Aus dem Institut für Zahn-, Mund- und Kieferheilkunde
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Characterization of the interactions between *Entamoeba gingivalis* and the oral mucosa to assess its pathogenic potential

Charakterisierung der Interaktionen zwischen *Entamoeba gingivalis* und der oralen Mukosa zur Feststellung des pathogenen Potentials

zur Erlangung des akademischen Grades
Doctor medicinae dentariae (Dr. med. dent.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von
Xin Bao
aus Shaanxi, China

Datum der Promotion: 18.12.2020

Table of contents

List of abbreviations IV

Abstract V

Synopsis 1

Introduction 1

1 Materials and methods 7

1.1 Subject recruitment and sample collection 7

1.2 Cultivation and detection of *E. gingivalis* 8

1.3 Histological staining 10

1.4 Primary gingival cell culture 10

1.5 Infection of gingival cells with *E. gingivalis* and *P. gingivalis* 10

1.6 Quantitative reverse transcription polymerase chain reaction (qRT-PCR) 11

1.7 *E. gingivalis* infection in *ex vivo* non-inflamed gingival biopsies 11

1.8 Cell proliferation assay 12

1.9 Statistical analysis 12

2 Results 14

2.1 Epidemiological study of *E. gingivalis* 14

2.2 Establishment of *E. gingivalis xenic* cultures 14

2.3 Detection of *E. gingivalis* within *in vivo* biopsies of inflamed gingival tissue 15

2.4 Pro-inflammatory cytokines were upregulated *by E. gingivalis* 15

2.5 *E. gingivalis* activates *MUC21 expression* in oral epithelial cells 17

2.6 *E. gingivalis* invaded wounded oral mucosa 18

2.7 *E. gingivalis* activates *MMP13* expression in oral fibroblasts 19

2.8 *E. gingivalis* inhibits cell proliferation and causes cell death 20

3 Discussion 20

Summary 25

References 26

Statutory declaration / Declaration of own contribution 31

Extract of the Journal Summary List 32

Publication 33

Xin Bao , Ricarda Wiehe, Henrik Dommisch , and Arne S. Schaefer; *Entamoeba gingivalis* Causes Oral Inflammation and Tissue Destruction; Journal of Dental Research; Feb, 2020; Vol. 99(5) 561–567 .

Curriculum vitae 40

Complete list of publications with impact factor 41

Acknowledgements 42

List of abbreviations

Abbreviation	English full name
ATCC	American type culture center
CCK-8	cell counting kit-8
cDNA	complementary DNA
Ct	cycle threshold
DMEM	dulbecco's Modified Eagle Medium
ECM	extracellular matrix
E. gingivalis	*Entamoeba gingivalis*
E. histolytica	*Enramoeba histolytica*
MMP	Metrix Metalloproteinase
MOI	multiplicity of infection
mRNA	messenger RNA
MUC	Mucin
PAS	Periodic acid–Schiff
PBS	phosphate Buffered Saline
PCR	polymerase chain reaction
P. gingivalis	Porphyromonas *gingivalis*
pGEC	Primary gingival epithelial cells
pGFB	Primary gingival fibroblast
qRT-PCR	quantitative real time polymerase chain reaction
rRNA	ribosome RNA

Abstract

Background: The frequency of the protozoan *Entamoeba gingivalis* is strongly increased in inflamed periodontal pockets, where it contributes the second-most abundant rRNA after human rRNA. Another Entamoeba species, *Entamoeba histolytica*, colonizes the gut where it causes amoebic dysentery that often causes inflammation and ulceration of the colon. This raised our concern about a putative pathogenic role of *Entamoeba gingivalis* in oral inflammation and the pathogenesis of periodontitis.

Aim: We aimed to evaluate the frequency of *E. gingivalis* in the oral cavity of periodontitis cases and healthy individuals. We assessed, if *E. gingivalis* used strategies to colonize the human host similarly to *E. histolytica* and compared the host adaptive immune response with the host response to colonization with the oral pathogenic bacterium *Porphyromonas gingivalis*.

Method: Subgingival plaque and buccal swabs were collected for detection and culture of *E. gingivalis*. Polymerase Chain Reaction (PCR) was performed after the DNA extraction of the collected samples with *E. gingivalis* specific primers. Primary gingival epithelial cells and fibroblasts were cultured from gingiva explants for the establishment of in vitro cell infection model. Quantitative real-time PCR was performed to test the related gene expression after the infection experiment. Histochemical staining was performed to prove the capability of *E. gingivalis* to invade inflamed human gingiva *in vivo* and in *ex vivo* biopsies. The cell proliferation rate of *in vitro* cultures of gingival epithelial cells was analyzed during *E. gingivalis* colonization.

Results: *E. gingivalis* was detected in 15% of health controls (n=107), 77% of inflamed gingival pockets and 22% of healthy sites in periodontitis group (n = 51). *MUC21* expression was upregulated in gingival epithelial cells after 2 h infection with *E. gingivalis* (7.7 fold, $P=7\times10^{-4}$). *E. gingivalis* infection of gingival epithelial and fibroblast cells increased *IL-8* expression 2000 fold ($P=2\times10^{-4}$) and 20 fold ($P=4\times10^{-5}$), respectively. In gingival fibroblast cells *E. gingivalis* increased *MMP13* expression 11 fold ($P=3\times10^{-4}$). *In vitro* infection further showed that *E.*

gingivalis inhibited cell proliferation, and induced cell death of gingival epithelial cells. Microscopy showed that *E. gingivalis* invaded the inflamed and wounded oral mucosa.

Conclusion: *E. gingivalis* colonizes inflamed periodontal pockets in a high frequency and it has the capacity to invade inflamed and wounded gingival tissue where able to feed on the host cell nuclei. *E. gingivalis* infection causes a strong adaptive immune response, inhibits host cell proliferation and causes cell death. Upregulation of *MUC21* and *MMP13* suggests similar invasion strategies as the related colonic parasite *E. histolytica*.

Key words: *Entamoeba gingivalis*; immune response; *MUC21*; *MMP13*; periodontitis

Zusammenfassung

Hintergrund: Das Protozoon *Entamoeba gingivalis* kolonisiert die Mundhöhle und ist besonders in entzündeten Parodontaltaschen sehr häufig. Hier trägt es nach menschlichen Zellen den größten Teil detektierbarer rRNA bei. *Entamoeba histolytica*, ein weiterer Vertreter der Gattung *Entamoeba*, kolonisiert den Darm und ist der Auslöser der Amöbenruhr, die oftmals durch Entzündung und Ulzeration des Darmgewebes gekennzeichnet ist. Ein möglicher Zusammenhang zwischen einer möglichen Pathogenität von *Entamoeba gingivalis*, oraler Entzündung und der Ätiologie der Parodontitis war zu Beginn der Arbeit nicht bekannt.

Ziele der Arbeit: In der vorliegenden Arbeit sollte die Häufigkeit von *E. gingivalis* in der Mundhöhle von Patienten der Parodontitis und gesunden Probanden untersucht werden. Es sollte geprüft werden, ob die von *E. gingivalis* verwendeten Mechanismen zur Kolonisierung des menschlichen Wirtes denen von *E. histolytica* ähnlich sind. Außerdem sollte die adaptive Immunantwort gegen *E. gingivalis* gegen eine Infektion mit dem oralen pathogenen Bakterium *Porphyromonas gingivalis* verglichen werden.

Methoden *E. gingivalis* wurde aus subgingivaler Plaque und Wangenabstrichen über einen DNA Nachweis mit der Polymerase-Ketten-Reaktion (PCR) detektiert. Zur Etablierung eines *in vitro* Zellmodells zur Infektion mit *E. gingivalis* wurden primäre gingivale Epithelzellen und Fibroblasten aus Zahnfleischbiopsien kultiviert. Die relative Genexpression wurde durch Quantitative Real-Time PCR bestimmt. Gewebefärbungen wurden durchgeführt, um die Fähigkeit von *E*. *gingivalis* zu testen, in Zahnfleischgewebe einzudringen. Die Zellproliferation gingivaler Epithelzellen wurde während der *in vitro* Kolonisierung mit *E. gingivalis* bestimmt.

Ergebnisse: *E. gingivalis* wurde in der Mundhöhle von 15% der gesunden Kontrollen nachgewiesen (N=107). 77% der entzündeten Zahnfleischtaschen und 22% der nicht-entzündeten Bereiche von Parodontitispatienten (N = 51) waren mit *E. gingivalis* kolonisiert. Infektion mit E. gingivalis erhöhte in gingivalen Epithelzellen signifikant die Expression des Gens *MUC21 (7.7 fach)* und *IL-8* (1000 fach). In gingivalen Fibroblasten war die Expression von MMP13 durch *E. gingivalis* 11 fach erhöht.

In vitro Infektion zeigte, dass direkter Zellkontakt mit *E. gingivalis* die Zellproliferation gingivaler Epithelzellen inhibiert und zu vorzeitiger Letalität führt. Gewebeschnitte zeigten, dass *E. gingivalis* in entzündetes und verwundetes Zahnfleisch eindringen, sich dort fortbewegen und ernähren kann.

Schlußfolgerung: *E. gingivalis* ist ein häufiger Kolonisierer entzündeter Parodontaltaschen und hat die Fähigkeit in entzündetes und verwundetes Zahnfleischgewebe einzudringen, sich dort fortzubewegen und zu ernähren. *E. gingivalis* löst eine starke Reaktion des adaptiven Immunsystems aus, inhibiert die Zellproliferation und trägt zu vorzeitigem Zelltod bei. Die signifikant erhöhte Expression von *IL-8*, *MUC21* und *MMP13* deutet Wechselwirkungen an, die der Invasion- und Abwehrmechanismen zwischen menschlichem Wirt und *E. histolytica* ähnlich sind. Bei *E. gingivalis* handelt es sich um einen pathogenen Parasiten der Mundhöhle mit einer möglichen Bedeutung für die Ätiologie der Parodontitis.

Synopsis

Characterization of the interactions between *Entamoeba gingivalis* and the oral mucosa to assess its pathogenic potential

Introduction

The oral cavity is colonized by numerous microorganisms that represent an important part of the human microbiota. The microbial communities at any one site contains ~50 species to a subset of ~1,000 species that are capable of oral colonization [1, 2]. Various kinds of commensal microorganisms with pathogenic potential have been identified in the oral cavity, which are associated with oral inflammatory disease, such as endodontic or periodontal diseases. Periodontal diseases are confined to the oral cavity and affect soft and hard tissues of the periodontium. They are characterized by progressive destruction of the tooth-supporting apparatus and loss of periodontal attachment. Biologically, periodontitis can partly be seen as the immune response to a disruption of the microbial homeostasis [2, 3], indicating misbalance of the complex network of interactions between the microbial community of oral cavity and the host epithelial and immune cells [4]. Severe periodontal diseases, which may result in tooth loss, are estimated to affect nearly 10 % of the global population [5]. Recently, a metagenomics analysis found that the oral protozoan *Entamoeba gingivalis* (*E. gingivalis*) contributed the second-most abundant rRNA after human rRNA in inflamed periodontal pockets [6].

The members of the protozoan genus Entamoeba, member of to the rhizopodian order Amoebida, are mostly parasitic in the intestines of many vertebrates, including humans. Five species of *Entamoeba* that colonize humans are described to date and include *E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. polecki*, and *E. gingivalis* [7]. *Entamoeba histolytica* (*E. histolytica*) is recognized as a leading parasitic cause of death worldwide and the causative agent of amebiasis. This infectious gastrointestinal disease is characterized by invasion of the amoeba into the lamina propria of the intestinal mucosa. However, only 10% to 20% of infections develop disease symptoms, with tremendous variation in clinical outcome, such as colitis,

diarrhea, vast intestinal tissue damage, and liver abscess [8, 9]. The wide variation in presentation of disease manifestations argues for additional susceptibility factors that determine parasite pathogenicity. The genetic constitution of the host and environment factors should also be considered which contributing to pathogen susceptibility and microbiome composition. *E. histolytica* virulence seems to both require and disrupt the microbiota during infection. Accordingly, characteristics of the host microbiota shape the virulence potential of the parasite [10, 11].

E. gingivalis is the single amoeba species that is known to colonize human oral cavity, and it has been investigated less. Its influence on the homeostasis of the oral microbiome or oral health has remained unknown. The bacterial load of subgingival biofilms from individuals with periodontitis accumulates with increasing clinical inflammation. Therefore, inflammation is an important ecologic change that drives the outgrowth of specific, periodontitis-associated microorganisms, which could serve as a food source and affect the prevalence of *E. gingivalis* and potentially also affects its virulence potential. It was shown that *E. gingivalis* can attach to human neutrophils and ingests the nuclei from the host cells through a channel-like pseudopodium[12], as already observed for trogocytosis in *E. hystolytica* [13].

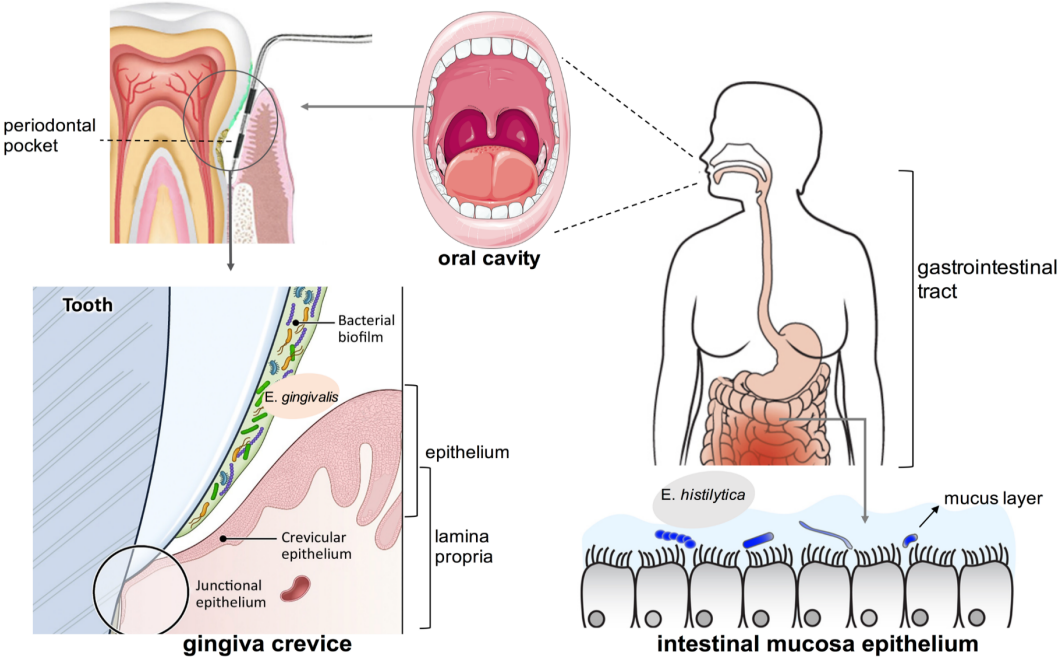

Figure 1. Locations of the different ecological niches of the oral and intestinal microenvironment that are colonized by *Entamoeba* (modified after [14-17])

The oral cavity is the entrance of the gastrointestinal tract. As the barrier to the external environment, they provide similar favorable ecological niches for a commensal, largely bacterial microbiota, that are both characterized by constant temperature, high humidity and a continuous supply with nutrients. Additionally, the intestines, and the inflamed periodontal pockets, which are colonized by *Entamoeba histolytica* and *Entamoeba gingivalis*, respectively, are largely anaerobic.

The pathogenesis of amebic inflammation and the strategy *E. histolytica* to invade the human intestinal mucosa is characterized by a sequel of steps [17, 18]. The first barrier that *E. histolytica* must overcome to invade the intestinal mucosa is the mucus layer that covers the intestinal epithelium. Here, the parasite modulates transcription of the colonic *MUC2* gene in host epithelial cells causing subsequent depletion and leading to breakdown of the mucus layer [19]. This results in direct cell contact, upon which the amoeba induces apoptosis of the epithelial cells, causing epithelial damage [20] and tissue invasion. The host responds to amoebic infection by tremendous upregulation of the inflammatory cytokine interleukin 8 (*IL-8*), which results in neutrophil infiltration [21, 22]. The invading amoeba also actively increases the production of host matrix metalloproteinases (*MMP*s) that break down the extracellular matrix (ECM) [23] (summarized in Figure 2). This results in tissue damage and translocation of intestinal bacteria into the tissue, which may also promote dissemination of bacteria to other organs with putative pathogenic consequences for systemic diseases. Notably, periodontitis is associated with other complex diseases, and long-lasting extensive oral inflammation may increase the risk for cardiovascular disease [24], rheumatoid arthritis [25], and oral cancer [26].

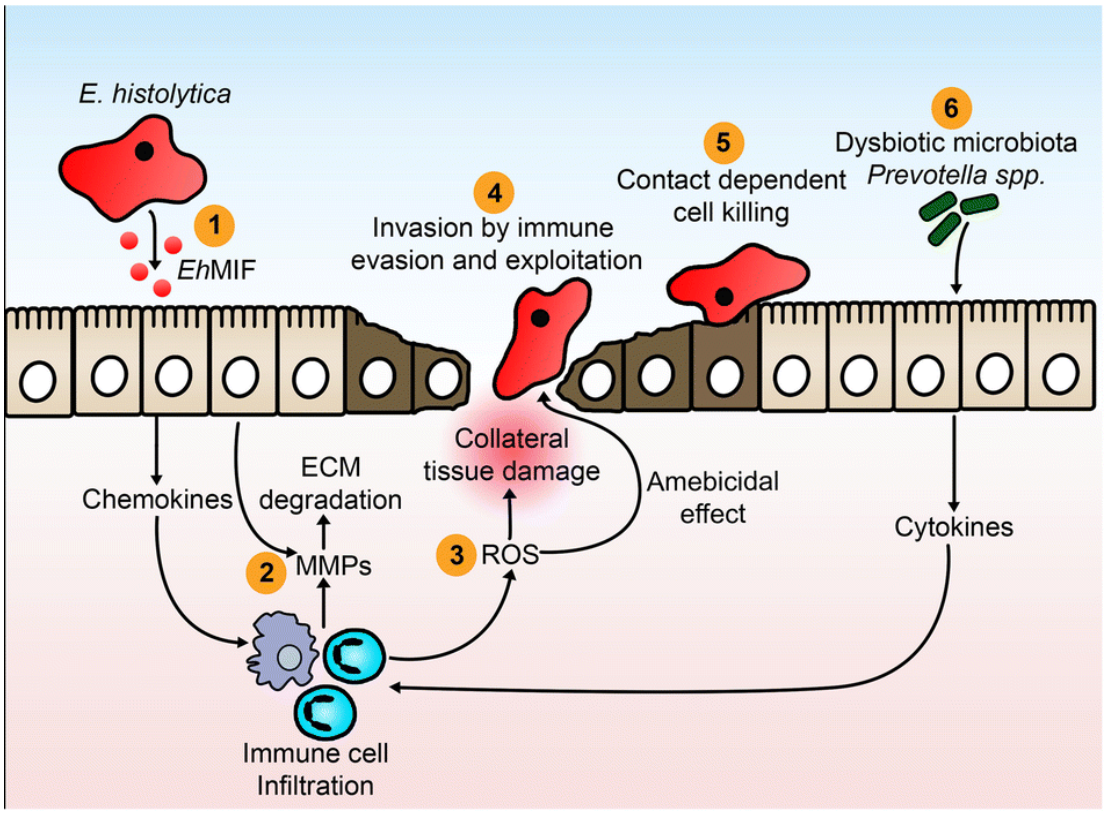

Figure 2. Pathogenesis of intestinal amebiasis (modified after [17])

Pathogenesis of intestinal amebiasis: 1. Secreted *E. histolytica* macrophage migration inhibitory factor (EhMIF) promotes mucosal inflammation; 2. *E. histolytica*–induced inflammation results in increased production in matrix metalloproteinases (MMPs) which break down extracellular matrix (ECM) in the gut to promote cell migration; 3. Infiltrating inflammatory cells generate oxygen free radicals (ROS) which are capable of killing parasites. Oxygen free radicals are also responsible for collateral tissue damage during the inflammatory period; 4. *E. histolytica* invades the intestinal mucosa by evading and exploiting the host immune system; 5. Contact-dependent cell killing by *E. histolytica*; 6. Elevated levels of *Prevotella copri* increases the risk of colitis.

The mucosal epithelium forms a physical and immunologic barrier and have direct antimicrobial activity. Mucin glycoproteins contribute to these functions and have the ability to opsonize microbes to aid clearance. However, they are also actively targeted by mucosal pathogens such as *E*. *histolytica* [27]. Mucins that are expressed in the oral mucosal epithelium differ from mucins of the gut mucus layer. In the intestines, *MUC2* constitutes the main component of the epithelial mucus layer, that is secreted by goblet cells. *MUC6* is found in gastric glands, *MUC1*, *MUC3*, *MUC4, MUC12, MUC13*, and *MUC17* are all recognized as transmembrane mucins in the gastrointestinal tract. However, numerous mucins are present in the oral cavity as secreted and as cell surface mucins. *MUC5B, MUC7, MUC19* are a gel-forming mucin secreted by the salivary glands. *MUC1, MUC4* are membrane bound mucins which are found virtually in all epithelia. The aberrant expression of this *MUC21* is associated with lung adenocarcinoma, meanwhile it is 1 out of 7 most increased in expression genes (≥30-fold change) during surgical wound healing of the oral mucosa.

The epithelium of the intestinal tract is covered by a mucus layer and is additionally moisturized by digestive juices. Similar to the intestines, the oral mucosa produces a mucus line that is specific to the oral cavity and is moisturized by saliva. Mucins protect the oral cavity through several different mechanisms that are influenced by their unique polymer structures. First, [28]. Second, mucins bind to microbes which repel them on the mucus surface and prevent them from directly interacting with the epithelium[29].

The parallels of the two distinct habitats of the gastrointestinal tract, the intestines and the oral cavity, the relationship between *E. gingivalis* and *E*. *histolytica*, and the abundance and stongly increased prevalence of *E. gingivalis* in the inflamed pockets let us consider whether *E. gingivalis* has a similar ability to cause inflammation and tissue destruction such as *E. histolytica*.

Porphyromonas gingivalis (*P. gingivalis*), a Gram-negative anaerobic bacterium, is frequently found in inflamed periodontal pockets and is a well-established colonizer of the oral epithelium, strongly associated with advanced periodontal lesions [2]. It produces a variety of virulence factors that can cause destruction to periodontal tissues either directly or indirectly by

modulating the host inflammatory response [30]. *P. gingivalis* was included in cell infection model as a reference value for the virulence potential of *E. gingivalis* that has not been described to date.

E. gingivalis colonizes the oral cavity as the preferred ecological niche. In response, colonization could initiate and drive inflammation and, similar to the situation of *E. histolytica* infection of the colon, the ECM might be degraded by the proteolysis of *MMPs*, contributing to tissue destruction. The hypothesis of this thesis is that *E. gingivalis* and the human host evolved infection and response strategies that are similar to those described for *E. histolytica*. To test this hypothesis and to better understand the underlying host-parasite interactions, the current study has the following aims.

Aims of the study

- Ascertainment of the prevalence of *E. gingivalis in the healthy and inflamed oral cavity.*
- Characterization of the potential of *E. gingivalis* to invade gingival tissues.
- Characterization of the host immune response caused by *E. gingivalis* infection
- Identification of the influence of *E. gingivalis* infection on cell proliferation and cell viability.

1. Material and methods

1.1 Subject recruitment and sample collection

The study sample consisted of two groups: patients who were diagnosed with periodontitis and controls who were free from periodontitis. The cases (n=51) and controls (n=107) were recruited at the Department of Periodontology and Synoptic Dentistry, Charité-University Medicine Berlin. The patients were clinically diagnosed with periodontitis according to the classification system published in 2018 [31]. The controls gave self-reports to be free of periodontal diseases and none were seeking dental medical care. No bleeding on probing and no signs of reddened or swollen tissue indicated absence of inflammation. A detailed description of the patient sample is shown in **Table 1**. Subgingival plaque was taken by a dentist with a sterile curette. For the detection and cultivation of *E. gingivalis*, a fraction of the subgingival plaque sample was incubated in lysis buffer and the leftover was placed directly into petri-dishes for *in vitro* culture. Sterile cotton swabs were used to collect *E. gingivalis* from the surfaces of the hard palate, both sides of the buccal mucosa, and the tongue to represent the uninflamed region. Each participant was informed about the study by the attending dentists prior to the sampling.

Table 1. Description of study population [33]

Traits		**Periodontitis (n=51)**	**Controls (n=107)**
female sex (n)		53% (27)	54% (58)
Mean age years (± SD)		61 (±15)	42 (±15)
Smoking % (n)	never smoked non-smoker(<2y) non-smoker(>2y) current smoker	53% (27) 18% (9) 16% (8) 14% (7)	ukwn. ukwn. ukwn. ukwn.

Systemic disease %(n)	no self-reported systemic disease	88% (45)	ukwn.
	diabetes	2% (1)	ukwn.
	Cardiovascular disease	10% (5)	ukwn.
Clinical attachment loss	mean % affected sites 3-4mm (±SD)	36% (±18)	N.A.
	Mean % affected sites >5mm (±SD)	36% (±24)	N.A.
Pocket depth	mean % affected sites 4-6mm (±SD)	26% (±13)	N.A.
	mean % affected sites >7mm (±SD)	7% (±13)	N.A.
Inflammation status	% BOP (n)	96% (49)	N.A.
	mean % sites with BOP (±SD)	33% (±28)	N.A.

1.2 Cultivation and detection of E. *gingivalis*

Because no axenic cultures of E. *gingivalis* exist to date, subgingival plaque samples containing the oral microflora were added into 1ml of TYGM9 medium without antibiotics in each petri-dish directly after sampling. A 35°C incubator and an anaerobic bacterial culture system were used to propagate the *E*. *gingivalis* xenic culture. The petri dish cultures were visually inspected for the presence of *E. gingivalis* by light microscopy every 2-3 days and half of the medium was replaced by fresh medium added once per week to avoid drying of the medium, high density of bacteria and depletion of nutrients.

For the detection of E. *gingivalis*, except for the routinely microscopic check, polymerase chain reaction (PCR) was performed by using an E. *gingivalis* specific primer [32] (forward: AGGAATGAACGGAACGTACA; reverse: CCATTTCCTTCTT-CTATTGTTTCAC) with the template DNA extracted from the subgingival plaque sample. To determine the success of DNA extraction, primers for the human house-keeping gene ß-Actin were used as a control reaction (forward: ATTTAGCGCCAATTCCCA; reverse: GGCGGGGTCTTTGTCTGA). PCR products were loaded with loading dye in a 2% agarose gel and run in a gel electrophoresis system for 45 min and the product size was controlled with a 50 bp DNA ladder. Gel documentation machine (Vilber, Germany) was used to visualize and save the gel pictures.

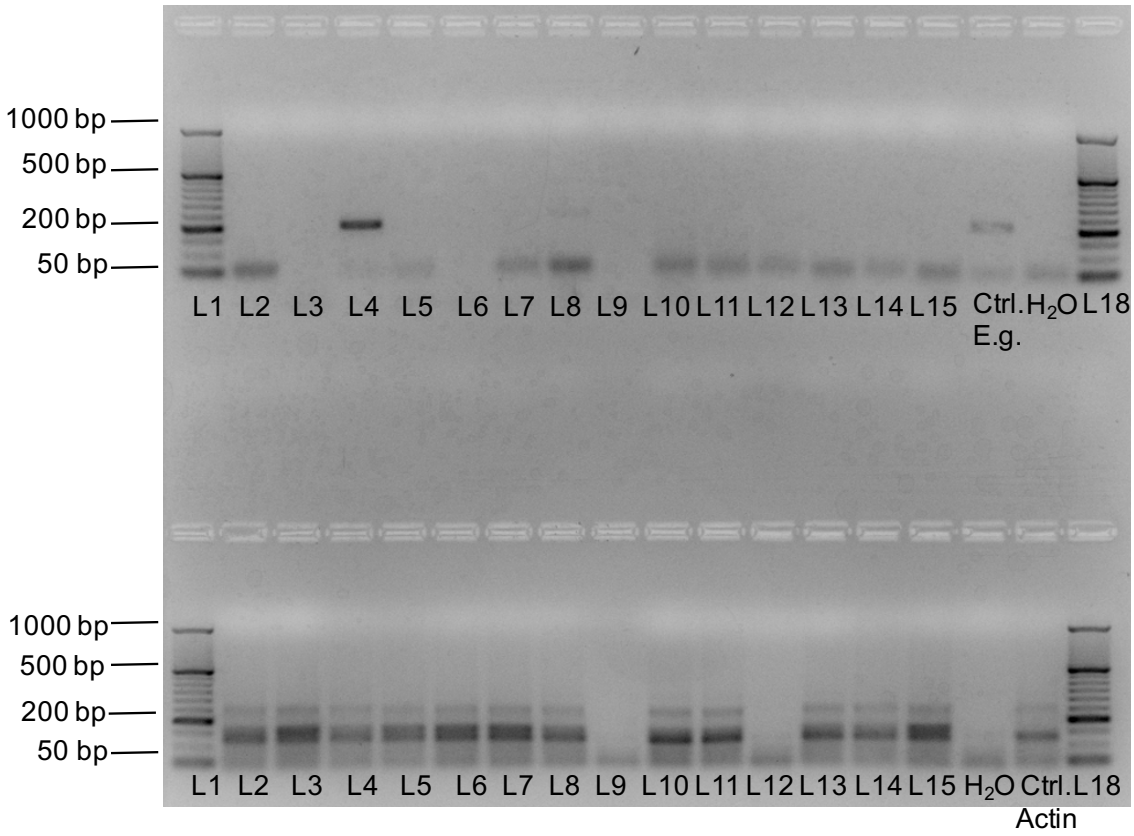

Figure 3. PCR-detection of *E. gingivalis* from buccal swabs and plaque samples ([33] appendix).

Upper lane: The PCR products were generated with *E. gingivalis* specific primers. Lane 4 and 8 show amplification of a DNA fragment of the expected (203 bp) size.

Lower lane: The PCR products (expected size: 122 bp) were generated with human *Actin* specific primers. Lane 9 shows no amplification indicating that no DNA was isolated. Such a sample was discarded from the analysis. (L = lane, L1 and L18 = size marker O'RangeRuler 50 bp (Thermo Scientific), Ctrl. E.*g.* = DNA template from E. *gingivalis* cultures, Ctrl. *Actin* = DNA template from human gingival cells, 2% agarose gel, 40min, 100V

1.3 Histological staining

Gingival biopsies were fixed in 4% paraformaldehyde for at least 48 h, and subsequently embedded in paraffin. Tissue sections (5 µm) were deparaffinized by dimethylbenzene and hydrated by graded ethanol. The slides were stained with a periodic acid–Schiff (PAS) staining

kit (ab150680; Abcam), according to the manufacturer's protocol. Stained slides were dehydrated through graded alcohols as 70%, 80%, 90% ,100%, and then cleared and mounted with for subsequent microscopic analysis and imaging.

1.4 Primary gingival cell culture

Primary gingival cells were cultured from fresh gingival tissue explants. Fresh gingival tissue was transferred into DMEM with 2× Penicillin-Streptomycin for 30 minutes to eliminate bacteria. To separate the epithelial layer from the lamina propria, the biopsies were incubated in 10ml DMEM with Dispase II (5mg/ml) on ice in a 4℃ refrigerator overnight. Primary epithelial cells were detached from the epithelial layer of the tissue by 5ml Trypsin/EDTA in a 37℃ 300 rpm shaker for 30min and neutralized by 10ml DMEM containing 10% FBS collected by 800 rpm 5min centrifugation. Primary gingival epithelial cells were cultured with Dermalife K keratinocyte Medium complete medium (LL-0007, LIFELINE), with 1× Penicillin-Streptomycin added. Lamina propria part was cut into <1×1 mm pieces by sterile scalpels and cultured with 500ul medium in T 25 cell culture flasks. Primary gingival cells were passaged when reaching 80% confluence. Primary gingival cells were only used before the fourth passage.

1.5 Infection of gingival cells with *E. gingivalis* and *P. gingivalis*

P. gingivalis (ATCC® 33277™) were bought from American Type Culture Collection. *P. gingivalis* were cultured on Columbia blood plates in an anaerobic chamber at 37℃ for 3-4 days. Gingival cells were counted and fresh culture medium without antibiotics was added before the infection experiment. *P. gingivalis* were collected at a multiplicity of infection (MOI) = 100 and added, diluted in 10μL PBS, to cultures of primary gingival epithelial cells (pGECs) and primary gingival fibroblasts (pGFBs). After 2h incubation, the cells were washed with PBS and collected for RNA extraction.

Because no axenic cultures of *E. gingivalis* exist to date, the petri dishes containing the amoebic cultures were placed on ice for 8 minutes to detach amoebae from the bottom. Subsequently, 500 µl of the medium were transferred to sterile 2 ml Eppendorf tubes and centrifuged for 10 minutes at 275g. The supernatant was discarded, the pellet was washed with 1.5 ml sterile 1×PBS. The pellet was dissolved by gentle pipetting and the washing was repeated 4× to

eliminate bacteria from the amoeba. E. *gingivalis* was collected at MOI=0.2 and added, diluted in 10µL PBS, to pGECs and pGFBs and the cells were co-incubated for 2 h. To generate the mock-infection medium, we used 10µl of the supernatant of the last washing step.

1.6 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

After 2 h of P. *gingivalis* / E. *gingivalis* infection and mock-infection, the cells were washed 3× with PBS. Cell disruption and total RNA extraction was carried out using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's specifications. Subsequently, qRT-PCR was performed with 500 ng total RNA using the High-Capacity cDNA Reverse Transcription Kit and oligo-(dT)-primers (Thermo Fisher Scientific, USA) in accordance to the manufacturer's instructions. Control reactions contained water instead of cDNA. qRT-PCR experiments were performed in technical triplicates using the CFX Connect System (Bio-Rad, USA) in combination with SYBR Select Master Mix (Thermo Fisher Scientific, USA) by following the manufacturer's instructions. The gene expression levels were normalized to the mRNA expression of GAPDH, and relative mRNA expression was calculated using the mathematical model delta delta ct using the statistical analysis software GraphPad Prism 6. GAPDH expression was unaffected of protozoan and bacterial infection. The Primers were manufactured from the company metabion GmbH (Planegg / Steinkirchen, Germany).

1.7 *E. gingivalis* infection in *ex vivo* non-inflamed gingival biopsies

For *E. gingivalis* infection of ex vivo biopsies of the healthy gingiva, the biopsies were placed upright on 4% agarose in 1.5 ml tubes and subsequently filled up to the epithelial surface with hand-warm agarose. To wound the upper epithelial layer, the biopsies were slightly punctured with a sterile needle or cut with a 2mm scalpel. *E. gingivalis* (3.5×10^4 in 250µl TYGM-9 Medium) was added to the biopsies and incubated with closed lids at 35℃ for 6h.

1.8 Cell proliferation assay

Approximately 1000 primary gingival cells in 100 µL medium without antibiotics were sown per well in a 96-well plate and cultured overnight. *E. gingivalis* and *P. gingivalis* were cultured before the experiment were added in 10µL PBS at MOI 0.2 and MOI 100. 10µL of WST-8 solution were added to each well of human cells and incubated at 37º C for 2 h. The measurement of the cell proliferation was performed at an optical density (OD) = 460 nm. A time-course including

5 measurements were performed to observe cell proliferation. The experiments were performed in biological duplicates from two different donors with 3 technical replicates.

1.9 Statistical analysis

Continuous normally distributed data are expressed as the means ± SDs. All statistical calculations were carried out using GraphPad Prism7 statistical software (GraphPad software, USA). For comparison of treated and control groups, data were analyzed via unpaired t-test with Welch's correction. To correct for multiple testing, Bonferroni correction was used to minimize type I errors. Bonferroni corrected P values with $P < 0.05$ were considered significant and marked with *, P values < 0.01 with **, P values < 0.001 with ***.

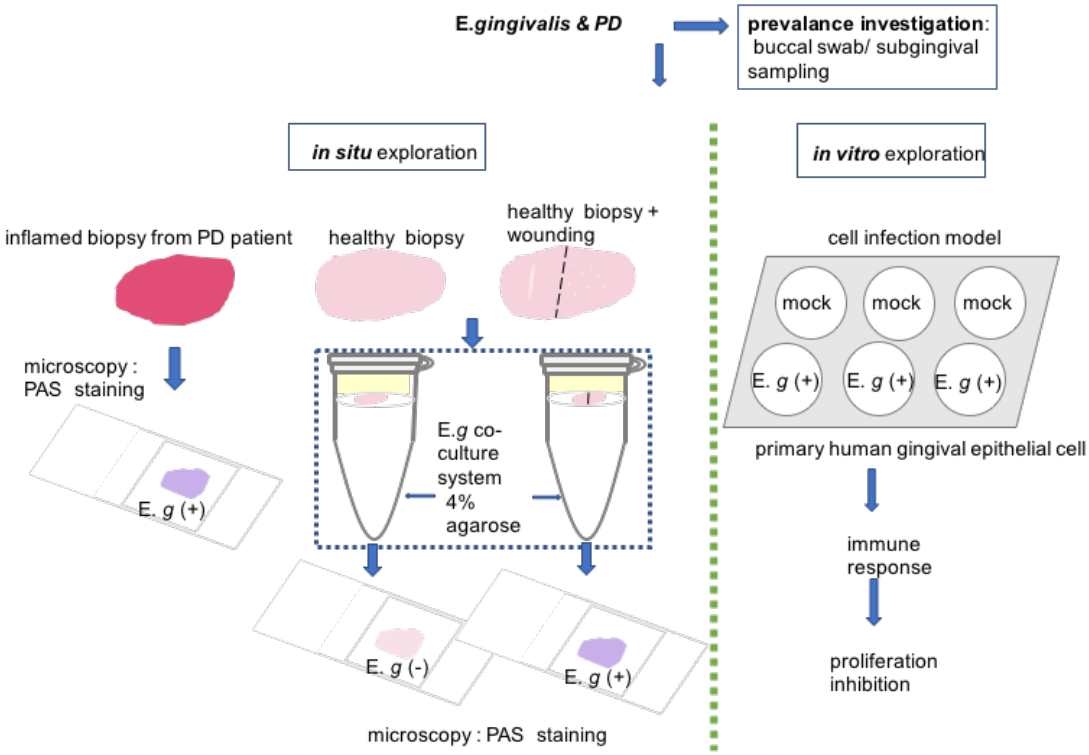

Figure 4. Schematic summary of the study([33] appendix)

To test the pathogenic potential of *E. gingivalis* in the oral cavity, prevalence investigation was done with oral samples following *in situ* and *in vitro* explorations. Histochemical staining was used in *ex vivo* biopsies and in an *E. gingivalis* co-culture system. Cell infection model was used to test the immune response and cell proliferation activity of gingival cells.

2. Results

2.1 Epidemiological study of *E. gingivalis*

77% of *E. gingivalis* was detected in the inflamed periodontal pockets of periodontitis patients. 22% and 15% of *E. gingivalis* detection was shown in the representative healthy areas of the patients and the controls, respectively.

Table 2. *E. gingivalis* frequency of the study population[33]

		Periodontitis (n=51)	Controls (n=107)
E. gingivalis detection	Inflamed pockets	77% (39)	N.A.
	Healthy sites	22% (11)	15% (16)

2.2 Establishment of *E. gingivalis* xenic cultures

Xenic cultures of *E. gingivalis* were successfully established from subgingival plaque samples, that contain a complex undefined bacteria flora (**Figure 5**). After 2 weeks' cultivation, *E. gingivalis* were harvested and pooled from 5 petri-dishes (35×10 mm) and a hemocytometer was used to count the cells. Normally 40 000-60 000 cells can be collected for later experiments.

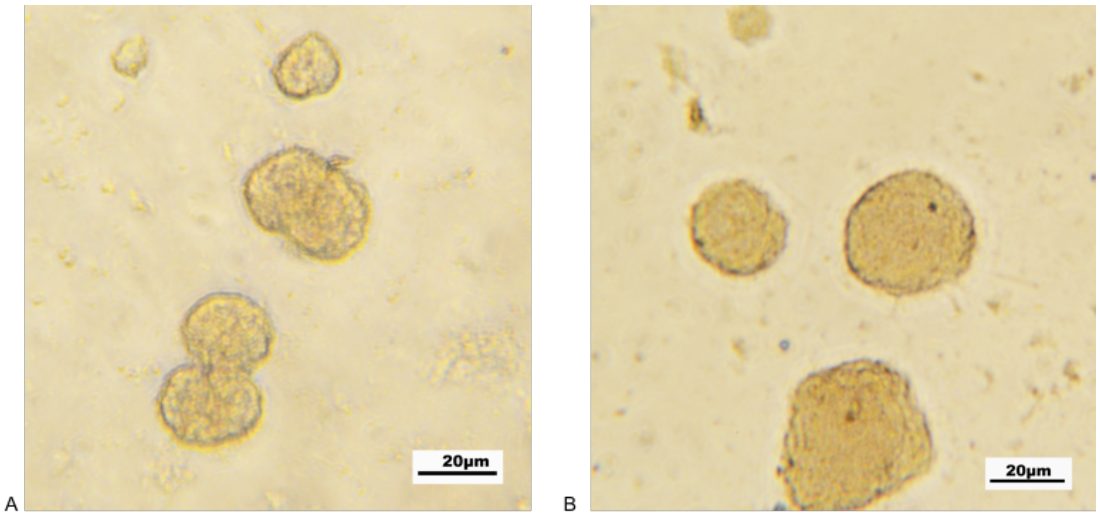

Figure 5. *E. gingivalis* as seen under the light microscope ([33], appendix)

E. gingivalis colonies from plaque cultures are visible under the light microscope after 2 days (culture in TYGM-9 Medium under anaerobic conditions at 35°C). *E. gingivalis* specimen show

differences in size (commonly between 10-30 µm in diameter). The specimen on the left pictures may show cell division by mitotic bipartion (32× magnification).

2.3 Detection of E. *gingivalis* within *in vivo* biopsies of inflamed gingival tissue

E. histolytica is commensal in 80% to 90% of infected individuals, but in some cases, it invades the colonic mucosa and causes amoebiasis. To see if *E. gingivalis* was capable to invade the oral mucosa, we microscopically analyzed an *in vivo* biopsy of inflamed gingiva from a female patient with severe generalized chronic periodontitis (**Figure. 6**). After PAS staining, we observed *E. gingivalis* within the gingiva surrounded by numerous infiltrated neutrophils.

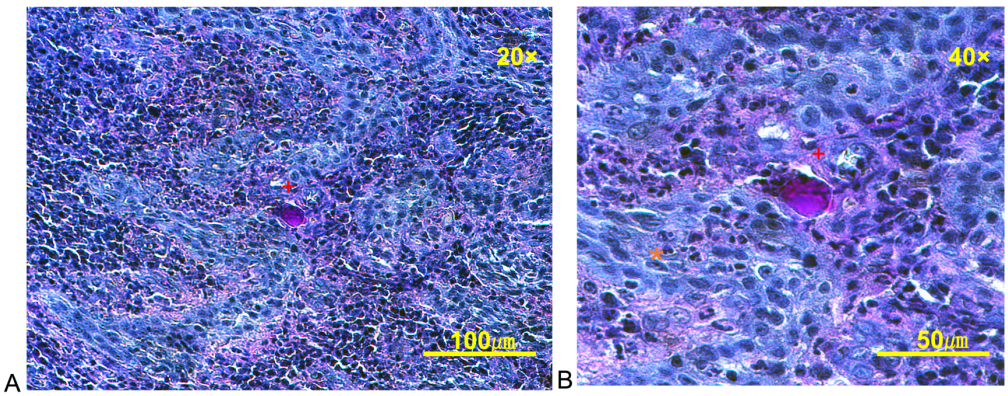

Figure 6. PAS staining of an *ex vivo* biopsy of the inflamed gingiva[33]

PAS staining revealed the presence of E. *gingivalis* in the inflamed gingiva of a patient with severe generalized chronic periodontitis. (A) E. *gingivalis* (indicated by the red cross) colonized the inflamed oral mucosa of a 36-year-old woman with severe generalized periodontitis (20× magnification). (B) The amoeba (red cross) was surrounded by abundant neutrophils, as indicated by their segmented nuclei (orange asterisk; zoom of panel A, 40× magnification).

2.4 Pro-inflammatory cytokines were upregulated by E. *gingivalis*

Because in colon epithelial cells, E. *histolytica* increased *IL-1β* expression moderately but increased *IL-8* expression >1,000-fold [34], I tested if E. *gingivalis* infection similarly induced *IL1-β* and *IL-8* expression in pGECs and pGFBs. *IL-8* was differentially expressed in pGECs and pGFBs, with a stronger expression in pGECs (Ct [cycle threshold] = 26.2) as compared with pGFBs (Ct = 30.5). After co-incubation of pGECs with E. *gingivalis*, the expression of IL1ß was increased 9.5-fold ($P = 6 \times 10^{-5}$; $P_{corrected} = 0.001$), and IL8 was increased 1,983.3-fold (P

= 2 × 10^{-4}; $P_{corrected}$ = 0.004; Fig. 4A). After infection of pGECs with P. *gingivalis*, *IL-8* expression was 7.7-fold increased (P = 0.002; $P_{corrected}$ = 0.041; Fig. 4B). In pGFBs, *IL-1β* expression in pGFBs was 11.9-fold upregulated after E. *gingivalis* infection (P = 1 × 10^{-4}; $P_{corrected}$ = 0.001), and *IL-8* was 17.9-fold upregulated (P = 4 × 10^{-5}; $P_{corrected}$ = 4 × 10^{-4}; Fig. 4C). After P. *gingivalis* infection, *IL-1β* was 15.0-fold upregulated (P = 9 × 10^{-5}; $P_{corrected}$ = 9 × 10–4), and *IL-8* showed 153.5-fold upregulation (P = 3 × 10–5; $P_{corrected}$ = 3 × 10–4; Fig. 4D).

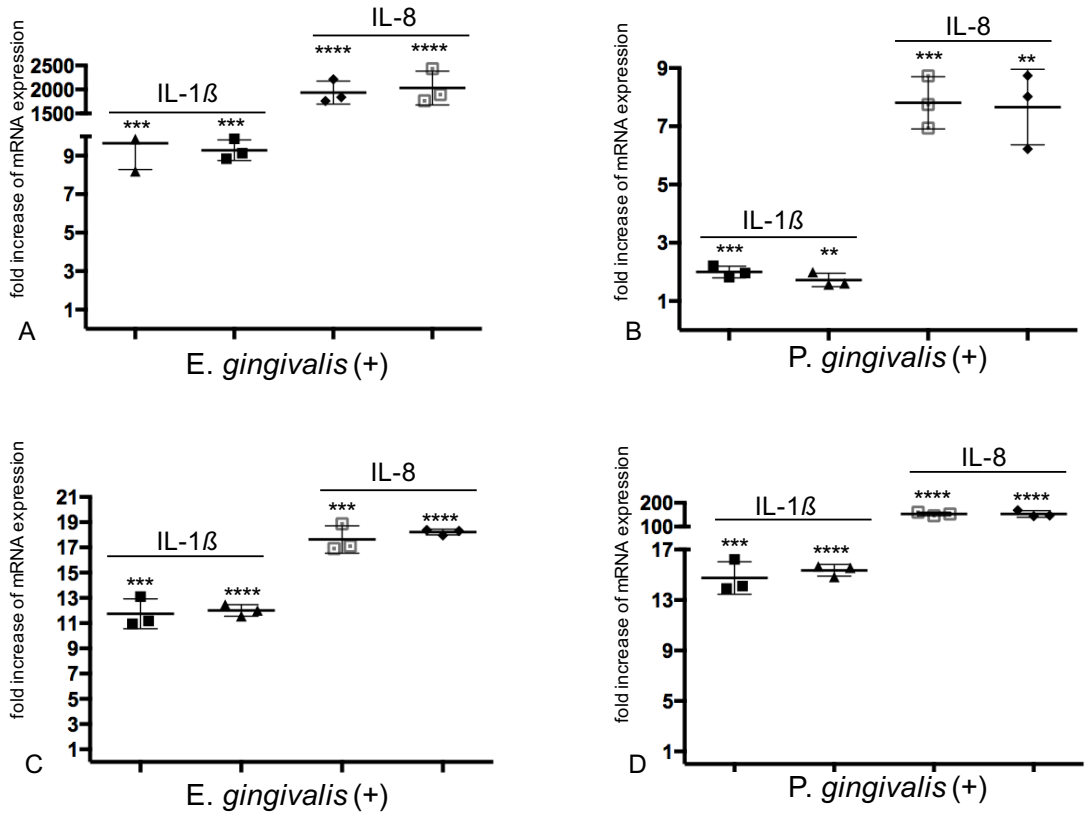

Figure 7. *IL-1β* and *IL-8* expression after *E. gingivalis* and *P. gingivalis* infection for 2h[33]

A-B. Primary gingival epithelial cells. C-D- primary gingival fibroblasts

For each gene, 2 biological replicates with 3 technical replicates were conducted. P values were corrected for multiple testing. Significant expression changes are marked by an asterisk. (A) *E. gingivalis* infection increased *IL-1β* expression 9.5-fold, *IL-8* 1,983.3-fold in pGEC (B) *P. gingivalis* infection increased *IL-1β* expression 1.9-fold, *IL-8* 7-fold in pGEC(C) *IL-1β* expression was 11.9-fold upregulated, *IL-8* 17.9-fold upregulated in pGFB (D) After P. *gingivalis* infection,

IL1β was 15.0-fold upregulated and IL8 showed 153.5-fold upregulation. Values are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

2.5 *E. gingivalis* activates *MUC21* expression in oral epithelial cells

To test if *E. gingivalis* was capable of modulating mucin gene expression after infection of oral epithelial cells, we systematically screened the expression of human mucin genes (*MUC1, -2, -3A, -4, -5B, -5AC, -6, -7, -12, -13, -15, -16, -17, -19, -20, -21*) in response to *E. gingivalis* infection in pGECs and pGFBs. *MUC1*, *-3A, -15, -4, -5B, -5AC, -6, -7, -13, -16, -19, -20,* and *-21* were expressed in pGECs at strong to moderate levels with Ct < 35. *MUC2, -12*, and *-17* were weakly expressed (Ct> 35). After E. *gingivalis* infection for 2 h, *MUC21* was significantly upregulated (7.7-fold, **Figure 8A**). *MUC1* showed nominal significant upregulation but the change of transcript levels was not significant after correction for multiple testing. The oral bacterium *P. gingivalis* did not induce *MUC*21 expression in oral epithelial cells but showed upregulation at a similar level as *MUC*1 as observed after *E. gingivalis* infection (**Figure 8B**).

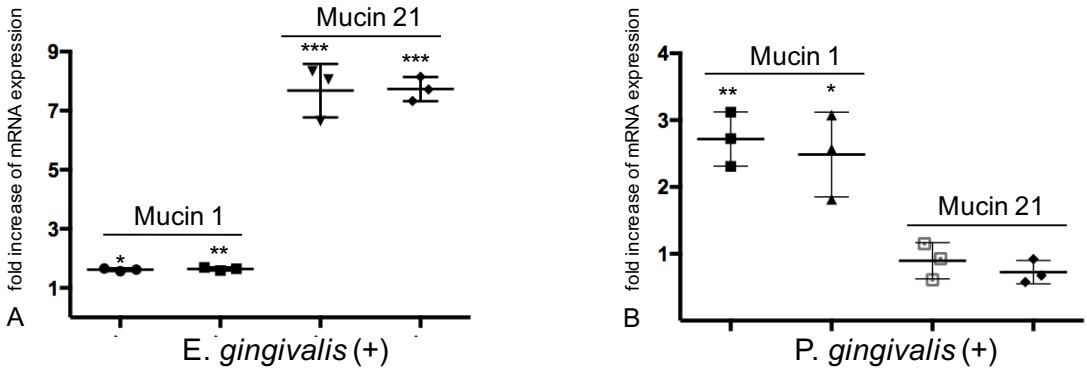

Figure 8. *MUC1* and *MUC21* expression in primary gingival epithelial cells [33]

(A) Of the tested mucin genes, *MUC*21 was 7.7-fold ($P = 7 \times 10^{-4}$; $P_{corrected} = 0.013$) upregulated after *E. gingivalis* infection. (B) *P. gingivalis* infection upregulated *MUC1* expression >2.5-fold ($P = 0.007$; $P_{corrected} = 0.126$). All experiments were performed in 2 biological replicates, and each has 3 technical replications. This effect was not validated after multiple correction in the replication.

2.6 *E. gingivalis* invaded wounded oral mucosa

Within the gingival epithelium of live, uninflamed *ex vivo* biopsies, microscope analysis did not reveal *E. gingivalis.* However, after wounding live, healthy gingival biopsies by slightly cutting the upper epithelial layer with a scalpel or puncturing it with sterile needles, we found the active and feeding stages of amoebic trophozoites after 6 h of incubation. Microscopy indicated moving of the amoeba within the gingival tissue, penetration into the cytoplasm of live host GECs and ingestion of fragments from the nuclei of the host cells (**Figure 9**). In the unharmed gingiva, we did not observe invaded amoeba after 6 h of incubation.

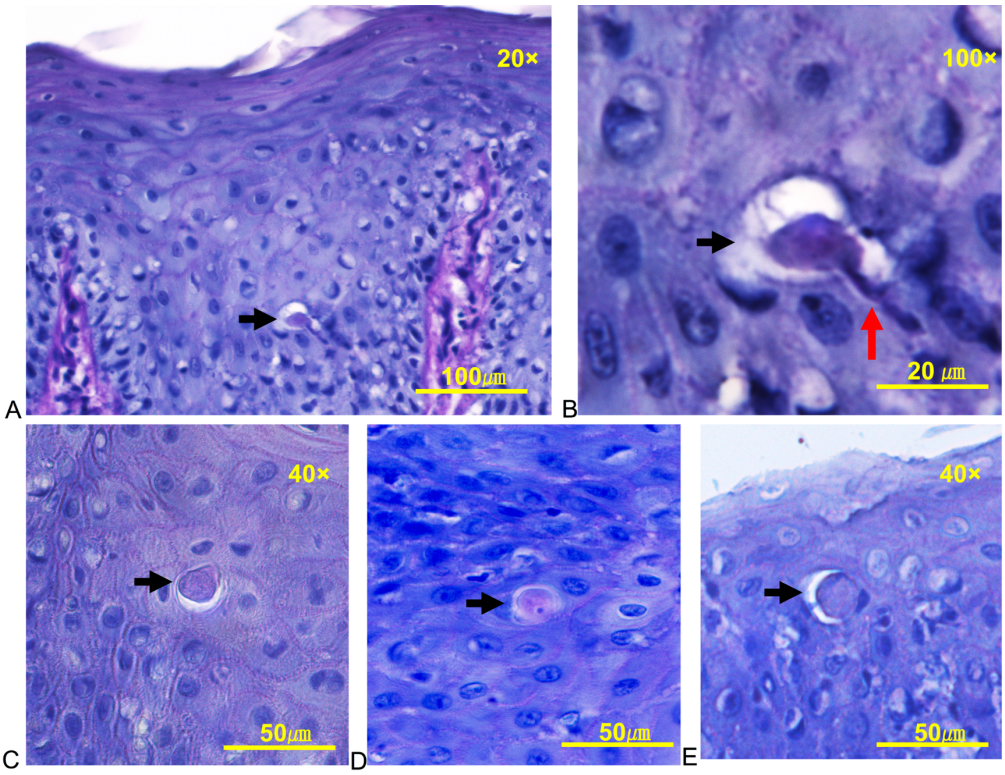

Figure 9. Human live explants of uninflamed, manually injured oral mucosa[33]

Moving and feeding amoeba are seen in various tissue layers after 6h E. *gingivalis* incubation (black arrows). The trophozoite is located below the squamous epithelium, left to the elongated keratinized papillae (A). The amoeba penetrated the cytoplasm of a cell. Internal material (possibly the nucleus) from the host cell elongated through a “channel” (red arrow) into the trophozoite. A hollow surrounds the amoeba, indicating that it modified cell morphology (B,

zoom of panel A). The trophozoite is moving within the gingival epithelium; the moving direction is indicated by the upward pseudopod (C). The pseudopod contacted a host cell, and its nucleus was half disintegrated and filled a food vacuole inside the amoeba (D). The amoeba contacted the nucleus of an endothelial cell, and streaks from another endothelial cell, possible chromatin, are observable in the hollow (E).

2.7 *E. gingivalis* activates *MMP13* expression in oral fibroblasts

To test if *E. gingivalis* is capable to modulate MMP expression in gingival fibroblasts, the main cell type of the oral ECM, we screened the expression of the *MMP* genes *MMP1*, *-2, -3, -7, -8, -9, -13*, and *-20* in pGFBs. *MMP1, -2,* and *-3* showed strong expression (Ct < 25). *MMP7, -8* and *-13* were moderately expressed with Ct between 26 - 34). *MMP9* and *MMP20* were not expressed (Ct > 35). After 2 h co-incubation of pGFBs with *E. gingivalis*, we observed 2.0-fold upregulation of *MMP3* ($P = 1.8 \times 10^{-3}$; $P_{corrected} = 0.018$; **Figure 10**) and 11.2-fold upregulation of *MMP13* ($P = 3 \times 10^{-4}$; $P_{corrected} = 0.003$). *P. gingivalis* infection upregulated *MMP3* and *MMP13* 2.8-fold ($P = 1 \times 10^{-4}$; $P_{corrected} = 0.001$) and 3.7-fold ($P = 5 \times 10^{-5}$; $P_{corrected} = 5 \times 10^{-4}$), respectively.

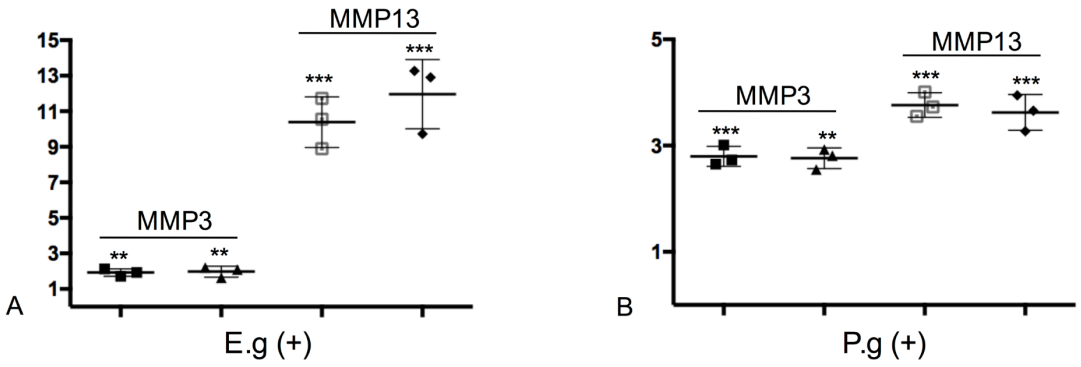

Figure 10. *MMP3*, *-13* expression in primary gingival fibroblasts[33]

(A) Of the tested *MMP* genes, *MMP3* expression was 2-fold upregulated after *E. gingivalis* infection with P = 0.002 ($P_{corrected} = 0.018$). *MMP13* showed 11.2-fold upregulation after *E. gingivalis* infection, P = 3 × 10–4 ($P_{corrected} = 0.003$). (B) P. *gingivalis* infection increased *MMP3* expression 2.8-fold, P =1 × 10–4 ($P_{corrected} = 0.001$). *MMP13* expression was increased 3.7-fold, $P = 5 \times 10^{-5}$ ($P_{corrected} = 5 \times 10^{-4}$).

2.8 *E. gingivalis* inhibits cell proliferation and causes cell death

E. histolytica adherence to colonic epithelial cells induces apoptosis [20]. Similarly, *P. gingivalis* adherence to GECs is associated with enhanced cell death through apoptosis [35]. We tested, if *E. gingivalis* also impaired growth of pGECs *in vitro*. The cell proliferation was inhibited by both *E. gingivalis* and *P. gingivalis* compared to the mock-infected control cells (**Figure 11**).

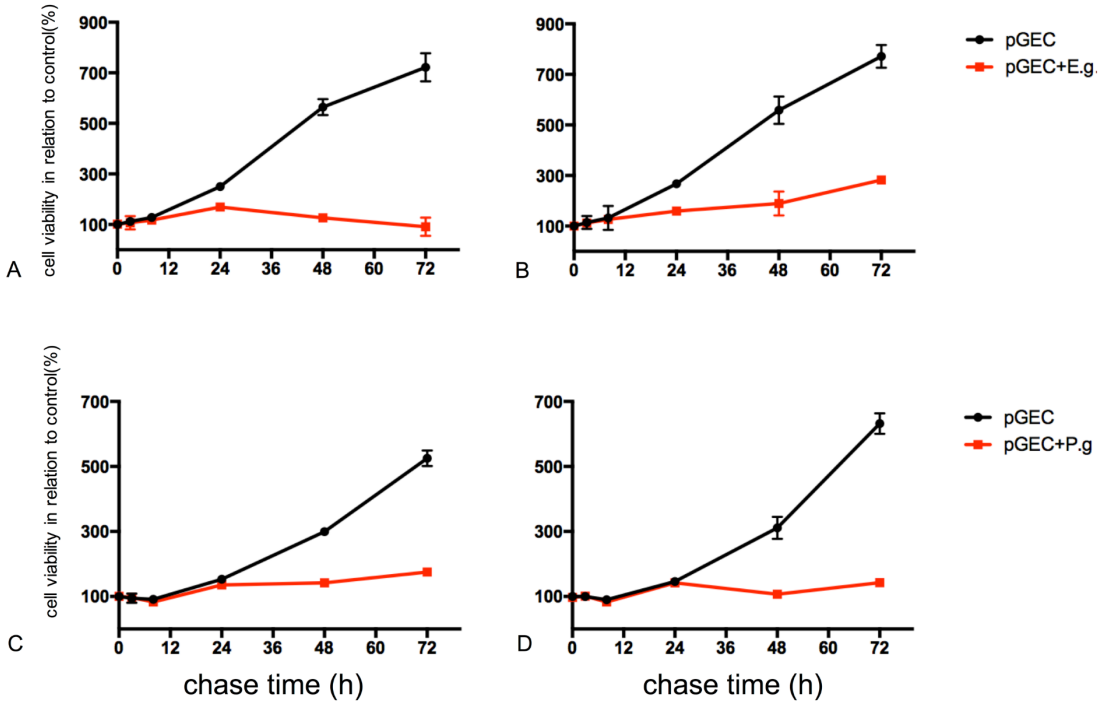

Figure 11. *E. gingivalis* and *P. gingivalis* inhibit proliferation of primary gingival epithelial cells (pGECs)[33]

Direct contact of *E. gingivalis* and *P. gingivalis* to pGECs impaired cell proliferation (Values are presented as mean ± SD).

3. Discussion

E. gingivalis is the first amoeba in humans to be described and regarded as an opportunistic amoebozoa, whilst less was known about its pathogenicity in oral diseases. In our study population, uninflamed areas of patients' oral mucosa and the controls showed similar frequency of *E. gingivalis*. However, this parasite was highly abundant in inflamed periodontal pockets compared with uninflamed areas, suggesting the inflamed periodontal pocket as the

preferred ecologic niche. However, microscopy of a severe chronic periodontitis patient gave evidence that *E. gingivalis* not only remains on the surface of the oral mucosa, but also invades into the deeper layers of the inflamed tissue. Here, it has the potential to trigger a strong adaptive immune response, because the biopsy revealed that *E. gingivalis* was surrounded by numerous infiltrated neutrophils. However, because it was previously shown that neutrophils likely are unable to kill *E. gingivalis* [12], but serve as a food source for this protozoan, neutrophils are probably not able to eliminate *E. gingivalis*, but increased neutrophil invasion will rather aggravate inflammation and tissue destruction. In an *ex vivo E. gingivalis* invasion model, within 6h infection time, *E. gingivalis* was only observed in the biopsy which was wounded slightly on the epithelium instead of the intact tissue. This may suggest that within the 2 h of the experiment, this protozoan did not actively invade the tissue but took the opportunity of impaired barrier integrity. In the case of *E. histolytica*, the high frequency of asymptomatic infection with *E. histolytica* indicates that invasion into the tissue of the human host is dispensable for *Entamoeba* survival, replication, and transmission.

The first line of innate host defense in preventing pathogen-induced epithelial injury is the mucin barrier. Mucins are molecules that bind to invading microorganisms, but they also act as a growth substrate and food source for commensal bacteria. Thus, some mucins are specifically upregulated by pathogens, and some are constitutively secreted. In the pathology of *E. histolytica* invasion, the first step of host defense involves upregulation of *MUC*2. *E. histolytica* colonizes the mucus layer by binding to galactose and *N*-acetyl-d-galactosamine residues on colonic *MUC2*, preventing parasite contact dependent cytolysis of goblet cells [36]. Our screen for differential expression of human mucins in response to *E. gingivalis* infection in gingival cells identified *MUC21* to have the strongest increase in expression. Noteworthy, a genome-wide expression screen that searched for differential transcription in the gingiva following surgical wounding identified *MUC21* as 1 of 7 genes of the transcriptome that showed ≥30-fold upregulation after wounding [37]. The observation of strong *MUC21* upregulation in response to disruption of the oral epithelium adds independent evidence to our finding that *MUC21* plays an important role in defense mechanisms of impaired oral barrier integrity. We note that the

oral bacterium *P. gingivalis* did not induce *MUC*21 expression in GECs, indicating mucin upregulation to be species-specific.

The immune response of oral cells to *E. gingivalis* infection was investigated, and at the same time, infection with the periodontal subgingival anaerobic bacterium *P. gingivalis* was included to obtain reference values of the inflammatory responses. The results showed that the two inflammatory cytokines *IL-1β* and *IL-8* were both upregulated after infection with *E. gingivalis* and *P. gingivalis*. This indicated that E. *gingivalis* does induce an inflammatory response and suggests *E. gingivalis* as a potential pathogen causing inflammation. *Entamoeba*, bacteria, and their human host coevolved and developed species and tissue-specific infection and defense mechanisms. A known example is the modulation of cytokine secretion of GECs by *P. gingivalis*. Generally, *IL-1β* is expressed early after bacterial challenge and then upregulates *IL-8*, which is the primary cytokine involved in the recruitment of neutrophils to the site of damage or infection[38]. However, GECs challenged with live *P. gingivalis* mount a primary cytokine response of *IL-1β* that is not followed by a secondary response of *IL-8* [35]. In the current study, we showed that infection of GECs with *E. gingivalis* increased *IL-8* expression 1,900-fold. This is similar to infection of HT-29 colon epithelial cells with *E. histolytica*, which also stimulated *IL-8* expression 1,000- fold [34]. In contrast, infection with *P. gingivalis* increased *IL-8* expression of GECs 7-fold. Here, an adaptive benefit of opposite modulations of immune response by both pathogens seems plausible. *P. gingivalis* is often part of the normal oral microbiota, and constitutive activation of IL8 in GECs would be dis-advantageous for both bacteria and host. However, *E. gingivalis* does not belong to the healthy oral microbiota and it is not likely that the host would have evolved strategies to balance *E. gingivalis* colonization. However, *E. gingivalis* seems to profit from increased neutrophil invasion and ECM breakdown, because it can use neutrophils as food source and can colonize the impaired ECM as a habitat. In contrast to the situation in epithelial cells, we noticed that *IL-8* expression in GFBs was ~100-fold higher after *P. gingivalis* infection. In the healthy situation, *P. gingivalis* is not found in the connective tissue of the healthy oral mucosa. Although speculative, the activated host response in pGFBs might be an advantageous immune response for the human host to fend off tissue invasion. However,

IL-8 transcript levels in pGFBs were still significantly lower after *P. gingivalis* infection as compared with E. *gingivalis* infection, indicating that *E. gingivalis* is perceived by the immune system as a serious invader.

Invasion through the ECM barrier is a complex, stepwise process involving cell adhesion, ECM proteolysis by *MMPs*, and migration of the invading cells. Evidence emerged that vesicular trafficking and secretion of *MMPs* is a crucial factor in the ECM degradation and cellular invasion. Our screen for differential expression of human *MMPs* in response to *E*. *gingivalis* infection identified *MMP13* as the most upregulated *MMP*, with >10-fold increase after 2h of stimulation. In contrast, *P*. *gingivalis* infection increased *MMP13* <4-fold. The strong activation of *MMP13*, which is expressed at exogenous levels in the healthy gingiva, indicates a role in the context of tissue invasion of *E. gingivalis*. It is of interest that the major cytokine to induce *MMP13* expression was *IL-1*, which induced a marked increase in *MMP13* mRNA in mouse calvarial bone cultures [39]. *MMP13* plays a special role in wound healing, tissue remodeling, cartilage degradation, bone mineralization, and ossification. Because of the unique role of *MMP13* in ossification and keratinocyte migration during wound healing, a candidate gene study quantified *MMP13* gene expression in patients with untreated periodontitis and showed that *MMP13* activity in gingival crevicular fluid was significantly increased in inflamed periodontal sites [40].

P. *gingivalis* adherence to GECs is associated with enhanced cell death [35]. Likewise, *E*. *histolytica* adherence to colonic epithelial cells induces apoptosis [20]. We showed that E. *gingivalis* impaired cell proliferation and induced cell death of GECs to a similar extent as *P*. *gingivalis*. The results indicated that amoebic infection impairs cell proliferation as severely as *P*. *gingivalis*. It should be noted that in the experiments of this study, the mock-infected cells of the *P*. *gingivalis* proliferation experiments grew slower compared with the mock-infected cells of the *E*. *gingivalis* experiments. This can be explained by a different age of these cells. Whereas the cells that were used for the amoeba experiments were collected from patients

prior to the experiments, the cells used for bacterial stimulation were stored 10 years at –80 °C. The long storage time might have affected the proliferation rate.

Conventional treatment of periodontitis is regularly mechanical removal of the subgingival calculus that eliminates the stimulatory factors that may cause inflammation. However, the severity of periodontitis differs and the recurrence cannot be predicted nor always avoided. Currently, it is an open question if the regular treatment methods are efficient to eliminate *E. gingivalis* colonization, which may change the outcome of periodontitis, or if the recurrence of periodontitis is related to re-colonization of *E. gingivalis.* Proof of causality and of the specific contribution to the recurrence of periodontal inflammation during periodontal therapy are important topics for future research to improve the existing measures of periodontitis treatment.

Summary

The oral protozoan *E*. *gingivalis* was found frequently in inflamed periodontal pockets but little was known of its pathogenicity in oral inflammatory diseases. We hypothesized that this parasite has pathogenic potential and developed invasion strategies similar to that of the intestinal amoeba *E*. *histolytica*. This study showed a frequency of *E*. *gingivalis* in 80% of inflamed periodontal pockets compared to 15% of uninflamed areas of the healthy oral cavity. Invasion of *E. gingivalis* into inflamed gingival tissues was detected in *ex vivo* biopsies. Subsequently, *E*. *gingivalis* co-culture experiments were performed in healthy biopsies, and resulted in *E. gingivalis* invasion of the biopsy which was wounded on the surface. In cell infection models, the innate immune response of gingival cells was activated by *E*. *gingivalis*, with *IL-8* showing >1000 fold induction. *E. gingivalis* infection inhibited cell proliferation and caused cell death.

In summary, this study showed that *E*. *gingivalis has* the ability to cause inflammation and tissue destruction and testified potential to contribute to the pathogenesis of oral inflammatory diseases.

References:

1. Abusleme, L., A.K. Dupuy, N. Dutzan, N. Silva, J.A. Burleson, L.D. Strausbaugh, J. Gamonal, and P.I. Diaz, *The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation.* ISME J, 2013. **7**(5): p. 1016-25.
2. Lamont, R.J., H. Koo, and G. Hajishengallis, *The oral microbiota: dynamic communities and host interactions.* Nat Rev Microbiol, 2018. **16**(12): p. 745-759.
3. Darveau, R.P., *Periodontitis: a polymicrobial disruption of host homeostasis.* Nat Rev Microbiol, 2010. **8**(7): p. 481-90.
4. Clemente, J.C., L.K. Ursell, L.W. Parfrey, and R. Knight, *The impact of the gut microbiota on human health: an integrative view.* Cell, 2012. **148**(6): p. 1258-70.
5. Marcenes, W., N.J. Kassebaum, E. Bernabe, A. Flaxman, M. Naghavi, A. Lopez, and C.J. Murray, *Global burden of oral conditions in 1990-2010: a systematic analysis.* J Dent Res, 2013. **92**(7): p. 592-7.
6. Deng, Z.L., S.P. Szafranski, M. Jarek, S. Bhuju, and I. Wagner-Dobler, *Dysbiosis in chronic periodontitis: Key microbial players and interactions with the human host.* Sci Rep, 2017. **7**(1): p. 3703.
7. Aurrecoechea, C., A. Barreto, J. Brestelli, B.P. Brunk, E.V. Caler, S. Fischer, B. Gajria, X. Gao, A. Gingle, G. Grant, O.S. Harb, M. Heiges, J. Iodice, J.C. Kissinger, E.T. Kraemer, W. Li, V. Nayak, C. Pennington, D.F. Pinney, B. Pitts, D.S. Roos, G. Srinivasamoorthy, C.J. Stoeckert, Jr., C. Treatman, and H. Wang, *AmoebaDB and MicrosporidiaDB: functional genomic resources for Amoebozoa and Microsporidia species.* Nucleic Acids Res, 2011. **39**(Database issue): p. D612-9.
8. Haque, R., P. Duggal, I.M. Ali, M.B. Hossain, D. Mondal, R.B. Sack, B.M. Farr, T.H. Beaty, and W.A. Petri, Jr., *Innate and acquired resistance to amebiasis in bangladeshi children.* J Infect Dis, 2002. **186**(4): p. 547-52.

9. Shirley, D.T., L. Farr, K. Watanabe, and S. Moonah, *A Review of the Global Burden, New Diagnostics, and Current Therapeutics for Amebiasis.* Open Forum Infect Dis, 2018. **5**(7): p. ofy161.
10. Burgess, S.L. and W.A. Petri, Jr., *The Intestinal Bacterial Microbiome and E. histolytica Infection.* Curr Trop Med Rep, 2016. **3**: p. 71-74.
11. Marie, C. and W.A. Petri, Jr., *Regulation of virulence of Entamoeba histolytica.* Annu Rev Microbiol, 2014. **68**: p. 493-520.
12. Bonner, M., M. Fresno, N. Girones, N. Guillen, and J. Santi-Rocca, *Reassessing the Role of Entamoeba gingivalis in Periodontitis.* Front Cell Infect Microbiol, 2018. **8**: p. 379.
13. Tanyuksel, M. and W.A. Petri, Jr., *Laboratory diagnosis of amebiasis.* Clin Microbiol Rev, 2003. **16**(4): p. 713-29.
14. Cao, X., *Intestinal inflammation induced by oral bacteria.* Science, 2017. **358**(6361): p. 308-309.
15. Flynn, J.M., D. Niccum, J.M. Dunitz, and R.C. Hunter, *Evidence and Role for Bacterial Mucin Degradation in Cystic Fibrosis Airway Disease.* PLoS Pathog, 2016. **12**(8): p. e1005846.
16. Moutsopoulos, N.M. and J.E. Konkel, *Tissue-Specific Immunity at the Oral Mucosal Barrier.* Trends Immunol, 2018. **39**(4): p. 276-287.
17. Ghosh, S., J. Padalia, and S. Moonah, *Tissue Destruction Caused by Entamoeba histolytica Parasite: Cell Death, Inflammation, Invasion, and the Gut Microbiome.* Curr Clin Microbiol Rep, 2019. **6**(1): p. 51-57.
18. Leon-Coria, A., M. Kumar, and K. Chadee, *The delicate balance between Entamoeba histolytica, mucus and microbiota.* Gut Microbes, 2020. **11**(1): p. 118-125.
19. Cornick, S., F. Moreau, and K. Chadee, *Entamoeba histolytica Cysteine Proteinase 5 Evokes Mucin Exocytosis from Colonic Goblet Cells via alphavbeta3 Integrin.* PLoS Pathog, 2016. **12**(4): p. e1005579.

20. Cornick, S., F. Moreau, H.Y. Gaisano, and K. Chadee, *Entamoeba histolytica-Induced Mucin Exocytosis Is Mediated by VAMP8 and Is Critical in Mucosal Innate Host Defense.* mBio, 2017. **8**(5).
21. Mortimer, L., F. Moreau, S. Cornick, and K. Chadee, *Gal-lectin-dependent contact activates the inflammasome by invasive Entamoeba histolytica.* Mucosal Immunol, 2014. **7**(4): p. 829-41.
22. Yu, Y. and K. Chadee, *Entamoeba histolytica stimulates interleukin 8 from human colonic epithelial cells without parasite-enterocyte contact.* Gastroenterology, 1997. **112**(5): p. 1536-47.
23. Thibeaux, R., P. Ave, M. Bernier, M. Morcelet, P. Frileux, N. Guillen, and E. Labruyere, *The parasite Entamoeba histolytica exploits the activities of human matrix metalloproteinases to invade colonic tissue.* Nat Commun, 2014. **5**: p. 5142.
24. Desvarieux, M., R.T. Demmer, T. Rundek, B. Boden-Albala, D.R. Jacobs, Jr., R.L. Sacco, and P.N. Papapanou, *Periodontal microbiota and carotid intima-media thickness: the Oral Infections and Vascular Disease Epidemiology Study (INVEST).* Circulation, 2005. **111**(5): p. 576-82.
25. Detert, J., N. Pischon, G.R. Burmester, and F. Buttgereit, *The association between rheumatoid arthritis and periodontal disease.* Arthritis Res Ther, 2010. **12**(5): p. 218.
26. Michaud, D.S., Z. Fu, J. Shi, and M. Chung, *Periodontal Disease, Tooth Loss, and Cancer Risk.* Epidemiol Rev, 2017. **39**(1): p. 49-58.
27. Linden, S.K., P. Sutton, N.G. Karlsson, V. Korolik, and M.A. McGuckin, *Mucins in the mucosal barrier to infection.* Mucosal Immunol, 2008. **1**(3): p. 183-97.
28. Frenkel, E.S. and K. Ribbeck, *Salivary mucins in host defense and disease prevention.* J Oral Microbiol, 2015. **7**: p. 29759.
29. Naughton, J., G. Duggan, B. Bourke, and M. Clyne, *Interaction of microbes with mucus and mucins: recent developments.* Gut Microbes, 2014. **5**(1): p. 48-52.

30. How, K.Y., K.P. Song, and K.G. Chan, *Porphyromonas gingivalis: An Overview of Periodontopathic Pathogen below the Gum Line.* Front Microbiol, 2016. **7**: p. 53.
31. Caton, J.G., G. Armitage, T. Berglundh, I.L.C. Chapple, S. Jepsen, K.S. Kornman, B.L. Mealey, P.N. Papapanou, M. Sanz, and M.S. Tonetti, *A new classification scheme for periodontal and peri-implant diseases and conditions - Introduction and key changes from the 1999 classification.* J Periodontol, 2018. **89 Suppl 1**: p. S1-S8.
32. Bonner, M., V. Amard, C. Bar-Pinatel, F. Charpentier, J.M. Chatard, Y. Desmuyck, S. Ihler, J.P. Rochet, V. Roux de La Tribouille, L. Saladin, M. Verdy, N. Girones, M. Fresno, and J. Santi-Rocca, *Detection of the amoeba Entamoeba gingivalis in periodontal pockets.* Parasite, 2014. **21**: p. 30.
33. Bao, X., R. Wiehe, H. Dommisch, and A.S. Schaefer, *Entamoeba gingivalis Causes Oral Inflammation and Tissue Destruction.* J Dent Res, 2020. **99**(5): p. 561-567.
34. Kim, J.M., H.C. Jung, K.I. Im, I.S. Song, and C.Y. Kim, *Synergy between Entamoeba histolytica and Escherichia coli in the induction of cytokine gene expression in human colon epithelial cells.* Parasitol Res, 1998. **84**(6): p. 509-12.
35. Stathopoulou, P.G., M.R. Benakanakere, J.C. Galicia, and D.F. Kinane, *The host cytokine response to Porphyromonas gingivalis is modified by gingipains.* Oral Microbiol Immunol, 2009. **24**(1): p. 11-7.
36. Kissoon-Singh, V., F. Moreau, E. Trusevych, and K. Chadee, *Entamoeba histolytica exacerbates epithelial tight junction permeability and proinflammatory responses in Muc2(-/-) mice.* Am J Pathol, 2013. **182**(3): p. 852-65.
37. Wang, Y. and D.N. Tatakis, *Human gingiva transcriptome during wound healing.* J Clin Periodontol, 2017. **44**(4): p. 394-402.

38. Eskan, M.A., M.R. Benakanakere, B.G. Rose, P. Zhang, J. Zhao, P. Stathopoulou, D. Fujioka, and D.F. Kinane, *Interleukin-1beta modulates proinflammatory cytokine production in human epithelial cells.* Infect Immun, 2008. **76**(5): p. 2080-9.
39. Kusano, K., C. Miyaura, M. Inada, T. Tamura, A. Ito, H. Nagase, K. Kamoi, and T. Suda, *Regulation of matrix metalloproteinases (MMP-2, -3, -9, and -13) by interleukin-1 and interleukin-6 in mouse calvaria: association of MMP induction with bone resorption.* Endocrinology, 1998. **139**(3): p. 1338-45.
40. Hernandez, M., M.A. Valenzuela, C. Lopez-Otin, J. Alvarez, J.M. Lopez, R. Vernal, and J. Gamonal, *Matrix metalloproteinase-13 is highly expressed in destructive periodontal disease activity.* J Periodontol, 2006. **77**(11): p. 1863-70.

Statutory Declaration

"I, Xin, Bao, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic **"Characterization of the interactions between *Entamoeba gingivalis* and the oral mucosa to assess its pathogenic potential / Charakterisierung der Interaktionen zwischen *Entamoeba gingivalis* und der oralen Mukosa zur Feststellung des pathogenen Potentials "**, independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date Signature

Declaration of your own contribution to the publications

Xin Bao contributed the following to the below listed publication:

Publication: Xin Bao, Ricarda Wiehe, Henrik Dommisch, Arne S. Schaefer; Entamoeba gingivalis causes oral inflammation and tissue destruction, Journal of Dental Research, Feb.5.2020.
Contribution: All the raw data related to the publication were obtained by my own; Figures and table were generated on the basis of my results of experiments and statistical evaluation; The clinical samples were collected by me with the help of dentists and oral hygienists practically who work in the Department of Periodontology and Synoptic Dentistry, Charité-University Medicine Berlin.

_____
Signature, date and stamp of first supervising university professor / lecturer

_____
Signature of doctoral candidate

Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCI
Selected Categories: **"DENTISTRY, ORAL SURGERY and MEDICINE"**
Selected Category Scheme: WoS
Gesamtanzahl: 90 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	PERIODONTOLOGY 2000	4,534	7.861	0.005740
2	JOURNAL OF DENTAL RESEARCH	20,078	5.125	0.021290
3	DENTAL MATERIALS	14,193	4.440	0.013220
4	JOURNAL OF CLINICAL PERIODONTOLOGY	14,049	4.164	0.013240
5	CLINICAL ORAL IMPLANTS RESEARCH	13,819	3.825	0.015930
6	ORAL ONCOLOGY	9,033	3.730	0.013340
7	INTERNATIONAL ENDODONTIC JOURNAL	6,767	3.331	0.006630
8	PEDIATRIC DENTISTRY	3,000	3.312	0.001950
9	JOURNAL OF DENTISTRY	8,950	3.280	0.011810
10	Clinical Implant Dentistry and Related Research	3,945	3.212	0.008420
11	Molecular Oral Microbiology	889	2.925	0.001800
12	JOURNAL OF ENDODONTICS	15,755	2.833	0.011700
13	JOURNAL OF PROSTHETIC DENTISTRY	12,350	2.787	0.008700
14	JOURNAL OF PERIODONTOLOGY	15,479	2.768	0.011520
15	International Journal of Oral Science	1,123	2.750	0.002290
16	Journal of Prosthodontic Research	937	2.636	0.001860
17	ORAL DISEASES	4,062	2.625	0.004740
18	JOURNAL OF PERIODONTAL RESEARCH	4,211	2.613	0.004310
19	JOURNAL OF THE AMERICAN DENTAL ASSOCIATION	6,822	2.572	0.004070
20	European Journal of Oral Implantology	1,127	2.513	0.002320

Xin Bao, Ricarda Wiehe, Henrik Dommisch, Arne S. Schaefer; *Entamoeba gingivalis* Causes Oral Inflammation and Tissue Destruction; Journal of Dental Research; Feb, 2020; Vol. 99(5) 561–567.

https://doi.org/10.1177/0022034520901738

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection

Complete list of publications

1 Entamoeba gingivalis causes oral inflammation and tissue destruction

Xin Bao, Ricarda Wiehe, Dommisch Henrik, Arne Schaefer. **J Dent Res**. 2020 Feb 5: 22034520901738 (**impact factor: 5.12**)

2. Proteolytic release of the p75NTR intracellular domain by ADAM10 promotes metastasis and resistance to anoikis

Xin Bao, Jianbo Shi, Wantao Chen, Zhiyuan Zhang and Qin Xu **Cancer Res**. 2018 May 1;78(9):2262-2276. (**impact factor: 8.38**)

3. Serum miR-626 and miR-5100 are Promising Prognosis Predictors for Oral Squamous Cell Carcinoma

Jianbo Shi*, **Xin Bao***, Zengying Liu, Zhiyuan Zhang, Wantao Chen, Qin Xu. **Theranostics** 2019; 9(4):920-931. (**impact factor: 8.06**)

4. Disruption and inactivation of the PP2A complex promotes the proliferation and angiogenesis of hemangioma endothelial cells through activating AKT and ERK.

Xie F, **Bao X**, Yu J, Chen W, Wang L, Zhang Z, Xu Q. **Oncotarget.** 2015 Sep 22;6(28):25660-76. (**impact factor:** Not available)

5. miR-300 inhibits epithelial to mesenchymal transition and metastasis by targeting Twist in human epithelial cancer.

Yu J, Xie F, **Bao X**, Chen W, Xu Q. Mol Cancer. 2014 May 24;13:121. **(impact factor: 10.68)**

Acknowledgments

I owe my sincere gratitude to my supervisor Univ.-Prof. Dr. Arne Schäfer, leader of the research group Molecular Genetics of Oral Inflammatory Diseases Charité - University Medicine Berlin, for supervising me my whole doctoral study. I am so grateful and honored to study and work with him for 3 years which I will cherish for my whole life. Prof. Schäfer is very supportive and helpful for my study and life in Germany as an excellent and thoughtful leader of the group. Because of his group, I learnt so much and enjoyed a lot as a foreign student.

I would also thank Univ.-Prof. Dr. Henrik Dommisch, director of the Department of Periodontology and Synoptic dentistry, for his kind help of sample collection and professional clinical advice for my study. I appreciate all the detailed sampling work which was done by every dentist and oral hygienist of our department. This dissertation would not have been possible without their supports.

My dear colleagues: Ricarda, Avi, and Gesa, who I spent the most time to work and study with, you gave me great help for my research and daily life. Because of you all, I forgot my homesick and enjoyed good working atmosphere for my 3 years in Germany.

I am very grateful to Chinese Scholarship Council who funded me for 3 years to study in Germany, and the help from embassy of China in Germany.

Last but not least, I would like to thank my parents, my friends who always give me the biggest support and love.

Sincere thanks to all of you!