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Bacterial adhesion to sulcular epithelium in periodontitis

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Abstract

The purpose of this study was to investigate, by electron microscopy, the type of bacterial attachment to the sulcular epithelium in periodontitis. Gingiva biopsies were observed in a transmission electron microscope using cytochemical staining with ruthenium red for glycocalyx visualisation. In addition, subgingival plaque samples and biopsies from the sulcular epithelium in periodontitis from the patients were estimated microbiologically. Aerobic bacteria only were estimated in the subgingival plaque and both aerobic and anaerobic bacteria in the gingival biopsies. No bacterial internalisation could be observed. Fimbria-mediated adhesion as the only type of bacterial attachment and a large diversity of bacterial glycocalyces were detected. As the fimbrial adhesins of putative periodontal pathogens are able in vitro to induce inflammation and bone resorption via stimulation of the proinflammatory cytokine production, the demonstrated fimbrial adhesins suggest the significant role of bacterial adhesion to sulcular epithelium in periodontitis.

Keywords: Sulcular epithelium; Transmission electron microscopy; Fimbria; Adhesin; Proinflammatory cytokine

1. Introduction

Recently, bacterial fimbria-mediated adhesion to the stratified squamous epithelium of the hard palate in denture stomatitis has been demonstrated [1]. Adhesion appears to be the first step in the bacterial colonisation process of the epithelial soft tissue. Many facultative pathogenic bacteria exhibit a strong correlation between adhesion and virulence [2–8], and mutation of adhesin genes causes a significant decrease in virulence [7,8]. In case of DPG3, a *Porphyromonas gingivalis* fimbria-deficient mutant, an 8-fold decrease of virulence compared to its parent, strain 381, has been reported [6]. Since bacteria, especially those from the dental plaque, are assumed to be responsible for the inflammation of the periodontium, the question arises whether bacterial adhesion to the gingival pocket tissue takes place. Such adhesion could play a significant role in the pathogenesis of the periodontitis, since the interaction between many adhesins and epithelial membrane receptors results in cytokine release that induces inflammation [4,9–11]. However, transmission electron microscope (TEM) data concerning the mode of bacterial attachment to the sulcular epithelium in vivo are lacking.

The purpose of this study was to examine by transmission electron microscopy the relationship between sulcular epithelium and adjacent bacteria in adult periodontitis in vivo using ruthenium red (RR) staining for detection of fimbrial adhesins [12,13]. The study focused on the important question whether bacterial attachment to the gingival tissue could be related to fimbria-mediated adhesion.

2. Materials and methods

Ten patients aged between 37 and 72 years (mean: 55 years) with adult (progressive chronic) periodontitis were selected for this study. In all patients serial extraction of the incisor teeth with the necessity to improve the contour of the alveolar bone by reducing bone prominences and hyperplastic soft tissue was indicated. Radiological exami-

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Table 1
Results of the microbiological investigation: yeast and bacterial species and density in patients 1–10

Patients	Age (years)	Sex	Sample	*Candida* spp.	Viridans str.	*Neisseria* spp.	*Haemophylus* spp.	*Enterococcus* spp.	Str. B	*S. epidermidis*	*Bacteroides fragilis Gr*	*Bacteroides* spp.	Anaerobic str.	*Gemella morbillorum*	*P. oralis*
1	60	m	plaque		+++	+	+								
			excision		+++	+	+				+				
2	37	f	plaque	++	++										
			excision	+	+				+						
3	69	m	plaque		++										
			excision		+++							+	+		
4	72	f	plaque		++		++								
			excision											+	
5	49	f	plaque		+++		++								
			excision		+++		++								+
6	49	f	plaque		+++										
			excision		++										
7	50	m	plaque	+	+					+					
			excision		+										
8	66	m	plaque		+++			+++							
			excision		+++										
9	49	f	plaque	+++	+										
			excision		+										
10	46	f	plaque		+++					++					
			excision		+++					+					

+, low yeast or bacterial density; ++, moderate density; +++, high density.

nation revealed irregular bone resorption of more than 50%, and depth-on-probing was more than 5 mm in all teeth. Exclusion criteria for patients were: systemic disease related to subacute bacterial endocarditis prophylaxis, diabetes mellitus, antibiotic therapy in the last 6 months, steroid therapy and radiotherapy. The study protocol was approved by the local ethics committee, and informed written consent was obtained from all patients.

Immediately after extraction, samples for microbiological investigation were taken with an excavator from about 2 mm^2 of the subgingival root plaque. Bacterial estimation was performed as described recently [1]. The *Candida* density was estimated as described previously [14].

The gingival tissue between two of the extracted teeth was excised with a scalpel and immediately washed in 50 ml saline by gently swaying for 20 s. The specimens were washed to remove all the substances in contact but not attached to the epithelial surface. All epithelial non-sulcular parts were removed from the biopsy with a scalpel and the remaining sulcular epithelium of the excision was divided into three pieces perpendicular to the vestibulo-lingual axis of the sample. Two pieces of each biopsy were processed for transmission electron microscopy, and the third one was prepared for microbiological investigation. One TEM sample of each biopsy was fixed with 1.2% glutaraldehyde (buffered at pH 6.5 with 0.1 M sodium cacodylate) for 2 h at 4°C. Postfixation of these control samples was performed with 1% osmium tetroxide (buffered at pH 6.5 with 0.1 M sodium cacodylate) for 2 h at 4°C. The other sample from each biopsy was fixed and postfixed by the fixatives mentioned above with addition of 0.05% RR in each case. All specimens were routinely embedded in Epon 812. 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. For microbiological investigation, 9 ml of Bouillon BBL® Schaedler Broth (Becton Dickinson and Company, Cockeysville, MD, USA) were inoculated with the third piece of each biopsy. Further microbiological estimations were performed as described above.

3. Results

Results of the microbiological investigation are summarised in Table 1. The predominant bacteria found in plaque as well as biopsy samples were streptococci (mainly viridans streptococci). Distinct differences concerning the microbial flora were detected between individuals. Anaerobic bacteria were isolated from biopsies only.

The samples fixed in the absence of RR showed no or a rather faint layer exterior to the cell wall (Fig. 1). In two samples scattered fibrin-like materials (Fig. 2) and formation of a biofilm both attached to the sulcular epithelium were apparent (Figs. 3 and 4) in TEM micrographs. The bacteria attached to epithelial membranes were located exclusively on the desquamated and loosed epithelial cells.

In samples of two patients desquamated and loosed epithelial cells could not be detected and no bacteria were observed by TEM analysis. Microbiological analysis of these samples revealed streptococci only. In all other biopsy samples different bacteria were detected microbiologically and TEM analysis revealed bacterial attachment to the sulcular epithelium. Bacteria attached to the epithelial membranes were located superficially, scattered on the epithelial surface. However, bacteria localised within the superficial sulcular epithelium cells could not be detected in the present TEM investigation. A great ultrastructural diversity of microbiota (cocci, cocci-like, and rod-shaped bacteria) were found attached to the outer surface of the epithelial cell membranes (mainly to the villi-like protrusions of the epithelial cells) (Figs. 3, 5 and 6), as well as to other bacteria (Figs. 7 and 8), to fibrin-like substances (Figs. 9 and 10), to vesicles (Fig. 8) or to cell debris (Fig. 11). Appearance of one or more layers within the bacterial cell wall was clearly perceptible (Fig. 12). Aggregations of groups of bacteria leading to formation of a bacterial multilayer (biofilm) (Figs. 3 and 8) were evident. In addition, spirochetes could be observed within the biofilm (Fig. 7). In all samples that revealed fibrin-like substances, bacterial attachment to these fibrin-like substances was evident (Figs. 9 and 10).

All bacteria stained with RR showed diverse glycocalyces with different morphology. Bacterial adhesion to epithelial cells (Figs. 3, 5 and 6), vesicles (Figs. 8, 11 and 12), or fibrin-like substances (Figs. 9 and 10), as well as coadhesion (Figs. 3 and 8) was exclusively mediated via fibrillar adhesins, i.e. the only type of bacterial adhesion was the fimbria-mediated one. The intimate contact between bacterial fimbriae and above mentioned structures was clearly apparent. A dense and a loose type of glycocalyces could conditionally be differentiated: dense and loose. The fimbriae of the dense glycocalyx are not ever clearly distinguishable and such a glycocalyx seemed often like a narrow, very electron-dense, continual layer (Figs. 6 and 9). The loose type of glycocalyx was frequently monitored as clearly distinguishable fimbriae and had a low electron density. Further loosely diffuse (Fig. 5) and loosely net-like (Fig. 8) glycocalyces could be differentiated. The width of the loose diffuse type varied considerable.

4. Discussion

The present study provides for the first time ultrastructural evidence for fimbria-mediated bacterial adhesion to sulcular epithelium in periodontitis. Fimbrial adhesins are

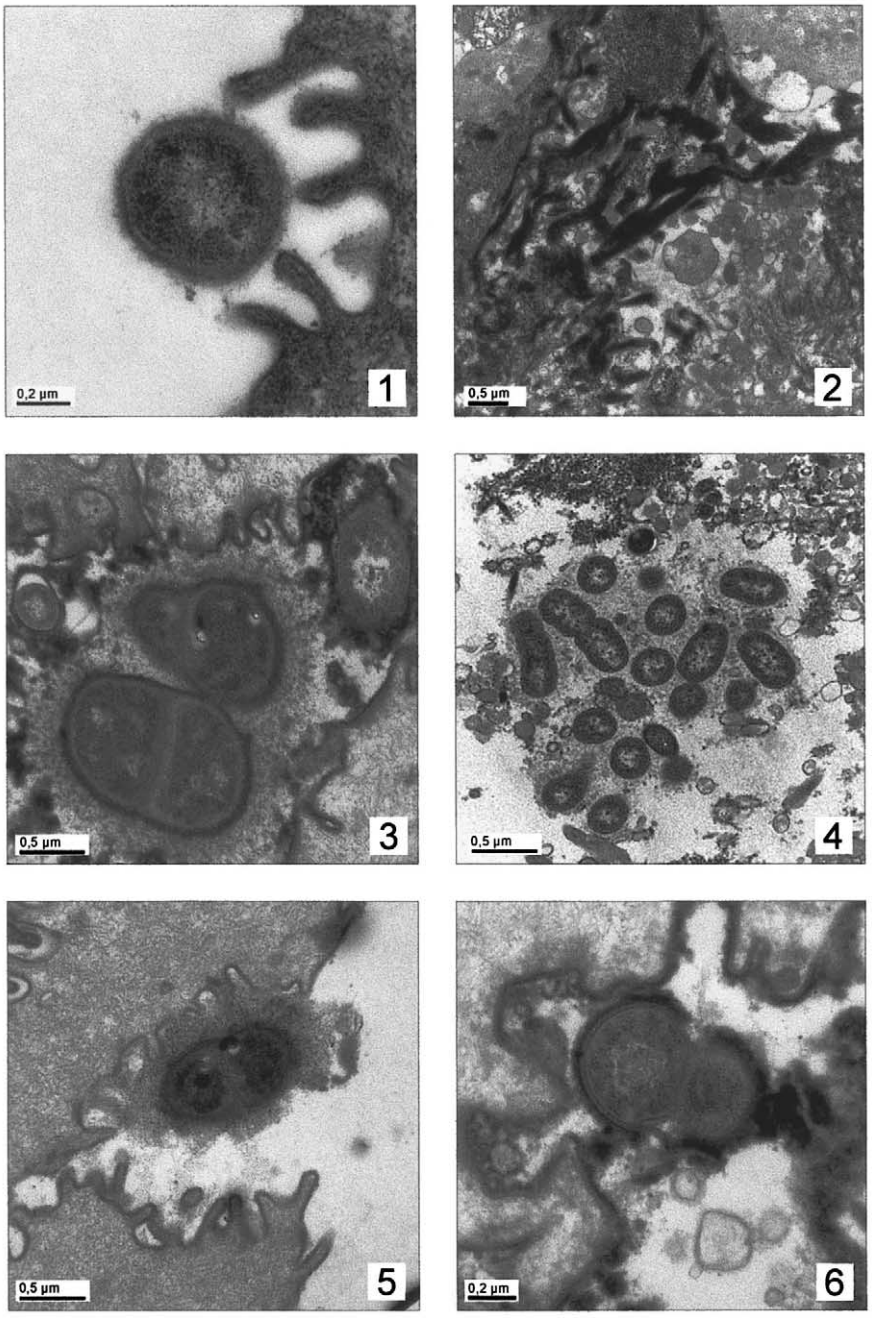

Fig. 1. Control staining. Coccus-like bacterium at some narrow distance from epithelial cell membrane. The bacterium exhibits a faint additional layer outside the bacterial wall.
Fig. 2. RR staining. Biofilm consisting of vesicles, fibrin-like substances and decay substances.
Fig. 3. RR staining. Adhesion to an epithelial cell membrane via differently sized glycocalyces, also bacterial co-adhesion. Begin of formation of a biofilm attached to sulcular epithelium.
Fig. 4. RR staining. Short rods with multiple layer wall with diffuse glycocalyces. Some of them are adhered to the remnants of decayed epithelial cells. Some vesicle-like structures with diverse sizes and electron densities are also visible.
Fig. 5. RR staining. Adhesion to an epithelial cell membrane via a large glycocalyx.
Fig. 6. RR staining. Dividing bacteria with narrow but very electron-dense glycocalyx adhering to an epithelial cell membrane. The glycocalyx ruptured at the dividing line

an integral part of the additional but not obligatory polysaccharide-rich surface layer of bacteria denoted as glycocalyx [13]. The glycocalyx is peripherally located to the outer membrane in Gram-negative bacteria and to the peptidoglycan in Gram-positive ones. Appearance of one or more layers within the bacterial envelope can be the consequence of both the fixation method and the environment-related distribution of wall components, and thus cannot be a valid criterion for distinction whether a bacterium is Gram-negative or Gram-positive [12]. The ability

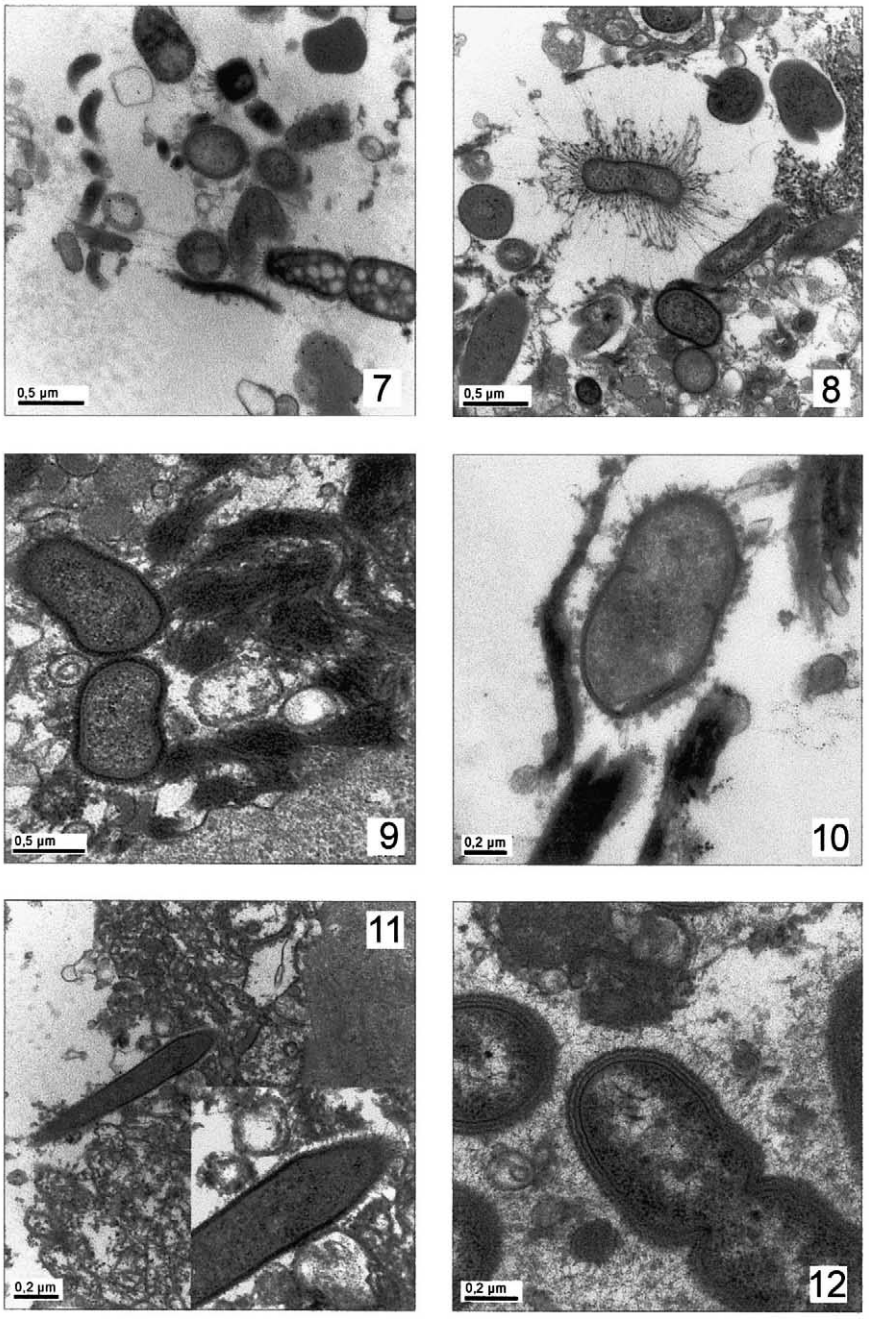

Fig. 7. RR staining. Large morphological diversity of bacteria building a gingival biofilm. Arrows: spirochetes.
Fig. 8. RR staining. Biofilm formed by different bacteria showing a large morphological diversity of glycocalyces. Central: a bacterium with net-like glycocalyx. The co-adhesion via fimbriae is clearly evident.
Fig. 9. RR staining. Fibrin-like clumps adhered to an epithelial cell membrane and two bacteria adhering via a dense glycocalyx to one of them. The fibrin-like clump serves as a link between the cell surface and the bacteria.
Fig. 10. RR staining. Bacterial adhesion via a loose glycocalyx to fibrin-like substances.
Fig. 11. RR staining. Rod-shaped bacterium adhering to vesicles and cell debris. Insert: A detail of bacterial wall with sparse glycocalyx.
Fig. 12. RR staining. A detail from Fig. 4. The biofilm matrix three-dimensional network adhering to the surrounding surfaces.

of the cationic RR to enhance staining of acidic glycosylated structures at the ultrastructural level is widely used for their visualisation [2,13,15,16]. The glycocalyces in periodontitis showed larger morphological differences than those in denture stomatitis [1]. The dense type of glycocalyx found in the present study resembles the glycocalyx of *P. gingivalis* [16] and of *Staphylococcus aureus* [17] observed in vitro. Bacterial adherence to gingival cells is based on interactions between bacterial adhesins and corresponding receptors, e.g. expressed on the epithelial membranes of eukaryotic cells [4,5,15,18–20]. Apart from bacterial attachment at the given site by adhesion, adhesins

play a substantial role in determining the outcome of prokaryotic–eukaryotic interactions [4]. Biological activities in inflammatory tissue destruction are governed by cytokines, which are also involved in the progress of periodontitis [21]. In vitro findings indicate stimulation of the production of proinflammatory cytokines by adhesins of periodontal pathogens. The production of interleukin-1 and granulocyte macrophage colony-stimulating factor is stimulated by fimbriae of *P. gingivalis* [10]. The adhesion of *Eikenella corrodens* stimulates oral epithelial cells to secrete interleukin-6 and interleukin-8 [22]. Two adhesins from oral viridans streptococci stimulate the production of interleukin-8 from epithelial cells [11]. Additionally, in vitro findings indicate that cytokines represent an important pathway of connective tissue destruction in human periodontitis, e.g. bone resorption was stimulated by fimbriae of *P. gingivalis* [10].

The demonstration of fimbria-mediated adhesion in periodontitis in the present TEM study, the ability of adhesins to stimulate cytokine production and bone resorption as well as the correlation between the level of proinflammatory cytokines and the periodontitis progression highlight the significance of bacterial adhesion for periodontal pathogenicity. As only few oral bacteria are considered to be strongly associated with periodontitis [23–26] and the loss of a certain adhesin causes a multifold decrease of the bacterial virulence [2], vaccination against adhesins [27] could reveal new possibilities for treatment of periodontitis.

Separate sampling from subgingival plaque and from saline washed excisions was made on the presumption that only particular bacteria which recognise epithelial receptors via adhesins should be able to adhere to the gingival pocket epithelium. The adhesion within the subgingival plaque could be a result of co-adhesion of some bacteria [1,28,29] to others already adhering to the root surfaces. The difference between gingival-attached bacteria and plaque bacteria is not ascertainable by the usual paper point technique [24–26,30]. Insertion of paper points into the gingival pocket causes attachment of desquamated and scraped off epithelial cells together with adhering bacteria as well as scraped off plaque bacteria to the paper point surface. The bacteria attached to the gingival pocket epithelium were both anaerobic and aerobic in contrast to the plaque microbiota which was aerobic only and consisted mainly of viridans streptococci. Consequently, it could be supposed that two different microbiotas are coexisting: (1) plaque bacteria, which are predominantly aerobic; (2) gingiva-attached aerobes and anaerobes. However, there is no clear dividing line between gingiva-attached and plaque-associated bacteria, since anaerobes and aerobes coaggregate particularly via *Fusobacterium nucleatum* [31], and/or by biofilm formation [13,32], so that a link between gingiva-attached microbiota and plaque microbiota could be expected. Recently, it has been shown that anaerobes are able to survive only when facultative or aerobic species were present. In the absence of such species, the anaerobes died quickly, and even biofilms could not provide an appropriate biotope for the anaerobes under these circumstances [33]. In vitro studies have demonstrated the survival of anaerobes in aerated environments in planktonic cultures of mixed flora [31]. Metabolism of aerobic and/or oxygen-tolerant species may reduce the concentration of oxygen to levels that are not toxic to obligate anaerobes [34]. The spatial limiting of the gingival pocket facilitates the establishment of anaerobic conditions via oxygen consumption by aerobes and facultative anaerobes. A spatial distribution of bacteria species depending on the pocket depth and thus probably related to depth distribution of oxygen has also been reported [35].

The microbiological estimation of the gingiva-attached microbiota revealed in some samples less species than morphological forms could be observed in TEM. One reason for that could be the coexistence of different morphological varieties of a bacterial strain or lack of growth of certain species under the growth conditions of mixed flora. Use of incision instead of paper points hindered homogenisation of the samples [24–26,30] and taking equivalent valuable specimens for further microbiological determination by selective media. In addition, the microbiological estimation of gingiva-attached pocket microbiota by in vitro cultivation depends strongly on duration of oxygen exposure [33] until the inoculation and should be subject of further improvements.

The attachment of vesicle-like structures to bacteria was probably managed by adhesion. The vesicle-like structures could be cellular debris (granules from PMN cells) or might represent salivary micelles which are able to agglutinate certain bacteria [36]. The vesicle-like structures revealed a very low electron density and relatively large dimensions compared to the observed spirochetes. These findings point against the probability that these vesicle-like structures could be spherical bodies of treponemes which have been described recently [37–39].

Appearance of fibrin is a normal finding in crevicular fluid in healthy [40] and diseased periodontium [40,41]. Not only intact fibrin, but also fibrin fragments were observed [42]. The fibrin-like substances detected in the present study were fibrillar, electron-dense clumps showing the characteristic electron microscopic feature of fibrin [43,44]. Nevertheless, the artificial formation of fibrin clumps during sampling by means of incision cannot be excluded with certainty. Bacterial adhesion to fibrin has been identified as the critical initial step in the pathogenesis of foreign body-related infections [17]. Bacterial adhesion to fibrin-like material could be considered as an additional opportunity for certain bacteria to colonise the periodontal pocket. *Staphylococcus epidermidis*, which was found on sulcular epithelium, is able to adhere to fibrin by means of glycocalyx [32], and also *Streptococcus parasanguis* can bind to fibrin [45].

The present TEM micrographs indicate that the slime, a

glycocalyx layer peripherally associated to the outer bacterial surface and commonly located exterior to the capsule, extends further into the intercellular environment and serves particularly for bacterial attachment to surfaces [13]. It is also considered to form a part of a polysaccharide fibrous network serving for bacterial community function enabling the formation of biofilms.

Internalisation of putative periodontal pathogens, e.g. *Prevotella intermedia* [46], *P. gingivalis* [16,47] or *Actinobacillus actinomycetemcomitans* [48], has been observed in vitro. In addition, bacterial invasion of the junctional epithelium of the gingival pocket has been demonstrated in acute ulcerative gingivitis [49] as well as in advanced cases of periodontal disease [50,51] by transmission electron microscopic investigations. The non-observation of internalisation of periodontal pathogens in the present work indicates that bacterial tissue invasion might not play a leading role in the pathogenesis of periodontitis. These findings are in accordance with TEM results published previously [52,53].

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