Killing of adherent oral microbes by a non-thermal atmospheric plasma jet

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Atmospheric plasma jets are being intensively studied with respect to potential applications in medicine. The aim of this *in vitro* study was to test a microwave-powered non-thermal atmospheric plasma jet for its antimicrobial efficacy against adherent oral micro-organisms. Agar plates and dentin slices were inoculated with 6 $\log_{10}$ c.f.u. cm^{-2} of *Lactobacillus casei*, *Streptococcus mutans* and *Candida albicans*, with *Escherichia coli* as a control. Areas of 1 cm^2 on the agar plates or the complete dentin slices were irradiated with a helium plasma jet for 0.3, 0.6 or 0.9 s mm^{-2}, respectively. The agar plates were incubated at 37 °C, and dentin slices were vortexed in liquid media and suspensions were placed on agar plates. The killing efficacy of the plasma jet was assessed by counting the number of c.f.u. on the irradiated areas of the agar plates, as well as by determination of the number of c.f.u. recovered from dentin slices. A microbe-killing effect was found on the irradiated parts of the agar plates for *L. casei*, *S. mutans*, *C. albicans* and *E. coli*. The plasma-jet treatment reduced the c.f.u. by 3–4 $\log_{10}$ intervals on the dentin slices in comparison to recovery rates from untreated controls. The microbe-killing effect was correlated with increasing irradiation times. Thus, non-thermal atmospheric plasma jets could be used for the disinfection of dental surfaces.

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INTRODUCTION

Plasma jets are ionized local gas flows, generated under normal pressure by means of microwaves, high frequency or pulsed direct current in so-called plasma-jet sources using noble gases. Reactive oxygen species are produced through an admixture of chemically active gases (O_2, N_2 and others), which are able to react with biological material or tissues (Kieft *et al.*, 2005). Furthermore, UV irradiation, ions and electrons are emitted in the plasma discharges, which also interact with biological material or tissues (Laroussi & Leipold, 2004; Vleugels *et al.*, 2005). In medicine, plasmas are used to sterilize surgical instruments and consumables (Masaoka, 2007). Another important medical application is argon-plasma coagulation (Manner, 2008). Plasmas can also be used to give hydrophilic properties to surfaces (Duan *et al.*, 2007).

Recently, the generation of local plasmas under atmospheric pressure with low temperatures of around 40 °C has been achieved. At the same time much more compact plasma sources were developed making them more attractive for medical applications (Coulombe *et al.*, 2006; Yu *et al.*, 2006; Sladek *et al.*, 2007). The antibacterial effect of cold plasmas has been shown for a variety of micro-organisms in numerous studies (Moisan *et al.*, 2001; Laroussi, 2002; Becker *et al.*, 2005; Laroussi & Lu, 2005; Vleugels *et al.*, 2005; Lee *et al.*, 2006; Yu *et al.*, 2006, 2007; Hong *et al.*, 2009). However, some studies have shown that bacterial DNA is not completely destroyed by cold plasma (Venezia *et al.*, 2008; Kim & Kim, 2006).

Methods for the decontamination and conditioning of intraoral surfaces are of great interest in the field of dentistry. Cold plasmas are of particular interest, as heat damage of the dental pulp must be prevented. Only a few papers have been published dealing with the application of plasma jets for biofilm and plaque removal in dentistry. For instance, the removal of carious dentin has been suggested as an alternative to conventional drilling (Stoffels *et al.*, 2002; Sladek *et al.*, 2004). The *in-vitro* disinfection of *Streptococcus mutans* grown on agar plates (Goree *et al.*,

Abbreviations: CHX, chlorhexidine; CI, confidence interval; SEM, scanning electron microscopy.

2006) and in biofilms has been demonstrated (Sladek *et al.*, 2007). However, due to the presence of dentin tubules, the disinfection of dentin poses special challenges during caries therapy. It must be achieved either by invasive removal, by chemical disinfection or by application of ozone. No data on the effect of plasma jets on microbially contaminated dentin are available yet.

The aim of this *in vitro* study was to test a non-thermal atmospheric plasma jet for its antimicrobial efficacy against important oral micro-organisms grown on agar plates or adherent to dentin slices. The experiments were performed with the caries-associated bacteria *Lactobacillus casei* (Badet & Thebaud, 2008) and *S. mutans* (Rupf *et al.*, 2008), as well as *Candida albicans* (Klinke *et al.*, 2009), which is frequently isolated from dentin caries lesions. *Escherichia coli* was included as a control in the study.

METHODS

Plasma jet. A non-thermal plasma source (microwave driven, 2.45 GHz) (Fig. 1) developed at the Leibniz Institute of Surface Modification was mounted on a computer controlled three-axis motion system to ensure reproducible time, distance and scanning parameters. Irradiation was carried out at a distance of 1.5 mm between plasma-jet nozzle and sample surface. The pulse width of the microwave was 5 µs at 300 W, and the process gas flow was $He/O_2/N_2$ at 2.0/1.2/1.5 l min^{-1}. The mean power was 2.5 W. Plasma treatment was carried out in a meander-like scanning mode with a line speed of 11, 16 or 30 mm s^{-1} and a step width of 0.1 mm. The temperature of the plasma jet on the surface of agar plates as well as on dentin slices was measured by means of infrared-camera thermoscopy (Optris PI; Optris). Temperature measurements were performed at room temperature with a thermal resolution of ± 0.1 °C at an optical frame of 160×120 pixels.

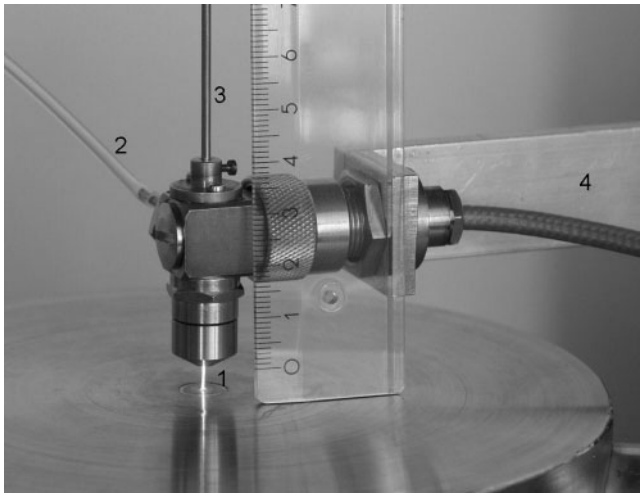

Fig. 1. Plasma source and plasma jet. 1, Plasma jet; 2 and 3, the gas supply of the plasma source consists of an outer (2) and an inner gas inlet (3); 4, the microwave to the plasma source.

Micro-organisms. The micro-organisms *E. coli* (XL1-Blue; Stratagene), *L. casei* (DSM 20011), *S. mutans* (DSM 20523) and *C. albicans* (DSM 1386) were cultivated overnight in liquid media (*E. coli* and *C. albicans*, in LB broth, *L. casei*, in MRS broth, *S. mutans*, in brain heart infusion (BHI) broth) (all from Sigma-Aldrich). Their concentration was determined by measurement of the OD_{600} and revealed 8.7 $\log_{10}$ c.f.u. ml^{-1} for *E. coli*, 8.7 $\log_{10}$ c.f.u. ml^{-1} for *L. casei* and 8.5 $\log_{10}$ c.f.u. ml^{-1} for *C. albicans*. The concentration of *S. mutans* was determined by plating tenfold dilution series on BHI agar and revealed 8.7 $\log_{10}$ c.f.u. ml^{-1}.

Agar plates and dentin slices. One-hundred microlitres of the four microbial suspensions were plated on (90 mm diameter) agar plates [*E. coli* on LB agar, *L. casei* on Rogosa agar, *S. mutans* on BHI agar (all from Sigma-Aldrich) and *C. albicans* on Sabouraud 4 % glucose agar (from Carl Roth)] and dried for 15 min at 37 °C. A density of approximately 6 $\log_{10}$ c.f.u. cm^{-2} resulted on the agar plates. The duration of the treatment of the agar plates was 33, 63 and 91 s in a meander-like scan of a surface area of 1 cm^2 resulting in irradiation times of 0.3, 0.6 and 0.9 s per mm^2, respectively. Agar plates were incubated for 48 h at 37 °C. The irradiated area was assessed in comparison to the untreated surrounding agar surface. Colonies were counted and inhibition zones were measured.

Dentin slices of 2 mm thickness with diameters of 8 to 10 mm were cut from the crowns of extracted human molars using a diamond rotating saw (Conrad Apparatebau). The exposed dentin was subjected to machine polishing (Metkon Gripo 2V; Metkon Instruments) with silicon-carbide grinding paper (Buehler) with decreasing grit size (grit P 120, 600, 800, 1200, 2500). Subsequently, the samples were cleaned ultrasonically for 15 min, autoclaved at 121 °C and stored in 0.9 % NaCl. Five microlitres of the four microbial suspensions were spotted on the centre of the dentin slices covering an area of approximately 20 mm^2. The bacteria were allowed to adhere in a humid atmosphere for 15 min until plasma treatment was carried out. Dentin slices were fixed on sterile glass plates by wax in a marked area of 10×10 mm and irradiated immediately. The area of 1 cm^2 was treated for 33, 63 and 91 s resulting in treatment times of 6 s (0.3 s mm^{-2}), 12 s (0.6 s mm^{-2}) and 18 s (0.9 s mm^{-2}) for the contaminated areas on the dentin slices, similar to the above described agar plate treatment. After plasma treatment dentin slices were vortexed for 1 min in 1 ml liquid media, and 100 µl of each of these samples were placed on agar plates in 10-fold dilution series. The agar plates were incubated for 48 h at 37 °C. After incubation, colonies on the agar plates were counted and the numbers of c.f.u. recovered from the dentin samples were calculated. The remaining 900 µl liquid medium containing one dentin slice was incubated as described above in order to assess the disinfective efficacy of the plasma treatment. Samples without microbial growth after 48 h of incubation were assumed to be completely disinfected.

Scanning electron microscopy (SEM). In order to visualize the destruction of the micro-organisms due to plasma-jet treatment dentin slices were contaminated with microbial suspensions and the contaminated areas were plasma treated as described above. After irradiation, the samples were fixed in glutaraldehyde (2.5 % in PBS; PAA Laboratories) for 2 h and rinsed 5 times for 10 min in PBS. Subsequently, the samples were dehydrated in an increasing series of ethanol (50–90 % 10 min each; 100 % 2×10 min). Finally, the samples were dried in 1,1,1,3,3,3-hexamethyl-disilazane (HMDS: Acros Organics). HMDS was vaporized at room temperature in a flowhood. Samples were mounted on SEM-sample stubs (Plano) and sputtered with gold/palladium (20 nm, Edwards Sputter Coater S 150B; Edwards). SEM assessment of the micro-organisms was performed with an ULTRA 55 scanning electron microscope (Carl Zeiss SMT). The dentin surfaces were scanned for bacterial remnants or intact bacteria.

Control samples. Negative control samples (agar plates and dentin slices) were irradiated with the pure process gases without plasma ignition for 0.9 s mm^{-2}. Additionally, areas of 1 cm^2 on agar plates were irradiated with the plasma jet for 0.9 s mm^{-2} followed by subsequent plating of the four microbial suspensions in order to detect possible changes of the agar that could result in microbial growth inhibition. In addition, dentin slices were contaminated with the four micro-organisms. Micro-organisms not subjected to plasma treatment were recovered from the dentin slices as described above for control purposes.

Agar-plated micro-organisms were incubated with chlorhexidine (CHX) solution (0.2 %) for 90 s as positive controls. The CHX was removed, and the surfaces washed three times with liquid media. Agar plates were incubated as described above and the number of c.f.u. was determined. Contaminated dentin slices were incubated with 10 µl CHX for 18 s, corresponding to 0.9 s mm^{-2} plasma treatment. The CHX was removed; the surface of the dentin slices was carefully rinsed with PBS and the dentin slices were further processed as described above.

For SEM, untreated dentin slices and those irradiated without plasma ignition (0.9 s mm^{-2}) were prepared as well.

Statistical analysis. Experiments were carried out five times, and the controls three times. All c.f.u. numbers were expressed as $\log_{10}$ values. The mean of the $\log_{10}$ values was computed together with 95 % confidence intervals (CIs).

RESULTS AND DISCUSSION

Temperature monitoring

During irradiation of the dentin slice by the plasma jet, the dentin surface temperature increased instantaneously and reached its maximum in the jet's centre within 5 s. A maximum temperature of 50.8 °C was measured on the dentin surface without movement of the plasma jet over the sample. No further increase in temperature was observed. The temperature decreased to room temperature at a distance of 5 mm around the centre of the jet. Movement of the plasma jet resulted in a decrease of the maximum surface temperature in the centre of the plasma jet to values of 39 to 44 °C. Lower temperatures were measured with increasing scan velocities. On the agar surface, the temperature of the plasma jet was 20 °C when unmoving and 14 to 20 °C under movement (Table 1).

Table 1. Temperatures measured on the surface of a dentin slice or agar plate without movement of the plasma jet, as well as under motion, with treatment times of 0.3, 0.6 or 0.9 s mm^{-2}

Values are the mean and SD of tenfold measurements.

Surface temp. (°C)	No movement	Movement (mm s^{-1})		
		11	16	30
Dentin slice	50.1 ± 0.6	43.6 ± 1.2	43.6 ± 1.3	39.1 ± 1.0
Agar plate	20.4 ± 1.1	19.5 ± 0.8	14.2 ± 0.7	14.8 ± 0.5

Plasma treatment of agar plates and dentin slices

The plasma treatment of the agar plates caused complete growth inhibition of *E. coli* and *L. casei* on the irradiated surface areas. Between one and four colonies were present on 30 % of the treated areas on agar plates inoculated with *C. albicans*. A time-dependent reduction of growth was observed on the irradiated surfaces for *S. mutans*. A reduction of colony density was observed after irradiation at 0.3 s mm^{-2} in comparison to untreated control areas. A mean of 1.3 $\log_{10}$ c.f.u. was present after 0.6 s mm^{-2}, whereas the c.f.u. number dropped to a mean lower than 1 $\log_{10}$ after 0.9 s mm^{-2} of irradiation (Fig. 2). Expanded inhibition zones surrounding the irradiated areas were observed. These inhibition zones were most distinctive for *L. casei*, whereas agar plates infected with *C. albicans* and *E. coli* demonstrated less widespread inhibition zones. Inhibition zones were smallest for *S. mutans*. The extent of the surrounding inhibition zones was correlated with the length of irradiation. Longer irradiation times resulted in extended inhibition zones (Table 2).

CHX treatment resulted in complete microbial growth inhibition on the agar. No inhibition of microbial growth was visible on the agar plates irradiated with only the process gases. Plasma treatment of the agar plates before microbial inoculation did not influence microbial growth (Table 2).

A mean of 2 $\log_{10}$ c.f.u. of *E. coli* and *L. casei* were recovered from dentin slices after plasma treatment for 0.3 s mm^{-2}. After 0.6 and 0.9 s mm^{-2} of treatment this number decreased to 1 $\log_{10}$ c.f.u. Independently of the irradiation time, a mean of 1 $\log_{10}$ c.f.u. was isolated for *C. albicans*. In contrast, a c.f.u. reduction dependent on the duration of irradiation was observed for *S. mutans*. Their mean numbers decreased from 3 $\log_{10}$ c.f.u. after 0.3 s

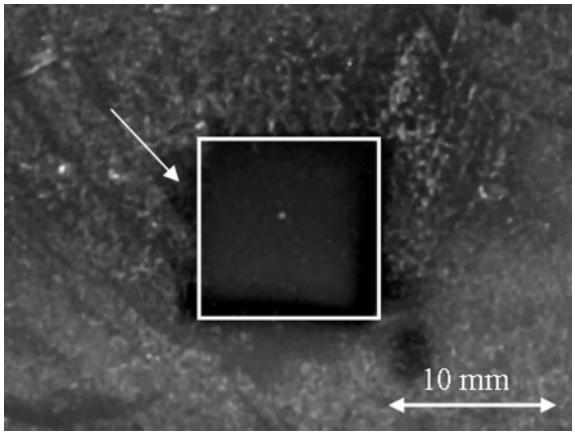

Fig. 2. Plasma treated (0.9 s mm^{-2}) area of 1 cm^2 (indicated by a white box) on an agar plate inoculated with *S. mutans* after 48 h incubation at 37 °C. The colony number in the treated area was 1 $\log_{10}$ c.f.u. Also an expanded growth inhibition zone of 1 to 2 mm surrounding the irradiation area is visible (indicated by an arrow).

Table 2. Effect of the plasma treatment (P) on agar plates inoculated with 100 µl of 8.7 $\log_{10}$ c.f.u. of the micro-organisms *E. coli*, *L. casei*, *S. mutans* and *C. albicans*, and results of CHX application, plasma treatment of agar before microbial plating (PB) and application of process gases without plasma ignition (G)

All treatments were carried out with treatment times of 0.3, 0.6 or 0.9 s mm^{-2} on agar areas of 1 cm^2, and all agar plates were incubated at 37 °C for 48 h. The mean number of colonies (mean $\log_{10}$ c.f.u.) in the treated areas ($n \pm 95\%$ CI) and mean expanded inhibition zones (expIZ) around the treated areas (mm ± SD) are given. NGI, No growth inhibition visible; NC, no colonies detectable.

Method	*E. coli*		*L. casei*		*S. mutans*		*C. albicans*	
	$\log_{10}$ c.f.u.	expIZ	$\log_{10}$ c.f.u.	expIZ	$\log_{10}$ c.f.u. (CI)	expIZ	$\log_{10}$ c.f.u. (CI)	expIZ
P (0.3 s mm^{-2})	NC	0.5 ± 0.8	NC	17 ± 2.7	2.9 (2.8–3)	0	0.1 (0–0.3)	0.5 ± 1.2
P (0.6 s mm^{-2})	NC	1.7 ± 0.9	NC	22 ± 4.5	1.3 (1–1.6)	0	0.1 (0–0.3)	3.5 ± 4.1
P (0.9 s mm^{-2})	NC	2.0 ± 1.0	NC	23 ± 4.5	0.3 (0–0.6)	3 ± 2.7	0.1 (0–0.3)	7 ± 4.5
CHX	NC	–	NC	–	NC	–	NC	–
PB	NGI	–	NGI	–	NGI	–	NGI	–
G	NGI	–	NGI	–	NGI	–	NGI	–

mm^{-2} to 2 $\log_{10}$ c.f.u. after 0.9 s mm^{-2} (Fig. 3). In correlation to longer plasma-jet irradiation times increasing numbers of dentin slices were completely disinfected, which was assessed by incubation of the dentin slices in liquid medium for 48 h. The plasma jet was most effective against *C. albicans* and *E. coli* after irradiation of 0.6 and 0.9 s mm^{-2}. No microbial growth was observed in up to four of five liquid samples. Only one of five dentin slices inoculated with *S. mutans* were found to be completely disinfected. CHX treatment resulted in comparable reductions of microbes on dentin slices. No c.f.u. of *C. albicans* were isolated from the dentin slices and the according liquid cultures were free of microbial growth. In the samples infected with *L. casei* 1 $\log_{10}$ c.f.u. were found, for *E. coli* between 1 and 2 $\log_{10}$ c.f.u. were found and for *S. mutans* 2 $\log_{10}$ c.f.u. were detected in one or two not

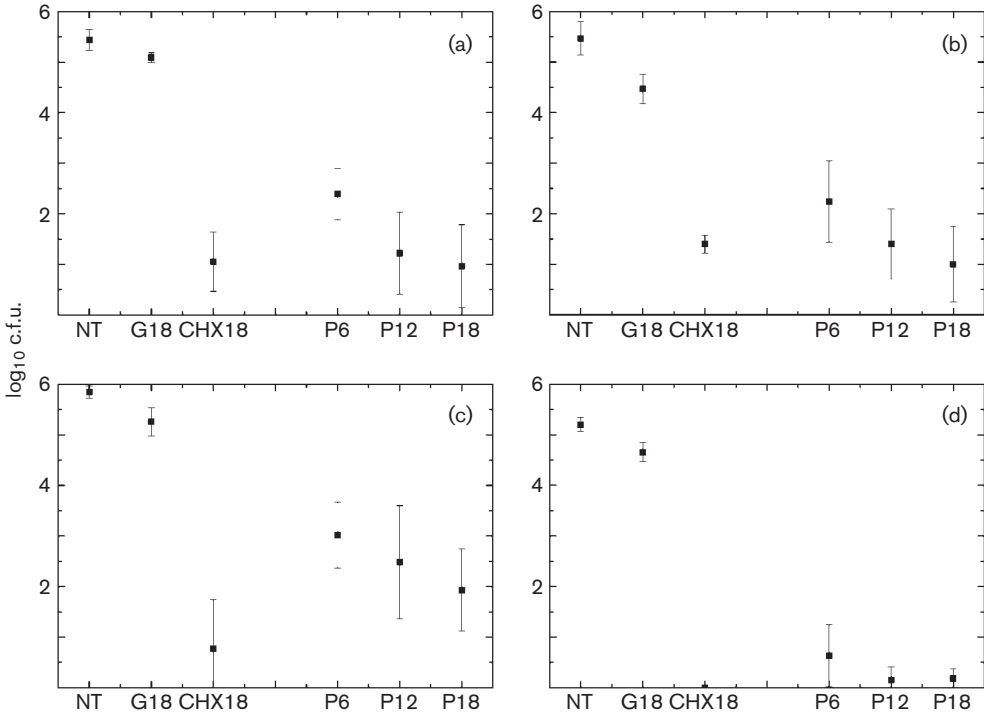

Fig. 3. Killing of adherent micro-organisms on dentin slices with a cold atmospheric plasma jet. The c.f.u. values are given in $\log_{10}$ c.f.u. of *E. coli* (a), *L. casei* (b), *S. mutans* (c) and *C. albicans* (d) recovered after plasma treatment. P6, P12, P18, indicate exposure to the plasma jet for 6, 12 or 18 s (0.3, 0.6 or 0.9 s mm^{-2}, respectively); G18, gas irradiation without plasma ignition for 18 s (0.9 s mm^{-2}); CHX18, exposure to CHX for 18 s; NT, no treatment. Mean values with 95% CIs are the mean of a fivefold determination for plasma-jet-treated samples and of triplicates for the controls.

completely disinfected dentin slices each. Between 4 and 5 $\log_{10}$ c.f.u. were recovered from the samples irradiated only with the process gases, and more than 5 $\log_{10}$ c.f.u. were isolated from the untreated controls. All liquid samples showed distinct microbial growth (Table 3, Fig. 3).

SEM analysis

Plasma treatment induced distinct micromorphological alterations of adherent micro-organisms. Only microbial remnants, cells with pores in their cell walls and a low number of intact micro-organisms were detected on the dentin surfaces after plasma-jet treatment independently of the application time (Fig. 4a, b, c, d). However, no complete removal of adherent micro-organisms from the dentin surfaces was observed. In contrast, intact micro-organisms dominated on dentin samples treated without plasma ignition or on untreated dentin surfaces, and no reduction of the number of adherent micro-organisms could be observed.

The present investigation demonstrated that a cold atmospheric plasma jet is suitable for substantially reducing oral micro-organisms on agar plates or adherent to dentin slices, as well as the bacterium *E. coli*. These results confirm previously published data on the efficacy of cold plasma jets for killing and removal of planktonic or adherent micro-organisms (Lee *et al.*, 2006; Sladek *et al.*, 2007; Hong *et al.*, 2009). Parts of agar plates contaminated with a density of 6 $\log_{10}$ c.f.u. cm^{-2} were nearly completely disinfected by plasma-jet treatment in the present study. Disinfection on directly irradiated areas was achieved at the shortest treatment time for *E. coli*, *L. casei* and *C. albicans*. For *S. mutans*, however, longer treatment times were necessary. Surrounding the directly irradiated areas of 1 cm^2, additional, irregular inhibition zones were recorded for all the micro-organisms, which expanded after longer plasma treatment. The appearance of inhibition zones outside of the directly irradiated area suggests an antimicrobial effect of the plasma jet beyond the directly treated areas. This extension of the inhibition zones might be caused by reactive oxygen species produced by the plasma jet (Hong *et al.*, 2009).

A substantial reduction of approximately 4 $\log_{10}$ intervals was observed on the dentin slices for the bacteria *E. coli*, *L. casei* and *S. mutans*, and even slightly higher killing rates for *C. albicans*, after plasma-jet treatment. The destructive power of the plasma jet against the four micro-organisms tested in the present study on dentin slices was visualized by SEM micrographs. On some of the dentin slabs, the adherent micro-organisms were completely destroyed. However, killing of the microbes by plasma jet on the dentin slices was not as effective as treatment of agar plates. This might be caused by the penetration of the micro-organisms into the dentin tubules before plasma treatment during sample drying. Additionally, micro-organisms located at the dentin tubules orifices might be transported into the inner dentin by the gas pressure of the plasma jet.

Distinct differences in the susceptibilities of the four investigated micro-organisms to cold plasma irradiation were detected in the present study. *S. mutans* revealed the strongest resistance to plasma-jet irradiation on agar plates, as well as on dentin slices. *S. mutans* is a Gram-positive, non-motile facultative anaerobe and small in size compared with the other investigated micro-organisms. Its relative resistance to plasma-jet treatment may be explained by its small size. Presumably it was transported into the dentin tubules due to the gas pressure of the plasma jet. Another explanation could be the structure of the cell wall of *S. mutans*, which is composed of highly cross-linked murein. The particular influence of reactive oxygen species or of the temperature of the plasma jet cannot be elucidated from this study. The killing rate on the dentin slices increased during irradiation of 0.9 s mm^{-2} with a related temperature of 44 °C, compared to an irradiation time of 0.3 s mm^{-2} with maximum temperature lower than 40 °C. The same correlation was observed for microbes on agar plates combined with expanding inhibition zones at longer irradiation times. In conclusion of these results, direct and indirect co-effects of the plasma jet caused its anti-microbial efficacy.

Undoubtedly, additional disinfecting effects up to a complete sterilization of surfaces may be achieved by a plasma jet at higher performances correlated with higher temperatures and extended treatment times. However, there are certain limitations concerning these parameters for the application of the plasma-jet technology under oral conditions, and especially when dentin is treated. The combination of gas stream, chemical radicals and electrons can cause severe desiccation of dentin resulting in cracks and damage of odontoblasts or pulp tissue. For these reasons, we chose experimental conditions simulating

Table 3. Number of completely disinfected dentin slices inoculated with 6.4 $\log_{10}$ c.f.u. after subsequent plasma-jet treatment (P) (exposure to plasma jet at 0.3, 0.6, 0.9 s mm^{-2}), CHX treatment for 18 s (CHX), application of process gases without plasma ignition for 18 s (G) or no treatment (NT)

Liquid medium containing dentin slices (n=5 for plasma treatment, n=3 for controls) was incubated for 48 h at 37 °C to detect bacterial growth.

Method	*E. coli*	*L. casei*	*S. mutans*	*C. albicans*
P (0.3 s mm^{-2})	0/5	1/5	0/5	2/5
P (0.6 s mm^{-2})	2/5	1/5	1/5	4/5
P (0.9 s mm^{-2})	3/5	2/5	1/5	3/5
CHX (18 s)	2/3	1/3	2/3	3/3
G (18 s)	0/3	0/3	0/3	0/3
NT	0/3	0/3	0/3	0/3

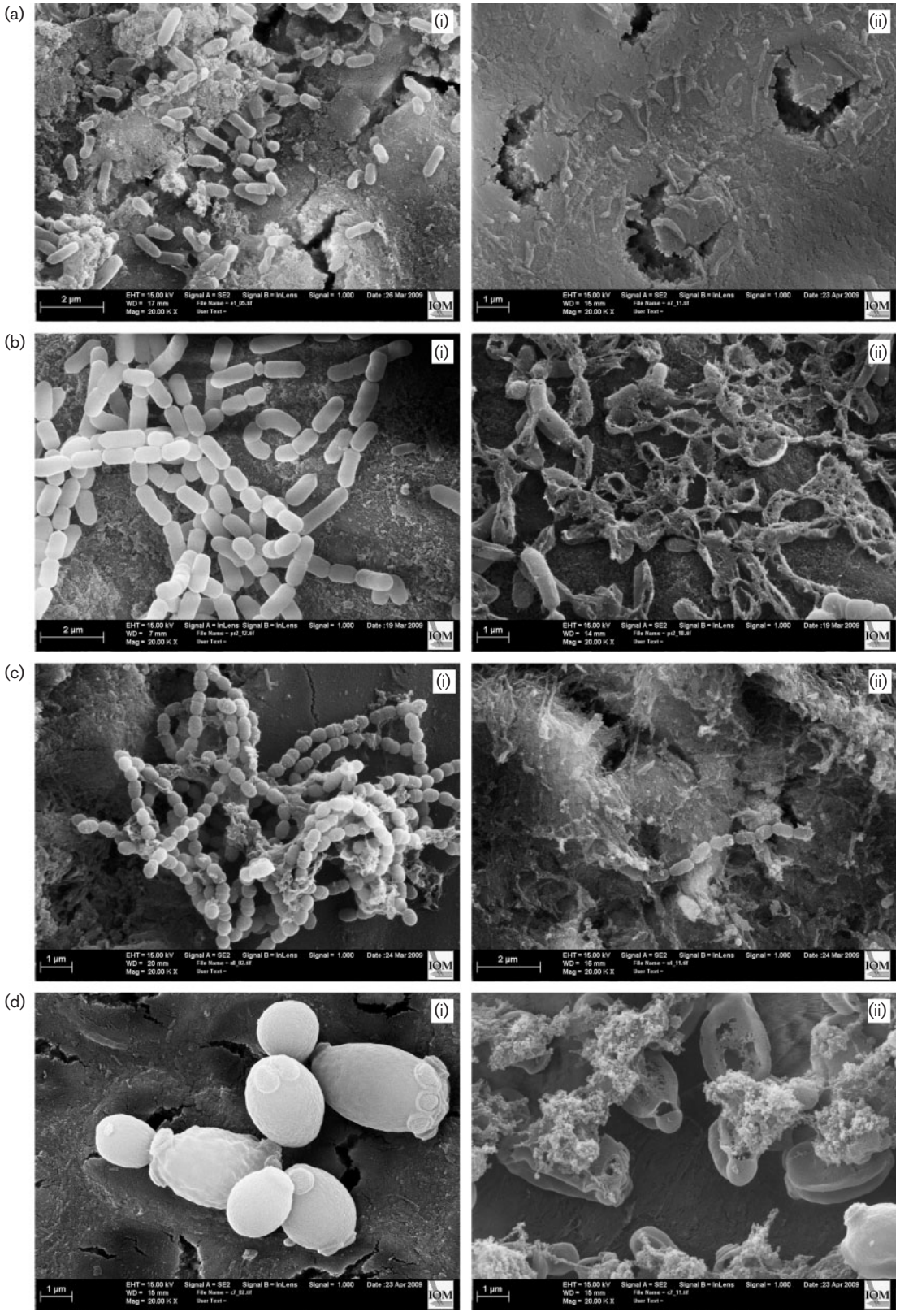

Fig. 4. Representative scanning electron micrographs of adherent micro-organisms on dentin slices: (i) controls without plasma treatment and (ii) after plasma treatment (0.9 s mm^{-2}). (a) *E. coli*, (b) *L. casei*, (c) *S. mutans* and (d) *C. albicans*. Magnification ×20 000.

realistic oral dental conditions to prevent heat-induced damage to the dentin and pulp. The plasma jet was applied under meander-like motion to prevent temperature increase and dehydration of the dentin. Application times of 5 s are common in dentistry, e. g. for air drying of cavities. Temperature rises of 5 °C in the pulp chamber have been reported for the use of light-curing units currently used in restorative dentistry (Hannig & Bott, 1999; Miletic *et al.*, 2009). Concerning these aspects, the plasma jet used in the present study appeared to be applicable to dental surfaces. An improvement of the plasma jet might be achieved by further optimization of the mixture of noble and reactive gases, as well as flow conditions by modification of the plasma source. These aspects are currently under investigation.

Despite all limitations shown in the present study, plasma-jet applications are of great interest for dentistry. The technology offers the benefits that were expected from ozone treatment but that so far have not been proven (Baysan & Beighton, 2007). The results of our investigation emphasize the potential of cold plasma jets for antimicrobial applications in dentistry. In particular, the disinfection of infected dentin after caries removal or of shallow root caries lesions by plasma-jet treatment might be an interesting option.

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