$4.0x10^3$	$3.8x10^2$

[a]The mean pH value is 6.8±0.6.

shown that patients I, L, N, and P were still candida-free 3 months later (data not shown).

DISCUSSION

In this work the saliva loads of *S. mutans*, lactobacilli and *Candida* spp. in children suffering from extensive open dental decay (in many instances the feeding-bottle syndrome) has been analyzed and compared with those of caries-free children. Remarkable differences were observed with higher microbial loads in subjects with open caries lesions than in caries-free subjects. Children affected by caries were treated for 2 weeks with an anti-caries mouthwash consisting in a twice-daily rinse with a solution mainly composed of 0.2% chlorhexidine and fluorine. It is worth noting that chlorhexidine has been shown to be active both *in vitro* and *in vivo* against *Candida spp*. (Ellepola and Samaranayake, 1999; Barasch *et al.*, 2004). At the end of the anti-caries treatment, the salivary pH mean value increased slightly, i.e. from 6.2 to 6.8. It is worth noting that the salivary pH was 7.3 (mean value) in the caries-free subjects. The evaluation of the salivary microbial load after anti-caries treatment showed a marked reduction in *S. mutans* counts, a slight decrease in lactobacilli, but a reduction in the *Candida* spp. load was observed in a few samples only. This probably reflects the fact that the treated subjects still had open carious lesions, in which high levels of lactobacilli could still be present. Therefore, with treatment with mouthwash alone and without adequate conservative therapy, as in the case for these very young subjects, this result would be expected. Lactobacilli and

TABLE 5 - *Values of Candida spp in saliva of selected subjects after treatment with nystatin consisting in thrice-daily oral administrations for 10 days. Subjects are part of those in Tables 1 and 4.*

Subject	*UFC/ml of Candida spp*		
	Prior to treatment	*After 1th treatment*	*After 2nd treatment*
B	$8.0x10^3$	$5.3x10^2$	80
C	70	0	NT
G	15	0	NT
H	10	10	0
I	$2.0x10^2$	0	NT
L	$2.0x10^3$	10	0
M	$1.4x10^3$	$8.5x10^2$	50
N	$6.3x10^2$	92	0
O	$1.6x10^3$	$8.4x10^2$	$5.1x10^2$
P	$3.8x10^3$	$3.0x10^2$	0

NT = not treated

Candida spp. were present in high loads and this means that dental decay is active and also the prognosis should be regarded as negative. The subjects that still showed high loads of lactobacilli and *Candida* spp. after anti-caries treatment were topically treated with an antifungal drug (nystatin). In some cases the treatment was repeated to obtain a drastic reduction or elimination of the yeast load. The absence of *Candida* spp. was confirmed 3 months later for several patients.

Taking into consideration the fact that *Candida* spp. are capable of colonizing the hard surface of the teeth, invading the dentinal tubules, participating in the formation of microbial biofilm (Sen *et al*., 1997) and producing large amounts of acids that are responsible for demineralization of tooth enamel (Samaranayeke *et al*., 1986; Peltroche-Llaesahuanga *et al*., 2001), and dissolution of hydroxyapatite (Nikawa *et al*., 2002; Maijala *et al*. 2007) it has been hypothesized that *Candida* spp. play a crucial role in the pathogenesis and, especially, in the progression of caries lesions. Our results support the active role of *Candida* spp. in dental caries.

At present, the relationship between *Candida* spp. and colonization and initiation or progression of caries lesions is still not clear. In other words, it is not clear whether and how the disease process precedes the adhesion of yeasts.

Our current knowledge suggests that high sugar consumption, together with inefficient oral hygiene, open caries lesions and, as in the case of babies, the extensive intake of sweet substances via the feeding-bottle are the factors mainly responsible for the high prevalence of this microorganism in the mouths of children (Galbiatti de Carvalho *et al.,* 2006). The oral cavity of children therefore becomes one of the main sources of transmission of this fungus and its eradication should start with a reduction in the concentration of this microorganism in the mouth (Akdeniz *et al.*, 2002). It seems, moreover, that approximately 50% of the subjects colonized by the yeast maintain it up to 36 months later in spite of regular dental care. It has been observed that children colonized by *Candida* spp. were twice as likely as uncolonized subjects to host lactobacilli with which a positive association is established (Starr *et al*., 2002; see also subject O in this study).

The phenomenon of dental decay is a well known process and this is widely documented in the literature. This aspect, however, is quite different in paediatric patients. Often their mouths are found to be in a very bad state, with multiple destructive dental decay and, possibly, abscesses (Milnes and Boweden, 1985). Prevention plays a crucial role in order to maintain the good health of the oral cavity of children and adolescents. In particular, the period of mixed dentitions constitutes a crucial phase because large lesions of the deciduous dentition can be transferred as carious lesions of permanent teeth.

The data obtained in this study allow us to conclude by saying that in many cases anti-caries therapy alone is not sufficient for resolving tooth decay in children, above all in those with extensive open decay, but need additional therapy with an antifungal mouthwash to eliminate any *Candida* spp. reservoir in order to prevent reinfection.

ACKNOWLEDGEMENT

This study was supported by funds ex 60% from the University of Verona to C.S and PRIN2005 from the Italian Ministry of University and Research to P.C. The technical assistance of Anna Bertoncelli is greatly appreciated.

REFERENCES

AKDENIZ B.G., KOPARAL E., SEN B.H., ATES M, DENIZCI A.A. (2002). Prevalence of *Candida albicans* in oral cavities and roots canals of children. *ASDC J. Dent. Child.* **69**, 289-292.

BARASCH A., SAFFORD M.M., DAPKUTE-MARCUS I., FINE D.H. (2004). Efficacy of chlorhexidine gluconate rinse for treatment and prevention of oral candidiasis in HIV-infected children: a pilot study. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **97**, 204-207.

BERMAN J., SUDBERY P.E. (2002). *Candida albicans*: a molecular revolution build on lessons from budding yeast. *Nat. Rev. Genet.* **3**, 918-930.

BOYD D.H., GREGG T.A. (1995). Candida infection of a persistent palatal ulcer in a 20-month-old child. *Int. J. Paediatr. Dent.* **5**, 109-11.

CANNON R.D., CHAFFIN W.L. (1999). Oral colonization by *Candida albicans*. *Crit. Rev. Oral Biol. Med.* **10**, 359-383.

COULTER W.A., MURRAY S.D., KINIRONS M.J. (1993). The use of a concentrated oral rinse culture technique to sample oral candida and lactobacilli in children, and the relationship between Candida and lacto-

bacilli levels and dental caries experience: a pilot study. *Int. J. Paediatr. Dent.* **3**, 17-21.

De Hooq G.S., Guano J., Genè J., Figueras M.J. (2000). Atlas of clinical fungi. Centralbureau voor Schimmelcultures - Universitat Rovira i Virgili.

Ellepola A.N.B., Samaranayake L.P. (1999). *In vitro* post antifungal effect (PAFE) elicited by chlorhexidine gluconate on oral isolates of *Candida albicans*. *Microb. Ecol. Health. Dis*. **11**, 143-148.

Ersin N.K., Eronat N., Cogulu D., Uzel A., Aksit S. (2006). Association of maternal-child characteristics as a factor in early childhood caries and salivary bacterial counts. *J. Dent. Child (Chic).***73**, 105-111.

Galbiatti De Carvalho F., Souza Silva D., Hebling J., Spolidorio L.C., Palomari Spolidorio D.M. (2006) Presence of mutans streptococci and *Candida* spp. in dental plaque/dentine of carious teeth and early childhood caries. *Arch. Oral. Biol.* **51**, 1024-1028.

Hazen K.C. (1995). New and emerging yeast pathogens. *Clin Microbiol Rev.* **8**, 462-478.

Hodson J.J., Craig G.T. (1972). The incidence of *Candida albicans* in the plaques of teeth of children. *Dent. Pract. Dent. Rec.* **22**, 296-301.

Jacob L.S., Flaitz C.M., Nichols C.M., Hicks M.J. (1998). Role of dentinal carious lesions in the pathogenesis of oral candidiasis in HIV infections. *J. Am. Dent. Assoc.* **129**, 187-194.

Maijala M., Rautemaa R., J*f*rvensivu A., Richardson M., Salo T., Tj*f*derhane L. (2007) *Candida albicans* does not invade carious human dentine. *Oral Dis.* **13**, 279-284.

Marchant S., Brailsford S.R., Twomey A.C., Roberts G.J., Beighton D. (2001). The predominant microflora of nursing caries lesions. *Caries Res*. **35**, 397-406.

Milnes A.R., Boweden G.H. (1985). The microflora associated with developing lesions of nursing caries. *Caries Res.* **19**, 289-297.

Moalic E., Gestalin A., Quinio D., Gest P.E., Zerilli A., Le Flohie A.M. (2001). The extent of oral fungal flora in 353 students and possible relationship with dental caries. *Caries Res.* **35**, 149-155.

Moore L.V.H., Moore W.E.C., Riley C., Brooks C., Burmeister J.A., Smibert R.M. (1993). Periodontal microflora of HIV positive subjects with gingivitis or adult periodontitis. *J. Periodontol.* **64**, 48-56.

Nikawa H., Yamashiro H., Makihira S., Nishimura M., Egusa H., Furukawa M., Setijanto D., Hamada T. (2003). In vitro cariogenic potential of *Candida albicans*. *Mycoses* **46**, 471-478.

Odds FC. (1998). Candida and candidosis. 2nd edn. Bailliére Tindal, London.

Peltroche-Llacsahuanga H., Hauk C.J., Kock R., Lampert F., Lütticken R., Haase G. (2001). Assessment of acid production by various human oral micro-organism when palatinose or leucrose is utilized. *J. Dent. Res.* **80**, 378-384.

Radford J.R., Ballantyne H.M., Nugent Z. Beighton D., Robertson M., Longbottom C., Pitts N.B. (2000). Caries-associated micro-organisms in infants from different socio-economic backgrounds in Scotland. *J. Dent.* **28**, 307-312.

Russell J.I., Macfarlane T.W., Aitchison T.C., Stephen K.W., Burchell C.K. (1991). Prediction of caries increment in Scottish adolescents. *Community Dent. Oral Epidemiol.* **19**, 74-77.

Samarayake L.P., Huhes A., Weetman D.A., Macfarlane T.W. (1986). Growth and acid production of *Candida* species in human saliva supplemented with glucose. *J. Oral Pathol.* **15**, 251-254.

Schaeken M.J., Van Der Hoeven J.S., Franker H.C. (1986). Comparative recovery of *Streptococcus mutans* on five isolation media, including a new simple selective medium. *J. Dent Res.* **65**, 906-908.

Sedgley C.M., Samaranayake L.P., Chan J.C., Wie S.H. (1997). A 4-year longitudinal study of the oral prevalence of enteric gram-negative rods and yeast in Chinese children. *Oral Microbiol. Immunol.* **12**, 183-188.

Sen B.H., Safavi K.E., Spangberg L.S.W. (1997). Colonization of *Candida albicans* on cleaned human dental hard tissues. *Arch. Oral Biol.* **42**, 513-520.

Starr J.R., White T.C., Leroux B.G., Luis H.S., Bernardo M., Leitao J., Roberts Mc. (2002). Persistence of oral *Candida albicans* carriage in healthy Portuguese schoolchildren followed for 3 years. *Oral Microbiol. Immunol.* **17**, 304-310.

Yang Y.L. (2003). Virulence factors of *Candida* species. *J. Microbiol. Immunol. Infec.* **36**, 223-228.

Wall-Manning G.M., Sissons C.H., Anderson S.A., Lee M. (2002). Checkboard DNA-DNA hybridisation technology focused on the analysis of Gram-positive cariogenic bacteria. *J. Microbiol. Methods.* **51**, 301-311.