10-2020

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Tissue Engineering Part B : Reviews, Vol. 26, No. 5 (October 2020): 443-460. DOI.

Three-Dimensional *In Vitro* Oral Mucosa Models of Fungal and Bacterial Infections

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Abstract

Oral mucosa is the target tissue for many microorganisms involved in periodontitis and other infectious diseases affecting the oral cavity. Three-dimensional (3D) *in vitro* and *ex vivo* oral mucosa equivalents have been used for oral disease modeling and investigation of the mechanisms of oral bacterial and fungal infections. This review was conducted to analyze different studies using 3D oral mucosa models for the evaluation of the interactions of different microorganisms with oral mucosa. In this study, based on our inclusion criteria, 43 articles were selected and analyzed. Different types of 3D oral mucosa models of bacterial and fungal infections were discussed in terms of the biological system used, culture conditions, method of infection, and the biological endpoints assessed in each study. The critical analysis revealed some contradictory reports in this field of research in the literature. Challenges in recovering bacteria from oral mucosa models were further discussed,

suggesting possible future directions in microbiomics, including the use of oral mucosa-on-a-chip. The potential use of these 3D tissue models for the evaluation of the effects of antiseptic agents on bacteria and oral mucosa was also addressed. This review concluded that there were many aspects that would require optimization and standardization with regard to using oral mucosal models for infection by microorganisms. Using new technologies—such as microfluidics and bioreactors—could help to reproduce some of the physiologically relevant conditions and further simulate the clinical situation.

Impact statement

Tissue-engineered or commercial models of the oral mucosa are very useful for the study of diseases that involve the interaction of microorganisms and oral epithelium. In this review, challenges in recovering bacteria from oral mucosa models, the potential use of these three-dimensional tissue models for the evaluation of the effects of antiseptic agents, and future directions in microbiomics are discussed.

Keywords:

3D tissue models, bacterial infection, biofilm, candidiasis, engineered oral mucosa, microbiomics, oral mucosa models

Introduction

The oral cavity contains a large number of microorganisms, most of which are part of the normal flora and have a commensalism relationship with the host tissues. In this diverse population of microorganisms, there are some opportunistic and also nonresident species, which can cause diseases.1 Oral mucosa is one of the barriers in the oral cavity with an important role in inhibition of microorganism's colonization. It consists of the epithelium—including stratified and differentiated keratinocytes—and the connective tissue layer, containing predominantly fibroblasts.2 Even though there is a harsh exposure to different microorganisms like *Streptococci*, *Actinobacillus*, *Porphyromonas*, *Tannerella*, *Fusobacterium*, *Prevotella*, *Campylobacter*, *Eikenella*, and *Treponema* species, the oral mucosa limits microflora colonization and protects the oral cavity from invasion of microorganisms with high turnover and shedding, and secretion of different types of cytokines and antimicrobial proteins, like defensins.3 However, in certain conditions, breakdown of homeostasis in the normal flora would result in change of commensalism relationship of normal flora to parasitism, increase in the number of opportunistic microorganisms, and invasion into the underlying tissues, leading to disease development.4 In periodontal diseases, invasion of oral epithelial cells by pathogens (like *Porphyromonas gingivalis* or *Fusobacterium nucleatum*), their survival and proliferation in the epithelial tissue, and their penetration to connective tissue cause some immune responses that have key roles in periodontal breakdown.5 In oral mucositis, following chemotherapy and radiotherapy in some patients, oral tissues encounter damage and pathogens can penetrate tissues and cause infection.6 In candidiasis, invasion of oral epithelium by *Candida albicans*—especially in immunocompromised patients—is responsible for infection.7

Study of the mechanism of disease development in periodontal tissue or infection of oral mucosa by fungi or bacteria—which leads to periodontal disease, mucositis, stomatitis, candidiasis, or other mucosal infections—requires *in vitro* tissue culture models containing microorganisms to simulate the *in vivo* situation. Although two-dimensional (2D) monolayer cell culture systems contributed to the progress of our knowledge of oral microbiome, a multilayer epithelium, which works as a barrier

against pathogen invasion and synergistic effects of fibroblasts and keratinocytes on secretion of cytokines, is missing from the monolayer cell culture systems.[2,8,9] Degradation of epithelial layer, direct exposure of the connective tissue to the oral biofilm, and active participation of fibroblasts in bacterially induced inflammation are some of the limitations of *in vitro* multilayer epithelium models.[10,11] Mimicking the *in vivo* condition requires models that reflect native tissue and their interactions with pathogens. For this purpose, many researchers use different types of oral mucosa equivalents as a relevant *in vitro* tool to investigate the interaction of microorganisms with oral mucosa, the process of epithelial layer's damage, and initial steps of infection, as well as treatment approaches.[12]

Isolation and expansion of epithelial and fibroblast cells from gingiva, buccal or palatal mucosa, seeding and culture of fibroblast in a suitable substrate, and finally, seeding of epithelial cells onto the engineered connective tissue layer is a common procedure for engineering of oral mucosa models. There are also commercially available oral mucosa models, which can be used for microbiological studies. Engineered or commercial models of oral mucosa are very useful for the study of diseases that involve interaction of microorganisms and oral epithelium.[13] Reducing animal experiments is one of the most advantages of using tissue-engineered models in microbiology.[14] This aspect is also considered in skin tissue engineering, using skin substitutes for *in vitro* infection modes, and engineering of intestinal functional models for application in food microbiology.[15,16] Interaction of oral microbiomes with other microbiomes in various sites of human body, their implications in systemic pathologies (like esophageal cancer, colorectal cancer, pancreatic cancer, and inflammatory diseases such as atherosclerosis, pneumonia, heart diseases, and rheumatoid arthritis), and its relationship to diabetes and Alzheimer's disease highlight the importance of engineering *in vitro* models that mimic oral cavity situation for better disease diagnosis and treatment.[17–20]

Two review articles have been published thus far that investigate *in vitro* and *in vivo* model systems' potential for studying the human microbiome, but not oral mucosa equivalents. Coenye and Nelis drew attention to the tools that could be used for understanding medically relevant biofilms, while Werlang *et al.* investigated the requirement of mucin mimetics for *in vitro* culture systems and modulation of microbial community structure.[13,21] The goal of this study was to answer the focused questions: what are the methods used for oral mucosa infection and which microorganisms are usually used for infection? Furthermore, the *in vitro* biological endpoint assessed as the outcome of the oral mucosa models' infection was evaluated.

Materials and Methods

The defined question of the study was used for the extraction of keywords. PubMed and Scopus databases were searched for the period time of 2000–2020 using the following separated or combined keywords: 3d oral mucosa, engineered oral mucosa, oral mucosa models, oral mucosa equivalents, bacterial infection, microbiology, microorganism, microbiota, *Candida albicans*, *Porphyromonas*, *Fusobacterium*, candidiasis, periodontal diseases, periodontitis, *Streptococcus*, and biofilms. Only English-language articles in which commercialized oral mucosa or full-thickness oral mucosa models were used for infection with one or multispecies bacteria were included. Studies on the interaction of microorganisms with monolayer cell cultures, epithelial cell 2D cultures, or epithelial cell sheets with lack of fibroblasts were excluded. Articles on the

investigation of oral mucosa models for other purposes like biocompatibility of dental materials, assessment of radiotherapy-induced mucositis, or cytotoxic evaluation of oral antiseptics were excluded as well. The bibliography of selected articles was checked to identify other relevant articles. The classification of articles was according to the bacterial strain used, culture condition, oral mucosa model, time of contact between microorganism and oral mucosa model, infection evaluation, and results. Finally, 43 articles were selected for the final analysis and review.

Results

Methods of oral mucosa infection

For infection of oral mucosa models, bacteria or fungi are cultured in an appropriate broth for 24–48h, and after centrifugation, the suspension of bacteria in appropriate media—such as phosphate-buffered saline, cell culture media, or special media of microorganism—at a defined concentration is prepared. Oral mucosa is washed in antibiotic-free medium (24, 48, or 72h before infection). Then, the desired concentration of microorganisms (respecting multiplicity of infection [MOI] of 100 bacteria per surface cell) in limited amount of appropriate media (20–50µL) is added onto the surface of epithelial layer (center of oral mucosa model). After incubation of infected and noninfected tissues (control group) at 37°C/5% CO_2 for different time points (24-, 48-, or 72-h incubation), the models are ready for analysis. The other option is producing biofilm of bacteria before infection.22–24 Also, one of the possibilities that should be considered in *in vitro* microbiological studies is producing damage to epithelial layer to provide a route for microbial invasion, as it occurs in some pathological conditions of the oral cavity.22,25 **Figure 1** shows the different steps and methods of oral mucosa infection.

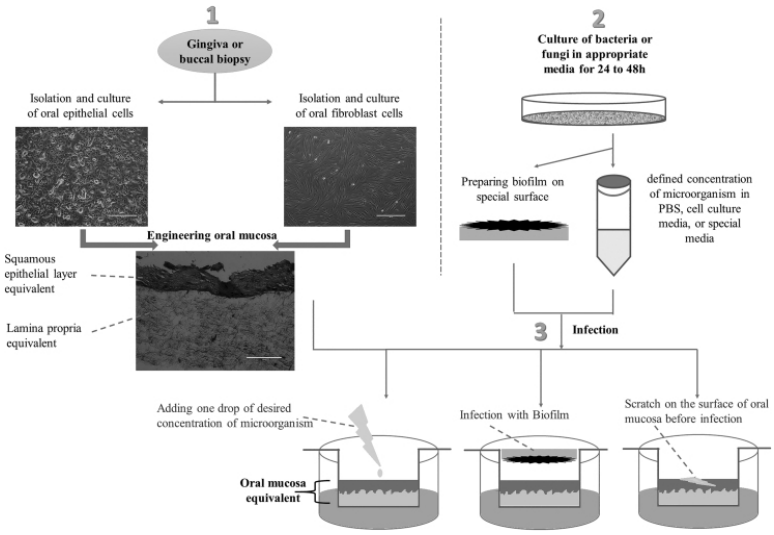

FIG. 1. Steps and methods of oral mucosa infection.

Candida

In the oral cavity, 85 species of fungi exist—one of the most important being *Candida*. Denture stomatitis and candidiasis are infections related to fungus, specially, *Candida albicans*. Although this microorganism is a part of commensal flora and is found normally in healthy individuals, because of its opportunistic nature, its colonization could switch it to a pathogen in some patients (like elderly or

immunocompromised hosts). Attachment of the yeast to mucosal cells by adhesins and invasion of cells by yeast–hyphal transition would result in mucosal inflammation. *C. albicans* is the most abundant yeast species in oral cavity, yet other species like *C. glabrata* or *C. famata* could co-infect with *C. albicans*, which can make the treatment more difficult. Even though single colonization of the cavity with *C. albicans* is possible, some other microorganisms—like oral *Streptococci* or *Staphylococci*—could help *Candida* in the production of biofilm. Coaggregation of these microorganisms as the primary colonizers of oral biofilm with *Candida* could enhance its filamentation and increase its pathogenicity.[26,27]

Table 1. Studies Related to Infection of Oral Mucosa Models with *Candida* Species Alone or in Association with Other Bacteria

Authors	**Bacteria strain**	**Culture condition**	**Oral mucosa model**	**Time of contact between *Candida* a nd Mucosa**	**Assays**	**Results**
Claveau *et al*.28	Original clinical isolate (*Candida*-associated stomatitis)	10^7*C. albicans*/mL of PBS (10^5/cm^2)	EHOM:NOKs seeded on the collagen-embedded NOFs	2, 4, 8, 24h	RT-PCR, Western blotting, Zymography, ELISA	*Candida* increase expression of laminin-5, type IV collagen, MMP-2 and MMP-9 genes; decrease type 2 matrix metalloproteinase tissue inhibitors (TIMP-2) by oral epithelial cells
Mostefaoui *et al*.29	*C. albicans* and *Streptococcus salivarius* (ATCC 25975)	Live and killed *C. albicans* (10^5/cm^2) or *S. salivarius* (10^6/cm^2)	EHOM:NOKs seeded on the collagen-embedded NOFs	2, 4, 8, 24, 48h	Epithelial cell viability, Masson trichrome staining, RT-PCR, ELISA	*C. albicans* or *S. salivarius*, induce release of proinflammatory mediators (IL-6, IL-8 and TNF-a) by oral epithelial cells (more efficiency of *S. salivarius*)
Mostefaoui *et al*.30	Original clinical isolate (*Candida*-associated stomatitis)	Live and heat-inactivated *C. albicans*: 10^8*C. albicans*/mL (10^5/cm^2)	EHOM:NOKs seeded on the collagen-embedded NOFs	2, 4, 8, 24, 48h	RT-PCR, epithelial cell viability, ELISA, Western blotting, bacteria count, H&E	Increased expression of IL-1b by oral epithelial cells in early stages of infection with live *C. albicans*
Green *et al*.31	*C. albicans* strains: SC5314, B311 (ATCC 32354), GDH2346, and M61	50µL *C. albicans*/PBS suspension (2×10^6 cells, 2×10^5 cells, or	RHE (SkinEthic, Nice, France) (TR146 cell lines cultured on	12, 24, 36, 48h	RT-PCR, SEM	Consistent detection of ALS genes in the *Candida* over time with progress

		2×10^4 cells/RHE model)	polycarbonate filters)			destruction of the RHE
Schaller *et al*.32	Clinical *C. Albicans* wild-type strain SC5314	50µL *C. albicans*/PBS suspension (2×10^6 cells total)	RHE (SkinEthic, Nice, France). (TR146 cultured on polycarbonate filters) Supplemented with PMN	12, 24h	LDH, killing assay, qRT-PCR, FACS	Increase expression of IL-8 and GM-CSF, and chemoattraction of PMNs following infection
Tardif *et al*.33	*C. albicans* LAM-1 (serotype A)	($1.5\times10^6/cm^2$) seeded onto the EHOMs using sterile swab	EHOM:NOKs seeded on the collagen-embedded NOFs	2, 4, 6, 12, 24, 48h	Spectro-Photometric Analysis, RT-PCR, Western blotting, ELISA	Increased secretion of IL-18 and IFNγ in response to *C. albicans*
Dongari-Bagtzoglou and Kashleva34	*C. albicans* strains: SC5314, efg1/efg1/cph1/cph1, rbt4/rbt4, rim101/rim101	50µL live *Candida*/KSFM (10^6 organisms/insert) (MOI of 1:1 fungal to surface epithelial cells), or 4mm diameter agar slices containing 10^3 yeast/mL on top of the epithelial layer	EpiOral (GIN-100, MaTek, Ashland, MA), NOKs over submucosa (containing NOFs), OKF6/TERT-2 cells over submucosa	48h	ELISA, LDH assay	Strain of *Candida* used for infection of oral mucosa influences the level of tissue invasion and damage infect oral epithelia
Samaranayake *et al*.35	PL^+ and PL^-*C. albicans* isolates	—	RHOE (Skinethic, Nice, France)	12, 24, 48h	PASS, Genomic PCR	Expression of phospholipase gene in *Candida* influences its growth and invasion in the RHOE model
Zakrzewski and Rouabhia36	Clinical *C. albicans* (*Candida*-associated stomatitis)	10^7 cells/mL in PBS	Nonkeratinized and keratinized EHOM (NOKs seeded on the	2, 4, 8, 24h	H&E, *C. albicans* count, Western blotting, IHC	Higher morphological change of *C. albicans* on

			collagen embedded NOFs)			nonkeratinized mucosa and significant disorganization of this mucosa following contact with *C. albicans*
Villar *et al*.37	12 strains of *C. albicans*	1×10^5 *C. albicans* cells in 100µL of airlift medium	EHOM (NOKs seeded on the collagen-embedded NOFs)	17–48h	IHC, CLSM, TEM	Degradation of E-cadherin in epithelial cells by *C. albicans* facilitates its penetration in mucosal tissues
Ohnemus *et al*.38	*C. albicans* strain ATCC 10231	10^5 CFU *C. albicans* diluted in 2µL PBS	*Ex vivo* PMOCM	24h infection, 48 or 96h treatment with nystatin	Evaluation of fungal growth, agar diffusion method, H&E, PASS	Equal efficiency of different dosage of Nystatin (230, 100, 20 IU) in *C. albicans* infection
Lermann and Morschhauser 39	*C. albicans* strains	Infection of RHOE with 5×10^5 *C. albicans* cells.	RHOE (Skinethic Lab, Nice, France)	48h	Light microscopy and staining, LDH activity, PCR	Invasion of RHE by *C. albicans* is not dependent to expression of the SAP1–SAP6 genes
Decanis *et al*.40	*C. albicans* isolated from *Candida*-associated candidiasis	Adjusted to 10^7/mL (10^6/cm^2)	EHOM: OKF6/TERT-2 cells seeded on the collagen embedded NOFs	4, 24h	qRT-PCR, ELISA	Increase of epithelial cell defense against *C. albicans* infection by using farnesol
Bahri *et al*.41	*C. albicans* (ATCC 10231) as a reference species, *C. famata* was isolated from water (various sites in the Mediterranean Sea)	Adjusted to 10^7/mL (10^6/cm^2)	EHOM:NOKs seeded on the collagen embedded NOFs	24h	H&E, qRT-PCR	*C. famata* activate local defenses of human epithelial cells
Diaz *et al*.42	*Candida albicans* SC5314, *Streptococ*	10^6 cells of *C. albicans* or 10^7 cells	Immortalized human oral	4, 16, 24h	CLSM, IF, FISH, RT-PCR	Stimulation of biofilm formation

	cus oralis 34 (provided by P.E. Kolenbrander), *Streptococcus gordonii* Challis CH1 (provided by J. M. Tanzer) and *Streptococcus sanguinis* SK36 (ATCC BAA-1455)	of *S. oralis* or a combination of both organisms in 500µL of salivary medium for biofilm formation	keratinocyte cell line (OKF6/TERT-2) seeded on collagen type I-embedded fibroblasts (3T3 fibroblasts)			of *Streptococci* in presence of *C. albicans*, increased invasion of oral mucosa by *C. albicans* in presence of *Streptococci*
Yadev *et al*.43	*C. albicans* wild-type strain (CAF2–1)	5×10^7 CFU/mL (100µL: 5×10^6 CFU)	RHOE (Skinethic Lab, Nice, France), EpiOral (GIN-100, MaTek,Ashland,MA), FTOM (NOKs seeded on the collagen embedded NOFs)	24h	ELISA, IHC, PASS	Similar damage in all models following infection; more cytokine release in FTOM
Rouabhia *et al*.44	Strains of *Candida albicans*: CAI4 wild-type, Δ*ipt1* mutant, *IPT1* revertant	10^7/mL in PBS (10^5 cells/cm^2)	EHOM:NOKs seeded on the collagen embedded NOFs	24h	qRT-PCR, ELISA	Reduced adhesion of *Candida* to epithelial cells in strains with disrupted *IPT1* gene
Silva *et al*.45	Six clinical isolates of *C. glabrata*, recovered from the oral cavity (strains D1 and AE2), vagina (strains 534784 and 585626) and urinary tract (strains 562123 and 513100); reference strain of *C. glabrata* (ATCC 2001)	2×10^6 cells/mL (infected only with *C. glabrata*, or simultaneously with *C. glabrata* and *C. albicans*)	RHOE (Skinethic Lab, Nice, France)	12h	PNA FISH, CLSM, LDH activity	Increased invasiveness of *C. glabrata* and increased LDH release by the RHOE in presence of *C. albicans*
Semlali *et al*.46	*C. albicans* (SC5314)	10^6 cells in 200µL of Sabouraud dextrose broth	EHOM:NOKs seeded on the collagen embedded NOFs	24h	qRT-PCR, Western blot, ELISA	No toxicity of KSL-W on epithelial cells and decrease of *Candida* virulence in its presence

Rouabhia *et al*.47	*Candida* strains: CAF2-parental strain, RML1, RML2, RML3, RML4	10^4 cells/cm^2 in a serum-free, antifungal-free DMEM medium	EHOM:NOKs seeded on the collagen embedded NOFs	24h	H&E, LDH assay, qRT-PCR, Western blot	Evidence on active role of *ECM33* gene in biofilm formation and tissue damage of *Candida*
Whiley *et al*.7	Denture stomatitis strain NCYC 1467, strain AC-1 from the saliva of a healthy subject, NCPF 8112 from vaginal candidosis, NCYC 1472 from an asymptomatic cervical smear	4×10^7 CFU/mL: 50µL=2×10^6 CFU)	Models of human buccal and vaginal epithelia (SkinEthic Lab, Nice, France)	4, 12, 24h	MTT, ELISA, H&E, PAS, PL assay, SAP assay	Different response of oral and vaginal epithelial cells to *C. albicans*
de Carvalho Dias *et al*.12	*C. albicans* SC5314 and *S. aureus* ATCC25923	1×10^7 cells/mL in RPMI 1640	ROMT (NOK-si seeded on the collagen-embedded fibroblast cell line)	8, 16h	H&E, LDH assay	Synergistic interaction of *C. albicans* and *S. aureus* in tissue damage and depth of infection in ROMT
Sobue *et al*.48	*C. albicans* strain SN425, *C. glabrata* strain GDH2269, *S. oralis* 34 (provided by Dr. P. Kolenbrander), and *S. mitis* 49456	20µL media containing 10^6 fungal (*C. albicans* or *C. glabrata*) or 10^7 bacterial (*S. oralis* or *S. mitis*) cells	Keratinocyte cell line (SCC15) seeded on collagen-embedded fibroblasts (3T3 cell line) pretreated with 5-FU for mucosal injury	6–16h	IF, FISH, ELISA	Intensification of the inflammatory response, but not significant effect on fungal or bacterial biofilm by using 5-FU
Morse *et al*.49	*C. albicans* ATCC 90028, *S. sanguinis* ATCC 10556, *S. gordonii* ATCC 10558, *Actinomyces viscosus* ATCC 15987,	Single or mixed-species biofilm grown on PMMA coupons inverted and placed in direct	RHOE, EpiOral, FTOM: TR146 or FNB6 keratinocytes seeded on	12h	H&E, Real-time qPCR, LDH activity	Increase in LDH activity and damage by *C. albicans*-only and mixed-species biofilms, higher

	and *A. odontolyticus* NCTC 9935)	contact with the OMMs	collagen-embedded NOFs			extent of damage in FTOM
Bertolini *et al*.50	*C. albicans* SC5314 and 529L, *C. albicans tup1Δ/Δ* homozygous deletion mutant, *E. faecalis* OG1RF	10^6 cells of *C. albicans* SC5314, 10^7 cells of *E. faecalis*, or a combination	SCC15 oral keratinocytes seeded on collagen-embedded fibroblasts (3T3) pretreated with 5-FU for mucosal injury	20h	CFU determinations, immuno-FISH	Pronounced fungal invasion in 5-FU-treated tissues infected with both organisms

5-FU, 5-fluorouracil; ATCC, American type culture collection; CFU, colony-forming unit; CLSM, confocal laser scanning microscopy; DMEM, Dulbecco's Modified Eagle Medium; EHOM, engineered human oral mucosa; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FISH, fluorescent *in situ* hybridization; FTOM, full-thickness oral mucosa; H&E, hematoxylin and eosin; IF, immunofluorescence; IFN, interferon; IHC, immunohistochemistry; IL, interleukin; LDH, lactate dehydrogenase; MOI, multiplicity of infection; MTT, (dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide); NOKs, normal oral keratinocytes; NOK-si, immortalized normal human oral keratinocytes; NOFs, normal oral fibroblasts; OMMs, oral mucosal models; PASS, periodic acid Schiff staining; PBS, phosphate-buffered saline; PMOCM, pig mucosa organ culture model; PL^-, undetectable phospholipase activity; PL^+, phospholipase positive; PMMA, poly-methyl methacrylate; PMN, polymorphonuclear leukocyte; PNA FISH, peptide nucleic acid fluorescent *in situ* hybridization; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RHE, reconstituted human epithelium; ROMT, reconstituted oral mucosa tissue; RHOE, reconstituted human oral epithelium; SAP, secreted aspartyl proteinase; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TNF, tumor necrosis factor.

Porphyromonas gingivalis

Although periodontitis is a multifactorial disease, an abundance of bacteria (like *P. gingivalis* and *Aggregatibacter actinomycetemcomitans*) and lower levels of some other bacteria in the oral cavity of patients with periodontitis show important interaction of these bacteria with the host. The Gram-negative, anaerobic bacterium *P. gingivalis* is considered the main agent in etiology of periodontitis. This bacterium has the ability to invade oral mucosa cells, which result in its escape from therapeutic and host immune agents. This bacterium produces dental plaque biofilm in combination with primary (*Streptococci*) and secondary colonizers (*Fusobacterium*).[51,52]

The studies related to the infection of oral mucosa models with *Porphyromonas* alone or in association with other bacteria are summarized in **Table 2**.

Table 2. Studies Related to the Infection of Oral Mucosa Models with *Porphyromonas* Alone or in Association with Other Bacteria

Authors	**Bacteria strain**	**Culture condition**	**Oral mucosa model**	**Time of contact between *P. gingivalis* and mucosa**	**Assays**	**Results**
Andrian *et al*.5	*P. gingivalis* ATCC 33277 and the derivative gingipain-null mutant KDP128	10^6 and 10^9 bacteria (ATCC 33277 or KPD128)/mL in DMEH, incubated in an anaerobic chamber	EHOM (primary epithelial and fibroblasts cells in collagen)	24h	TEM, ELISA	Higher penetration of nonmutant form in lamina propria; high secretion of cytokines from oral mucosa models after infection
Kimball *et al*.25	*P. gingivalis* (ATCC 33277 or strain 861), *S. gordonii* DL-1, and *Fusobacterium nucleatum* ATCC 25586	6×10^6 bacteria in 10–50µL bacterial growth medium (MOI of 100:1 bacteria per surface cell)	EpiOralTM (MatTek Corporation, Ashland, MA)	24–72h	H&E, IHC, qRT-PCR	Increase of hBD2 expression after infection
Andrian *et al*.53	*P. gingivalis* ATCC 33277 or its derivative gingipain-null mutant (KDP128)	100µL of 10^9 bacteria/mL in DMEH, in an anaerobic chamber	EHOM (primary epithelial and fibroblasts cells in collagen)	4, 8, 24h	RT-PCR, ELISA	Increase activation of TIMP-2 and expression of MMP-2 and MMP-9 by oral mucosa following infection
Wayakanon *et al*.54	Clinical strains (A245br) of *P. gingivalis*	MOI=100	OMM (NOK or TR146 cells on collagen containing NOFs)	18h	Bacteria count, IHC	Reduced number of intracellular *P. gingivalis* in presence of polymersome-encapsulated metronidazole or doxycycline
Belibasakis *et al*.55	*P. gingivalis* ATCC 33277T, *Campylobacter rectus* (OMZ 697), *F. nucleatum* (OMZ 596), *Prevotella intermedia* ATCC	10-species "subgingival" biofilm model grown on sintered hydroxyapatite	EpiGing, (MatTek, Ashland, MA)	3–24h	qPCR, LDH activity, ELISA	Upregulation of IL-8 gene expression and secretion after 3h in both biofilms, in the

	25611T, *Tannerella forsythia* OMZ1047, *Treponema denticola* ATCC 35405T, *Veillonella dispar* ATCC 17748T, *Actinomyces oris* (OMZ 745), *S. anginosus* (OMZ 871), and *S. oralis* SK 248 (OMZ 607)	discs placed onto OMM				presence of the "red complex"
Pinnock *et al*.56	*P. gingivalis* strains NCTC 11834 and W50	MOI of 100 (monolayer) or 2×10^7 cells/300mL	OMMs with either NOK or the H357 cell line on collagen containing NOFs	1.5 or 4h	Antibiotic protection assay, IF, IHC, chemokine array	Higher intracellular survival of *P. gingivalis* in mucosal models compared with monolayer cultures
Thurnheer *et al*.57	*P. gingivalis* ATCC 33277T, *S. oralis* SK248 *S. anginosus* ATCC 9895, *Actinomyces oris* (OMZ 745), *F. nucleatum* subsp. *Nucleatum* OMZ 598, *Veillonella dispar* ATCC 17748T, *Campylobacter rectus* OMZ 698, *Prevotella intermedia* ATCC 25611T, *T. forsythia* OMZ 1047, and *Treponema denticola* ATCC 35405	Subgingival biofilm formed on hydroxyapatite discs put upside-down on the OMM	EpiGing (MatTek, Ashland, MA)	24, 48h	IF, CLSM, SEM, histological staining	Colonization of OMM by "red-complex" species, a colonization of *Streptococci* on the gingival epithelia, in the absence of all three "red complex" bacteria from the biofilm
Bao *et al*.58	*Porphyromonas gingivalis* W50 (OMZ 308), *Prevotella intermedia* ATCC 25611T, *A. actinomycetemcomitans* JP2 (OMZ 295), *Campylobacter rectus* (OMZ 398), *Veillonella dispar* ATCC 17748T, *F. nucleatum subsp. Nucleatum* (OMZ 598), *S. oralis* SK248 (OMZ 607), *Treponema denticola* ATCC 35405T, *Actinomyces oris* (OMZ	11-species biofilm formed on hydroxyapatite discs co-cultured with the OMM in the bioreactor	Immortalized epithelial cells, fibroblasts, and a monocytic cell line perfused through 3D collagen scaffold into	24h	Proteomic, LC-MS/MS analysis, gene ontology (GO) analysis	Identification of 896 proteins in the supernatant and 3363 proteins in the biofilm lysate, significant regulation of the levels of *F. nucleatum*, *Actinomyces oris*, and *Campylobacter rectus* proteins

	745), *S. anginosus* ATCC 9895, and *Tannerella forsythia* (OMZ 1047)		the bioreactor			
Bao *et al*.59	*Porphyromonas gingivalis* W50 (OMZ 308), *Prevotella intermedia* ATCC 25611T, *A. actinomycetemcomitans* JP2 (OMZ 295), *Campylobacter rectus* (OMZ 398), *Veillonella dispar* ATCC 17748T, *F. nucleatum subsp. Nucleatum* (OMZ 598), *S. oralis* SK248 (OMZ 607), *Treponema denticola* ATCC 35405T, *Actinomyces oris* (OMZ 745), *S. anginosus* ATCC 9895, and *Tannerella forsythia* (OMZ 1047)	11-species biofilm formed on hydroxyapatite discs co-cultured with the OMM in the bioreactor (37°C, 2% O_2 and 5% CO_2)	Immortalized epithelial cells (HGEK-16), fibroblasts (GFB-16), and a monocytic cell line perfused through 3D collagen scaffold into the bioreactor	24h	qPCR, quantification of cytokine secretion, Masson's Trichrome Staining, SEM	Reduced growth of *Campylobacter rectus*, *Actinomyces oris*, *S. anginosus*, *Veillonella dispar*, and *P. gingivalis* in the presence of OMM; upregulation of cytokine release in cell culture supernatants in presence of the biofilm
Bugueno *et al*.60	*P. gingivalis* strain 33277	MOI=100	3D microtissue of TERT-2 OKF-6 cell line on 3D spheroid of NOFs	2–24h	Antibiotic Protection Assay, qRT-PCR, IF, SEM, TEM	Invasion of the fibroblastic core and increased apoptosis after infection
Brown *et al*.61	*P. gingivalis* W83, *S. mitis* NCTC 12261, *S. intermedius* 20753, *S. oralis* NTCC 11427, *F. nucleatum* ATCC 10596, F. spp. *vincentii* DSM 19507, *Act. naeslundii* DSM 17233, *Veillonella* NCTC 11831, *Prevotella intermedia* DSM 20706, and *A. actinomycetemcomitans* ATCC 43718	Three multispecies oral biofilms representative of a "health associated" (3 species), "gingivitis-associated," (7 species), and "periodontitis associated" (10 species) grown on coverslips	HGE (Episkin, Skinethic, Lyon, France) + PBMC/CD14 + monocytes	1–2 days	H&E, LDH assay, qRT-PCR, ELISA	High viability of HGE exposed to all multispecies biofilms, more differential inflammatory response in immune cells cultured with epithelium stimulated by "gingivitis-associated" biofilm

		attached to the underside of inserts, and then placed into inserts containing the HGE tissue				

3D, three dimensional; PBMCs, peripheral blood mononuclear cells; HGE, human gingival epithelium; MS, mass spectrometry.

Other microorganisms

In the oral cavity, some bacteria are involved in pathogenesis of dental caries (Gram-positive *Streptococcus mutans*), while others are responsible for periodontal diseases (Gram-negative *Actinobacillus actinomycetemcomitans* and *F. nucleatum*). Bacteria in the oral cavity—and especially in dental plaque—often interact with each other and are associated together in the procedure of disease progression. It is important to consider primary and second colonizers, as well as the third colonizers.

The studies considering infection of oral mucosa models with microorganisms other than *Candida* and *Porphyromonas* are summarized in **Table 3**.

Table 3. Studies Considering Infection of Oral Mucosa Models with Microorganisms Other Than *Candida* and *Porphyromonas*

Authors	Bacteria strains	Culture condition	Oral mucosa model	Time of contact between biofilm and mucosa	Assays	Results
Gursoy *et al*.23	Two strains of *F. nucleatum:* ATCC25586 and AHN9508 (clinical oral isolate)	Two groups: anaerobically grown biofilm on a semipermeable membrane placed upside-down on OCC, 10µL (3×10^6 CFUs/PBS) of planktonic bacteria	HaCaT epithelial cells grown on a fibroblast collagen matrix (OCC model)	24h	H&E, Ki-67, PASS, LDH release	Invasion of the collagen matrix by one of the strains; more cytotoxicity and invasiveness of biofilm in comparison to planktonic bacteria
Dabija-Wolter *et al*.62	Four strains of *F. nucleatum*: ATCC 10953, ATCC 25586, and two other clinical isolates: AHN 8158 and MRC-23	5×10^7 unstained or FITC-labeled *F. nucleatum* in 20–30µL FAD medium, in anaerobic atmosphere for 3h and then at 37°C in aerobic conditions	3D engineered models of human gingiva using primary gingival keratinocytes and fibroblasts	24, 48h	CLSM, IHC, qRT-PCR	Penetration of *F. nucleatum* to gingival epithelium without causing permanent damage
Pollanen *et al*.63	*F. nucleatum* (ATCC) 25586	Biofilm grown on semipermeable nitrocellulose membranes placed on OMM	HaCaT cells seeded on collagen fibroblast gels and a tooth piece placed on top	≤24h	IHC	Epithelial migration and altered epithelial proliferation pattern
De Ryck *et al*.22	Microbiota derived from a swab of the inner cheek	Microbiota grown on an agar/mucin layer positioned on top of oral mucosa	TR146, HaCaT, or normal keratinocyte cells grown on collagen layer containing NIH-3T3 fibroblasts	72h	Oral scratch assay, Pyrosequencing, PCR-DGGE analysis, live/dead staining, flow	Reduced healing in the presence of microbiota, no reduction of the proliferation index, no increase of

					cytometry, SCFA, MTT, SRB, LDH, Western blot, lactate analysis, Van Gieson, Alcian Blue, E-cadherin, Ki67, H&E	apoptotic or necrotic cells
Buskermolen *et al*.64	Three biofilm types: commensal, gingivitis, and cariogenic	10µL of 10^5, 10^6, or 10^7 CFUs/equivalent diluted in HBSS	Immortalized human keratinocyte (KC-TERT) and fibroblast (Fib-TERT) embedded in collagen hydrogel	24h	IHC, FISH, fluorescence resonance energy transfer, ELISA	Increased expression of elafin, secretion of the antimicrobial cytokine and inflammatory cytokines in the gingiva epithelium
Shang *et al*.24	From healthy human saliva, consists of typical commensal genera *Granulicatella* and major oral microbiota genera *Veillonella* and *Streptococcus*	10^7 CFU of biofilm cells diluted in 10µL HBSS, dripped onto the surface of the RHG	RHG: immortalized human keratinocyte (KC-TERT) and fibroblast (Fib-TERT)-populated hydrogel	1, 2, 4, or 7 days	ELISA, RT-PCR, CFU count, H&E, FISH	Increased epithelial thickness, stratification, keratinocyte proliferation, and production of anti-microbial proteins in biofilm exposed RHG
Rahimi *et al*.65	*Streptococcus mutans* (strain UA-159)	Injection of 2µL of bacterial solution (with optical density between 0.2 and 0.3) into the keratinocyte-containing channel of the device	Microfluidic mucosal model-on-a-chip: fibroblast cell line-laden collagen, followed by a keratinocyte	24h	DiI fluorescence staining, TEER	Some infiltration in collagen layer, lower TEER after bacterial exposure

			cell line (Gie-No3B11) layer			
Shang *et al*.66	Commensal, gingivitis, or cariogenic biofilms from human healthy saliva	Biofilms cultured in the AAA model diluted as 1×10^7 CFU biofilm cells in 10µL HBSS	RHG: keratinocyte (KC-TERT, OKG4/bmi1/TERT) on collagen-embedded fibroblast (Fib-TERT)	24h	FISH, H&E, RT-PCR, Western blotting	Upregulation of gene expression involved in TLR signaling by commensal biofilm, and suppression of some by cariogenic biofilm; no significant damaging effect on RHG morphology
Ingendoh-Tsakmakidis *et al*.67	Biofilm of *S. oralis* (DSM 20627) on polyethersulfone membrane, biofilm of *A. actinomycetemcomitans* JP2 strain on coverslip	*S. oralis* or *A. actinomycetemcomitans* bio film facing the peri-implant oral mucosa model with direct contact to titanium disk	Peri-implant oral mucosa model assembly: OKF6/TERT-2 seeded on titanium disks-HGF-collagen matrix	24h	Microarray data analysis, ELISA, IHC	Induction of a protective stress response by *S. oralis.* downregulation of genes involved in inflammatory response by *A. actinomycetemcomitans*
Beklen *et al*.68	*A. actinomycetemcomitans* strain D7S	*A. actinomycetemcomitans* bio film cultured on porous filter discs added on top of OMM	Immortalized human gingival keratinocyte cells seeded on fibroblast-collagen matrix	24h	IHC, TEM	Thick necrotic layer and decrease of keratin expression in epithelium following infection

AAA-model, Amsterdam active attachment model; AHN, anaerobe Helsinki negative; DGGE, denaturing gradient gel electrophoresis; FITC, fluorescein-isothiocyanate; HaCaT, human adult low-calcium high-temperature; HBSS, Hank's Balanced Salt Solution; KC-TERT, telomerase reverse transcriptase-immortalized human keratinocyte; OCC, organotypic cell culture; RHG, reconstructed human gingiva; SCFA, short-chain fatty acid; SRB, sulforhodamine B colorimetric assay; TEER, transepithelial electrical resistance; TLR, toll-like receptor.

Discussion

Monolayer culture of epithelial cells is considered to be a deficient model to study the interaction of pathogenic bacteria with host cells. In contrast, the potential of 3D models of human oral mucosa for histological analysis of the process of infection—and observation of the tissue invasion—makes these models very relevant and informative for microbiomics.69 In this study, we summarized the studies using 3D models of oral mucosa optimized for fungal pathogenesis and bacterial-derived oral infections. It seems that there are many aspects that require optimization and standardization with regard to using oral mucosal models (OMMs) for infection by microorganisms.

Equivalents of oral mucosa

Engineered oral mucosa includes a connective tissue layer containing fibroblasts as lamina propria covered by epithelium containing epithelial cells.8,70 The substrate used for cell culture in most of the engineered oral mucosa models used in this review was collagen. Ease of extraction and manipulation, reproducibility, and high growth of epithelial cells on its surface are the reasons for choosing this material to load fibroblast cells.71 The potential role of the scaffold as a barrier against infection has been mentioned by researchers.72 However, with advancing tissue engineering, scaffold-free approaches are now starting to be utilized in engineering of oral mucosa.73 One study prepared a 3D spheroid model of oral mucosa by hanging-drop method and infected it with *P. gingivalis*.60 However, lack of keratinization is a limitation of this micro-tissue model.

Cells used for oral mucosa models include primary cells—NOKs (human-derived normal oral keratinocyte cells from oral mucosa) or cell lines such as TR146 (oral squamous cell carcinoma cell line), HaCaT (immortalized keratinocyte cell line), H357 (cell line from squamous cell carcinoma of the tongue), OKF6/TERT-2, 20 (normal oral epithelial cell line, immortalized by forced expression of telomerase), and Gie-No3B11 (immortalized gingival keratinocytes). Upregulation of genes in tumor-derived cells suggests more suitability of normal or immortalized cells for OMM production.3 On the other hand, primary cells have short life span, and their growth rate and response to infection are different based on various donors.34

The engineered oral mucosa for investigation of oral microbiomics has been used since 2004.5,28,30,31 Based on this review, 29 studies used engineered oral mucosa, while 14 studies used commercialized models. Reconstituted human oral epithelium (RHOE, SkinEthic) model is a multilayered epithelium consisting of TR146 cells on a polycarbonate transwell insert. EpiOral (MaTek) is based on primary oral keratinocytes grown in Millipore Millicell inserts. Although these models are inexpensive, easily handled, and reproducible, the absence of fibroblast-embedded collagen layer in both of these models raises concerns about their reliability. Mimicking steps of keratinocyte differentiation requires their culture on a connective tissue layer.74 More cytokine release and

expression of defensin from full-thickness engineered oral mucosa in comparison to split-thickness models suggest that they are better representative of *in vivo* conditions.43 Among articles reviewed in this study, only one study used porcine *ex vivo* oral mucosa model based on structural similarity to human oral mucosa.38

In native oral mucosa, many other cells besides fibroblasts and epithelial cells exist, including immune cells, endothelial cells, and melanocytes.75 Presence of neutrophils within biofilms was confirmed in different studies.76,77 In this review, one study used RHOE supplemented with polymorphonuclear leukocytes to study oral candidiasis.32 Another study used co-culture of immune cells (peripheral blood mononuclear cells and CD14+ monocytes), human gingival epithelium (Skinethic), and multispecies biofilms.61 Bao *et al.* used a monocytic cell line in their oral mucosa-infected model.59 Interaction of oral epithelial cells with immune cells in response to infection has been reported in many studies.78–80 To simulate the *in vivo* situation as closely as possible, engineering of more complex oral mucosa models that are vascularized or contain immune cells would be indicated for microbiomics.

Oral mucosa infection

Long-term co-culture of bacteria and oral mucosa model is challenging, because each of them requires different culture media. Time of infection of oral mucosa with pathogen microorganisms in different studies varies between 1.5 and 48h. De Ryck *et al.* used 72-h bacterial exposure of oral mucosa model.22 Determination of time course of infection is important in different bacteria, because some microorganisms, like *P. gingivalis*, need anaerobic incubation, which compromises epithelial viability after 24h.81 Contact of *C. albicans* with epithelium after 8 and 24h causes tissue disorganization as well,30 but visible damage caused by *S. salivarius* is reported after 48h contact.30 Shang *et al.* showed that commensal oral microbiota from healthy saliva could be in contact with oral mucosa model for 7 days.24

MOI used in most studies was 100. Groeger *et al.*, reported no difference in the transepithelial electrical resistance at an MOI of 100, even after 48h.82 Higher MOI could result in destruction of cell–cell contacts.

Another aspect of oral mucosa infection is the atmosphere of culture for producing optimum results. While *Candida* and *Streptococcus* could grow in aerobic conditions, *Fusobacterium* and *P. gingivalis* require an anaerobic atmosphere. However, prolonged incubation of oral mucosa model in this condition destroys its structure.81 Researchers showed that there is no significant difference in bacterial viability between anaerobic and aerobic incubation over 4-h infection of oral mucosa model.83 Gursoy *et al.* also showed that bacterial viability does not alter after change of the environment from anaerobic to aerobic.23

Beside oxygen, the effect of temperature on the growth of bacteria is important. Although the temperature of body is about 37°C, increase of temperature in some conditions—like inflammation in periodontitis—is reported, which must be considered in future studies. Dynamic environment of the oral cavity and shear forces by saliva also should be considered in infection of oral mucosa. In the study by Bao *et al.*, a closed dynamic perfusion bioreactor system was used for the creation of continuous

sheer forces.59 Mimicking temperature, atmosphere, and shear stress of the natural environment and simulating the environment of periodontal pocket or oral cavity are now possible by using bioreactors.

Biofilm versus non-biofilm design

Most studies concerning microbiomics of oral mucosa used single species and planktonic bacteria (non-biofilm design). Buskermolen used saliva-derived commensal and pathogenic biofilms for oral mucosa exposure,64 and Shang *et al.* used multispecies commensal biofilm,24 both from healthy human saliva. While these two studies used 10µL of determined concentration of oral biofilm, De Ryck *et al.* used oral biofilm derived from swabs wiped along the inner cheek and after growth of this biofilm on an agar/mucin layer, it was placed on top of oral mucosa model with no direct contact.22 Gursoy *et al.* in their study by placing a biofilm of *F. nucleatum* on top of OMM, investigated direct contact between single-species biofilm and oral mucosa.23 Using poly-methyl methacrylate and hydroxyl apatite disc for producing oral biofilm before contact with oral mucosa has also been proposed in other studies.49,59 Microorganisms in the oral cavity have an affinity to form multispecies biofilm, and the behavior of them in a biofilm-embedded by matrix is very different from their planktonic form. Higher resistance of bacteria in biofilm to antibacterial agents and different gene expression by them highlight the importance of *in vitro* biofilm design.84

Another relevant aspect to consider in producing biofilm is the role of saliva containing mucin and acquired pellicle. Only one study used saliva as supplement of biofilm growth medium.42 Using natural or artificial saliva rather than culture media in co-culture of bacteria-OMM is a possible option for mimicking the condition of the oral cavity.85

Survival and penetration of microorganism in oral mucosa model

Survival of microorganisms in oral epithelial cells over different time periods was investigated in different studies. Studies related to *C. albicans* showed that transformation to the hyphal form, which begins 8h after infection, could result in the decrease of colony-forming units (CFUs).30 Yeast transition is reduced in keratinized form of oral mucosa in comparison to nonkeratinized form.36 Although Samaranayake *et al.* reported no penetration of *C. albicans* into the connective tissue layer at 48h,35 Whiley *et al.* and Dongari-Bagtzoglou and Kashleva showed that penetration into the submucosa was dependent on the strain used for infection.7,34 Association of *C. albicans* with other microorganisms, like *Staphylococcus aureus* or *S. oralis*, could result in deeper invasion into subepithelial collagen matrix.12,42 Hyphal transformation was not detectable in *C. famata*; however, its penetration to the lamina propria of the oral mucosa model was reported after 24h of infection.41 Invasion of *F. nucleatum* to collagen matrix is also strain dependent and is enhanced in the biofilm form of *F. nucleatum* compared to the planktonic form.23 *P. gingivalis* penetration into the connective tissue has been demonstrated.54 Andrian *et al.* showed the contribution of *P. gingivalis* gingipains in its potency to penetrate the connective tissue.5 Pinnock *et al.* reported that submerged OMM with a thin epithelium allows penetration of bacteria into the connective tissue, while airlifted OMM with thicker epithelium prohibits its penetration to lamina propria. They also showed that the viability of this bacterium in OMM decreases over time.56

While almost all studies showed disorganization of epithelial layer after infection with pathogenic bacteria, Shang *et al.* reported higher epithelial thickness and keratinocyte proliferation in oral mucosa

models exposed to biofilm that was composed of multispecies commensal microorganisms from healthy human saliva after 7 days.24 It seems that commensal oral bacteria act as an antagonist against potential pathogens and help in maintenance of oral mucosa health.9

Recovering bacteria from OMM

Different methods have been used for the release of bacteria from the infected oral mucosa models. One method is using tissue dissociator for dissociation of tissue, following by sonication.24 The second method is using homogenizer, lysing the keratinocyte plasma membrane, and robustly pipetting to release intracellular bacteria.54,56 One other option is treating tissue with lysis buffer and strictly mixing it.36 Scraping, or using the cycle of sonication and vortexing, was also suggested by Heersink.86 Hamilton *et al.*, in their study of different methods of collecting biofilm cells from surfaces, emphasized the importance of using similar methods of harvesting biofilm for acceptable result of comparison.87

Further consideration in this step is the possible disorganization of epithelial cells over time and release of cells containing bacteria in culture media, which could result in false report of reduction of bacteria over time. Standardization of the techniques used for recovering bacteria from OMM is very important.

Methods of evaluation of infected OMM

Extent of bacteria proliferation or oral mucosa damage can be evaluated by different methods. Most of studies use qualitative/semiquantitative analyses for description of oral mucosa infection. Histology staining, (dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT) and lactate dehydrogenase activity measurement were the most common methods for analysis of epithelial cell damage.7,22,39,76

Conventional plate count and CFU-based quantitation, which have been used in different studies, only consider the number of bacteria on the surface of OMM and not the bacteria in deeper layer. Also, this method is not useful for viable but nonculturable organisms.24,30,36,54 Alternative methods like crystal violet staining and resazurin staining can be used for biofilm research.88 Five studies used confocal laser scanning microscopy (CLSM) to investigate various aspects of microbial biofilm formation.37,42,45,57,62 One other approach for visualization of biofilm is using fluorescein isothiocyanate-labeled bacteria and flow cytometry.22,62 Flow cytometric cell sorting is also a useful tool for separation of bacteria.32 Because of concerns regarding dissociation of biofilm during handling and preparation for staining, Pittman *et al.* proposed using low-melting agarose on the surface of infected oral mucosa.89

One of the best quantitative methods for evaluation of barrier integrity of cells is the measurement of transepithelial electrical resistance (TEER)/transendothelial electrical resistance.65 This noninvasive method can reflect changes in tight junction proteins. Reduced TEER of keratinocytes after infection with bacteria was reported in several studies.3,90 When using TEER for comparing different models, it is important to consider the influencing parameters—like porosity and material of the model, and the medium used for the measurement.91

Fluorescent *in situ* hybridization (FISH) is also a useful technique that was used in seven studies for detection of microorganisms in OMMs.[24,42,45,48,50,64,66] Combination of different methods, like FISH and CLSM, could help to better determine interaction between oral mucosa and biofilm.[92]

Effect of antibacterial agents

OMMs are suitable and relevant *in vitro* test systems for evaluating antibacterial products. The effect of an antibacterial agent on bacteria should be considered in combination with its biosafety for oral tissues. Effect of different dosage of a commercially available topical Nystatin suspension on an *ex vivo* model of oral mucosa infected with *Candida* was studied by Ohnemus *et al.*[38] They proved that, while a dosage of 0.25 IU Nystatin was efficient in agar diffusion model, it had no confirmed activity at dosage of 10 and 0.1 IU on infected oral mucosa, suggesting the closer properties of OMM to the *in vivo* situation.[38] Biocompatibility of synthetic antimicrobial decapeptide KSL-W and its antibacterial effects against *C. albicans* was investigated by Semlali *et al.* using OMM. They showed its safety for epithelial cells and its negative effect on the growth of *Candida*.[46] Wayakanon *et al.* investigated the effect of metronidazole-, doxycycline-, and gentamicin-encapsulated polymersome on biocompatibility of keratinocyte cells and reduction of intracellular *P. gingivalis* load in OMMs.[54] Effects of plasma treatment on reduction of the biofilm of *C. albicans* and *Staphylococcus aureus* without toxic effects on OMM have also been reported by Delben *et al.*[93]

Considering the importance of quorum-sensing and presence of adhesins for adhesion of bacteria to mucosal surfaces, future antibacterial approaches could be focused on the alteration of quorum-sensing or blocking of adhesins in combination with stimulation of defensin release from OMM. Finally, using oral mucosa-on-a-chip could be very helpful to study the reciprocal effects of antibacterial agents on bacteria and oral mucosa.[65]

Conclusion

Invasion of oral bacteria to tissue-engineered oral mucosa is dependent on the strains of bacterium and can be influenced by the type of cells and culture conditions used. The methods used for tissue processing and assessment of the effects of bacteria on oral mucosa can be potentially invasive and may alter the cells or bacteria. Therefore, data reported in the literature regarding invasion of oral mucosa by bacteria must be interpreted with caution.

Although OMMs are more relevant and more informative than monolayer cultures of epithelial cells, they lack some other types of cells present in the normal human oral mucosa. Other limitations of OMMs include nonconstant desquamation, absence of saliva consisting mucin, deficiency in the number of present bacteria and immune responses, and static environment, which make it difficult to extrapolate the data from the *in vitro* experiments to the clinical situation. Using new technologies, such as microfluidics and bioreactors, could help to reproduce some of these physiologically relevant conditions.

Acknowledgments

This research is supported by National Institute of Dental and Craniofacial Research (NIDCR) of the National Institutes of Health (NIH) under award number R15DE027533.

Disclaimer

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Disclosure Statement

No competing financial interests exist.

Funding Information

This study was funded by the National Institute of Dental & Craniofacial Research (NIDCR) of the National Institutes of Health (NIH) (award no. R15DE027533).

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