***Entamoeba gingivalis* Causes Oral Inflammation and Tissue Destruction**

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Appendix Methods

E. gingivalis collection, culture and detection

All procedures are described in detail in the appendix. To test the frequency of *E. gingivalis* in inflamed and healthy areas of the oral cavity, we collected the samples at different sites. From each patient, we collected plaque in a single inflamed periodontal pocket with a sterile curette. The plaque was transferred to TYGM-9 Medium and cultured under anaerobic conditions at 35°C for two days. The presence of *E. gingivalis* in the growth medium was visually examinded with a microscope prior to PCR analysis (**Appendix Figure 1**). Uninflamed periodontal pockets have, at the most, small volumes. Thus, restriction to the gingival margins to collect plaque samples from uninflamed sites would result in reduced sensitivity to detect the amoeba. Thus, we included all uninflamed areas at the buccal mucosa, hard palate, tongue, and the upper and lower dentitions. Inclusion of these areas increased the sensitivity to detect the total absence or presence of *E. gingivalis* in the uninflamed oral cavity, although these areas differed from the inflamed periodontal pockets. We stroked each area three times with an oral swab. This swab was subsequently used for DNA extraction. DNA was isolated directly from both, growth medium and oral swabs using phenol-chloroform extraction (Rosenbaum et al. 2019). DNA was amplified by PCR using *E. gingivalis* specific primers (Bonner et al. 2014). Primers for amplification of DNA sequences of the human *Actin* gene were used as positive control for DNA isolation (**Appendix Figure 2**).

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

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 was 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* at multiplicity of infection (MOI) 0.2 was added in 10µL PBS to primary gingival epithelial cells (pGECs) and primary gingival fibroblasts (pGFBs) and co-incubated for 2 hours. To generate the mock-infection medium, we used 10µl of the supernatant of the last washing step. *P. gingivalis* was infected at MOI 100 in 10µL PBS.

For *E. gingivalis* infection of healthy live gingiva, *ex vivo* biopsies were placed upright on 4% agarose in 1.5ml 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 2 mm scalpel. *E. gingivalis* (3.5×10^4 in 250µl TYGM-9 Medium) was added to the biopsies and incubated with closed lids at 35°C for 6h.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Gene expression was quantified by determining the quantitative reverse transcription polymerase chain reaction (RT-qPCR) quantification cycle (C_q) values. The procedure of all RT-qPCR experiments and the primer sequences are described in the Appendix. RT-qPCR data were analysed with Student's t test for $2^{-\Delta\Delta Cq}$ values with *GAPDH* as reference gene. P-values were corrected for multiple testing by the method of Bonferroni. The RT-qPCR experiments that screened for differential expression of mucin and interleukin genes in pGECs and MMP genes and interleukins in pGFBs were regarded as independent experiments and correction was performed in each of the two experiments for the number of the 16 and 10 tested genes, respectively.

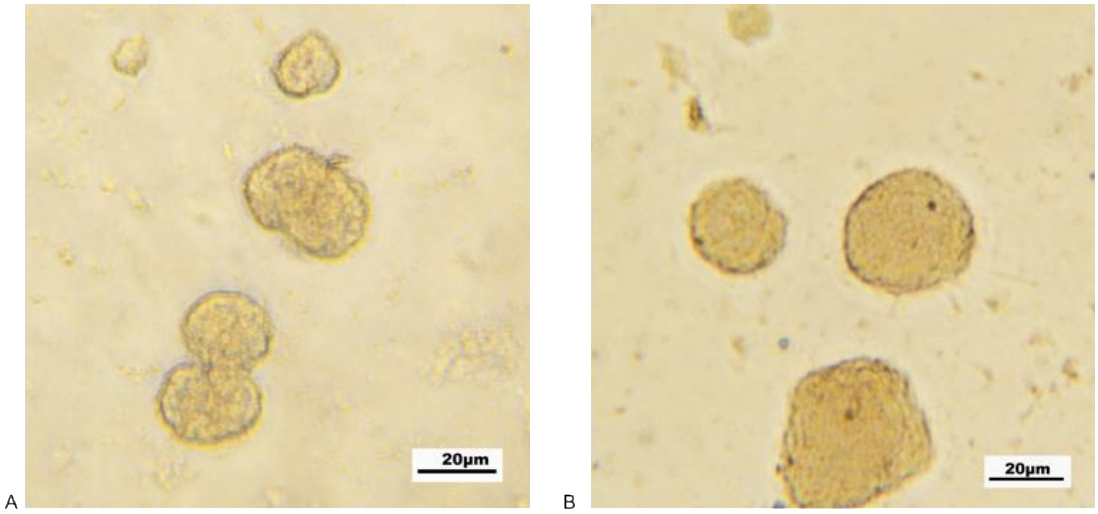
After 2 hours of *E. gingivalis* infection or mock-infection, the cells were washed 3× with PBS. Cell disruption and total RNA extraction was carried out using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's specifications. Subsequently, quantitative reverse transcription polymerase chain reaction (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) 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 (GraphPad Prism 6). GAPDH expression was unaffected of protozoan and bacterial infection. The primers were manufactured from the company metabion GmbH (Planegg/Steinkirchen, Germany).

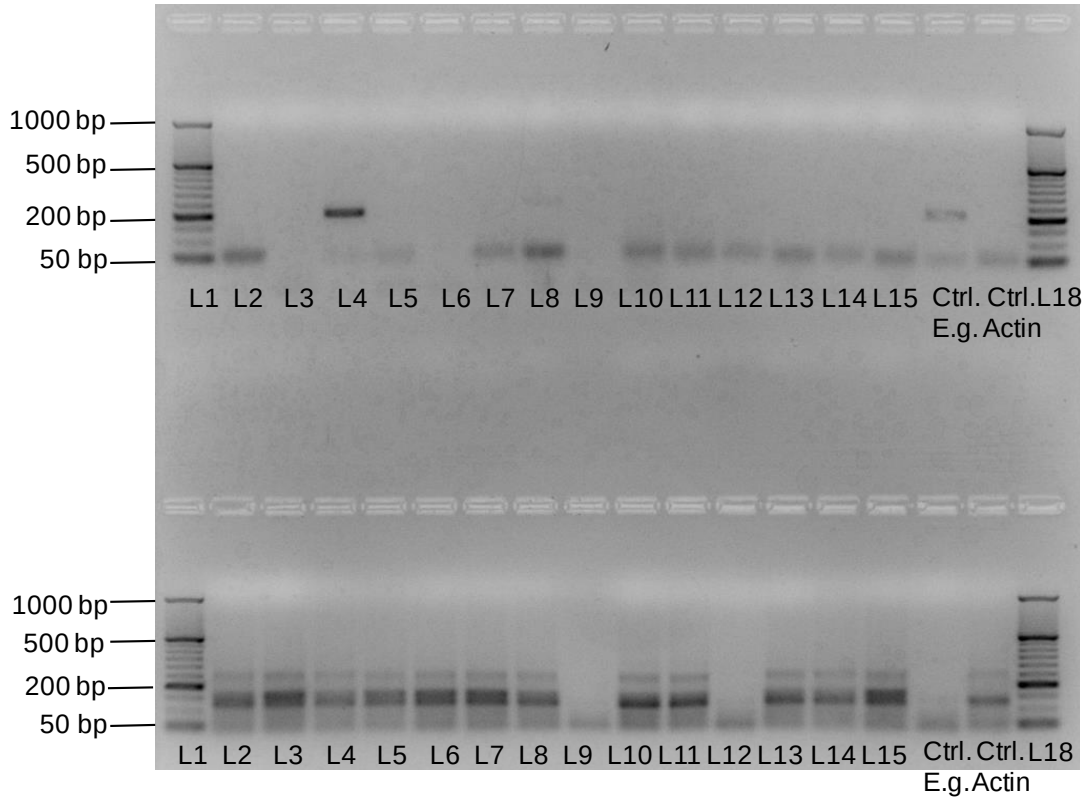
Cell proliferation assay

Cell proliferation was quantified using the Cell Counting Kit-8 (Millipore Sigma, USA). ~2000 pGEC cells per well were plated one day before the experiment. pGECs were treated with *E. gingivalis* at multiplicity of infection (MOI) 0.2 (Cornick et al. 2017), with *P. gingivalis* at MOI 100 in 10µL PBS (Dommisch et al. 2007), and mock-infected, followed washing with PBS for 3 times at each time point, and CCK-8 solution incubation for 1.5 hours. Each experiment was performed in three technical replications. The absorbance was measured at 450nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Promega).

Appendix Figures



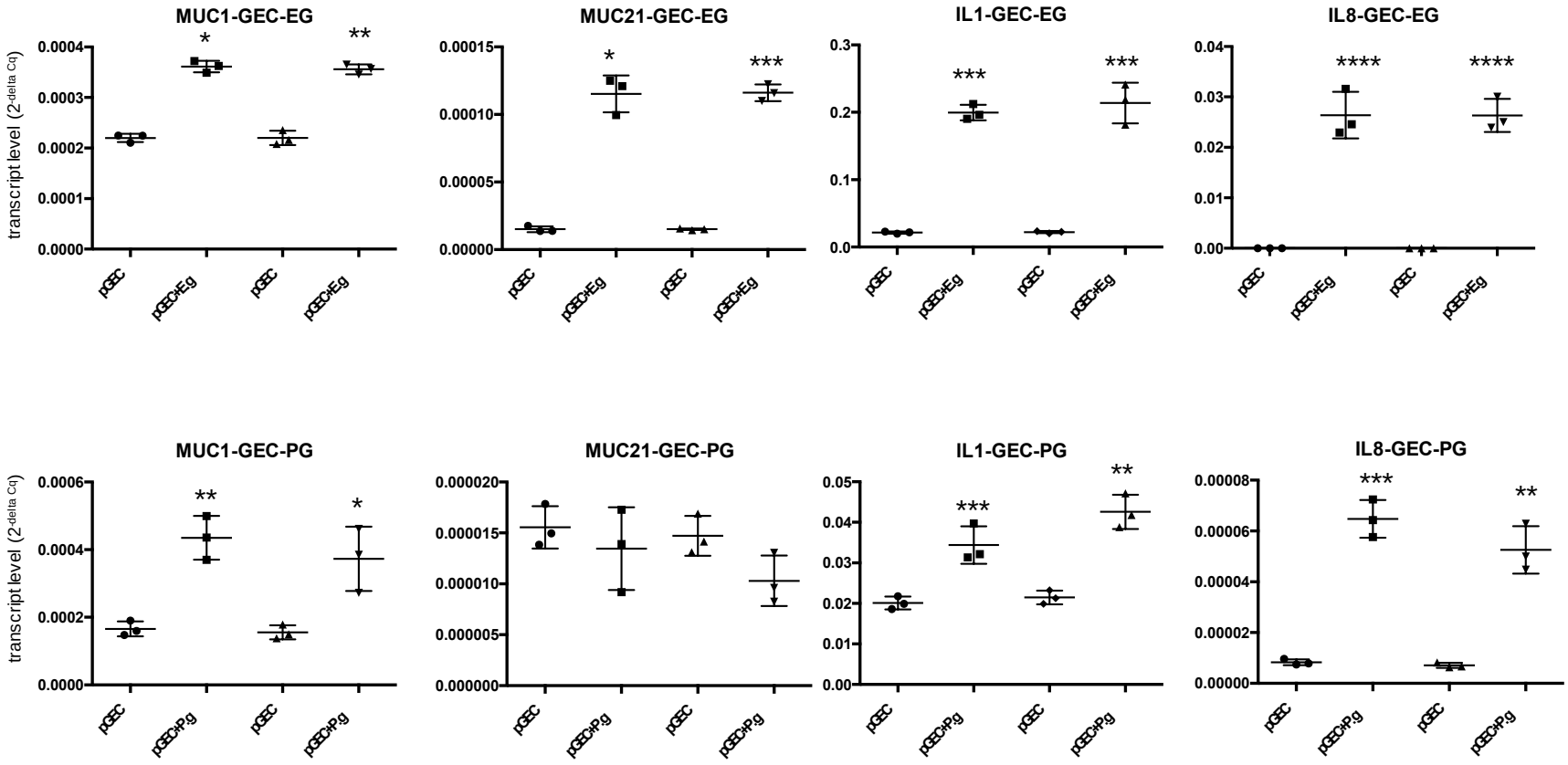
Appendix Figure 1. Appearance of *E. gingivalis* under the light microscope.
E. gingivalis cultures were obtained using plaque samples from periodontal pockets that were collected with a curette. The plaque was transferred to TYGM-9 Medium and cultured in anaerobic conditions at 35°C for 2 days. *E. gingivalis* was 32 × magnified.



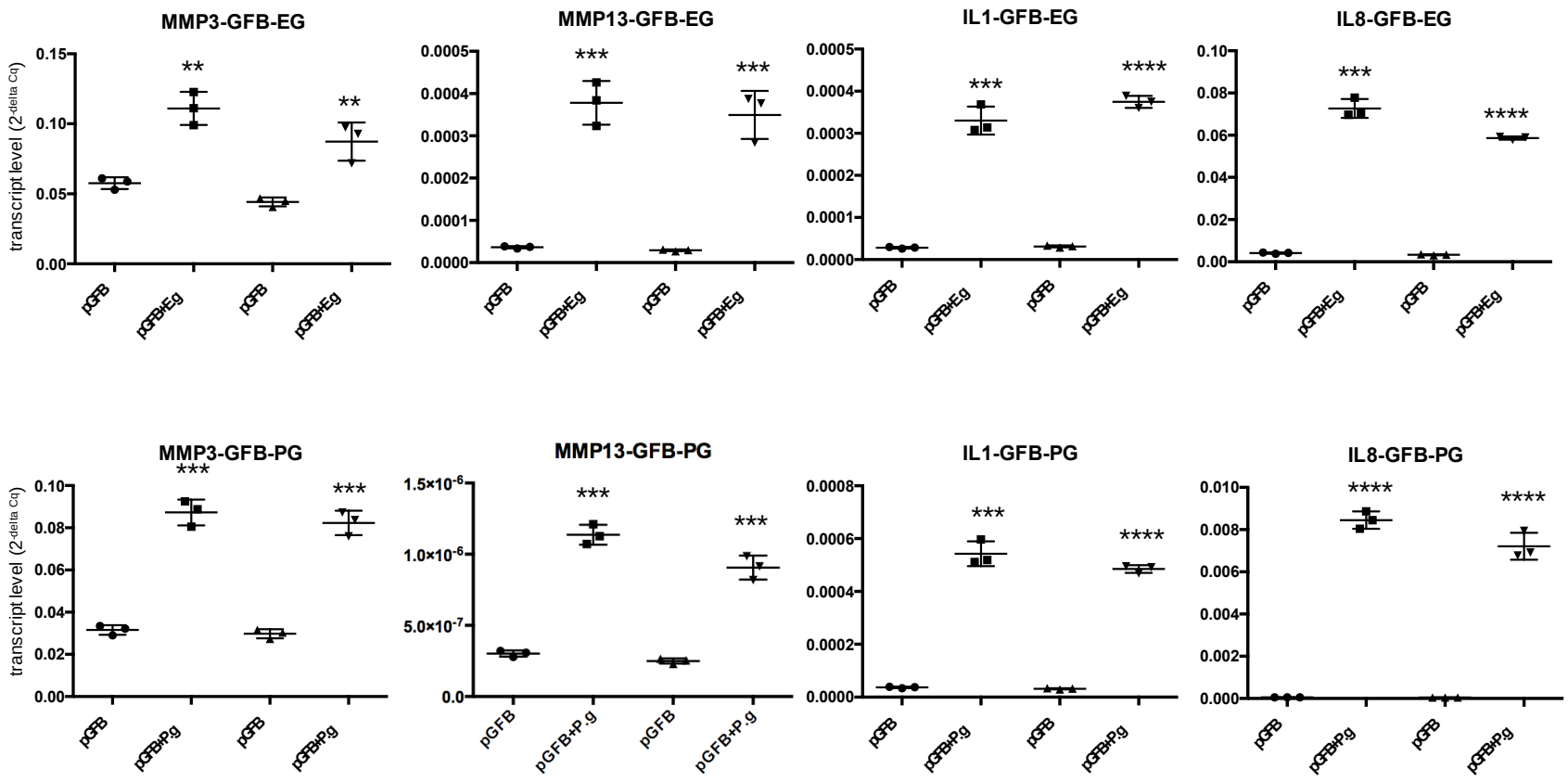
Appendix Figure 2. PCR-detection of *E. gingivalis* from buccal swabs.

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

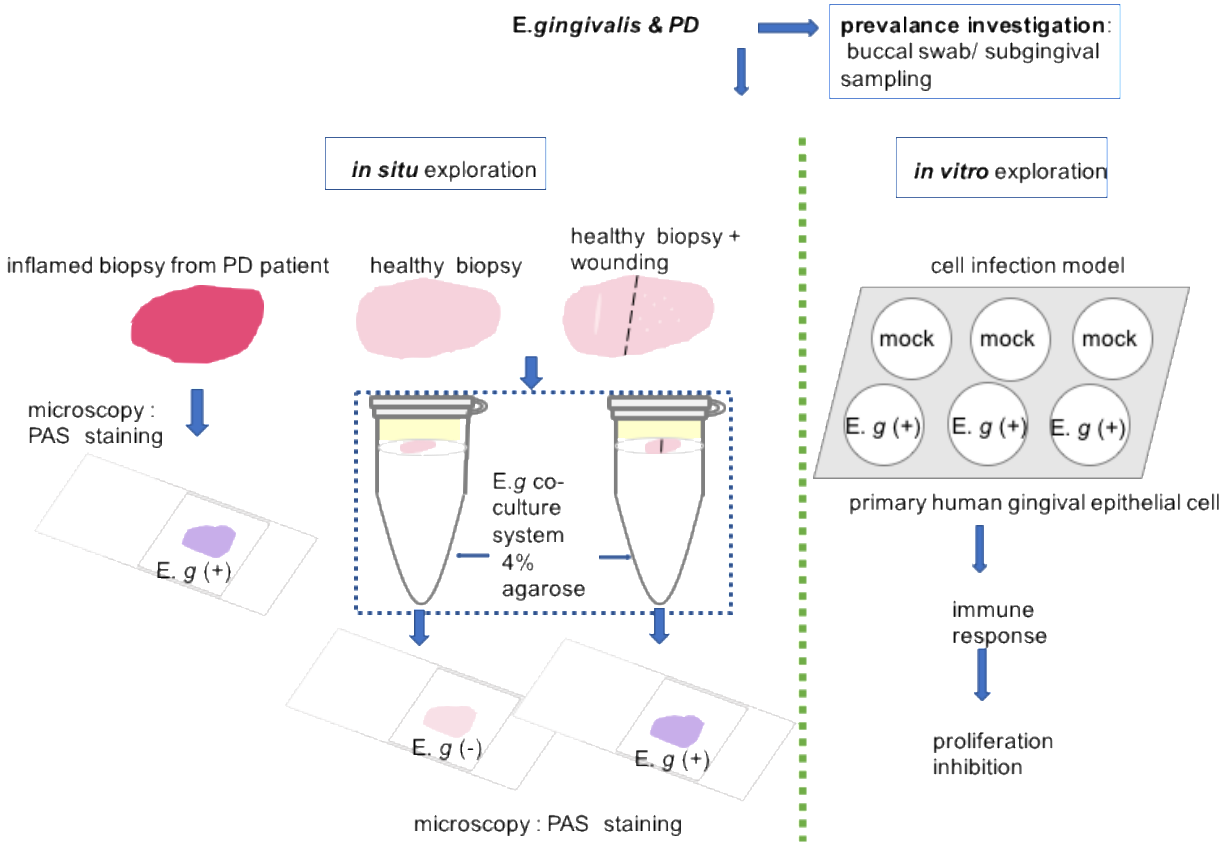
Lower lane: The PCR products were generated with human *ACTIN* specific primers. Lane 9 shows no amplification indicating that no DNA was isolated. Such a sample was discarded from the analysis. (Lane 1 and 18 = O'RangeRuler 50 bp DNA, Ctrl. E.g.= DNA template from E.g. cultures (the presence of E.g. was additionally validated by microscopy), Ctrl. *ACTIN* = DNA template from human gingival cells, 2% agarose gel, 30 minutes, 100V)



Appendix Figure 3. *MUCIN 1, -21, IL1β, -8* expression in primary gingival epithelial cells with *E. gingivalis* or *P. gingivalis* infection.



Appendix Figure 4. *MMP3, -13, IL1β, -8* expression in primary gingival fibroblasts with *E. gingivalis* or *P. gingivalis* infection



Appendix Figure 5. Schematic representation of the study

Appendix Table. PCR and qRT-PCR Primers

IL primers	
IL1B-fw	AACACGCAGGACAGGTACAG
IL1B -rev	GAGCAACAAGTGGTGTTCTC
IL8-fw	TTGGCAGCCTTCCTGATT
IL8-rev	AACTTCTCCACAACCCTCTG
***Mucin* primers**	
MUC1-fw	GTCATGCAAGCTCTACCCCA
MUC1-rev	GAGCTGGGCACTGAACTTCT
MUC2-fw	TGCTGATCAAGACCGTGCAT
MUC2-rev	CCTCCAGCCCGTACTTCTTG
MUC3A-fw	TACCATAGTCCCTGCCTCCC
MUC3A-rev	GTTAATGGGGTGGTGGGAGG
MUC4-fw	ACTCACCTCATCCTTACCGC
MUC4-rev	CAGCCTTCAGTCACCTTCCC
MUC5A/C-fw	GCTGTGTCCCTGTGTCAAAG
MUC5A/C-rev	CAAACGTTGAGAAGTGGGCA
MUC5B-fw	GCGGGGACATGGAAACTTTT
MUC5B-rev	CACACGCACGTTGTAGTTGA
MUC6-fw	CTTCTCTCACCACCCGAGTT
MUC6-rev	TGACTGGAGAGGTGGGGATA
MUC7-fw	TAGACCCATCATCCGCCTCA
MUC7-rev	AGGTGTGGTAATTGGGGCAG
MUC12-fw	TCTGCATCCACACCCAGTTC
MUC12-rev	TGCAGTTGAGCCCGTGATAA
MUC13-fw	AACCAGACTGCGGATGACTG
MUC13-rev	TTGCAGGCATCAGGACACTT
MUC15-fw	CAGCAGAGCATTCTTTGGGC
MUC15-rev	GCTATGGATCAAGGGAGGGC
MUC16-fw	TCAACCAACAAGCAGCTCCA
MUC16-rev	CTGGGCTTTGTCCTGGGAAT
MUC17-fw	CCGCCACAGGTACAACATCT
MUC17-rev	TCATCTCAGGGTTGGTGCTG
MUC19-fw	CCTCTGGGGTATCTCTTGCC
MUC19-rev	AGAACTTTGCCAGAGGTGCC
MUC20-fw	CTTTGACACCCTTTGCACG
MUC20-rev	GGAGGTGTGAGCCAATGTCA
MUC21-fw	TCTACTGCAGTGAGTGAGGC
MUC21-rev	AGGTGTTTCTCAGGGACAGG

***MMP* primers**	
MMP1-fw	GTGATGTTCAGCTAGCTCAGGA
MMP1-rev	GGCTGGACAGGATTTTGGGA
MMP2-fw	TCATGATCAACTTTGGCCGC
MMP2-rev	CATGAGCCAGGAGTCCGTC
MMP3-fw	TTGTTAGGAGAAAGGACAGTGGT
MMP3-rev	ATCAGCCTCTCCTTCATACAGC
MMP7-fw	TGCCAACAGTTTAGAAGCCAA
MMP7-rev	TGATGTCAGCAGTTCCCCAT
MMP8-fw	TCACCAGGATCTCACAGGGA
MMP8-rev	CATGAGCAAGGATTCCATTGGG
MMP9-fw	GAGACGCCCATTTCGACGAT
MMP9-rev	GATGAAGGGGAAGTGGCAGG
MMP13-fw	TCTGAAGTCGAAAAGGCATTCA
MMP13-rev	TGTCCAGGTTTCATCATCATCAA
MMP20-fw	CTCCTGCTCTTCAAGGACCG
MMP20-rev	GGTAATAGTGCTGGGCCGAA

***E.g* primer**	
E.gingivalis-fw	AGGAATGAACGGAACGTACA
E.gingivalis-rev	CCATTTCCTTCTTCTATTGTTTCAC

human actin primer	
IP-actin 1-fw	ATTTAGCGCCAATTCCCA
IP-actin 1-rev	GGCGGGGTCTTTGTCTGA

Appendix References

Cornick S, Moreau F, Gaisano HY, Chadee K. 2017. Entamoeba histolytica-induced mucin exocytosis is mediated by vamp8 and is critical in mucosal innate host defense. MBio. 8(5).

Dommisch H, Chung WO, Rohani MG, Williams D, Rangarajan M, Curtis MA, Dale BA. 2007. Protease-activated receptor 2 mediates human beta-defensin 2 and cc chemokine ligand 20 mrna expression in response to proteases secreted by porphyromonas gingivalis. Infect Immun. 75(9):4326-4333.