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Ex vivo gingival-biofilm consortia

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ABSTRACT

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Aims: To develop a protocol for harvesting *ex vivo* samples of gingival-biofilm consortia and to investigate their basic characteristics.

Methods and Results: Gingival epithelial cells with attached biofilm were collected from healthy subjects by taking a smear. The bacterial viability was estimated via the alteration of the membrane permeability and metabolic activity via the double/single-stranded nucleic acid ratio using a confocal laser-scanning microscope. Morphological analysis was performed by scanning and transmission electron microscopy. Additionally, microbiological estimations were made. The electron microscopy revealed fimbriae-mediated adhesion and the formation of a biofilm matrix. Most bacteria were viable and had a high metabolic activity.

Conclusions: The presented study offers an easy to follow approach for harvesting samples of gingival-biofilm consortia. The latter differs considerably from the supragingival plaque in viability and zonal distribution. Related to free-living and *in vitro*-grown biofilms, the gingiva-associated biofilm revealed an atypically high metabolic activity.

Significance and Impact of the Study: Biofilm fragments should possess the basic features of the entire gingiva-associated biofilm; which as yet cannot be simulated *in vitro*. Thus, samples of *ex vivo* gingival-biofilm consortia can be used to investigate the resistance of oral biofilms against antibiotics and biocides.

Keywords: biofilm immaturity, biofilm matrix, biofilm sampling, metabolic activity, viability.

INTRODUCTION

Both most spread diseases in man, caries and periodontitis, are caused by biofilms denoted as supra- and subgingival plaque. The biofilm is not a simple aggregation of bacteria, but a complex microbial community, which is characterized by features not established in planktonic bacteria: primitive homeostasis and metabolic cooperativity (Costerton *et al.* 1995), cell-to-cell signalling (Davies *et al.* 1998), 500 times higher resistance to antibiotics (Hoyle and Costerton 1991; Brown *et al.* 1995; Costerton *et al.* 1995), dissemination ability and resistance to the host humoral as well cellular defence (Peters *et al.* 1981; Kharazmi 1991). The common view is that planktonic bacteria must be exposed to deleterious agents to develop a protected mode of growth, i.e. biofilm that allows survival in a hostile environment (Costerton *et al.* 1999). Hence, the host's effects are the crucial factor, which as yet cannot be simulated *in vitro*, but essentially determines the character of the oral biofilm. Biofilm bacteria express another phenotype (Loo *et al.* 2000; Donlan and Costerton 2002) than the planktonic ones. Consequently, a small piece of oral biofilm should possess the basic features of the entire biofilm (Gilbert and Brown

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1995; Hoiby *et al.* 1995). *In situ* biofilm growth on enamel pieces attached to the dentition of volunteers enables harvesting of supragingival plaque samples (Zaura-Arite *et al.* 2001; Watson *et al.* 2004). Because of the physiological gingival exfoliation, gingival cell samples (i.e. microbiopsies) with attached biofilms could be also used for collection of *in vivo* biofilms without harming the donating subjects or damaging the biofilm structure. However, up to now, no methods for harvesting *ex vivo* samples of the gingival-biofilm consortia, e.g. in order to investigate susceptibility of oral biofilms to biocides, have been described.

The aim of this work was to create an approach enabling the collection of undistributed fragments of naturally formed gingival-biofilm consortia and to investigate their basic characteristics.

MATERIALS AND METHODS

Gingival epithelial cell collection

Superficial epithelial cells form the vestibular site of the attached gingiva of the upper canine and incisor teeth were collected from 10 donors for each analysis. All cell donors were healthy and non-smokers. Exclusion criteria for the donors were: systemic diseases related to subacute bacterial endocarditis prophylaxis, diabetes mellitus, any antibiotic, chlorhexidine, steroid or radiotherapy in the last 6 months. The study protocol was approved by the local ethics committee and informed written consent was obtained from all donors. The last teeth brushing by the donors took place between 8:00 h and 9:00 h. After renunciation of teeth cleaning before going to bed, the sampling took place on the next day between 8:00 h and 9:00 h, previous to the donors' teeth brushing or breakfast. Thus, the age of the expected biofilm should be nearly up to 24 h. A smear was taken from *c.* 10 mm^2 of the vestibular attached gingiva with a swab and immediately suspended in 2 ml of saline. For the microbiological estimation, a smear was taken from *c.* 5 mm^2 of the vestibular attached gingiva with swabs (Copan Transystem®; Copan Italia, Brescia, Italy).

Transmission electron microscopy

As the fimbriae and biofilm matrix are highly glycosylated, only special staining techniques are useful to demonstrate bacterial adhesion via transmission electron microscopy (TEM) (Vitkov *et al.* 2001, 2002). One millilitre of cell suspension was fixed with 1·2% glutaraldehyde and 0·05% Ruthenium Red (RR; Sigma-Aldrich, Steinheim, Germany) buffered at pH 6·5 with 0·1 mol l^{-1} cacodylate (sodium cacodylate trihydrate; Merck-Schuchardt, Hohenbrunn, Germany) for 2 h at room temperature. Subsequently these fixed epithelial cells were centrifuged at 3000 *g* for 5 min and resuspended into 0·1 mol l^{-1} cacodylate buffer (pH 6·5) three times in succession. After a subsequent centrifugation at 2000 *g* for 5 min, postfixation was performed with 1% osmium tetroxide (OsO_4; Serva Feinbiochemica GmbH & Co., Heidelberg, Germany) and 0·05% RR (buffered at pH 6·5 with 0·1 mol l^{-1} sodium cacodylate) for 2 h at room temperature. The fixed epithelial cells were centrifuged at 2000 *g* for 5 min and resuspended into 0·1 mol l^{-1} cacodylate buffer (pH 6·5) three times in succession. The samples were routinely embedded in Epon 812 (Serva Feinbiochemica, Heidelberg, Germany). Ultrathin sections were cut on an ultra-microtome (Reichert Ultracut S, Optische Werke C, Reichert, Vienna, Austria), contrasted with LKB 2168 Ultrostainer (LKB Produkter AB, Bromma, Sweden) and examined in a transmission electron microscope (LEO EM 910; LEO Elektronenmikroskopie Ltd, Oberkochen, Germany) operating at 80 kV.

Scanning electron microscopy

One-tenth millilitre of the cell suspension diluted in 3 ml of saline was filtered through a 5-μm filter (Minisart NML; Sartorius, Göttingen, Germany) and afterwards washed with 10 ml of saline. Subsequently the filters were fixed with 1·2% glutaraldehyde (buffered at pH 6·5 with 0·1 mol l^{-1} sodium cacodylate) for 2 h at room temperature. Postfixation of these control samples was performed with 1% OsO_4 (buffered at pH 6·5 with 0·1 mol l^{-1} sodium cacodylate) for 2 h. The postfixed filters were dehydrated in ascending series of ethyl alcohol, critical-point dried in a drying equipment CDP 030 (BAL-TEC, Balzers, Liechtenstein) and subsequently sputtered with gold (*c.* 10 nm). The specimens were examined in an environmental scanning electron microscope (ESEM XL30; FEI Company, Philips, Eindhoven, the Netherlands) operating at 20 kV.

RNA–DNA relationship

Acridine orange is a metachromatic intercalator used extensively in histochemistry to differentiate double- from single-stranded nucleic acid by the emission of green and red fluorescence, respectively, under short-wave light (McFeters *et al.* 1991; Bancroft and Cook 1994; Rychlik *et al.* 2000; Lauretti *et al.* 2003). To analyse the RNA–DNA relationship in the oral bacteria, 0·1 ml of the cell suspension was dropped upon a clean glass microscope slide and allowed to dry. No fixation was made. The microscope slide was rinsed with 1% acetic acid for a few seconds and subsequently rinsed twice with bi-distilled water for over 1 min. Staining was performed with 0·01% acridine orange in phosphate buffer at pH 7·0 for 3 min. The slide was rinsed with phosphate buffer at pH 7·0 for 1 min. Subsequently a differentiation with 0·1 mol l^{-1} calcium chloride

was made for 30 s. Washing with phosphate buffer at pH 7·0 for 1 min and thereafter with bidistilled water for over 1 min completed the staining procedure. All specimens were examined in the confocal laser scanning microscope (CLSM) (Zeiss LSM 510 Meta UV; Carl Zeiss GmbH, Vienna, Austria). The staining of the nuclei of the exfoliated human mucosal epithelial cells served as controls. The exfoliated mucosal epithelial nuclei are characterized by the presence of DNA and the absence of RNA, which indicated an absence of metabolic activity (Bancroft and Cook 1994). The excitation and emission for RNA was 458 and 560 nm; the excitation and emission for DNA was 488 and 525 nm respectively.

Bacterial viability

The viability kit (Live/Dead BacLight^{TM} kit L-13152; Molecular Probes Inc., Eugene, OR, USA) has been developed to differentiate between live and dead bacteria based on plasma membrane permeability. This kit comprises two fluorescent nucleic acid stains: SYTO9 and propidium iodide. SYTO9 (excitation 488 nm and emission 525 nm) penetrates both viable and nonviable bacteria, while propidium iodide (excitation 488 nm and emission 560 nm) penetrates bacteria with damaged plasma membranes only, quenching the green SYTO9 fluorescence. Therefore, bacteria incubated in the presence of both stains simultaneously will fluoresce either green (i.e. viable) or red (i.e. dead), depending on the intact or damaged membrane permeability.

A 0·1-ml aliquot from the cell suspension and the same volume of the double stock solution of the viability kit Live/Dead were simultaneously mixed on a glass microscope slide. The cells were allowed to incubate with the dyes in the dark for 15 min at room temperature before the analysis. The assays do not require washing. All specimens were examined in the same CLSM. The thermally killed bacteria (heated at 100°C for 15 min) served as controls.

Microbiological estimation

For aerobic culturing, each swab was plated on Columbia sheep blood agar (Biotest AG, Dreieich, Germany), Columbia CNA agar (Becton Dickinson, Franklin Lakes, NJ, USA) and MacConkey agar (Biotest AG) and incubated at 37°C for 18 h. For anaerobic culturing, the swabs were plated on Schaedler agar (Biotest AG) and Schaedler KV agar (Becton Dickinson) and incubated at 37°C for 7 days under anaerobic conditions. Microbiological differentiation was performed as follows:

Viridans streptococci were identified by colony morphology, alpha-haemolysis and the use of a commercially available optochin test. Nonhaemolytic streptococci were identified by colony morphology, lack of haemolysis and the optochin test. Enterococci were identified by colony morphology and antibiotic susceptibility. Staphylococci were identified by colony morphology, colony colour and testing of coagulase activity with Staphaurex (Abbott Laboratories, Abbott Park, IL, USA). *Hemophilus* spp. were identified by colony morphology, Gram staining and biochemical identification procedures API NH (Biomerieux SA, Marcy l'Etoile, France). *Neisseria* spp. were identified by colony morphology, Gram staining and positive oxidase reaction. Gram-negative bacilli (*Escherichia coli*, *Klebsiella*, *Enterobacter*, etc.) as well as *Lactobacillus casei* were identified by the use of a biochemical identification system (Crystal System; Becton Dickinson).

For semiquantitative estimation of the bacterial density the following counting procedure was applied: negative bacterial growth was characterized by one to four colonies per agar plate; low bacterial density was defined by the growth of at least five colonies per agar plate; moderate bacterial density was defined by the growth of over half of the agar plate; high bacterial density was defined by confluent bacterial growth.

RESULTS

Electron microscopy

The TEM revealed multilayers of bacteria attached to the outer surface of the gingival epithelial cells and to each other (Fig. 1a,b). RR staining visualized both the individual bacterial glycocalyces and a sparse amount of fibrillar matrix substances as a morphological base of the biofilm (Fig. 1b). Many bacterial cells lay deeply invaginated on the epithelial cells, but no complete internalization could be observed. In addition, bacterial division within the biofilm was frequently evident. The average thickness of the biofilm was *c.* 10 μm.

Scanning electron microscopy analysis revealed a formation of bacterial biofilm on the outer surface of the gingival epithelial cells (Fig. 1c,d).

RNA–DNA relationship, bacterial viability and biofilm architecture

The bacteria showed high quantity of RNA indicating strongly metabolic activity. By contrast, the nuclei of the exfoliated epithelial cells lacked RNA completely and exhibited the presence of DNA only (Fig. 2a–d). Thus, the specific staining of the nuclei of the exfoliated epithelial cells was used to control the validity of the RNA/DNA staining in the bacteria.

The staining with Live/Dead reveals that most of the bacterial cells in the biofilm on the epithelial cells were viable (Fig. 2e–h). The double staining of the epithelial nuclei indicates the transition to cell death. Randomized

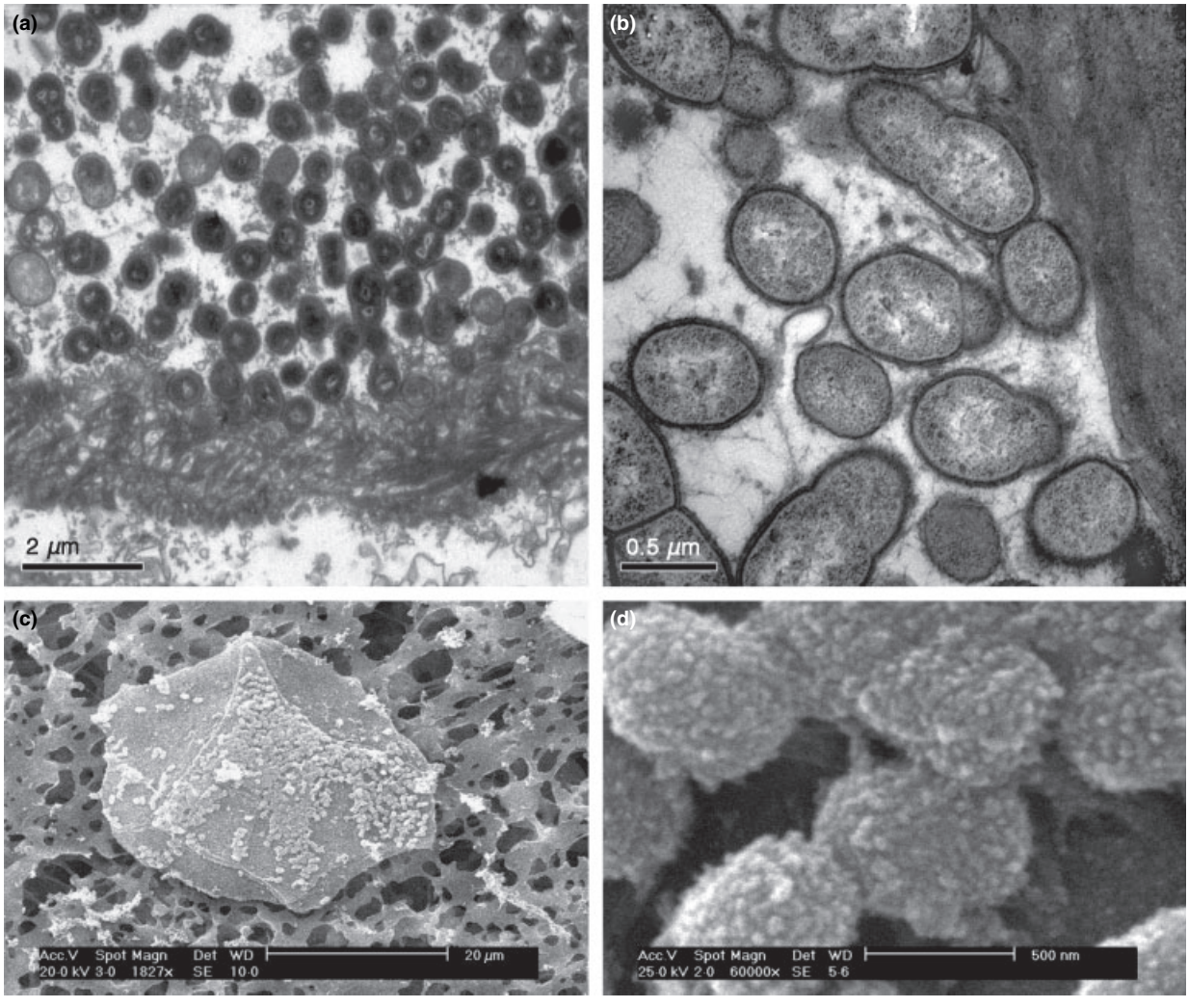

Fig. 1 (a) Transmission electron microscopy: a multitude of bacteria attached to the outer surface of the gingiva cells forming a multilayer. The bacterial glycocalyx consists of a multitude of fimbriae (pili), which are long, thin, filamentous, multimeric macromolecules attached on the outer bacterial surface, ending with an adhesin. The bacterial adhesions bind selectively receptors expressed on the host surface. Bacteria adjacent to the epithelial cell membrane are invaginated in it. All bacterial laying in the membrane invaginations are coated with glycocalyx. (b) Transmission electron microscopy: high magnification. Sparse biofilm matrix (fine fibrils connecting the bacteria) is evident in the intercellular spaces of the biofilm. Some bacteria are in the process of division. (c) Scanning electron microscopy: an epithelial cell trapped on a 5-μm filter. The outer cell surface is covered by a multitude of bacteria. The biofilm cover pattern of the epithelial cell suggests that the biofilm may occupy vast gingival surface. (d) Scanning electron microscopy: high magnification. Bacteria on the top of the biofilm reveal 'granulated' surface, as they are covered by glycocalyx and partly by the biofilm matrix. Hence, the bacteria appear to be in direct contact with each other. These highly hydrated substances partly shrank because of the exsiccation procedure

space distribution of the dead bacteria was observed (Fig. 3). The thermally pretreated controls at 100°C for 10 min either on or outside oral epithelial cells revealed only read-fluorescing, i.e. dead bacteria. The average thickness of the biofilm was *c.* 10 μm (Fig. 3). The surface of the biofilm fragments corresponded to the surface of an epithelial cell. Additionally, a multitude of dispersed single bacteria and tiny biofilm fragments were also observed.

Microbiological estimation

The predominant bacteria found in all subjects were viridans streptococci with moderate or high bacterial density. In addition, *Neisseria* spp. with low or moderate bacterial density were estimated in some subjects. The colony forming units achieved from the biofilm samples are shown in Fig. 4.

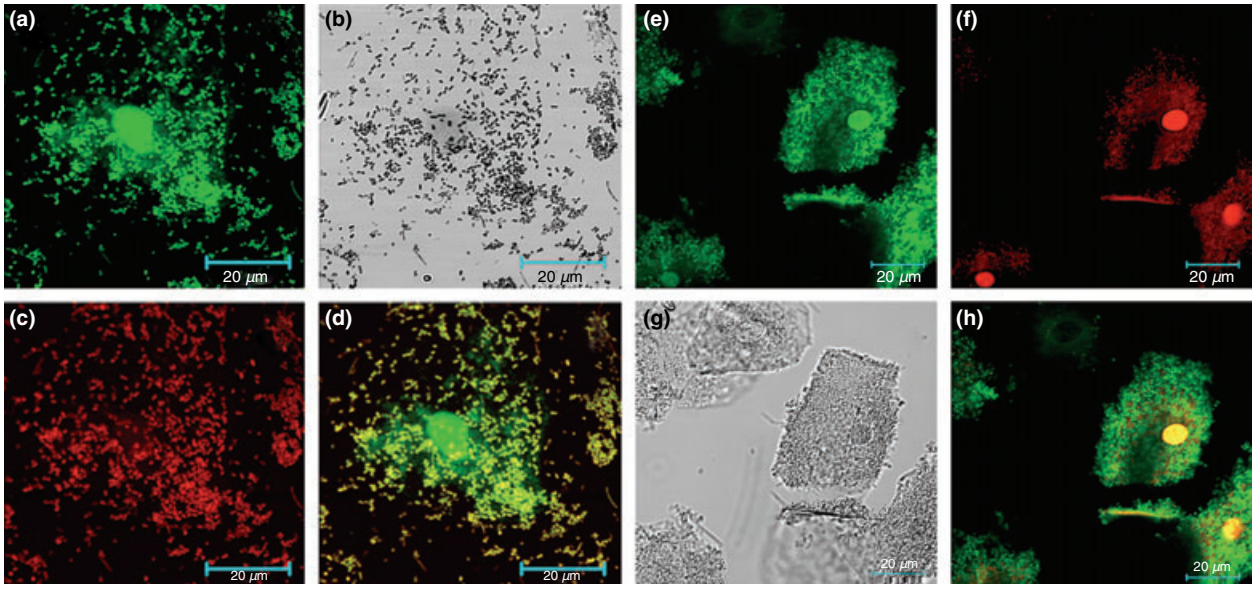

Fig. 2 Confocal laser scanning microscope: (a) acridine orange staining, 525-nm emission. The bacteria and the nucleus of the epithelial cell fluoresce green indicating the presence of DNA; (b) phase contrast image of the same object. The nucleus of the epithelial cell is visible via the slightly different diffraction; (c) the same object as in (a). Acridine orange staining, 560-nm emission. The red fluorescence of the bacterial cell indicates the presence of high quantity of RNA. On the contrary, the nucleus of the epithelial cell does not fluoresce indicating the complete lack of RNA; (d) merged image of (a) and (c). The nucleus is green but the bacterial cells appear yellow-orange – a sign of metabolic activity; (e) SYTO9 staining. Only the vital bacteria being in the focus plane are stained. The epithelial cell nuclei are stained too; (f) propidium iodide staining of the same object. A small quantity of dead bacteria and the epithelial cell nuclei in the focus plane are stained; (g) phase contrast image. The same object as observed in (e) and (f). The LM focus plane is wider and more details become evident; (h) merged images of (e) and (f). The viable bacteria are vastly prevailing. The exfoliated epithelial cell nuclei are stained with both SYTO9 and propidium iodide

DISCUSSION

The morphological key hallmark of the biofilm is the matrix – a highly glycosylated three-dimensional net-like skeleton of the biofilm. The matrix of the gingiva-associated biofilm was very loose, with relatively large intracellular spaces. The weak bacterial binding within the soft tissue biofilm may result in an increase of dissemination ability. The lack of bacterial internalization could be a consequence either of a mechanical retention by the biofilm matrix, which may hinder the engulfment of solitary bacteria or the apoptosis of the epithelial cells bearing the biofilm. However, the invagination of solitary bacteria is an indication of their pathogenicity, as the invagination is a preliminary stage of internalization. The balance in the host–biofilm interactions is probably kept by a clearance via apoptosis and subsequent exfoliation. A commensal way of life of the gingiva-associated biofilm is facilitated by two circumstances: (i) the ability of some indigenous oral bacteria to utilize saliva as their sole nutrient source and (ii) the nutrition mutualism enabling other bacteria to grow (Palmer *et al.* 2001). In addition, the gingival-biofilm consortia may serve as a breeding ground for periodontal pathogens and caries implicated bacteria. Thus, anaerobes as *Porphyromonas gingivalis* in association with *Fusobacterium nucleatum* in biofilm can survive oxygen concentrations, which are toxic for individual anaerobe bacteria (Bradshaw *et al.* 1997, 1998; Diaz *et al.* 2002).

Bacterial metabolism in the biofilm is often lowered and is supposed to be a significant factor for the increased biofilm resistance against antibiotics and bactericides (Costerton *et al.* 1999; Xu *et al.* 2000). The linear relationship between the relative RNA content and growth rate in bacteria has been documented for a number of species (Schaechter *et al.* 1958; Neidhardt and Magasanik 1959; Gausing 1977; Bremer and Dennis 1987). This relationship can also be visualized microscopically. The ability of acridine orange to bind with bacterial RNA and DNA and to emit red (in single-stranded nucleic acid) and green (in double-stranded nucleic acid) fluorescence, respectively, is well known (Bruno *et al.* 1996) and routinely used to analyse the bacterial metabolism (McFeters *et al.* 1991; Rychlik *et al.* 2000), as bacteria growing actively could be distinguished from cells in the stationary phase. Cells in the log phase emit red fluorescence, whereas those in the stationary phase emit a green one. This acridine orange ability has also been used to analyse the bacterial metabolism in planktonic cells (McFeters *et al.* 1991; Rychlik *et al.* 2000) and in biofilms

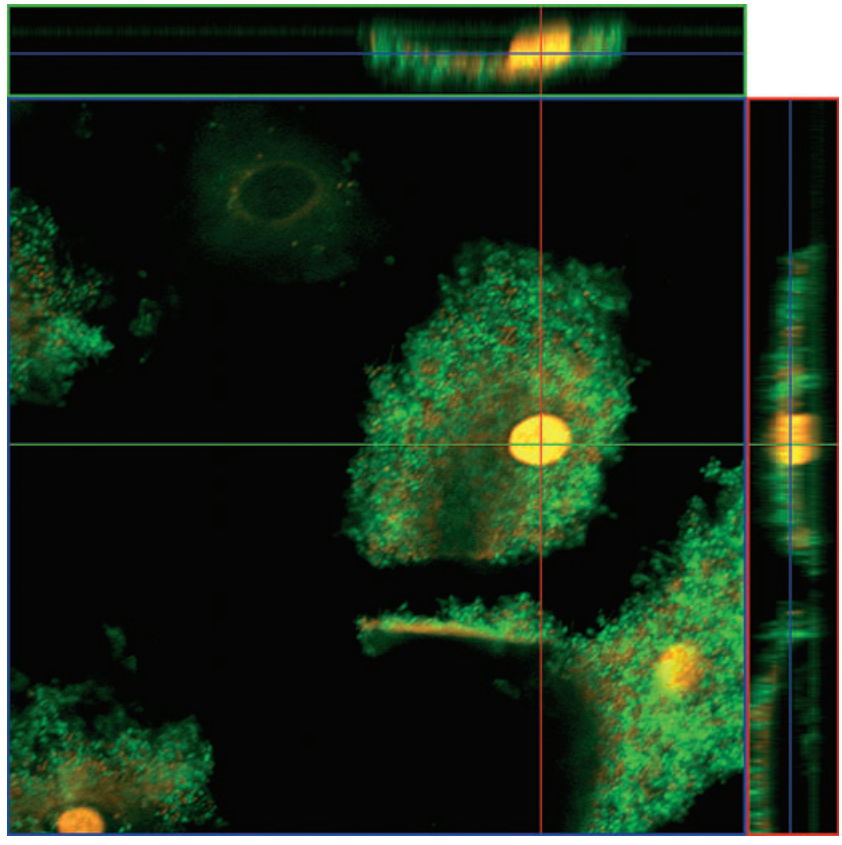

Fig. 3 Confocal laser scanning microscope: the central figure depicts the merged image of two nucleated epithelial cells and the biofilm on them. On the right the *z* perspective runs through the nucleus of the left epithelial cell and at the top the *x* perspective runs through the same nucleus. The few dead bacteria are randomly distributed within the biofilm

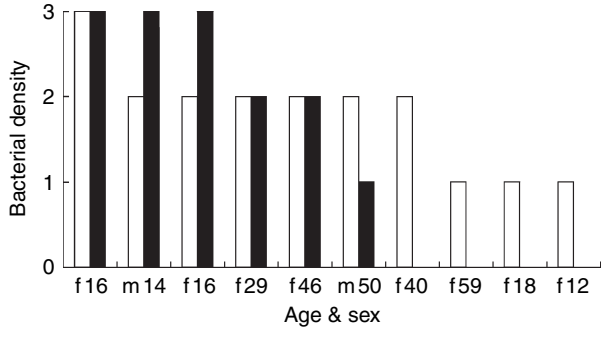

Fig. 4 Bacterial species and bacterial density recovered from smears taken from the attached gingiva of volunteers. The ordinate represents the bacterial density: 1, low; 2, moderate; 3, high bacterial density. Volunteers' sex and age are shown on the abscissa (f, female; m, male). □, Streptococci; ■, *Neisseria* spp.

(Xu *et al.* 2000). The gingiva-associated biofilm showed very high quantity of RNA in the biofilm cells that provides evidence for high metabolic activity. However, the lack of comparable metabolic investigations concerning naturally formed oral biofilms restricts the evaluation of this finding. Related to free-living and *in vitro*-grown biofilms, this could be a sign of biofilm immaturity, despite the multilayer structure and developed matrix. The frequent bacterial division and the very low numbers of dead bacteria in the gingival-biofilm consortia compared with the dental plaque (Zaura-Arite *et al.* 2001) confirm this statement. The biofilm immaturity might be explained with the relatively short period of biofilm development of no more than 12–24 h (in fact limited by the exfoliation) and an excess of nutrients from saliva (Palmer *et al.* 2001), however, this requires further investigations.

Contrary to the supragingival plaque, some aspects of the host–parasite relationship (e.g. bacterial invagination and a possible internalization) can be studied by means of *ex vivo* gingival-biofilm consortia. The gingiva-associated biofilm differs considerably from the supragingival plaque in viability and zonal distribution. These differences might also result in a different bactericidal susceptibility. In addition, the metabolic activities of other oral biofilms may be studied via the double/single-stranded nucleic acid ratio. Thus, a possible relationship between metabolic activity and bactericidal susceptibility in oral biofilms could be investigated.

The presented biofilm sampling revealed substantial applicatory advantages: (i) individual and uncomplicated sampling is also possible within the bounds of a microbiological sampling; (ii) a relatively uniform biofilm fragmentation because of the nearly equal epithelial cell surface; (iii)

subjecting to biocides and a subsequent evaluation is possible without special appliances because of the small dimensions of the biofilm fragments. The approach of harvesting *ex vivo* samples of gingival-biofilm consortia provides a new perspective on further investigations, particularly on biofilm resistance against antibiotics and biocides.

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