**In vitro** study of the antibacterial properties and microbial colonization susceptibility of four self-etching adhesives used in orthodontics

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SUMMARY

OBJECTIVES: 1. To determine the **in vitro** antibacterial effectiveness of the orthodontic bonding Transbond XT (3M Unitek) and four self-etching adhesives with possible use in orthodontic bonding (Clearfil Protect Bond, CPB; Clearfil Self-etching Bond, CSB; Transbond Plus Self-Etching Primer, TSEP; iBond) against *Streptococcus mutans* and *Lactobacillus gasseri* in order to compare that capacity among the adhesives and with respect to Transbond XT; 2. To determine the bacterial adhesion capacity of the above mentioned microorganisms to the tested adhesives.

MATERIALS AND METHODS: The inhibitory effects of the adhesives against *S. mutans* and *L. gasseri* were examined using the agar diffusion method with Whatman No.1 5 mm disks loaded with 15 μl of adhesive, UV polymerized, layered on previously inoculated BHI and MRS plates incubated microaerobically for 48 hours at 37 degree C. Data were analysed with Kruskal–Wallis (<0.05) and Mann–Whitney tests, applying the Bonferroni correction (<0.003). Bacterial adhesion was studied using scanning electron microscopy.

RESULTS: Only CPB and iBond produced a clear growth inhibition halo against *S. mutans* and *L. gasseri* (<0.0001). iBond was the only tested product to which the bacteria adhere profusely, particularly *S. mutans*.

CONCLUSIONS: CPB has shown antimicrobial properties **in vitro**, and, provided the limitations of an **in vitro** study, the use of this self-etching adhesive may contribute to reduce microbial decalcification, making the use of this self-etching adhesive an attractive option for bracket bonding.

Introduction

The placement of fixed orthodontic appliances increases the level of streptococci and lactobacilli in saliva and dental plaque (Scheie, 1984; Lundström and Karasse, 1987; Huser et al., 1990; Rosenbloom and Tinanoff, 1991; Arneberg, 1984; Chang et al., 1997; Øgaard et al., 2001). This increase occurs during the second week following placement, and bacterial levels returning to normal once braces are removed (Rosenbloom and Tinanoff, 1991).

The high incidence of decalcification following orthodontic treatment is caused by the increase in *S. mutans*, favoured by the low pH (≤4.5) found in bacterial plaque around brackets and bands (Øgaard et al., 2001; Øgaard and Rolla, 1993). The application of fluoride reduces the risk of caries (Zachrisson, 1975; Fejerskov, 1996; Hamilton, 1996; Rolla, 1996; ten Cate, 1996). However, the acidic environment around brackets slows down remineralization, and the introduction of additional fluoride into the oral medium will not necessarily have a cariostatic effect (Zimmer et al., 2004).

Orthodontic adhesive resins used for bracket bonding on tooth enamel also contribute to demineralization as it has a rough surface ideal for colonization by oral microorganisms (Weitman and Eames, 1975). For this reason, rigorous elimination of resin remains and optimum oral hygiene during orthodontic treatment are recommended. It has also been shown that there are 10 μm gaps at the enamel–adhesive interface around the bracket base causing microfiltration and an accumulation of bacteria (Sukontapatipark et al., 2001). This fact highlights the importance of the technique used to bond fixed appliances and the fundamental role that orthodontic adhesives play as risk factors in enamel demineralization, given that demineralization most frequently occurs at the enamel–adhesive interface (Sukontapatipark et al., 2001; Gwinnett and Ceen, 1979).

In spite of advances in orthodontic materials and techniques in recent years, the incidence of enamel decalcification and caries around fixed orthodontic appliances continues to be problematic, and prevention is
crucial as both problems can compromise smile aesthetics. With this objective, self-etching adhesives with supposedly antibacterial properties have been introduced into the market. One such material is Transbond Plus Self Etching Primer (TSEP, 3M Unitek, Monrovia, California, USA), a self-etching fluoride-releasing orthodontic adhesive. There are also other self-etching adhesives used in conservative dentistry such as iBond Gluma Inside (iBond, Heraeus Kulzer GmbH). iBond contains glutaraldehyde, allowing the material to act as a desensitizer as well reducing or eliminating bacterial levels in cavity prepararations (Feltion et al., 1989). Clearfil Protect Bond (CPB, Kuraray Medical Inc., Okayama, Japan), a more advanced version of the self-etching adhesive Clearfil SE Bond (CSB, Kuraray Medical Inc.), differs from its predecessor in that it contains the antibacterial monomer MDPB in its primer and sodium fluoride in the bonding.

Several studies have attributed the antibacterial effect of self-etching adhesives to their low pH (Emilson and Bergenholtz, 1993; Meiers and Miller, 1996; Başeren et al., 2005), which is equivalent to etching with phosphoric acid (Settembrini et al., 1997). Nevertheless, it is difficult to demonstrate the antibacterial properties, if any, of self-etching adhesives with antibacterial monomers or fluoride salts (Imazato et al., 2002). Whichever be the origin of the inhibitory ability of the self-etching adhesives towards S. mutans and L. gasseri (low pH, fluoride release, specific antimicrobial molecules present in the material) it adds extra protection against microbes in bracket bonding.

This study has been designed with two main objectives: 1. To determine, using the agar diffusion test, the antibacterial properties against S. mutans and L. gasseri (the main cause of dental caries), of various self-etching adhesives: a conventional one (CSB), a fluoride-releasing self-etching adhesive (TSEP), a self-etching adhesive with antibacterial properties (iBond) and an self-etching adhesive having both, fluoride release and antibacterial properties (CPB) and 2. To study the adhesion of S. mutans and L. gasseri to these polymerized adhesives by means of scanning electron microscopy (SEM).

Materials and methods
Adhesives

An adhesive of conventional use in Orthodontics, Transbond XT® (3M Unitek Dental Products, Monrovia, California), and four self-etching adhesives were used: Clearfil Protect Bond® (CPB, Kuraray Medical Inc., Okayama, Japan), Clearfil SE Bond® (CSB, Kuraray Medical Inc., Okayama, Japan), Transbond Self Etching Primer® (TSEP, 3M Unitek Dental Products, Monrovia, California) e iBond Gluma Inside® (iBond, Heraeus Kulzer GmbH) (Supplementary Table 1).

Bacteria

The antimicrobial properties of adhesives where tested against two dental caries producing bacteria: S. mutans ATCC 55677 and L. gasseri ATCC 29601.

Bacterial suspensions

S. mutans and L. gasseri were cultured in Brain Heart Infusion (BHI) broth and Man–Rogosa–Sharpe (MRS) medium respectively. After incubation at 37 degree C in a jar with a microaerophilic atmosphere enriched with 5 per cent CO₂ (Oxoid Campygen), L. gasseri reached the initial stationary phase after 24 hours of cultivation OD₆₅₀ = 0.35, and S. mutans reached the same growth phase point after 48 hours (OD₆₅₀ = 0.30), measured with a Spectronic 20 (Milton Roy Company). These microbial suspensions were used to inoculate the agar diffusion test plates and perform the adhesion assays.

Agar diffusion test

The Kirby–Bauer test was adapted to the characteristics of the study. The substances to be tested were deposited on sterile (Whatman No.1) filter paper disks of 5 mm in diameter and 1.5 mm thickness. Each disk received (in aseptic conditions) 15 µl of each of the adhesives (Table 1), with the exception of CPB and CSB, from which 7.5 µl of primer and 7.5 µl of bonding were used, because these components came in different containers. The adhesives were polymerized on site with a halogen light-curing unit (Ortholux XT, 3M Unitek Dental Products, Monrovia, Calif.) for the duration indicated by the manufacturer (10 seconds for each adhesive except 20 seconds for iBond), verifying after that time the complete polymerization of the adhesives. Distilled water impregnated disks were used as controls.

Petri plates with 20 ml of BHI agar or MRS agar were surface inoculated with S. mutans and L. gasseri suspensions using a sterile swab to spread the inoculum. Twelve plates were prepared with each strain of bacteria. Each plate contained only three disks, either three different adhesives or two adhesives and one control. In every test, each strain of bacteria was exposed to 36 disks (12 plates), six disks per each adhesive and six controls (6 x 6). The plates were incubated for 48 hours at 37 degree C in a microaerophilic atmosphere enriched with 5 per cent CO₂ (Oxoid Campygen). The diameters of the inhibition halos were measured with a manual caliper (Leone p. 1560.15 (Nr 7452/186). The assay was repeated four times: 48 plates, 24 disks for each adhesive and control and each bacterial strain.

Evaluation of bacterial adherence to polymerized adhesives

Six tubes with 0.5 ml of early stationary phase back bacterial suspension were prepared for each strain at an OD₅₅₀ of 0.35
**Table 1** Inhibition halo sizes (mm) observed with the agar diffusion test produced by the different adhesives on each bacterial strain.

<table>
<thead>
<tr>
<th>Adhesive groups</th>
<th>Bacteria</th>
<th>Mean ± standard deviation (SD)</th>
<th>Median</th>
<th>Range</th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transbond XT (n = 24)</td>
<td><em>Streptococcus mutans</em></td>
<td>0 ± 0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Clearfil Protect Bond (n = 24)</td>
<td></td>
<td>16.50 ± 6.27e</td>
<td>16.50</td>
<td>21</td>
<td>13.54 ± 6.40e</td>
<td>11.50</td>
<td>27</td>
</tr>
<tr>
<td>Clearfil Self-etching Bond (n = 24)</td>
<td></td>
<td>11.33 ± 4.78e</td>
<td>10.00</td>
<td>24</td>
<td>12.25 ± 8.81e</td>
<td>14.00</td>
<td>27</td>
</tr>
<tr>
<td>Transbond Plus Self-Etching Primer</td>
<td></td>
<td>10.58 ± 2.08e</td>
<td>10.50</td>
<td>12</td>
<td>9.50 ± 1.38e</td>
<td>9.00</td>
<td>5</td>
</tr>
<tr>
<td>iBond (n = 24)</td>
<td><em>Lactobacillus gasseri</em></td>
<td>11.67 ± 1.63e*</td>
<td>11.50</td>
<td>6</td>
<td>13.92 ± 2.10e</td>
<td>14.00</td>
<td>9</td>
</tr>
<tr>
<td>Control (n = 24)</td>
<td></td>
<td>0 ± 0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Mann–Whitney test ($P < 0.05$), applying the Bonferroni correction ($P < 0.003$) showed for *S. mutans* significant differences of groups marked * compared with Transbond XT and control, and significant differences of groups marked † compared with Clearfil Protect Bond. For *L. gasseri*, groups marked ‡ showed significant differences compared with Transbond XT and control; groups marked § showed significant differences compared with iBond. Mann–Whitney test ($P < 0.05$) showed for TSEP and iBond significant differences in inhibition halo sizes (mm) when comparing the two bacterial strains, *S. Mutans* and *L. Gasseri*, marked with *.

(L. gasseri) and 0.30 (S. mutans). Each of the adhesives was introduced into vials and then were photopolymerized using an Ortholux XT® curing light (3M Unitek Dental Products, Monrovia, California) following the instructions supplied by the manufacturer of each product. The polimerized adhesives were cut using a scalpel blade (≠12), in 1–2 mm size solid cubic particles. Three solid particles were placed in each of six tubes, five with viable bacterial suspensions and the sixth containing a pasteurized inactivated bacterial suspension in order to evaluate the nonspecific adherence of the microbial cells.

The tubes were incubated at 37 degree C for 20 hours in a 5 per cent CO$_2$ enriched atmosphere. After the incubation time, each adhesive particle was washed three times in sterile culture medium and agitated for 10 seconds at 1400 rpm using an SA 8 Stuart®, UK, vortex mixer to release not firmly adhered bacteria. The adhesive particles were fixed, with glutaraldehide and osmium tetroxide, subjected to critical point drying and gold covered with a sputter evaporator (SEM Coating System, Bio-Rad. Polaron Division, East Grinstead, UK). Samples were observed with a Jeol 6100 (Tokyo, Japan) scanning electron microscope operating at 30 Kv.

**Statistical analysis**

The Kolmogorov–Smirnov normality test and the Levene variance homogeneity test were applied to the inhibition halos data. As there was neither homogeneity of variances nor a normal distribution, significant difference among the inhibition halos data produced by the different adhesives upon each bacterial strain was evaluated using the Kruskal–Wallis test ($P < 0.05$), finding those groups which were significantly different with the Mann–Whitney test for two independent samples. In order to avoid an accumulation of errors due to multiple comparisons, the significance level was modified dividing this ($P < 0.05$) by the number of comparisons made (Bonferroni Correction, $n = 15$) and the resulting $P < 0.003$ was considered significant.

The existence of significant differences between halo sizes produced by each adhesive in both bacterial strains was evaluated using the Mann–Whitney test ($P < 0.05$).

Data were subjected to statistical analysis using SPSS 14.0 for Windows statistical software (SPSS 14.0, Inc. Chicago, Illinois, USA).

**Results**

*Agar diffusion test*

Neither Transbond XT nor control disks impregnated with distilled water inhibited either *S. mutans* or *L. gasseri* growth. CPB and iBond produced clear growth inhibition halos with both bacterial strains. CSB showed a minor halo against *S. mutans* and a diffuse one against *L. gasseri* (Figure 1).

In general, and for both bacterial strains, the four self-etching adhesives showed inhibition halos data statistically significant with respect to the control and the Transbond XT adhesive. CPB showed a significantly greater inhibiting capacity on *S. mutans* than CSB ($P < 0.0001$) and TSEP ($P < 0.0001$) and in the case of *L. gasseri*, iBond showed an inhibiting capacity significantly greater than TSEP ($P < 0.0001$, Table 1).

When the inhibition halos produced by each self-etching adhesive were evaluated comparing the two bacterial strains, significant differences were found between those produced by TSEP and iBond ($P < 0.0001$) against both, *S. mutans* and *L. gasseri* (Table 1).
Evaluation of bacterial adherence to polymerized adhesives

The two tested microbial strains did not show specific adherence to most of the polymerized adhesives except iBond, on which \textit{L. gasseri} showed an average of 35 cells in 10 microscopic fields observed (600 $\mu$m$^2$ each, Figure 2a); nevertheless, a few areas were more colonized (see below). \textit{Streptococcus mutans} presented an even better adhesive capacity on iBond than \textit{L. gasseri}. A minimum of 290 bacteria and up to uncountable cell numbers were observed out of 10 microscopic fields quantified (Figure 2c). Exceptionally heavy colonized areas of iBond with cell filaments of \textit{L. gasseri} (Figure 3a) or chains of \textit{S. mutans} were observed (Figure 3b). Adherence of dead cells (control) to iBond (Figure 2b and d) showed that \textit{S. mutans} had a greater passive adherence capacity than \textit{L. gasseri} to the resin.

Discussion

The antibacterial action of adhesive systems is affected by the materials’ inherent properties such as pH, viscosity, diffusion capacity, the presence of antibacterial agents and factors related to the dentinal substrate (thickness and permeability, Başeren et al., 2005; Imazato et al., 2002; Imazato et al., 1998a; Imazato et al., 1998b; Imazato et al., 2004; Schmalz et al., 2004; Türkip et al., 2006; Feuerstein et al., 2007).

CPB has an antibacterial and a fluoride-releasing capacity due to the primer’s antibacterial monomer MDPB and the bonding’s sodium fluoride content. MDPB (Imazato et al., 1994) is a derivative of a quaternary ammonium molecule synthesized by combining dodecylpyridinium bromide with a methacryloxy group. The antibacterial agent is immobilized in the composite matrix by the co-polymerization of MDPB along with other monomers. The effect of the immobilized antibacterial component is mainly bacteriostatic and bacterial anti-adhesive (Imazato et al., 1998b; Imazato et al., 1995; Imazato et al., 2003a; Imazato et al., 2003b) as the agent cannot penetrate the cell wall or membrane as free antimicrobial agents do. MDPB’s antibacterial activity cannot reach the area around the orthodontic bracket wings, a fact that limits its antibacterial action, although it may be effective against the demineralization.
that occurs beneath brackets resulting from microleakage following polymerization (Kawabata and Nishiguchi, 1988). Besides, the effect of the bonding’s fluoride content extends around the bracket. Considering all of the above it is no surprise that we observed halos of inhibition surrounding the CPB disks.

Self-etching adhesives have an acidic pH. The primers’ acidity has been considered a key factor for bacterial inhibition (Bageren et al., 2005; Imazato et al., 1998a; Scheie, 1989; Kourai et al., 1994), although there are several recent studies (Imazato et al., 2002; Imazato et al., 2003a; Li et al., 2009) that did not find a significant relation between the acidity of self-etching adhesives and their antibacterial effects. 2-methacryloyloxyethyl phenyl hydrogen (Phenyl-P), 4-methacryloyloxyethyl trimellitate anhydride (4-META) and 10-methacryloyloxy-decyl dihydrogenphosphate (MDP), are the main acidic monomers incorporated in the formula of self-etching adhesive systems; they are able to simultaneously demineralize and infiltrate the dentinal substratum. According to Ohmori et al. (1999), the acid monomer in CSB (MDP) has a stronger microorganism-inhibiting action than Phenyl-P. Despite CSB’s MDP content, its lack of antibacterial action may be attributed to the hydrophobic molecules in its composition,
which impede MDP diffusion into the agar medium (Imazato et al., 2002). With regard to CPB’s antibacterial effect, it has been said above that it is based mainly on its MDPB antibacterial monomer component. This antibacterial effect could not be due only to its acidic pH as it has been shown that the monomer is clearly inhibitory against lactobacilli, which are acid-tolerant (acidophilic) bacteria (Korkmaz et al., 2008), nor should it be due to fluoride release, given that it has been shown that the 450µg/g of F− that it releases in vitro over 1 month (Imazato, 1998) are insufficient to inhibit cariogenic bacterial growth. We have been unable to find published studies concerning the antimicrobial properties of TSEP with which to compare our results; in any case, TSEP showed the lowest antibacterial properties among the products tested in the present study.

The antimicrobial properties shown by iBond in the agar diffusion test may be attributed to its glutaraldehyde content. Some dental materials containing glutaraldehyde have been shown to be effective against Streptococcus, Lactobacillus, and Actinomyces, a result of infiltration into dentinal tubules, which depends on the glutaraldehyde released by the cured materials (Meiers and Miller, 1996; Arhun et al., 2006). As glutaraldehyde does not polymerize within the resin matrix, its antibacterial effect persists after polymerization of the resin because the remaining free molecules diffuse into the surrounding environment.

When bacterial adherence to polymerized adhesive materials was evaluated, both S. mutans and L. gasseri adhered only to iBond. Streptococcus mutans adhered more than L. gasseri. Inactivated S. mutans retained a certain capacity to adhere to iBond probably due to the streptococci capsular polysaccharides. The apparent contradiction between the formation of clear inhibition halos by iBond in the agar diffusion test and the high levels of bacterial adhesion to the material’s surfaces in the nutrient-rich medium may be explained because the medium rich in amino-acids and peptides and the glutaraldehyde diffusing from the particles reacts irreversibly with these compounds neutralizing their toxicity (Hopwood, 1972; Eltoum et al., 2001). The surviving microorganisms would proliferate and actively recolonize the broth and the particle surface on which no toxicity remains. In the agar diffusion test, the microorganisms that constitute the bacterial lawn stay in place on the agar surface because S. mutans and L. gasseri are non-motile bacteria. Those microorganisms close to the iBond disks from which glutaraldehyde diffuses freely would get ‘fixed’ and die, and in this area a permanent inhibition halo is observed. Cells further away from the area of glutaraldehyde diffusion can proliferate freely (Hayat, 2000).

According to what has been discussed in the previous paragraph, iBond’s antimicrobial activity would depend on the free glutaraldehyde that diffuses into the environment from the solid polymerized adhesive particle but its toxicity is quickly and irreversibly neutralized as soon as it reacts with cells or molecules having free amino groups (Hayat, 2000; Hopwood, 1972).

With the exception of iBond, bacterial adhesion was not found with the other adhesives tested and none of the fluoride-releasing self-etchers, CPB and TSEP, showed any bacterial adhesion. This coincides with results obtained by Badawi et al. (2003), who found that biofilms growing on fluoride-releasing dental materials did not show colonization of S. mutans. However, Lim et al. (2008) did not observe significant differences in bacterial adhesion between fluoride-releasing composites and non-releasing ones and declared that bacterial adhesion depends largely on the material’s surface roughness. On the basis of our results, it cannot be stated that fluoride-releasing properties of dental materials influence bacterial adhesion. Furthermore, as we have been unable to locate other studies dealing with bacterial adhesion to fluoride containing self-etching adhesives it is not possible to compare our results with those of other researchers. Nevertheless, the incorporation of fluoride into adhesive systems and dental materials has more to do with inhibiting the demineralization process and optimizing remineralization than with antibacterial action (Hara et al., 2005). Besides, although many fluoride-releasing materials initially release large amounts of fluoride, this soon decreases (Wiegand et al., 2007) and may not be sufficient to prevent decalcification during orthodontic treatment (Benson et al., 2005).

Conclusions

1. CPB and iBond showed antibacterial activity in vitro against S. mutans and L. gasseri, as derived from the results of the agar diffusion test.
2. Evaluation of bacterial adhesion showed L. gasseri and, to a greater extent, S. mutans are both able to adhere to particles of polymerized iBond.

Supplementary material

Supplementary material is available at European Journal of Orthodontics online.

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