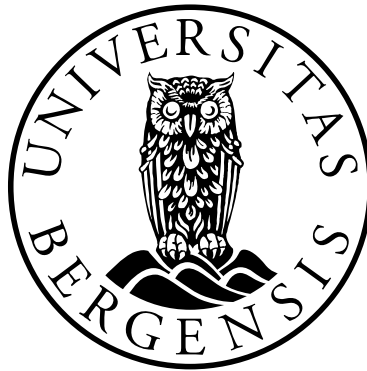


Interactions between *Fusobacterium nucleatum* and Primary Human Oral Cells

In Vitro Studies in Monolayer and Organotypic Culture Models

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To my family

Scientific environment

The Gade Institute

Section for Microbiology and Immunology

The Faculty of Medicine of Dentistry

University of Bergen

Norway

The Gade Institute

Section for Pathology

The Faculty of Medicine of Dentistry

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Summary

F. nucleatum is a Gram-negative bacterium, member of normal flora in the oral cavity, which has a major role in the formation of the subgingival biofilm. The adhesins expressed on the cell wall outer membrane confer *F. nucleatum* remarkable adhesive properties. *F. nucleatum* can bind a wide array of oral bacteria, including periodontal pathogens, but also can attach to and enter oral epithelial and endothelial cells and oral tissues, triggering release of molecules that contribute to the periodontal tissue breakdown. The main goal of this study was to investigate the interactions between different oral strains of *F. nucleatum* and normal human oral cells by use of *in vitro* cell culture models.

The entrance of *F. nucleatum* into oral fibroblasts of gingival and periodontal ligament origin was the first time investigated in this study. Both type of fibroblasts were invaded by *F. nucleatum* in a strain-dependent manner, process starting approximately after 1 h of co-culture and continuing for a couple of hours. The periodontal ligament fibroblasts were consistently more loaded with fusobacteria than donor-matched gingival fibroblasts, but the reasons behind this finding remain open to further research.

The invasion of *F. nucleatum* into an organotypic model of gingival mucosa, constructed with primary gingival epithelial cells on top of a collagen matrix containing gingival fibroblasts, was limited to the superficial epithelial layers. Although exposure to *F. nucleatum* induced strong shredding of the superficial epithelial layers and presence of caspase-3 positive cells in the epithelial compartment, the organotypic tissues kept their proliferative potential after fusobacterial challenge. These findings suggest that fusobacteria were rather efficiently eliminated by the gingival mucosa models, than inducing permanent damage to the organotypic tissues.

With the aim of using such models in future comparative and in depth studies on bacterial–host tissue interactions, a range of organotypic models resembling junctional

epithelium, sulcular epithelium and gingival epithelium have been successfully developed. It was determined that the type of fibroblasts used in the collagen matrix, together with the time allowed for growth and differentiation have strong impact on the phenotype of epithelium compartment of the organotypics. The periodontal ligament fibroblasts influenced significantly the CK 19 expression and pattern of distribution of proliferating cells in organotypic cultures grown for 5 days, reflecting the closest the phenotype of JE native tissue

The findings described in this study confirm the invasive potential of *F. nucleatum* documented by other studies in other type of cells and suggest the use of relevant tissue models, developed by use of primary oral cells, for further research in this field.

List of publications

This thesis is based on the following original papers. The papers are referred to in the text by their roman numbers.

Paper I

Dabija-Wolter, G., Cimpan, M. R., Costea, D. E., Johannessen, A. C., Sornes, S., Neppelberg, E., Al-Haroni, M., Skaug, N., and Bakken, V. 2009.
Fusobacterium nucleatum enters normal human oral fibroblasts *in vitro*. *J. Periodontol* 80:1174-1183.

Paper II

Dabija-Wolter G., Sapkota, D., Cimpan, M. R., Neppelberg, E., Bakken, V., Costea, D. E. 2012. Limited in-depth invasion of *Fusobacterium nucleatum* into *in vitro* reconstructed human gingiva. *Arch Oral Biol* 57:344-351.

Paper III

Dabija-Wolter G., Bakken, V., Cimpan, M. R., Johannessen, A. C., Costea, D. E. *In vitro* reconstruction of human junctional and sulcular epithelium. (manuscript, to be submitted to *J Oral Pathol Med*).

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List of Abbreviations

ATCC	American Type Culture Collection
CK	Cytokeratin
CLSM	Confocal laser scanning microscopy
DAPI	4',6-diamidino-2-phenylindole
FITC	Fluorescein-isothiocyanate
GFs	Gingival fibroblasts
(H)GECs	(Human) gingival epithelial cells
hBD	Human beta-defensin
HE	Hematoxylin and eosin
IL	Interleukin
JE	Junctional epithelium
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
NF- κ B	Nuclear factor kappa B
OMP	Outer membrane protein
OT	Organotypic
PBS	Phosphate buffered saline
PLFs	Periodontal ligament fibroblasts
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TG-I	Keratinocyte transglutaminase
TLR	Toll-like receptor
TNF	Tumor necrosis factor

1. Introduction

1.1. *Fusobacterium nucleatum*

1.1.1. General aspects

One of the most abundant bacterial species found in the oral cavity is *Fusobacterium nucleatum*, a Gram-negative, anaerobic microbe, described as non-motile and non-spore-forming bacterium, lacking fimbriae or pili (12, 31, 114). Although it has been isolated from infections and abscesses in different sites of the body (15, 19, 63), *F. nucleatum* is mostly associated with initiation and progression of periodontal disease (88).

F. nucleatum includes five subspecies (ssp.): *F. nucleatum* ssp. *nucleatum* American Type Culture Collection (ATCC) 25586, *F. nucleatum* ssp. *polymorphum* ATCC 10953, *F. nucleatum* ssp. *fusiforme* NCTC 11326, *F. nucleatum* ssp. *vincentii* ATCC 49256, and *F. nucleatum* ssp. *animalis* NCTC 12276 (41). The genomes of *F. nucleatum* subspecies *nucleatum* ATCC 25586 and *F. nucleatum* subspecies *vincentii* ATCC 49256 have been fully sequenced (76, 77). The distribution of *F. nucleatum* subspecies varies in periodontal disease (134) and between populations (44).

The bacterial dimensions vary within the same population and also between subspecies (*ssp. polymorphum* might include bacterial cells over 10 µm while *ssp. nucleatum* have a size of 2-4 µm), these dimensions making *F. nucleatum* one of the biggest microbe in the oral cavity. Although it is described as strictly anaerobe, *F. nucleatum* is able to tolerate up to 6% oxygen atmosphere (12) and responds by physiological alterations and increased pathogenicity to oxidative stress (23, 155). Evidently, *F. nucleatum* may tolerate aerobic conditions much better when organized in biofilm than in planktonic form (48).

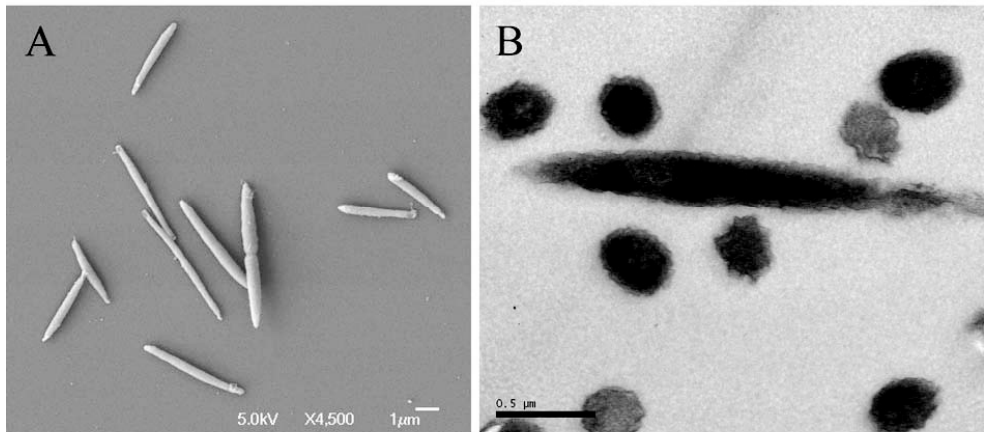


Figure 1. *F. nucleatum* ATCC 25586 by A) scanning electron microscopy (SEM) and B) Transmission electron microscopy (TEM). (Source: G Dabija-Wolter).

1.1.2. *F. nucleatum* and periodontal diseases

Periodontitis is a chronic inflammatory disease affecting the supporting tissues of the teeth, leading ultimately to tooth loss. Periodontitis is initiated by accumulation of the dental plaque biofilm and also because of abnormal host response to bacterial challenge. *F. nucleatum* has been associated with periodontal diseases due to the following facts:

- i. *F. nucleatum* is found in higher levels in chronic periodontitis as compared to gingivitis and periodontal health (114, 177).
- ii. *F. nucleatum* has a key role in biofilm formation, bridging between early colonizers, represented mainly by streptococci and actinomyces (97), and late colonizers, represented by a variety of Gram-negative bacteria (88, 89). Among the late colonizers are the periodontal pathogens included in the ‘red complex’ (158): *Porphyromonas gingivalis*, *Tannerella forsythia* (formerly *T.*

forsythus or *B. forsythus*) (148) and *Treponema denticola*, strongly associated with periodontal lesions (50, 177). *F. nucleatum* coaggregation with a wide plethora of bacteria (84, 86, 87, 135, 152) is mediated by adhesins, which are outer membrane proteins (OMPs).

iii. *F. nucleatum* seems to have a protective role towards obligate anaerobes (14). The association between *F. nucleatum* and *P. gingivalis* results in higher levels of oxygen toleration (28, 29), enhanced biofilm formation (99, 146) and increased pathogenicity (37) with greater destruction of soft tissue and alveolar bone (109, 127, 128). Apparently, *F. nucleatum* contributes to colonization by *Prevotella intermedia* of periodontal pockets, as this microbe was never detected in a site without the presence of *F. nucleatum*. Complexes of *F. nucleatum*, *P. intermedia* and *Campylobacter rectus* were detected in periodontally affected sites refractory to treatment.

iv. Possession of virulence factors with the potential to contribute to periodontal tissue destruction – will be reviewed in the following subchapter.

1.1.3. Virulence factors in *F. nucleatum*

- Ability of adhere to and invade into host cells demonstrated *in vivo* and *in vitro* (30, 48, 138, 140, 142-144, 171).
- Immunosuppressive factors – the FipA protein of *F. nucleatum* determines coaggregation of lymphocytes and apoptosis in peripheral blood mononuclear lymphocytes (PBMCs) and polymorphonuclear cells (PMNs) (26). The PMNs constitute the first line of cell defense of cellular innate immunity in the periodontal pocket.
- Endotoxins: lipopolysaccharide (LPS) (24) and hemolysin (96). The *F. nucleatum* LPS induces production of plasminogen activator by gingival fibroblasts (175).

- *F. nucleatum* triggers release of interleukin (IL)-1 β , IL-6, IL-8 and tumor necrosis factor alpha (TNF- α) from different types of cells, cytokines known to promote inflammation and stimulate bone and periodontal tissue destruction (45). Compared to healthy individuals, periodontally affected subjects may have significantly increased levels of IL-1 β , IL-6, IL-8 in gingival crevicular fluid (9, 43), which were strongly correlated with presence of clinical signs of periodontal disease and higher proportions of bacterial species belonging to ‘orange’ and ‘red complex’ (163).
- It stimulates production of metalloproteinases (MMPs) as MMP-2, -9, and -13 in oral epithelial cells (47, 168, 169). MMPs are enzymes involved in degradation and remodeling of the extracellular matrix in pathological conditions, including periodontitis (11). It was suggested that altered levels of MMPs are involved in periodontal tissue destruction, as periodontally affected subjects express higher levels of MMP-2, -8, -9, 13, -14 in gingival tissue and gingival crevicular fluid (81, 93, 94, 164).
- Tissue-toxic metabolic by-products such as butyrate, propionate and ammonium (12) which also seem to be involved in inhibiting the human gingival fibroblasts’ proliferation (179) interfering in this way with wound healing.
- Possession of a 65 kDa serine protease able to degrade the extracellular matrix proteins fibrinogen and fibronectin as well as collagen I and collagen IV (6).

Considering the above enumerated characteristics, it appears that although commensal, *F. nucleatum* has the profile of an ‘opportunistic pathogen’. A recent study proposes that *F. nucleatum* is involved in initiation and progression of periodontal diseases by colonization of periodontal tissues, followed by depletion of PBMCs and recruiting of other pathogenic bacteria such as *P. intermedia*, *B. forsythus* and *C. rectus* (154).

More frequently isolated from subgingival plaque of patients with periodontitis were strains assembling to *F. nucleatum* ssp. *nucleatum* ATCC 25586 and ssp. *vincentii* (50, 124), whereas strains belonging to *F. nucleatum* ssp. *polymorphum* ATCC 10953 and

ssp. *fusiforme* NCTC 11326 were more associated with healthy sites in the oral cavity (42). The distribution of *F. nucleatum* subspecies varies in periodontal disease (134) and between populations (44).

1.1.4. Local and systemic implications of *F. nucleatum*

In vivo, periodontal bacteria were frequently detected within gingival tissues and even in connective tissue in periodontally affected patients (2, 17, 105, 141-143). As sole microbe or in mixed infections, *F. nucleatum* may be involved in infections and abscesses in oral cavity or other parts of the body. It was isolated from endodontic and periapical infections (39, 147, 156). *F. nucleatum* may get in the blood stream and cause bacteremia and septic shock (118, 180) and eventually colonize other organs. There have been reported cases of acute appendicitis (161), brain abscess (52) and adverse pregnancy outcomes (53, 63) having *F. nucleatum* as causative agent, and recently the bacterium was found to be prevalent in colorectal carcinoma (16). *In vitro* studies showed as well that *F. nucleatum* is able to colonize placenta and determine a strong inflammatory response following TLR-4 activation, having as outcome fetal death in pregnant mice (55, 98). The *F. nucleatum* ability to migrate and induce infections elsewhere in the body might be supported by a recent report showing that *F. nucleatum* is able to bind CD46 (103), a complement regulatory protein which is expressed on the surface of nearly every cell type.

F. nucleatum is one of the oral bacteria involved in halitosis (oral malodour) due to different substances such as ammonia, hydrogen sulfide, butyric acid, and methyl mercaptan resulted from bacterial metabolic activity (12, 74, 99).

1.2. Human oral host tissue – structure of periodontium

The epithelia lining the oral cavity differ structurally according to their location and function, each type of epithelial mucosa protecting in a particular way against mechanical and microbial insults (151). The gingival epithelium (GE) changes gradually its phenotype towards the sulcular epithelium (SE) which lines the gingival sulcus, and further apically to the highly specialized epithelium, i.e. the junctional epithelium (JE) that is attached to the dental surface via hemidesmosomes. These three types of epithelium have different function and thus different structure (130, 136), that can influence the way they respond to different bacterial stimuli (67). The GE is a stratified squamous parakeratinized epithelium that, due to its highly differentiated structure, fulfills the barrier function. The SE is non-keratinized stratified squamous epithelium lacking the stratum granulosum present in GE, which makes the transition towards the JE. The unique features of JE include exceptionally high cellular turnover (13, 151) and widened intercellular spaces (62), allowing fluid and leukocytes from the gingival connective tissue to transmigrate to the gingival sulcus. The degree of differentiation in these epithelia is reflected by their cytokeratin profile (101, 121, 131, 132), allowing a quite clear demarcation to be made between these epithelia (Fig. 2).

The cytokeratins (CK) are major structural proteins of keratin-containing intermediate filaments, part of the cytoskeleton of epithelial cells. According to their pI they are classified as type I or acidic cytokeratins and type II, neutral or basic cytokeratins (112, 131). Their expression is organ or tissue specific, which allows classification of all epithelia upon their CK expression profile. In tissues, CK are found in pairs of a type I together with a type II cytokeratin.

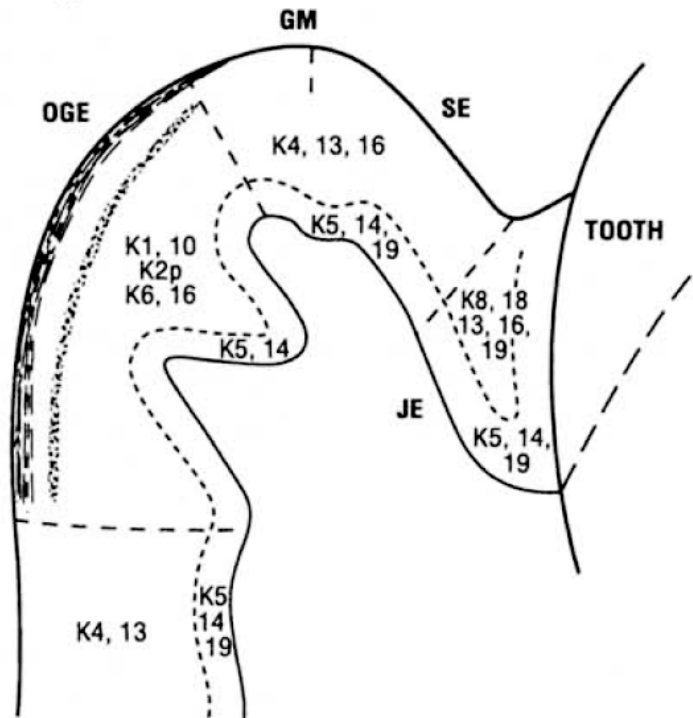


Figure 2. The cytokeratin (K) profile in periodontium. OGE = oral gingival epithelium; GM = gingival margin; SE = sulcular epithelium; JE = junctional epithelium. Source: Presland and Dale, *Crit Rev Oral Biol Med* 11(4):383-408, copyright © 2000 by SAGE Publications. Reprinted by Permission of SAGE Publications.

1.3. Interactions between host cells and oral bacteria

In a variety of *in vitro* studies, oral bacteria in relationship with host cells were investigated using mainly oral epithelial cells (primary or immortalized) but also other cell types, as: endothelial cells, fibroblasts, PBMCs and PMNs.

1.3.1. Adhesion to and invasion of oral epithelial cells by oral bacteria

Adhesion of bacteria to host cells is an important step, a prerequisite for subsequent invasion. The strength of adherence, followed by invasion of bacteria in host cells, varies among bacterial strains and is also dependent on the cell type and host organism response. Bacterial structures involved in adhesion: capsule, fimbriae or fibrils, flagella, cell wall, curli (133).

The bacterial entrance in host epithelial cells is an active process that involves both bacteria and the host cell. Oral bacteria may interact with a multitude of pattern recognition receptors situated on the cell surface, but also in the cytoplasm or endosomal compartments of host cells. These receptors recognize specific molecular patterns in the bacterial structures. β -integrins and cadherins may function as surface receptors, their stimulation by different bacteria may result in intracellular signals affecting the cytoskeleton followed by bacterial uptake (20). Bacteria may enter epithelial oral cells by ‘persuading’ the host cell to induce cytoskeleton rearrangements so that the bacterium is engulfed by the cell. In case of periopathogens *P. gingivalis* and *A. actinomycetemcomitans*, the entrance in host cells is done by receptor-mediated endocytosis (149, 159). Prior exposure of host cells to cytochalasin D, which disrupts actin filaments and inhibits actin polymerization, or to nocodazole, an inhibitor of microtubule formation, results in significantly reduced invasion of *P. gingivalis* indicating that both microfilament and microtubule activity are required for invasion of this periopathogen in HGECs. Energy metabolism is involved in invasion process both for eukaryotic and prokaryotic cells (95).

In vitro studies showed that certain bacteria are non-invasive (e.g. *Streptococcus cristatus*), however they might gain entrance into host cells due to coaggregation with invasive microorganisms (33). Other oral bacteria (e.g. *P. intermedia*, *Eikenella corrodens*, *T. forsythia*) invade at very low percentages (56). Dead bacteria were not found internalized at significant levels, and bacteria lacking adhesive properties are

also deficient in adhesion and invasion (56, 70), underlining that bacterial invasion is a double-sided process.

1.3.2. Consequences of host epithelial cells – oral bacteria interactions

After internalization, both host cell and bacteria have to adapt to their association. The changes induced upon bacterial uptake in host cells may be strong and highly organism specific especially when involving oral pathogens as *P. gingivalis* or *A. actinomycetemcomitans* (58), while the response to commensal organisms *F. nucleatum* and *S. gordonii* is of reduced magnitude (61), suggesting that possibly the host is able to discriminate commensals from pathogens (36). Downstream signaling events in the host cell may be expected to impact a wide array of cellular processes, such as cytoskeletal arrangements, cytokine and antimicrobial molecules production, cell cycle, proliferation and migration or possibly cell commitment to apoptosis.

Little is known on bacterial gene expression after host cell invasion. A study investigating *P. gingivalis* internalized in gingival epithelial cells (123) showed that bacteria adapt to the new environment by regulating the expression of membrane transporters such as *pepO*, encoding an endopeptidase, genes encoding an ATP-binding cassette (ABC) transporter and a cation-transporting ATPase.

Stimulation of cytokines and β -defensins production

Toll-like receptors (TLR) are pattern recognizing receptors present on the cell surface (TLR 1, 2, 4, 5, 6) or in the cytoplasm (TLR 3, 7, 8, 9). All TLRs except TLR-10 were found expressed in gingival epithelial cells (10) but the most investigated were TLR-2 which recognize bacterial lipoproteins, glycolipids and peptidoglycans and TLR-4, which recognize bacterial LPS. The GFs and PLFs also express constitutively mRNA of TLR-2 and TLR-4 and CD14 (which functions as a co-receptor). The stimulation of TLRs by bacteria have a pivotal role in initiating and modulating the production of pro-inflammatory cytokines, such as interleukin (IL)-8 and of other antibacterial molecules (defensins, cathelicidins) with essential role in innate immunity (173).

Apparently, Gram-negative oral bacteria stimulate predominantly TLR-2 rather than TLR-4 (60, 82). The signaling pathway leading to expression of hBDs or IL-8 may be different for various oral bacteria (18).

Human beta defensins (hBD) and cathelicidins are small cationic antimicrobial peptides, constitutively expressed or inducible in different epithelia, able to directly destroy the microorganisms (173). hBD-1 is normally expressed in most of human epithelia, while hBD-2 and -3 are expressed upon bacterial stimulation. The gingival epithelium expresses hBD-2 constitutively, probably due to the constant presence of oral bacteria. Commensal bacteria (e.g. *S. gordonii*, *P. intermedia*, *F. nucleatum*) seem to have significant stimulating effect on hBD-2, -3 and IL-8 production, contributing to a permanent, low-grade stimulation of innate immune response in the periodontal pocket (70).

P. gingivalis has very small or no effect on hBD-2 and IL-8 production (170); furthermore, is able to decrease the level of IL-8 induced by other bacteria such as *F. nucleatum* phenomenon named ‘local chemokine paralysis’ (25). *A. actinomycetemcomitans* may determine cytokines and hBDs production in a manner correlated with the pathogenicity of different serotypes – the serotype c (found predominantly in periodontally health sites) induces a stronger production of hBDs and IL-8 than the other serotypes (170).

It was documented that live bacteria induce a better production of these molecules, as they invade in significant numbers and gain access to cytoplasmic receptors in host cells with role in modulating hBDs production (72).

The fate of bacteria and host cells subsequent to invasion

In vivo, microbial consortia including *P. gingivalis*, *A. actinomycetemcomitans* and *F. nucleatum* were found internalized in live buccal cells of healthy individuals (137-140). The study employed the fluorescence *in situ* hybridization method (FISH),

method which uses 16S rRNA oligonucleotide probes targeting bacterial ribosomal RNA. A stronger fluorescence signal is obtained from bacteria with larger number of ribosomes, therefore the microbes detected by FISH are likely to be alive (3).

In vitro studies have documented that *A. actinomycetemcomitans* is engulfed by the epithelial cells within membrane-bound vacuoles from which it is able to escape, move through the cytoplasm and may also spread to adjacent cells by using the microfilaments of the host cell (38, 110). After invasion, which is completed in 20 minutes, *P. gingivalis* survives intracellularly being localized mainly around the nucleus (95), can multiply within the host cells (102), also exit them and spread via cell-to-cell (181). In case of microorganisms residing in the oral cavity, internalized bacteria may evade the immune response and/ or the antibiotic therapy, and may constitute a reservoir for recolonization of dental sites after treatment (34, 73). Furthermore, the oral cavity is considered the portal of entry for various plaque-associated bacteria that via bloodstream might be able to colonize other body sites (59, 90, 91).

Internalized *P. gingivalis* is able to influence the apoptotic pathways towards extension of host epithelial cell survival (suppressing apoptosis), thus taking advantage of the intracellular environment (116). The periopathogen *A. actinomycetemcomitans* may directly induce apoptosis in host cells (104). Internalized bacteria are much more efficient in turning on the cell death machinery than adhering ones.

1.3.3. *F. nucleatum* attachment to and invasion of host cells

The studies done *in vitro* to investigate the abilities of *F. nucleatum* to adhere to different types of cells have shown that this organism can bind to epithelial cells, lymphocytes (174), erythrocytes, polymorphonuclear leukocytes, fibroblasts and HeLa cells (122). *F. nucleatum* can invade human primary epithelial cells (56, 174), human

immortalized gingival keratinocytes (61), epithelial cell lines KB, TERT-2, HaCaT cells (33, 46) and endothelial cells (55).

F. nucleatum can transport intracellularly non-invasive bacteria such as *Str. cristatus* (33) and enhance the adhesion and invasion of *P. gingivalis* (108, 145).

***F. nucleatum* adhesins**

The outer membrane proteins (OMPs) of *F. nucleatum* integrate multifunctional adhesion molecules functioning as receptors involved in adherence to oral bacteria (co-aggregation) and salivary proteins, as well as to host cells (32, 54, 115, 162). The adhesive properties might differ among fusobacterial strains (176). The *F. nucleatum* putative adhesins might be divided in two distinct types, based on their inhibition either by D-galactose or L-arginine:

1) Lectin-like adhesins mediate galactose-sensitive attachment to eukaryotic cells and co-aggregation with other bacterial strains (56, 84, 86, 174).

2) L-arginine-sensitive adhesion involved in co-aggregation (corn-cob formation) with streptococci strains such as *Str. cristatus* or hemagglutination (32, 162).

The co-aggregation of fusobacteria with Gram-positive microbes might be inhibited by L-arginine and is probably mediated by radD adhesin (79), while adherence to Gram-negatives (involving another OMP with significant role in co-aggregation, FomA) seem to be hindered by D-galactose (87, 99).

FadA was identified as an adhesin associated with the outer membrane found common to oral fusobacterial species including *F. nucleatum*, *Fusobacterium periodonticum*, and *Fusobacterium simiae*, but was absent from the nonoral fusobacteria. Mutation in *fadA* gene and complementation of the *fadA* mutation demonstrated that FadA is exposed on the bacterial cell surface and plays a major role in adhesion and probably also in invasion of host cells, as well as colonization of placenta (54, 65). FadA is used

both in nonsecreted form (pre-FadA, 13.6 kDa) and secreted form (mFadA, 12.6 kDa) by *F. nucleatum* attachment to epithelial cells (54, 117).

Another major OMP of circa 40-42 kDa, FomA, was identified in more *F. nucleatum* strains (7, 84) and characterized as a porin, effectively controlling the permeability of the outer membrane (85). FomA is a D-galactose inhibitable adhesin, with significant importance in bacterial co-aggregation and biofilm formation, mediating the co-aggregation of *F. nucleatum* with Gram-negative bacteria (84, 99).

The L-arginine inhibitable adhesion RadD has high molecular weight (360-370 kDa) and was identified as a member of the autotransporter family of proteins (32).

Autotransporters are proteins located on the bacterial surface associated with a range of virulence functions, including host cell adhesion (125). Several genes belonging to the autotransporter family have been identified in the genome of *F. nucleatum* ssp. *polymorphum* (80). RadD is involved in *F. nucleatum* co-aggregation with Gram-negatives and has major role in biofilm formation (79).

Receptors on host cells

Although the adhesins on *F. nucleatum* outer membrane have been investigated and characterized, it is not clear at present which are the cognate receptors on the membrane of host cells involved in bacterial internalization. While the invasion of the periopathogen *P. gingivalis* in oral epithelial cells depends on integrin receptors (181), this does not seem to be the case for *F. nucleatum*, as indicated by the finding that all integrins were downregulated in oral epithelial cells exposed for 2 h to *F. nucleatum* and paxillin and FAK (pivotal molecules associated with cell migration, adhesion of cells to extracellular matrix, activation for actin-cytoskeleton formation) were not transcriptionally modulated by *F. nucleatum* (57). The same study reported that *F. nucleatum* upregulated Rac protein, member of Rho family, involved in several cellular processes including endocytosis (57).

Recently it was reported that in endothelial cells the receptor binding FadA fusobacterial adhesion is represented by the vascular endothelial cadherin (35).

The mechanisms of bacterial entrance in host cells

The mechanism of invasion of fusobacteria into host cells is not yet fully elucidated. *F. nucleatum* was characterized as a non-motile microorganism, lacking fimbriae or pili (12). However, more recent studies suggested that FadA structure (pre-FadA-mFadA complex) functions as a filament anchored in the inner membrane of *F. nucleatum* and its retraction might lead to the invasion of the microorganism into host cells (117, 178).

As other oral bacteria, *F. nucleatum* lacks the type III secretion which ensures the bacterial uptake following bacterial injection of bacterial toxins directly into the cytosol of their eukaryotic host cells (e.g. *Yersinia* spp., *Salmonella* spp., *Shigella* spp., enteropathogenic *Escherichia coli*). However, *F. nucleatum* is 'suspected' to use type 4 secretion system, typically associated with piliation/fimbriation. Type 4 pilus (Tfp) confers twitching motility in microorganisms such as *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae* is also involved in colonization and biofilm formation. It was formerly reported that *F. nucleatum* undergoes gliding motility (1). *F. nucleatum* was shown to possess genes encoding protein components of the Type II secretion and Tfp, which appears closely associated with secretion pathway involved in biogenesis of Tfp, but it was reported that the system was incomplete (77). However, by using bioinformatic tools Desvaux *et al.* (27) demonstrated that Type 4 piliation/fimbriation secretion system is present in *F. nucleatum* ssp. *nucleatum* (ATCC 25586) and *vincentii* (ATCC 49256) and suggested that *F. nucleatum* might be able to assemble the Tfp. According to the mentioned study, also the type V secretion is present in *F. nucleatum*.

The entrance of *F. nucleatum* in epithelial cells tested *in vitro* revealed that bacteria were present intracellularly after 3-4 h aerobic co-incubation with primary GECs (56) or immortalized epithelial cells (33, 46, 65). Among the inhibitors tested, targeting

either host cell or *F. nucleatum*, staurosporine (broad-spectrum inhibitor of protein kinases) and sodium azide inhibited best the fusobacterial invasion in HGECs, suggesting that protein phosphorylation and energy metabolism of the host cell are important for the invasion process. Other inhibitors such as cytochalasin D and nocodazole reduced *F. nucleatum* invasion in GECs, but to a lesser extent. Bacterial protein synthesis, inhibited by cloramphenicol, is also a prerequisite for fusobacterial invasion (56).

1.3.4. The fate of *F. nucleatum* after host cell - bacteria interplay

A. Fusobacteria – dead or alive

In vitro, present intracellularly as single bacterial species, *F. nucleatum* was reported to survive up to 12 h in HaCaT (immortalized skin epithelial cells) (46), in agreement with another study documenting the destruction of *F. nucleatum* in HOK-16B (immortalized oral epithelial cells) as soon as 12 h after infection (71). The survival of *F. nucleatum* in other type of cells was not investigated.

F. nucleatum is highly sensitive to antimicrobial peptides such as h β D-3 and LL37, although there is a variable susceptibility among species (69). On the other hand, the fact that *F. nucleatum* binds the CD46 complement regulatory protein expressed by oral epithelial cells (103) and secretory immunoglobulin A (32) suggests the ability of this microorganism to evade host immune response.

B. Bacterial multiplication and further invasion

Multiplication of fusobacteria within epithelial cells shortly after invasion was reported and described as requiring participation of host actin filament cytoskeleton. Apparently, fusobacteria are also able to exit host epithelial cells and survive in cell culture medium for limited periods of time. However, further invasion in other cells was not ruled out (46). The spread of fusobacteria towards deeper layers was reported in organotypic (OT) models challenged with both *F. nucleatum* in suspension (30) or

in biofilm (48). Fusobacteria were observed advancing through intercellular spaces rather than cell-to-cell spread (30).

1.3.5. Host cells' responses to *F. nucleatum* stimulation

A. Receptors expression on the cell surface

F. nucleatum is capable of activating multiple pattern recognition receptors, including but not limited to TLR2 and TLR4 (98). TLR-4 expression after 4 h co-culture in H400 oral epithelial cell line was upregulated upon *F. nucleatum* as well as *P. gingivalis* stimulation (111).

By increasing the expression of MHC Class II and CD54 (ICAM-1) on the cell surface (51), *F. nucleatum* contributes to activation of innate immune response. (ICAM-1) is a member of immunoglobulin supergene family and is expressed on epithelial and endothelial cells, having important role in leukocyte recruitment at infection sites.

B. Production of cytokines or other proinflammatory molecules

F. nucleatum is a strong inducer of cytokine production (such as IL-1 β , IL-6, IL-8, TNF- α , IFN- γ) from epithelial cells, a summary of *in vitro* studies on this matter is presented in Table 1. *In vivo* studies on a mouse chamber model confirmed that *F. nucleatum* infection determines a robust proinflammatory response by stimulating TNF- α and IL-1 β production (127, 128). These cytokines function as chemoattractants for neutrophil cells. The interaction between *F. nucleatum* and GECs appears to involve CD14 (25), co-receptor recognizing bacterial LPS and key mediator of innate host defense. The invasion of fusobacteria in GECs is accompanied by IL-8 production of host cells, which is dependent on TLR-2 stimulation (5). IL-8 production involves activation of NF- κ B, JNK and p38 (64). In contrast to the above mentioned, another study reported that TLR-2 and -4 were not transcriptionally modulated in oral epithelial cells stimulated with *F. nucleatum*, together with downregulation of IL-8 (57).

Table 1. Cytokines involved in periodontal tissues destruction released from different types of cells following *F. nucleatum* stimulation in vitro.

Cytokine	<i>F. nucleatum</i> components	Host	Reference
IL-1 β	Killed bacteria	HOK-18A	(51)
	Live and dead bacteria	HGECs	(160)
	Live bacteria	HGECs in multilayer	(30)
IL-6	Live bacteria	HIGKs	(61)
	Live bacteria	GMSM-K	(103)
IL-8	Live bacteria	HGECs	(25)
	Live bacteria	HGECs	(56)
	Cell wall extract & LPS	HGECs	(92)
	Live bacteria	HGECs from pocket epithelium	(170)
	Live bacteria	HIGKs	(61)
	Live bacteria	HaCaT cells	(47)
	Live bacteria	HOK-18A and HGF-1 cells	(157)
TNF- α	Cell wall extract & LPS	HGECs	(92)
	Live bacteria	HGECs in multilayer	(30)

HGECs = human gingival epithelial cells; HOK-18A, GMSM-K = immortalized human oral epithelial cell lines; HaCaT = immortalized skin epithelial cells; HGF-1 = immortalized human gingival fibroblasts; HIGKs = human immortalized gingival keratinocytes

The production of hBD-2 and hBD-3 may be induced in epithelial cells following stimulation by *F. nucleatum* (49, 70, 72, 83). Similarly to IL-8 production, TLR-2 (together with the cytoplasmic receptor NALP2) and the signaling pathways NF- κ B, JNK and p38 (72) are involved in hBD-2 and hBD-3 induction. However, it appears

that regulation of IL-8 and hBD-2 production may be done by different mechanisms in gingival epithelial cells (167).

Fusobacteria may affect wound healing and tissue remodeling by stimulation of metalloproteinases MMP-2, -9 and -13 production (47, 103, 169) and also of plasminogen activator by gingival fibroblasts (175). MMP-13 (collagenase 3) has a wide substrate specificity and may contribute to the connective tissue destruction in periodontitis (11). In one study it was suggested that MMP-9 production in oral epithelial cells upon *F. nucleatum* stimulation is associated with IL-6 and IL-8 production (103).

C. Cell survival, growth and proliferation

As early as 2 h after exposure to *F. nucleatum*, in human immortalized gingival keratinocytes the signaling pathways MAPK and NF- κ B were activated (61). MAPK pathway is involved in physiological processes as cell growth, proliferation, migration, differentiation, survival, development and innate immunity. Similarly, cell survival, proliferation and migration was documented by Uitto *et al.* (169) in a study using HaCaT cells stimulated for 2 h with *F. nucleatum*, in a wound closure model. The protein kinases investigated in this study pointed out towards a cell survival more than to apoptosis. Even a longer exposure (24 h) did not result in a significant effect on cell viability (46, 71).

D. Apoptosis

F. nucleatum putative apoptosis-inducing proteins Fap2 and RadD expressed on the outer membrane are reported to induce cell death in Jurkatt cells (immortalized lymphocyte cells) (78, 79). Jewett *et al.* (68) reported apoptosis induced by live and formaldehyde-treated *F. nucleatum* in peripheral blood mononuclear cells, but not any significant impairment of cell survival on Cal27 and SCC4 oral keratinocytes cell lines. On periapical fibroblasts, *F. nucleatum* exert a direct cytotoxic effect (179).

2. Problem statement and aims of the study

Invasion of host cells is considered a virulence factor for oral bacteria. The ability of *F. nucleatum* to enter, survive and multiply within keratinocytes raised the interest of investigating whether this bacterium is also able to enter other type of cells. It was demonstrated that *in vivo* *F. nucleatum* may reach the connective tissue in some situations; but it was lacking an *in vitro* study to investigate whether *F. nucleatum* is able to enter the fibroblasts, the main cell type in the connective tissue. Such study was relevant to be done even before addressing the question of *F. nucleatum* invasion of and advancement into deeper layers of a multilayered model. The invasion of *F. nucleatum* in reconstructed 3D tissue models *in vitro* has been to date investigated in only two other studies, none of them published at the time when this work was initiated. The need of performing such invasion studies by use of multilayer OT model was obvious, as well as the need for developing new, relevant investigation tools, such as *in vitro* JE or SE organotypic models.

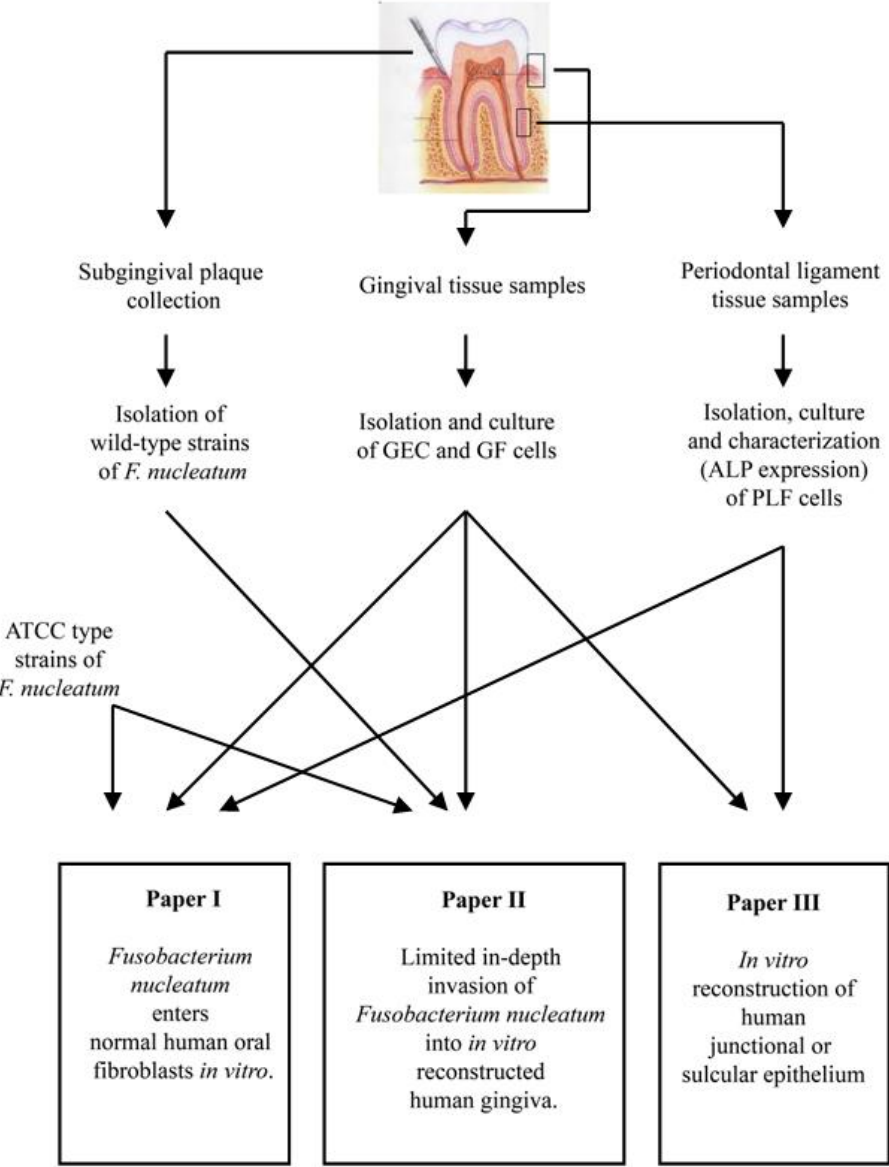
General objective

To investigate the interactions between different oral strains of *F. nucleatum* and normal human oral cells by use of *in vitro* cell culture models.

Specific objectives

1. To study the internalization of *F. nucleatum* in oral fibroblasts with gingival and periodontal ligament origin
2. To assess the effect of *F. nucleatum* on the epithelial layers by, using an organotypic model of gingival mucosa
3. To develop a organotypic model having the characteristics of sulcular/ junctional epithelium

3. Study design



4. Methodological considerations

4.1. *F. nucleatum* strains used in the study

In our experiments (Paper I and II) we used live fusobacteria. From the ATCC type strains we chose *F. nucleatum* ssp. *polymorphum* which was isolated mainly from healthy gingival sulci, also *F. nucleatum* ssp. *vincentii* and *nucleatum*, often associated with periodontally diseased sites (12, 42, 50, 124). The last mentioned strain was also reported to attach to and invade epithelial cells much more efficiently than the first strain; and able to efficiently multiply intracellularly (46). For means of comparison, two wild-type isolates were included in the study.

4.1.1. Plaque sampling and primary cultures

The collection of wild strains was done after informal and written consent of each individual. We have used sterile paper points nr. 50 – each one was inserted to the deepest possible point of the periodontal pocket or gingival sulcus and kept for 20 seconds. Samples were taken from three different periodontal sites from each subject and then transported to the laboratory in appropriate transport medium. The samples from each individual were pooled in a sterile vial containing 1.5 ml PBS and three sterile glass beads and vortexed for 2 minutes. From each sample suspension 10 µl were plated on crystal violet erythromycin agar plates, which is an enriched differential medium for the isolation and presumptive identification of *F. nucleatum* (172). Further analysis for identification of *F. nucleatum* was based on Gram staining, growth anaerobic conditions, stereomicroscopic colony morphology and biochemical profile, by use of miniaturized enzymatic tests (Rapid ID 32 A system, Biomerieux SA, France).

Fusobacteria from frozen stocks were first grown on fastidious agar plates (Lab M, UK) and then one colony was further inoculated on agar. Bacterial suspensions from

each strain were prepared in liquid growth medium, brain heart infusion (BHI) with hemin (5 mg/l) and menadione (1 mg/l), by use of optical density readings (approximately OD = 0.02 at 600 nm) and incubated anaerobically overnight, until circa OD = 1 was reached. Then, bacterial suspensions were adjusted for density according to the requirements of the experiments (usually ratio 100 : 1 of fusobacteria to one host cells), washed in PBS and resuspended in specific medium, then co-cultured with the host cells. In some cases *F. nucleatum* were fluorescently labeled with fluorescein-isothiocyanate (FITC) prior to experiments.

4.1.2. *F. nucleatum* fluorescent labeling (Papers I and II)

The goal of direct observation and localization the microbes when co-cultured with the primary cells triggered the necessity of using fluorescently labeled *F. nucleatum* in our experiments. Antibodies against *F. nucleatum* are not widely produced to be made commercially available and obtaining them is costly and time-consuming. Another alternative of fluorescently labeling of bacteria, by use of Bacterial Viability Kit LIVE/DEAD BacLight Kit (L7012) for microscopy and quantitative assays (Invitrogen) did not give in our hands the expected results. The main problem was that the stain was leaking also into the host cells, making thus difficult to establish the location of fusobacteria when observed by CLSM, or quantification of internalized microorganisms by flow cytometry. We attempted to tag *F. nucleatum* with green fluorescent protein (GFP) and in order to achieve this a collaborative study was initiated and run under supervision of Prof. Wim Crielaard and postdoc Dongmei Deng at Academisch Centrum Tandheelkunde Amsterdam (ACTA) and Vrije Universiteit (VU - Amsterdam, The Netherlands). Although a shuttle plasmid containing GFP was successfully elaborated and delivered into *F. nucleatum* by electroporation, the transformants did not express GFP, probably due to resistance of *F. nucleatum* to genetic manipulation (Appendix). Therefore, direct labeling with a fluorescent dye such as FITC rose as a convenient method to use in our studies. Pilot experiments were performed in order to calibrate efficient fluorescent staining.

Various concentrations of FITC were tested in order to obtain a good bacterial fluorescence without affecting the viability of *F. nucleatum* (Fig. 3).

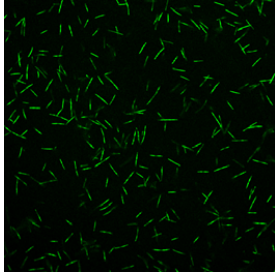


Figure 3. FITC-labeled *F. nucleatum* ATCC 25586 in suspension observed by CLSM.
(Source: G Dabija-Wolter).

4.2. Primary cells and organotypic models

All the gingival tissues and teeth samples used in this work were collected from individuals undergoing third molar extraction at Department of Clinical Dentistry – Oral Surgery and Oral Medicine, Faculty of Medicine and Dentistry, Bergen Norway. All the patients were informed upon the purpose of the studies and signed consent forms. The studies were approved by the Committee of Medical Ethics in Research (REK Vest 177.04) at the University of Bergen.

4.2.1. The rationale for using primary cells

The primary cells mimic closely the *in vivo* state and generate more physiologically relevant data. The immortalized cells are transformed cells, thus genetically different from normal cells and may present various genetic abnormalities, such as mutations and chromosomal rearrangements. Although they are genetically unstable and thus not entirely representative for normal cells, they might be used as models of normal cells.

Advantages for using an OT model in studying host-bacterial interactions

- Supporting a more realistic vision, closer to the *in vivo* situation
- This model was previously established (22) and is now already standardized in our laboratory and allows its use for different experiments, including exposure to oral bacteria
- Allows studying penetration of invasive bacteria from the surface epithelial cells into the underlying tissue layers, as it takes place *in vivo*
- Offers possibility to investigate the tissue response to bacterial aggression in a controlled, standardized (serum free) environment, repeatable setup
- Starting point for further development of similar OT culture models with a different phenotype

A continuous supply of tissue samples for generating new batches of primary oral epithelial and fibroblast cells was sometimes difficult to achieve. To overcome this issue, frozen stocks of primary cells were prepared, most of them in passage one. When necessary, cells were thawed and reseeded in culture flasks, for use in experiments.

4.2.2. Oral fibroblasts (Paper I - III)

In invasion studies using cells in monolayer and also for the reconstruction of OT models, oral gingival fibroblasts (GFs) and periodontal ligament fibroblasts (PLFs) were used. Both types of fibroblasts were grown in the same culture medium (DMEM with 10% FCS) and explant technique was used for isolation of the cells from connective tissue samples. Although not always noticeable, we have observed differences in the growth rates between GFs and PLFs collected from the same donor. In general, GFs started to migrate from the connective tissue explant in 2-4 days, while approximately a week was necessary for the PLFs. Usually GFs grew faster and up till higher passages than PLFs. We have experienced a higher failure rate with PLF than

GF, therefore was not always possible to obtain matched GF-PLF from the same donor.

4.3. Invasion studies

The *F. nucleatum* invasion was tested on primary cells in monolayers (GFs, PLFs – Paper I) and on *in vitro* reconstructed model of human gingiva, comprised of multilayered, differentiated epithelial layers on top of a collagen matrix populated with GFs (Paper II). The protocol for the construction of a novel OT model with phenotype similar to junctional epithelium (JE) or sulcular epithelium (SE) was set up in Paper III. This culture model may serve as an investigation tool in further studies, including invasion studies.

4.3.1. Imaging methods and the antibiotic protection assay (Paper I, II)

Imaging methods (TEM, SEM, CLSM) were chosen for direct observation at different time intervals, of live or sometimes fixed host cells in monolayers co-cultured with bacteria. These methods are more costly and time-consuming than other methods, but they have the advantage of rendering evident the physical association between microorganisms and host cells (Fig. 4). The quantification of internalized (considering both dead and alive) fusobacteria was assessed by flow cytometry.

The visualization of fusobacteria in epithelial layers by CLSM (Leica) was also tried by using the entire piece of OT tissue. After challenging with FITC-labeled fusobacteria the OTs were stained for 1-2 h with Cell Tracker red (Molecular Probes Europe, Leiden, The Netherlands), as described in the protocol for the fluorescently labeling of tissue slides (Paper II). Each entire piece of OT tissue was placed upside-down in a depression glass slide (Sciencelab.com, Inc., Houston, TX, USA), covered up by mounting medium, and a cover slide of 0.2 mm thickness was placed on top. When such assembly was investigated by CLSM, the laser was directed

perpendicularly on the OT tissue, from the top of the epithelial layers towards deeper layers. However, due to the high number of detaching epithelial cells, the uneven staining of the deeper epithelial layers and the limited penetration of the confocal laser (circa 50 μm), the depth to which fusobacteria reached into the epithelial layers could not be correctly estimated. Therefore, the imaging results were not evaluated as satisfactory and for further investigation exposed OT models were cut in 5-7 μm slices and processed as described in Paper II, then visualized by CLSM (Fig. 4).

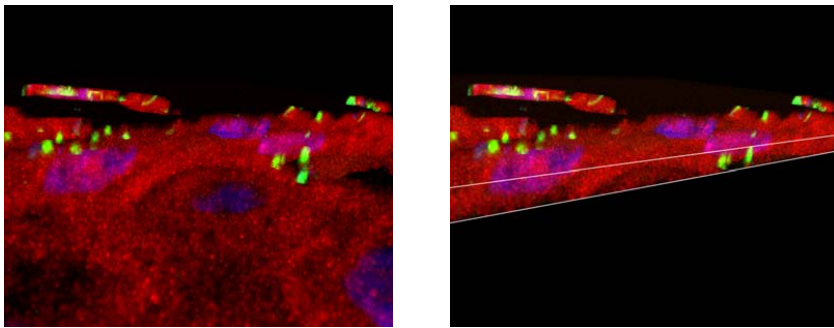


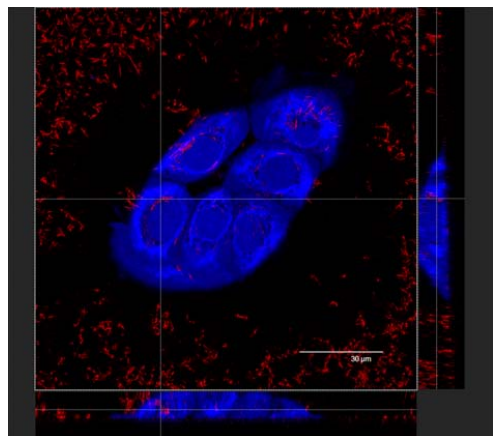
Figure 4. Invasion of *F. nucleatum* ssp. *nucleatum* in OT model of gingival mucosa. Fusobacteria are present as green fluorescent structures in contact with epithelial cells (red). The host cell nuclei are stained with DAPI (blue). The image taken by CLSM was processed to render 3D (Imaris software, Bitplane AG) and by using the ortho-slicer function, the position of *F. nucleatum* related to epithelial cells may be observed. (Source: G Dabija-Wolter).

In preliminary tests, we have also performed the antibiotic protection assay following already published protocols (56), in order to check the ability of *F. nucleatum* to enter the host cells and to quantify the internalized bacteria. Briefly, cells in monolayer were exposed to fusobacteria in suspension in cell culture medium for defined time periods (between 1 h and 24 h). After the exposure interval, the bacteria-containing cell culture medium was discarded, the cells were rinsed with PBS and a solution of cell culture medium containing antibiotics (usually metronidazole and gentamicin) were applied for a period of time (between 1-3 h, depending on the antibiotic concentration). The used antibiotics were supposed not to penetrate into the host cells within the mentioned interval, but annihilate only the extracellular bacteria, letting alive those which entered the host cells. After rinsing away the antibiotic solution and lysing the

host cells (by use of sterile distilled water), the cell lysate was fold-diluted and plated on fastidious agar plates. The internalized bacteria in host cells were quantified by counting the colony forming units resulted on agar plates. However, due to the following drawbacks, we decided that the antibiotic protection assay was not suited to be used in our study:

- The extent of antibiotic solution action cannot be accurately controlled, whether penetrates or not the host cells, partially or totally influencing the viability of internalized bacteria.
- We have observed that *F. nucleatum* adhering on the cell culture dish bottom were not completely killed by the usual antibiotic treatment applied as in the antibiotic protection assay and there were still recovered live microorganisms able to generate colony forming units on agar plates. We hypothesize that in such case *F. nucleatum* switched to a ‘quiescent-mode’ and did not respond properly to antibiotic treatment since metronidazole acts on metabolically active microorganisms.
- Dead bacteria found intracellularly (taken up actively by the host cells, as shown in Fig. 5 or eventually affected by the antibiotic treatment) cannot be quantified by use of this method.

Figure 5. Heat killed *F. nucleatum* ssp. *nucleatum* stained with propidium iodide co-cultured with live GECs for 24 h. Some fusobacteria (red) have been internalized by host cells (stained with Cell Tracker blue) and are localized intracellularly mainly around the cell nuclei. (Source: G Dabija-Wolter).



4.3.2. Invasion of *F. nucleatum* into OT model (Paper II)

The assessment of fusobacterial invasion was done by use of CLSM, observing the localization of FITC-labeled *F. nucleatum* on slices cut from 24 h - challenged OT models. Slices from both paraffin-embedded and frozen OTs were used, but the first ones gave the best images.

At the time when we started our laboratory experiments there were no published studies referring to an optimal period of exposure of a three-dimensional tissue to fusobacteria. In our preliminary experiments on 3D tissues, the aerobic conditions successfully used in other studies using 2D monolayer cultures (33, 46, 56, 65) did not seem optimal for fusobacterial invasion into a multilayered model. Not only that the apical part of our models was comprised of confluent epithelial cells that have been shown to be more resistant to bacterial invasion (46), but also the fusobacteria (planktonic, not biofilm) placed on the top of tissues in our model were directly exposed to the surrounding atmosphere and thus more exposed to O₂, being in a very small quantity of 20-30 µl medium (Fig. 6).

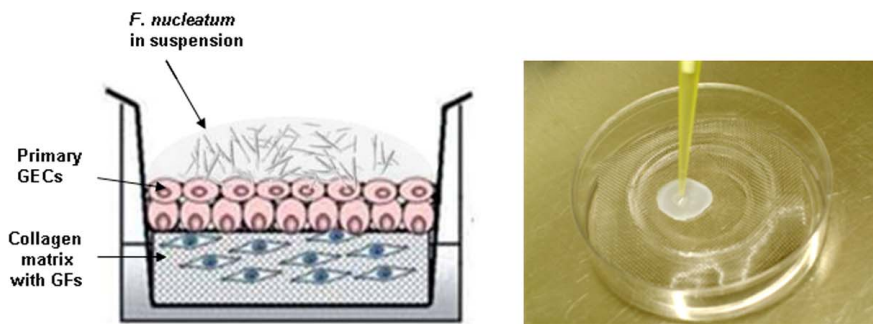


Figure 6. The OT model comprised of epithelial layers on top of collagen matrix with GFs exposed to *F. nucleatum*. **A.** Schematic representation. (Source: DE Costea). **B.** Placing the drop (20-30 µl) of bacterial suspension on top of the epithelial layers. (Source: G Dabija-Wolter).

On the other hand, we have found that a longer period of anaerobic incubation such as 24 h was too stressful for the OT models. Therefore we have arbitrarily chosen to limit the anaerobic conditions to 3 h, to allow *F. nucleatum* to survive and possibly invade the epithelium; and at the same time minimize the negative effects of anaerobic environment on the OT tissues.

4.3.3. Quantitative real time RT-PCR (Paper II)

In order to assess the mRNA expression only in epithelial cells, the epithelial components of the OT models, exposed to bacteria or controls, were collected and used for this analysis. Frozen OT tissues were selected and melted at room temperature, one by one. Each OT was briefly washed in PBS, then the epithelial component was gently peeled off the collagen matrix by help of fine tweezers and placed immediately in 200 µl RNA-Stat. The epithelium was lysed by repeated pipetting and stored at -80 °C until further processing.

4.3.4. Immunohistochemistry (Papers II, III)

Sections of formalin-fixed and paraffin-embedded OT or control tissues were cut, dewaxed and rehydrated in xylene and graded ethanol. For antigen retrieval microwave treatment was used (900 W for 8 min, then 450W for 15 min). The Tris-EDTA buffer was used for most antibodies, except TG-I where 10 mM citrate buffer gave better results. To reduce unspecific binding, 10 % goat serum diluted in 3% bovine serum albumin (BSA) in TBS was applied on tissues for 30 min. Primary antibodies were applied then for 60 min, following quenching the activity of endogenous peroxidase by use of H₂O₂ for 10 min. The appropriate secondary antibodies were applied for 30 min. Diaminobenzidine (DAB+) was used as chromogen, following counter-staining with haematoxylin, dehydration and mounting with non-aqueous mounting medium Eukitt (O. Kindler GmbH & Co., Freiburg, Germany). The peroxidase block, secondary antibodies and DAB were from

EnVision™ kit, which was used in all cases. Unless otherwise indicated, all reagents were purchased from DAKO A/S, Golstrup, Denmark. In some cases the protocols were optimized, for example overnight incubation at 4°C and antibody 1/100 dilution gave better results than 60 min incubation at room temperature with 1/250 diluted caspase-3 antibody.

Table 2. Information on antibodies and their titration used for immunohistochemical staining.

Antibody	Type	Clone/ number	Titration	Source	Tissue sections	Buffer
CK 8	mouse monocl.	-	1 : 5	CRUK*	P	TE
CK 10	mouse monocl.	-	1 : 5	CRUK	P	TE
	IgG1	-	1 : 50	DAKO	P	TE
CK 13	IgG1	KS-1A3	1 : 50	Novocastra	P	TE
CK 16	mouse monocl.	-	1 : 5	CRUK	P	TE
CK 19	mouse monocl.	-	1 : 5	CRUK	P	TE
Cleaved caspase-3	rabbit monocl	-	1 : 250	Cell Signalling Technology	P	Citrate
E-cadherin	mouse monocl.	-	1 : 9000	R & D Systems	F	TE
Ki-67	IgG1	MIB-1	1 : 25	DAKO	P	TE
Filaggrin	IgG1	15CID	1 : 50	Monosan	P	TE
MMP-13	mouse monocl.	-	1 : 30	R & D Systems	F	TE
TG-I	rabbit polycl.	pab0061	1 : 250	Covalab	P	Citrate

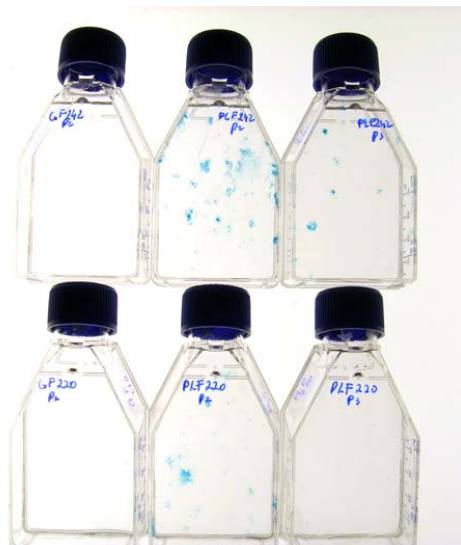
* CRUK = Cancer Research United Kingdom (kind gift from Prof. I.C. Mackenzie, Institute for Cell and Molecular Science, London, UK). P = paraffin; F = frozen sections. TE = Tris-EDTA buffer.

4.4. Issues of clinical relevance in this study

A number of wild-type strains were isolated from patients affected by periodontal diseases but also from clinically healthy individuals. This was done in order to investigate whether strains collected from diseased sites have an enhanced virulence compared to the ATCC type strains, or those collected from healthy patients, such as it was demonstrated for *P. gingivalis* (66). For this reason the wild-type strain AHN 8518, ampicillin-resistant *F. nucleatum* ssp. *polymorphum*, was included in our work. However, due to the limited number of cultures of primary cells (GECs or GFs) only few wild strains from those collected were used in experiments.

Previous studies have indicated that, in culture, primary cells may lose their original phenotype after several passages (100). Therefore, in our experiments only primary cells in early passages were used. The GECs were in their 1st-4th passage when used, the GFs in 1st-5th passage, whilst PLFs were taken in use maximum to their 3rd passage. In the construction of JE / SE models GFs and PLFs were matched from the same donor as often as this was possible.

Figure 7. The ALP expression in fibroblasts at passage 2, almost absent in GF and strong in PLF, decreases in intensity in PLF already by passage 3. (Source: G Dabija-Wolter).



5. Summary of results

Paper I

According to our knowledge, this was the first study to document the entrance of fusobacteria in primary normal human oral fibroblasts. We have established that all three studied strains of *F. nucleatum* (ssp. *nucleatum*, *polymorphum* and *vincentii*) begun their invasion into either GFs or PLFs during the first hour of co-culture. A higher number of bacterial-like structures were observed by SEM and TEM in the following hours of co-culture in the process of entering the fibroblasts or already internalized. Flow cytometry tests revealed a significant increase in the fluorescent signal derived from bacteria internalized in fibroblasts exposed for 3 hours compared with the baseline ($P < 0.001$) and a further increase at 5 hours. The highest bacterial load found in exposed fibroblasts of both origins was of *F. nucleatum* ssp. *polymorphum* and the smallest was of *F. nucleatum* ssp. *vincentii*. The bacterial load in PLFs was higher than in GF, estimated at 3 and 5 h of exposure to same fusobacterial strain.

Paper II

The invasive abilities of two laboratory strains and two clinical isolates of FITC-labeled *F. nucleatum* were investigated by placing bacteria in suspension on top of OT models of gingival mucosa. All fusobacterial strains used penetrated the superficial epithelial layers of OT tissues exposed for 24 h, but were not detected in the collagen matrix. The challenged OTs showed accentuated shedding of superficial layers and increased number of cleaved caspase-3 positive cells (7.82 ± 2.44 cells per field) compared with controls (2.29 ± 1.62) localized in spinous and basal layers. *F. nucleatum* ssp. *nucleatum* was the strongest inducer of caspase-3 activation in 24 h samples, while at 48 h the clinical isolates determined higher number of apoptotic cells. More positive cells for the proliferation marker Ki-67 were found in exposed tissues than in controls both at 24 and 48 h, although not statistically significant. The levels of E-cadherin and MMP-13 mRNA in epithelial cells were investigated both in challenged OT models and monolayers. Gene

transcript levels for E-cadherin and MMP-13 mRNA were not significantly altered in OTs. A variable and disproportionate response of MMP-13 mRNA level resulted in challenged primary keratinocytes in monolayers, compared to multilayer OT culture.

Paper III

The construction of a novel OT model was designed with the aim of obtaining a culture model with phenotype similar to JE or SE, to be used further for oral bacterial invasion studies. The time in culture allowed for the OT models to grow and differentiate had significant impact on the epithelium phenotype. The epithelial component in 3 and 5 days old OTs showed limited differentiation and expressed cytokeratins (CK) 8, 13, 16, 19, transglutaminase and Ki-67 in a fashion closest to native JE samples. The type of oral fibroblasts used in the collagen matrices of OT tissues had also impact on the phenotype of the culture model. Periodontal ligament fibroblasts (PLFs) supported better than GFs a higher expression of CK19 and suprabasal proliferation, although statistically significant only at day 5. The differentiation of epithelial layers was noticeable in cultures of 7 and 9 days; and the percentage of CK19-expressing cells was significantly decreased, as was the rate of proliferating cells in basal layers. Concluding, early harvested OTs (days 3-5) constructed with PLFs were proposed as suitable for JE models, while those with either GFs or PLFs grown to day 7 may be considered as SE models; and day 9 OTs with GFs are suitable for GE models.

6. General discussion

Periodontitis is the most common infectious disease in humans. Considered by some authors as commensal, or presented by others as ‘opportunistic pathogen’, *F. nucleatum* have a definite role in periodontal disease, due to remarkable adhesive properties and ‘promiscuous’ association with periodontal pathogens (145, 153). Since *F. nucleatum*, among oral bacteria associated with periodontal disease, are the most prevalent in clinical infections of other body sites, the studies linking oral bacteria to systemic conditions placed *F. nucleatum* under a new light. It was suggested that a ‘friend’ microorganism in one place might be a ‘foe’ in another place (36). Hence, the studies assessing the abilities of *F. nucleatum* to invade, survive within host cells and eventual spread to deeper layers, are highly relevant.

Invasion of *F. nucleatum* in fibroblasts

To the best of our knowledge, we were the first ones to show that *F. nucleatum* is able to enter GF and PLF, the major cell type in connective tissue. Since the maintenance of the connective tissue is crucial for the integrity of periodontium, the interactions of fibroblasts with oral bacteria are of high interest. We have documented that *F. nucleatum* may start invading oral fibroblasts within the first hour of co-culture and continues for a few hours (Paper I).

Although we have not investigated in depth this hypothesis, based on the images taken by SEM we suggested that *F. nucleatum* employ the zippering mechanism for entrance into oral fibroblasts (Fig. 8). This mechanism was extensively studied on bacteria such as *Yersinia* ssp., and *Listeria monocytogenes*; it describes the situation when the bacterium gets in very close contact with the cell surface and then ‘sinks’ into the cytoplasmic membrane. Han *et al.* (56) reported *F. nucleatum* as first oral bacteria to use zippering mechanism when invading oral epithelial cells. The receptors on the epithelial cells binding fusobacterial adhesins are not identified at present; however the receptor for *F. nucleatum* FadA in endothelial cells was identified as vascular

endothelial cadherin (35). This fact might be not surprising, considering that *F. nucleatum* and *L. monocytogenes* share the same type of invasion mechanism, and E-cadherin was known as receptor for *Listeria* ssp. (107). We consider that the hypothesis of cadherins functioning in different types of cells as receptors for *F. nucleatum* worth of further investigation.

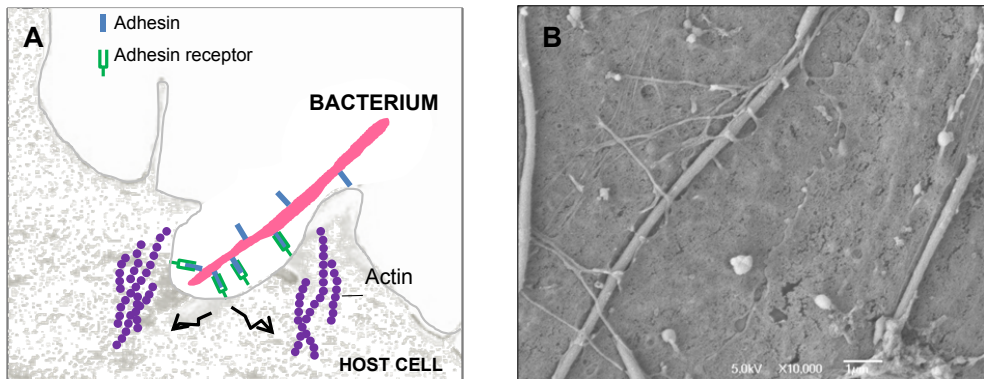


Figure 8. A. Schematic representation of *F. nucleatum* internalization into a host cell by zipper mechanism; **B.** *F. nucleatum* ssp. *polymorphum* on the surface a gingival fibroblast observed by SEM, after 8 h of co-culturing. (Source: G Dabija-Wolter).

The ability of bacteria to escape from vacuoles into the cytosol (such in case of *A. actinomycetemcomitans*) is regarded as a virulence factor. In our TEM images we have observed internalized *F. nucleatum* within vacuoles but also without vacuoles within the cytoplasm of fibroblasts (Paper I). Another author made the observation that about 70% internalized fusobacteria resided within actin surrounded vacuoles (46). The hypothesis that, fusobacteria may escape from vacuoles, was suggested but not tested up to present. Another study investigating the hBDs production by *F. nucleatum*-stimulated epithelial cells proposed that live fusobacteria, internalized in significantly higher numbers than heat killed ones, could access NALP2 cytoplasmic receptor involved in hBD-3 expression by eventual escape from vacuoles (72).

We have determined that the highest bacterial load in fibroblasts was of *F. nucleatum* ssp. *polymorphum*; but whether this one was the most invasive among the tested strains, is questionable. *F. nucleatum* ssp. *polymorphum* has a bigger size than ssp. *nucleatum* (about 10 µm length and circa 3 µm, respectively), therefore a stronger fluorescence signal may be derived from internalized ssp. *polymorphum* when assessed by flow cytometry. On the other hand, in the genome of *F. nucleatum* ssp. *polymorphum* were identified adhesins (two proteins containing von Willebrand type A domains) and in addition ten autotransporter genes belonging to the Type V secretion system, that were not found in the genomes of *F. nucleatum* ssp. *nucleatum* or *vincentii* (80). These particularities might render ssp. *polymorphum* more adhesive and invasive than the other two strains.

It was shown that in epithelial cells, *F. nucleatum* may undergo bacterial division (46), however, the possibility that fusobacteria multiplies within oral fibroblasts requires further investigation. More often than in case of the other two tested strains, *F. nucleatum* ssp. *nucleatum* were observed by SEM as having the mark of binary fission, suggesting bacterial division may take place after bacterial internalization (personal observation of the autor).

In our study, PLFs were consistently more invaded by fusobacteria than donor-matched GFs, but the reasons behind this finding remain open to further research. Neither the receptors on the fibroblasts binding fusobacterial adhesins, nor the mechanism of *F. nucleatum* uptake into these host cells are described at present.

Invasion of *F. nucleatum* in OT model

We wanted to assess the invasive abilities of *F. nucleatum* into a multilayer oral tissue model, since at that time when we have undertaken this study, all the reports on fusobacteria invading oral cells were done by using cells in monolayer, but not in OT model. We have used a standardized serum-free model of gingival mucosa starting from an OT model developed and characterized in our laboratory (21, 22). Our GE

model (Paper II) displayed a stratified, differentiated epithelial compartment, expressing CK 13 and 16 (characteristic for stratified epithelia), also CK 10 and TG-I (markers of terminal differentiation), as shown in Fig. 9. CK 19 is normally present in basal layers of GE *in vivo*, but also *in vitro* cultures gain expression of this marker (40, 100).

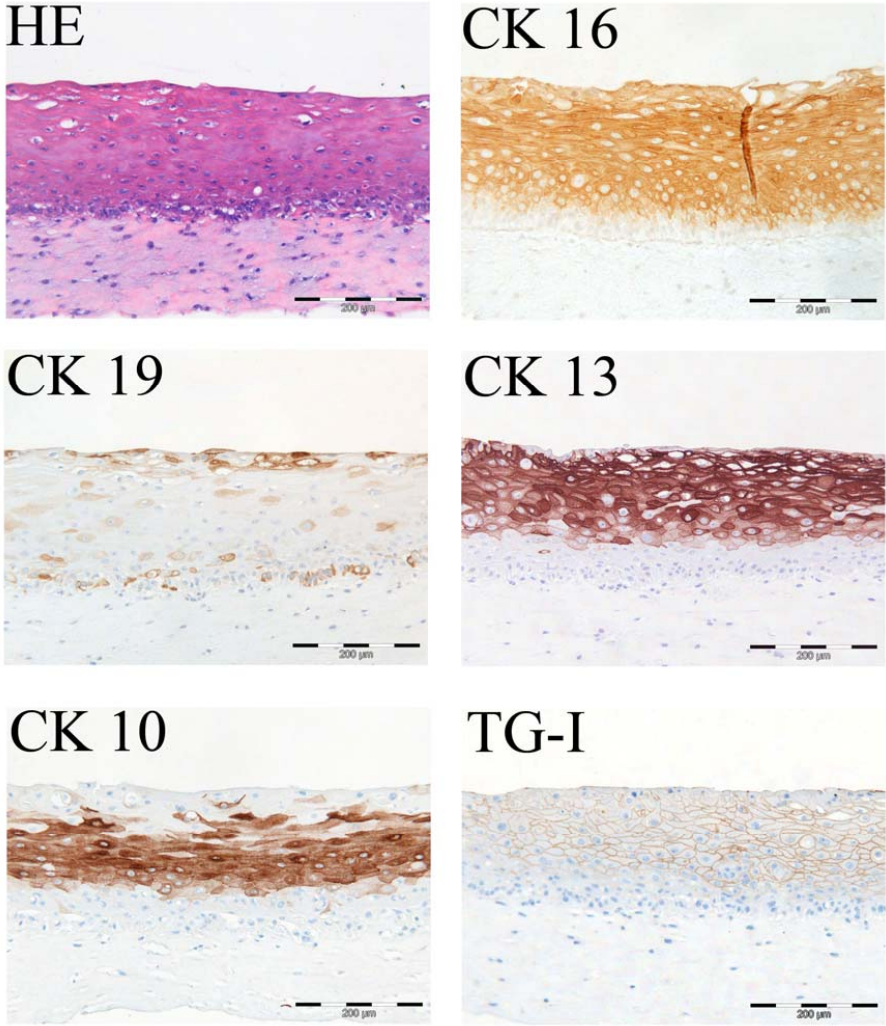


Figure 9. Sections through the *in vitro* reconstructed model of gingival mucosa stained with haematoxylin-eosin (HE) and by IHC for different differentiation markers. (Source: G Dabija-Wolter).

We have determined that all studied fusobacterial strains invaded superficially the OT model and no bacterial structures were found within collagen matrices. We could not observe differences between ATCC and wild-type fusobacterial strains regarding the penetration depth in the epithelial component. Because of the exfoliated superficial epithelial layers, it was very difficult to estimate the depth reached by fusobacteria in the epithelium. However, we determined that the number of cleaved caspase-3 positive cells was higher in OTs exposed to wild-type strains than to ‘standard’ ones after 48 h challenge (Fig. 10), although at 24 h no difference could be observed. A better survival in time of the clinical isolates than the ATCC strains might be the reason of apoptosis triggered at a later time point in invaded host tissue. The fate of different clinical isolates subsequently penetration into host tissue would be relevant to investigate in further studies *in vitro* by using OT models.

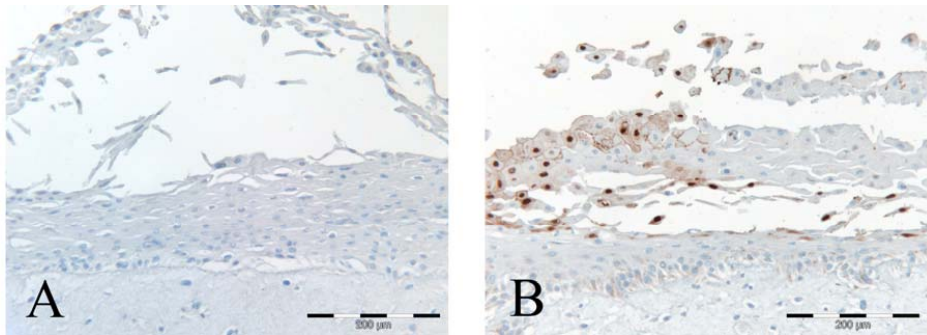


Figure 10. Cleaved caspase-3 induced in superficial epithelial cells of an OT model exposed for 48h to A. Cell culture medium alone; B. *F. nucleatum* AHN 8518. (Source: G Dabija-Wolter).

Although OTs exposed to *F. nucleatum* showed important shedding of superficial epithelial layers, the overall tissue homeostasis was not significantly perturbed, as reflected by the level of apoptosis and cell proliferation in the tissue. Bacterial-induced cell death is a wide and interesting subject, since apoptotic cells were found *in vivo* at periodontally affected sites (8, 75, 165). The apoptosis induced in reconstructed OT

models following exposure to *F. nucleatum* is at present a matter of debate. While cell death was induced by fusobacteria both in suspension (30) and biofilm (48) in two different models, another study demonstrated lack of apoptosis, stimulation of cell proliferation and migration (129). It is worth to mention that, although all authors characterized their study model as similar to junctional epithelium, these OTs differed to a great extent. In two studies immortalized skin keratinocytes were used (HaCaT cells) for construction of different JE-like culture models challenged with the same fusobacterial strains in biofilm. Interestingly, one of these studies reported significant apoptosis in the epithelial compartment induced by a wild-type fusobacterial strain (48), whilst the other suggested apoptosis-suppressing abilities of the same fusobacterial strain (129). The heterogeneity of models used in all above mentioned studies, including our Paper II, would explain the variations and inadvertence in the results. Furthermore, none of these reports used a second method to validate the fusobacterial-induced apoptosis in their models.

Interestingly, the loss of integrity of superficial layers of the epithelium compartment following fusobacterial challenge was not reflected by the level of E-cadherin mRNA. One explanation might be that other cell adhesion molecules are involved stronger than E-cadherin in fusobacteria – epithelial tissue interaction. Another reason might be that significant changes at transcriptional level should be observed at an earlier time point than 24 h.

Monolayer versus multilayer culture model

We have investigated MMP-13 mRNA production in epithelial cells in mono and multilayer as response to *F. nucleatum* stimulation. MMP-13 was reported to be expressed by the cells of gingival pocket epithelium, suggesting the role of this enzyme in the progression of the epithelium into the connective tissue (168). In our Paper II we have determined that MMP-13 was upregulated in *F. nucleatum* - stimulated GECs in monolayers, but a disproportionate response was obtained when an OT model was used. In the literature, many cases revealed differences related to the *in*

vitro study model. For example, the expression of hBD2 assessed by Kimball *et al.* (83) in monolayers was remarkably higher than in tissue model, suggesting that the OT model is less responsive to bacteria than the submerged cultures. They also concluded that a three-dimensional model similar to oral mucosa has also a barrier function (83), which does not come in discussion when 80% confluent cells in monolayers are used as a culture model. *F. nucleatum* is a potent stimulator of IL-8 production from different types of cells in monolayers, also of IL-6 (see Tabel 1). However, the levels of IL-6 and IL-8 were not found significant when the response of a multilayered model of epithelial cells was assessed after *F. nucleatum* stimulation (30). This underlines the relevance of using OT culture models for investigation of bacteria – host cell interactions, rather than host cells in monolayers.

The choice of study model in investigating oral bacteria – oral tissue interactions

The contribution of oral bacteria to periodontal tissue breakdown in periodontitis was studied over the last decades with increasing interest, and after establishing important steps by using cells in monolayers, multilayer models started to be taken in consideration for this purpose (4, 30, 49, 83, 129, 150).

We agree with the suggestion of Tribble and Lamont that, for studying *in vitro* the dynamics of periodontitis, it is relevant to use an appropriate model, such as OT models resembling either JE or SE (166), preferably constructed by use of primary cells. Unlike the immortalized ones, the primary epithelial cells may preserve, at least at early passages, the phenotype of the tissue of origin (100), they differentiate and become senescent (119, 120), mimicking closer the *in vivo* situation. The immortalized cells may be easier to handle and may be used at early stages of investigating a certain issue, however since they are genetically modified, they might not express the same receptors on the cell surface (5, 51), which may result in activation of signaling pathways in a different way than in primary cells. The response upon stimulation with oral bacteria might differ (cytokine secretion, expression of surface receptors) between different cell lines (106), and oral bacteria may possibly have greater effects on oral

versus skin (HaCaT) epithelial cells (51). Handling primary cells may be cumbersome at times and the known patient-to-patient variations need to be overcome by use of a higher number of samples. In addition, engineering of OTs is costly and time-consuming. However, despite their limitations, such models are the best to use in *in vitro* studies aiming to clarify the mechanisms through which bacteria undermine the periodontal tissues in periodontal disease.

Since the differentiation status of the host cells and tissue of origin affects bacteria-epithelium interactions (106, 113, 126), we have used fibroblasts of different origins to construct our OT models (Paper III). We have reported how, by use of GF or PLF and different growth periods, the phenotype of OTs could be modulated. A range of OT models was obtained: a non-differentiated, highly proliferating epithelium with CK profile similar to JE, then a more transitional model, in which epithelial stratification CK expression seemed closer to SE, and finally a mature epithelium with strong expression of CK of terminal differentiation, mimicking the GE.

7. Concluding remarks

Based on the findings of this study, the following conclusions could be drawn:

- *F. nucleatum* was able to invade in a strain-dependent manner both gingival and periodontal ligament fibroblasts, process starting approximately after 1 h of co-culture (Paper I)
- The periodontal ligament fibroblasts exhibited a higher load of internalized fusobacteria than donor-matched gingival fibroblasts, although not statistically significant (Paper I)
- *F. nucleatum* invaded the superficial epithelial layers of a differentiated, stratified gingival epithelium reconstructed *in vitro* and triggered accentuated shredding of the superficial epithelial layers (Paper II)
- A range of *in vitro* models of junctional epithelium, sulcular epithelium and gingival epithelium have been successfully developed and may be used as a relevant working tool for further comparative and in depth studies on bacterial–host tissue interactions (Paper III)
- The time allowed for growth and differentiation of organotypic models had a major impact on the phenotype of epithelium compartment in culture (Paper III)
- The periodontal ligament fibroblasts influenced significantly the CK 19 expression and pattern of distribution of proliferating cells in organotypic cultures grown for 5 days, reflecting the closest the phenotype of JE native tissue (Paper III)

8. Future perspectives

- Identify the receptors on epithelial and fibroblast cells involved in *F. nucleatum* uptake in host cells
- Characterize the interactions between *F. nucleatum* adhesins and the cognate receptors on the host cell surface and their subsequent events
- Comparative studies to examine the impact of *F. nucleatum* in monospecies or multispecies biofilm on the range of OT models with the phenotype resembling to JE /SE /GE
- Assess the viability in time of more clinical isolates of *F. nucleatum* in the above mentioned tissue models by using fluorescence *in-situ* hybridization (FISH)

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