Treponema denticola Induces Epithelial Barrier Dysfunction in Polarized Epithelial Cells

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Abstract

Treponema denticola, an anaerobic spirochete found mainly in the oral cavity, is associated with periodontal disease and has a variety of virulence factors. Although in vitro studies have shown that T. denticola is able to penetrate epithelial cell monolayers, its effect on the epithelial barrier junction is not known. Human gingival epithelial cells are closely associated with adjacent membranes, forming barriers in the presence of tight junction proteins, including zonula occludens-1 (ZO-1), claudin-1, and occludin. Tight junction proteins are also expressed by Madin-Darby canine kidney (MDCK) cells in culture. In this study, the MDCK cell profile was investigated following infection with T. denticola (ATCC 35405) wild-type, as well as with its dentilisin-deficient mutant, K1. Basolateral exposure of MDCK cell monolayers to T. denticola at a multiplicity of infection (MOI) of 10⁴ resulted in a decrease in transepithelial electrical resistance (TER). Transepithelial electrical resistance in MDCK cell monolayers also decreased following apical exposure. The effect on the TER was time-dependent and required the presence of live bacteria. Meanwhile, MDCK cell viability showed a decrease with either basolateral or apical exposure. Immunofluorescence analysis demonstrated decreases in the amounts of immunoreactive ZO-1 and claudin-1 in association with disruption of cell-cell junctions in MDCK cells exposed apically or basolaterally to T. denticola. Western blot analysis demonstrated degradation of ZO-1 and claudin-1 in culture lysates derived from T. denticola-exposed MDCK cells, suggesting a bacteria-induced protease capable of cleaving these tight junction proteins.

Key words: Treponema denticola — Epithelial cell — Transepithelial electrical resistance — ZO-1 — Claudin-1
Introduction

Bacterial biofilm on periodontal tissues is the primary etiological cause of periodontal disease. The gingival epithelium is interconnected by tight junctions (zonula occludens), adherens junctions, desmosomes, and gap junctions, which provide an important barrier against bacterial invasion. Hallmark tight junction proteins and junction-related structures have recently been identified in the gingival epithelium. The periodontopathogenic bacterium Porphyromonas gingivalis increases the paracellular permeability of the gingival epithelium by degrading tight junction proteins, such as claudin-1, occludin, and E-cadherin. Other periodontal pathogens, including Treponema denticola, may also facilitate intercellular invasion by impairing tight junction structures.

Polarized epithelial cells develop distinct apical and basolateral surfaces that form selective permeability barriers between biological compartments. The apical and basolateral membrane domains are distinguished by unique assemblies of proteins that form tight junctions, lipids, and other macromolecules and structures, such as layers of secreted mucous. Tight junctions, the most apical component of the intercellular junctional complex, serve to seal the paracellular space and polarized epithelia, while gap junctions are specialized intercellular connections between cells. Previous studies have shown that periodontal pathogens like P. gingivalis and T. denticola have the ability to invade human gingival epithelial cells, fibroblasts, and keratinocytes. The epithelium possesses cell-cell junction-complexes, such as tight junctions and gap junctions, which function as a mechanically protective barrier against invasion by pathogenic organisms. As mentioned above, tight junctions are the most apical component of the intercellular junctional complex, serving to seal the paracellular space and polarized epithelia, while gap junctions are specialized intercellular connections between cells. Earlier studies have shown that T. denticola DNA is detectable in human brain from donors with Alzheimer’s disease, and mouse brain from severe-combined-immunodeficiency mice infected with red-complex organisms. To the best of our knowledge, however, no studies to date have investigated T. denticola translocation from the blood to the central nervous system, and it is unclear how circulating T. denticola crosses the blood-brain barrier (BBB). Tight junctions between cerebral vascular endothelial cells are the basis of the BBB, where they form an impermeable seal.

The purpose of the present study was to determine the effects of T. denticola on epithelial cell barrier function and tight junction integrity in polarized epithelial cell monolayers using Madin-Darby canine kidney (MDCK) cells in an in vitro model. Madin-Darby canine kidney cells have been extensively used in...
studies of epithelial barrier function, because they form a well-polarized epithelial monolayer. In addition, polarized MDCK cells express tight junction-associated proteins, such as ZO-1, claudin-1, and occludin. Furthermore, a previous study indicates that *T. denticola* can penetrate periodontal tissue\(^2\), and that the chymotrypsin-like protease dentilisin plays a crucial role in this process by altering cellular tight-junctions\(^5,10\). In the present study, the effects of dentilisin on MDCK cell barrier-associated proteins ZO-1, claudin-1, and occludin were also investigated by comparing a dentilisin-deficient mutant with its parent strain.

### Materials and Methods

1. **Bacteria**

   *Treponema denticola* ATCC 35405 wild type and the dentilisin-deficient *T. denticola* mutant K1 were used. The microorganisms were grown and maintained in tryptone-yeast extract-gelatin-volatile fatty acids-serum (TYGVS) medium under anaerobic conditions at 37°C for 4 days\(^19,31\). *Escherichia coli* HB101 was used as a negative control for transepithelial electrical resistance (TER) measurements. The *E. coli* was cultured in Luria-Bertani broth at 37°C, overnight. The bacteria were harvested, washed in phosphate buffered saline (PBS), and centrifuged at 6,000 × g for 20 min. The number of bacteria in the suspension was spectrophotometrically determined according to optical density absorbency at 600 nm. The bacteria were then re-suspended in antibiotic-free minimum essential medium (MEM) supplemented with 10% fetal calf serum.

2. **Cell culture**

   Madin-Darby canine kidney cells (NBL-2) derived from dog kidney epithelial cells were obtained from the Health Research Resources Bank (Osaka, Japan) and maintained in MEM supplemented with 10% fetal calf serum and gentamicin/amphotericin B (Kurabo, Osaka, Japan) at 37°C in humidified 5% CO\(_2\). To prepare polarized MDCK cell monolayers, 0.4 × 10\(^5\) trypsinized cells were seeded onto BD Falcon cell culture inserts containing 0.33 cm\(^2\) filter membranes with 3.0 μm pores in a 24-well configuration (BD, Franklin lakes, NJ, USA). The cells were incubated until they reached confluency. Cell monolayers were used for experiments after 4 days of culture.

3. **Measurement of transepithelial electrical resistance**

   Monolayers were grown to allow the development of intercellular tight junctions and for the TER to reach >300 Ω cm\(^2\). Prior to infection with bacteria, the cell culture medium was changed to antibiotic-free medium. The MDCK cells were infected apically or basolaterally with *T. denticola* at a multiplicity of infection (MOI) of 10\(^2\) or 10\(^4\). The TER was then measured in units of Ω cm\(^2\) using a Millicell ERS Epithelial Volt-Ohm-Meter (Millipore, Bedford, MA, USA) with chopstick electrodes at 0, 2, 4, 6, 16, 24, and 48 hr post infection. Cell culture inserts with no MDCK cells (blank) or with *T. denticola* only were used to determine baseline levels. All experiments were performed in triplicate, and all values expressed represent the mean. Prior to some experiments, basolateral compartments were filled with medium treated with 10% formalin-fixed *T. denticola* (24 hr) or boiled *T. denticola* (100°C for 30 min). Samples of the formalin-fixed and heat-killed bacteria were cultured in TYGVS to confirm lack of bacterial viability.

4. **Confocal immunofluorescence microscopy**

   Madin-Darby canine kidney cell monolayers grown on culture inserts and exposed to *T. denticola* were fluorescently labeled as previously described\(^15\) with some modifications. Briefly, the infected cells were washed once with PBS and fixed with 4% formaldehyde in PBS for 20 min. The cells were washed 3 times with PBS and quenched with 50 mM glycine in PBS for 30 min at room temperature (approximately 23°C). Cells were washed twice with PBS and permeabilized with 0.2%
(w/v) Triton X-100 in PBS for 15 min, and then washed again 3 times with PBS. The cells were blocked with PBS containing 1% bovine serum albumin for 30 min, and incubated with 1:100 mouse anti-occludin monoclonal antibody (mAb) from Thermo Fisher Scientific (Rockford, IL, USA), 1:100 mouse anti-ZO-1 mAb (Thermo Fisher Scientific), 1:100 mouse anti-claudin-1 mAb (Thermo Fisher Scientific), or 1:100 mouse anti-claudin-2 mAb (Thermo Fisher Scientific). Following incubation with their respective primary antibodies, the cells were washed with PBS and incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (Thermo Fisher Scientific) for 2 hr at 37°C. The culture filters containing the cells were removed from the inserts, rinsed 3 times with PBS, and embedded with 95% glycerol in PBS. Fluorescence images were obtained with an LSM510 confocal laser-scanning microscope (Carl Zeiss, Jena, Germany). The experiments were repeated 3 times, with the results of a typical experiment being presented.

5. Electrophoresis and Western blotting

Madin-Darby canine kidney cells were grown on culture inserts as described above. Treponema denticola wild-type was added to the apical or basolateral compartments of the culture wells at an MOI of $10^4$. As a control, a third set of wells received no bacteria. After 2 hr or 16 hr incubation, the infected and uninfected cells were lysed in Complete Lysis-M reagent (Roche Diagnostics, Mannheim, Germany) and centrifuged for 20 min at 12,000 $\times$ g at 4°C. The soluble cell lysates were added to sodium dodecyl sulfate (SDS) loading buffer containing 1:20 (v/v) dithiothreitol at a ratio of 1:4, boiled for 5 min, and separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using Mini-PROTEAN TGX precast gels (Bio-Rad Laboratories, Hercules, CA, USA). Following electrophoretic separation, the proteins were transferred onto Immobilon-P membranes (Millipore). Blotting was performed using the SNAP i.d. Protein Detection System (Millipore). The membranes were incubated with the following primary antibodies (Thermo Fisher Scientific): anti-occludin mAb (1:1,000), anti-ZO-1 mAb (1:1,000), and anti-claudin-1 mAb (1:1,000). Mouse anti-β actin mAb at 1:5,000 (Sigma-Aldrich, St. Louis, MO, USA) was used as an internal loading control. All blots were then incubated with a secondary anti-mouse IgG antibody conjugated with horseradish peroxidase (Thermo Fisher Scientific). Immunoreactive signals were detected using TMB Membrane Peroxidase Substrate (1-Component) as the colorimetric substrate (KPL, Gaithersburg, MD, USA). Quantification was performed with ImageJ 1.44o software (Wayne Rasband, NIH, http://rsb.info.nih.gov/ij). Western blot intensity measurements were recorded and are presented as the ratio of the intensity of the tight junction protein band relative to the intensity of the corresponding β-actin band from the same sample. Protein concentrations were measured with Pierce BCA Protein Assay reagent (Thermo Fisher Scientific).

6. Cytotoxicity assay

Epithelial cell cytotoxicity was determined by colorimetric quantification of lactate dehydrogenase (LDH) release, using the CytoTox-ONE homogeneous membrane integrity assay (Promega, Mannheim, Germany) according to the manufacturer’s instructions, and as previously described, with the following modifications: MDCK cells grown in transwells were infected with T. denticola wild-type at the apical or basolateral surface, and 100 μl supernatant collected at 16 hr post infection from the apical chamber. To determine total amounts of LDH, residual intracellular LDH levels were measured by adding 2 μl lysis solution to the mock-infected cells. Residual intracellular LDH levels were used as a control baseline, which was set as 100%. Levels of LDH in the supernatants of the T. denticola-treated samples were measured as described below and normalized to the control baseline values. The absorbance of the samples was measured at 490 nm on a SpectraMax 340PC plate reader using SOFTmaxPro software in order to quantitate the amount of LDH.
Assays were carried out in triplicate, and the results are reported as the averages for 3 experiments.

7. Data and statistical analysis

The data are presented as the mean and standard error of the mean. A comparison of variance for multiple groups was performed using an ANOVA; the Bonferroni test was used for post hoc comparisons. All statistical analyses were performed using Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Values of p < 0.05 were considered statistically significant.

Results

1. Change in transepithelial electrical resistance

Apical exposure of MDCK monolayers to T. denticola at an MOI of 10^2 increased the TER after 6 hr, while apical exposure at an MOI of 10^4 greatly diminished the TER between 6 hr and 16 hr (Fig. 1A). In contrast, basolateral exposure of MDCK monolayers to T. denticola at an MOI of 10^4 decreased the TER in a time-dependent manner (Fig. 1B). A more rapid decrease was seen when bacteria at an MOI of 10^5 were used (data not shown). These findings indicate that the apical surface is more resistant to disruption of the epithelial barrier than the basolateral surface. Neither apical exposure nor basolateral exposure to E. coli HB101 had an effect on the TER during the first 24 hr post infection (data not shown). Treatment of MDCK monolayers with formaldehyde-fixed or heat-killed bacteria abolished the T. denticola-induced decrease in TER, indicating that live bacteria were required for the observed decrease in TER (Fig. 2).

2. Treponema denticola alters zonula occludens-1 and claudin distribution in Madin-Darby canine kidney cell monolayers

The effect of T. denticola on the epithelial cell barrier proteins in the MDCK monolayers was evaluated by immunofluorescence, using antibodies against ZO-1, claudin-1, and occludin. The MDCK cells were incubated with T. denticola wild type or K1 mutant at an MOI of 10^4 added to either the apical or basolateral surface for 16 hr. The uninfected control cells...
showed intact continuous rings at points of intercellular contact for ZO-1, claudin-1, and occludin (Figs. 3A, B, and C). Compared to uninfected control MDCK monolayers, no difference in the immunofluorescence levels of occludin was detected following exposure of the apical surfaces of cells to \textit{T. denticola}, but more gradual decreases were observed following exposure of the basolateral surfaces of cells to \textit{T. denticola} (Figs. 3F and I). In contrast to in the uninfected controls, no continuous rings of ZO-1 or claudin-1 were detected after exposure of the apical or basolateral surfaces of the cells to \textit{T. denticola}, and some aggregation was observed (Figs. 3D, E, G, and H). When the apical or basolateral surfaces of the monolayers were exposed to the dentilisin-deficient \textit{T. denticola} mutant bacteria K1, intact rings of ZO-1, claudin-1, and occludin were detected (Figs. 3J, K, L, M, N, and O).

3. Tight junction protein-expression profiles following \textit{Treponema denticola} infection

To complement the findings from confocal imaging, tight junction protein expression in whole cell extracts was quantified using immunoblotting (Fig. 4). After 2 hr of \textit{T. denticola} exposure, the ZO-1 signal was lost. No decrease was observed in the amounts of either claudin-1 or occludin following exposure of the monolayers to \textit{T. denticola} for 2 hr, however. After 16 hr of incubation, detectable levels of claudin-1 disappeared in cells treated by either basolateral or apical exposure to \textit{T. denticola}.

4. Cytotoxicity

To assess whether cytotoxicity was also involved in epithelial barrier disruption, the viability of the MDCK monolayers was assessed following exposure to \textit{T. denticola} wild-type for 24 hr by analyzing levels of released LDH (Fig. 5). Exposure of \textit{T. denticola} wild-type to either the apical or basolateral surface of the MDCK monolayers significantly increased the amount of LDH released in comparison with in the controls (apical treatment $= 14.45 \pm 4.95\%$, basal treatment $= 12.46 \pm 2.5\%$, control treatment $= 1.04 \pm 0.44\%$).

Discussion

The integrity of periodontal epithelium plays an important role in host defense against pathogens. The ability of periodontal pathogens to disrupt the epithelial barrier and penetrate deeper tissue may contribute significantly to the initiation and progression of periodontal diseases. \textit{Treponema denticola} invaded gingival keratinocyte HOK-16B cells by resisting endolysosomal degradation for many hours\textsuperscript{37}. In earlier experiments using non-polarized HEp-2 cells, \textit{T. denticola} strains disrupted epithelial cell monolayers\textsuperscript{38}. However, HEp-2 cells are not known to express the tight junction proteins occludin or claudin-1 in their intracellular spaces. In the present study, MDCK cells (NBL-2) formed a well-polarized epithelial monolayer and expressed the tight junction proteins ZO-1, occludin, and claudin-1, but not claudin-2 (data not shown). The difference was likely caused by the specific properties of the different cell types. For example, a subclone of MDCK cells...
(MDCK I) achieved a high TER and lacked detectable claudin-2, while the MDCK subclone MDCK II showed a low TER and expressed claudin-2\(^{12}\).

The destructive effect of *T. denticola* on the barrier function of polarized MDCK cell monolayers has been studied *in vitro*. It has been previously reported that a decrease in the TER of MDCK cells was associated with changes in the epithelial cell barrier\(^{38}\). In the present study, a decrease was observed in the TER of MDCK cells within 6 hr post exposure of their basolateral surface to *T. denticola* at an MOI of 10\(^4\) (Fig. 1B). Exposure of the apical surface to *T. denticola* at an MOI of 10\(^4\) did not significantly affect the TER during the first 6

\[\text{Fig. 3} \quad \text{Immunofluorescence analysis of tight junction proteins zonula occludens-1 (ZO-1) (A, D, G, J, M), claudin-1 (B, E, H, K, N), and occludin (C, F, I, L, O) in MDCK cells following exposure of apical or basolateral surfaces to either *T. denticola* wild-type or dentilisin-deficient mutant K1 at MOI of 10}\(^4\) \text{for 16 hr. (A, B, C) Uninfected MDCK cells. (D, E, F) Apical exposure to *T. denticola* wild-type. (G, H, I) Basolateral exposure to *T. denticola* wild-type. (J, K, L) Apical exposure to *T. denticola* K1. (M, N, O) Basolateral exposure to *T. denticola* K1. Marker = 20 µm.}\]
hr (Fig. 1A), however. These results suggest that basolateral surfaces are more susceptible than apical surfaces to the effects of *T. denticola*. One study noted that the apical and basolateral surfaces of polarized MDCK cells were involved in defense against *Pseudomonas aeruginosa*, and that *P. aeruginosa* colocalized with heparan sulfate-rich areas on the basolateral surface, but with complex N-glycans at the apical surface. Another study found that basolateral exposure to *Bacteroides fragilis* toxin affected epithelial cell morphology and rapidly decreased TER. Other research has shown that apical exposure of MDCK cells to *P. gingivalis* (10^10 bacteria) has little effect, and that the epithelial cell barrier remains intact. In the present study, exposing MDCK cells to a lower number of *T. denticola* had no detectable effect on the TER of the monolayers, indicating that a threshold concentration of *T. denticola* is required in order to disrupt the epithelial barrier (data not shown). An earlier study found that treating MDCK monolayers with a lower number of *P. gingivalis* (10^7 bacteria) had no effect on the TER. Taken together, these findings indicate that a threshold of bacteria is required to impose a detrimental effect on maintenance of the epithelial cell barrier.

In the present study, the nature of the host response to infection was also investigated by focusing on intercellular tight junction morphology and protein expression. Results from confocal immunofluorescence microscopy and Western blot analysis were consistent in that degradation of tight junction proteins ZO-1 and claudin-1 was apparent following incubation of the MDCK monolayers with *T. denticola* (Figs. 3, 4). Although ZO-1 was almost completely degraded at 2 hr post infection with *T. denticola*, the TER of the MDCK monolayers was maintained. An earlier study found that *Escherichia albertii* also caused disruption of ZO-1 in both MDCK-1 and T84 cells, and again, polarization was maintained. *Treponema denticola* produces several proteolytic enzymes, such as dentilisin, oligopeptidase, and dentipain. It is possible that these proteases were involved in the decrease in TER and disruption of tight junction proteins at 16 hr post infection of the cells, either basolaterally or apically, observed here. An elastase derived from *P. aeruginosa* causes disruption of ZO-1, and a decrease in the TER of MDCK cells. *Citrobacter rodentium*, a related mouse pathogen, produces the large toxin...
lymphostatin, which contains two enzymatic motifs, a glucosyltransferase and a protease. This toxin also disrupts ZO-1 and occludin, and induces a decrease in the TER of Caco2 cells. Furthermore, the TER of primary gingival keratinocytes and immortalized human gingival keratinocytes is disrupted by the presence of gingipains from P. gingivalis. One study reported that outer membrane extracts of wild-type T. denticola 35405 disrupted the TER of non-polarized HEp-2 cell monolayers, while the extracts from the dentilisin deletion mutant K1 had no effect. Another reported that HEp-2 cells infected with 5 × 10^9/ml wild-type T. denticola 35405 for 24 hr showed a decrease in staining for ZO-1 and a decrease in TER, whereas K1 showed only a slight decrease in ZO-1 staining and only a small decrease in TER. Recently, it has been demonstrated that dentilisin induces activation of host-derived MMP-2. An earlier study by the present group also found that the dentilisin activity of T. denticola played an important role in invasion of and survival within human gingival epithelial cells. The proteases of T. denticola may be responsible for these observed results. The present results demonstrate that, while wild type T. denticola ATCC 35405 can disrupt epithelial layers, formaldehyde-fixed or heat-killed bacteria, and the dentilisin-deficient mutant K1 abolishes T. denticola-induced decrease in TER (Fig. 2). This apparent difference may be explained by dentilisin’s proteolytic activity with the tight junction proteins. Further study is needed to clarify the specific T. denticola virulence factors involved in affecting the integrity of the epithelial cell barrier, including dentilisin.

In conclusion, to the best of our knowledge, this is the first study to demonstrate the effect of T. denticola infection on polarized epithelial cells using an in vitro model. The results suggest that T. denticola invades deeper tissues via a paracellular route by degrading the tight junction proteins ZO-1 and claudin-1. These mechanisms may provide new insight into the role of T. denticola in the pathogenesis of periodontitis. Increased penetration may play a significant role in the initiation and progression of periodontal disease. Once a substance that makes a positive effect on tight junction protein expression is identified, it may prove helpful in the treatment of people with gingival epithelial tight junction deficiency, and in the prevention of periodontitis. Furthermore, these results support the hypothesis that T. denticola dissemination in the body is promoted by its ability to penetrate the tight junctions of epithelial monolayers, including those of the BBB. Further studies are being considered in an effort to explore the relationship between T. denticola and Alzheimer’s disease.

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**References**

1) Azghani AO (1996) *Pseudomonas aeruginosa* and epithelial permeability: role of virulence factors elastase and exotoxin A. Am J Respir Cell Mol Biol 15:132–140.
2) Babbin BA, Sasaki M, Gerner-Schmidt KW, Nusrat A, Klapproth JM (2009) The bacterial virulence factor lymphostatin compromises intestinal epithelial barrier function by modulating rho GTPases. Am J Pathol 174:1347–1357.
3) Bucior I, Mostov K, Engel JN (2010) *Pseudomonas aeruginosa*-mediated damage requires distinct receptors at the apical and basolateral surfaces of the polarized epithel-
4) Chambers FG, Koshy SS, Saidi RF, Clark DP, Moore RD, Sears CL (1997) Bacteroides fragilis toxin exhibits polar activity on monolayers of human intestinal epithelial cells (T84 cells) in vitro. Infect Immun 65:3561–3570.

5) Chi B, Qi M, Kuramitsu HK (2003) Insertional inactivation of the prtP gene encoding a prolyl-phenylalanine-specific protease (dentilisin). Infect Immun 71:2336–2349.

6) Chukkapalli SS, Easwaran M, Rivera-Kweh MF, Velsko IM, Ambadapadi S, Dai J, Larjava H, Lucas AR, Kesavalu L (2017) Sequential colonization of periodontal pathogens in induction of periodontal disease and atherosclerosis in Apoe<sup>-/-</sup> mice. PLoS One 12:e0143291.

7) Chukkapalli SS, Velsko IM, Rivera-Kweh MF, Zheng D, Lucas AR, Kesavalu L (2015) Polymicrobial oral infection with four periodontal bacteria orchestrates a distinct inflammatory response and atherosclerosis in Apoe<sup>-/-</sup> mice. PLoS One 10:e0143291.

8) Donato KA, Zareie M, Jassem AN, Jandu N, Ailingary N, Carusone SC, Johnson-Henry KC, Sherman PM (2008) Escherichia coli and Hafnia alvei are candidate enteric pathogens with divergent effects on intercellular tight junctions. Microb Pathog 45:377–385.

9) Eckardt-Michel J, Lorek M, Baxmann D, Grunwald T, Keil GM, Zimmer G (2008) The fusion protein of respiratory syncytial virus triggers p53-dependent apoptosis. J Virol 82:3236–3249.

10) Ellen RP, Ko KS, Lo CM, Grove DA, Ishihara K (2000) Insertional inactivation of the prtP gene of Treponema denticola confirms dentilisin’s disruption of epithelial junctions. J Mol Microbiol Biotechnol 2:581–586.

11) Foschi F, Izard J, Sasaki H, Sambri V, Prati C, Müller R, Stashenko P (2006) Characterization of the outer sheath of Treponema denticola. J Bacteriol 188:407–435.

12) Furuse M, Furuse K, Sasaki H, Tsukita S (2001) Conversion of zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. J Cell Biol 153:263–272.

13) Gavard J, Gutkind JS (2006) VEGF controls endothelial-cell permeability by promoting the β arrestin-dependent endocytosis of VE-cadherin. Nat Cell Biol 8:1223–1234.

14) Groeger S, Domann E, Chakraborty T, Meyle J (2010) Effects of Porphyromonas gingivalis infection on human gingival epithelial barrier function in vitro. Eur J Oral Sci 118:582–589.

15) Groeger SE, Meyle J (2015) Epithelial barrier and bacterial infection. Periodontal 2000 69:46–67.

16) Hartsock A, Nelson WJ (2008) Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. Biochim Biophys Acta 1778:660–669.

17) Holt SC, Ebersole JL (2005) Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia: the “red complex”, a prototype polybacterial pathogenic consortium in periodontitis. Periodontol 2000 38:72–122.

18) Inagaki S, Kimizuka R, Kobu E, Saito A, Ishihara K (2016) Treponema denticola invasion into human gingival epithelial cells. Microb Pathog 94:104–111.

19) Ishihara K, Kuramitsu HK, Miura T, Okuda K (1998) Dentilisin activity affects the organization of the outer sheath of Treponema denticola. J Bacteriol 180:3837–3844.

20) Ishihara K, Miura T, Kuramitsu HK, Okuda K (1996) Characterization of the Treponema denticola prtP gene encoding a prolyl-phenylalanine-specific protease (dentilisin). Infect Immun 64:5178–5186.

21) Ishihara K, Wawrzonk K, Shaw LN, Inagaki S, Miyamoto M, Potempa J (2010) Dentipain, a Streptococcus pyogenes IdeS protease homolog, is a novel virulence factor of Treponema denticola. Biol Chem 391:1047–1055.

22) Katz J, Sambandam V, Wu JH, Michalek SM, Balkovetz DF (2000) Characterization of Porphyromonas gingivalis-induced degradation of epithelial cell junctional complexes. Infect Immun 68:1441–1449.

23) Katz J, Yang QB, Zhang P, Potempa J, Travis J, Michalek SM, Balkovetz DF (2002) Hydrolysis of epithelial junctional proteins by Porphyromonas gingivalis gingipains. Infect Immun 70:2512–2518.

24) Kazmierzak BI, Mostov K, Engel JN (2001) Interaction of bacterial pathogens with polarized epithelium. Annu Rev Microbiol 55:407–435.

25) Kimizuka R, Kato T, Ishihara K, Okuda K (2003) Mixed infections with Porphyromonas gingivalis and Treponema denticola cause excessive inflammatory responses in a mouse pneumonia model compared with monoinfections. Microbes Infect 5:1357–1362.

26) Lux R, Miller JN, Park NH, Shi W (2001) Motility and chemotaxis in tissue penetration of oral epithelial cell layers by Treponema denticola. Infect Immun 69:6276–6283.

27) Mäkinen KK, Mäkinen PL, Loesche WJ, Syed SA (1995) Purification and general properties of an oligopeptidase from Treponema denticola ATCC 35405—a human oral spirochete. Arch Biochem Biophys 316:689–698.

28) Miao D, Fennon JC, Timm JC, Joo NE, Kapila
YL (2011) The Treponema denticola chymotrypsin-like protease dentilisin induces matrix metalloproteinase-2-dependent fibronectin fragmentation in periodontal ligament cells. Infect Immun 79:806–811.

29) Miao D, Godovikova V, Qian X, Seshadrinathan S, Kapila YL, Fenno JC (2014) Treponema denticola upregulates MMP-2 activation in periodontal ligament cells: interplay between epigenetics and periodontal infection. Arch Oral Biol 59:1056–1064.

30) Mostov KE, Verges M, Altschuler Y (2000) Membrane traffic in polarized epithelial cells. Curr Opin Cell Biol 12:483–490.

31) Ohta K, Makinen KK, Loesche WJ (1986) Purification and characterization of an enzyme produced by Treponema denticola capable of hydrolyzing synthetic trypsin substrates. Infect Immun 53:213–220.

32) Okuda K, Ishihara K, Nakagawa T, Hirayama A, Inayama Y, Okuda K (2001) Detection of Treponema denticola in atherosclerotic lesions. J Clinical Microbiol 39:1114–1117.

33) Okuda K, Kato T, Ishihara K (2004) Involvement of periodontopathic biofilm in vascular diseases. Oral Dis 10:5–12.

34) Okuda K, Kimizuka R, Abe S, Kato T, Ishihara K (2005) Involvement of periodontopathic anaerobes in aspiration pneumonia. J Periodontol 76:2154–2160.

35) Paris L, Tonutti L, Vannini C, Bazzoni G (2008) Structural organization of the tight junctions. Biochim Biophys Acta 1778:646–659.

36) Riviere GR, Riviere KH, Smith KS (2002) Molecular and immunological evidence of oral Treponema in the human brain and their association with Alzheimer’s disease. Mol Oral Microbiol 17:113–118.

37) Shin J, Choi Y (2012) The fate of Treponema denticola within human gingival epithelial cells. Mol Oral Microbiol 27:471–482.

38) Wine E, Chan VL, Sherman PM (2008) Campylobacter jejuni mediated disruption of polarized epithelial monolayers is cell-type specific, time dependent, and correlates with bacterial invasion. Pediatr Res 64:599–604.

39) Zinicola M, Higgins H, Lima S, Machado V, Guard C, Bicalho R (2015) Shotgun metagenomic sequencing reveals functional genes and microbiome associated with bovine digital dermatitis. PLoS One 10:e0139674.

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