

Photobiomodulation of gingival cells challenged with viable oral microbes

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Word count: 3270

Reference count: 40

Figure/Table count: 5

Abstract

The oral cavity, a unique ecosystem harboring diverse microorganisms, maintains health through a balanced microflora. Disruption may lead to disease, emphasizing the protective role of gingival epithelial cells (GECs) in preventing harm from pathogenic oral microbes. Shifting GECs' response from pro-inflammatory to anti-microbial could be a novel strategy for periodontitis. Photobiomodulation therapy (PBMT), a non-pharmacologic host modulatory approach, is considered an alternative to drugs. While the host cell response induced by a single type of pathogen-associated molecular patterns (PAMPs) was widely studied, this model does not address the cellular response to intact microbes that exhibit multiple PAMPs that might modulate the response. Inspired by this, we developed an in vitro model that simulates direct interactions between host cells and intact pathogens and evaluated the effect of PBMT on the response of human gingival keratinocytes (HGKs) to challenge viable oral microbes at both the cellular and molecular levels. Our data demonstrated that LED pre-treatment on microbially-challenged HGKs with specific continuous wavelengths (red: 615 nm and near-infrared: 880 nm) induced the production of various antimicrobial peptides (AMPs), enhanced cell viability and proliferation, promoted reactive oxygen species (ROS) scavenging, and down-modulated pro-inflammatory activity. The data also suggest a potential explanation regarding the superior efficacy of near-infrared light treatment compared with red light in enhancing antimicrobial activity and reducing cellular inflammation of HGKs. Taken together, the findings suggest that PBMT enhances the overall barrier function of gingival epithelium while minimizing inflammation-mediated breakdown of the underlying structures.

Keywords: *Photobiomodulation therapy, anti-microbial proteins, pro-inflammation, gingival keratinocytes, host-microbe interaction*

1 **Introduction**

2 The oral cavity harbors a complex and diverse multitude of microorganisms. Commensals
3 indirectly and directly prevent infections by supplying the host with essential nutrients, enhancing
4 host defense mechanisms and/or suppressing the emergence of pathobionts in oral biofilms,
5 limiting their conversion to virulent biofilms.(Negrini, 2021) The transition from health to disease
6 involves the increased presence of “keystone pathogens” that, although at relatively low abundance,
7 are capable of evading the host response and creating an environment that favors the growth of
8 inflammophilic organisms, a process known as dysbiosis.(Hajishengallis, 2014) These organisms
9 subsequently induce an exuberant inflammatory response that mediates the breakdown of the
10 tooth-supporting structures and manifests itself as periodontitis. Such alteration of the commensal
11 steady-state microflora and disruption of the equilibrium between the microbes and host response
12 are key events in the pathogenesis of periodontitis.(Costalonga, 2014; Hajishengallis, 2014;
13 Hajishengallis, 2020)

14 GECs play a significant role in protecting the underlying structures that support teeth from
15 the deleterious effects of oral microbes.(Andrian, 2006) In addition, GECs serve as sentinels for
16 the presence of pathogenic microbes.(Lee, 2021) This is mediated through the interaction between
17 microbial molecules known as PAMPs with pattern-recognition receptors on host cells.(Amarante-
18 Mendes, 2018) For GECs, recognition of PAMPs on pathogens leads to the release of pro-
19 inflammatory and anti-microbial molecules.(Jain, 2010) Additionally, it results in an enhanced
20 expression of adhesion molecules on the surfaces of GECs, facilitating the directed migration of
21 inflammatory cells toward the detected pathogens.(Jain, 2010) Collectively, these events represent
22 the initiation of the host’s innate gingival immune response to prevent microbial colonization of
23 the underlying tissues. Modulation of the response of these cells to microbial challenge from one

1 that is predominantly pro-inflammatory to one that is primarily anti-microbial could represent a
2 novel approach for preventing or treating periodontitis.

3 In recent years, the administration of certain drugs (e.g., matrix metalloproteinase
4 inhibitors and non-steroidal anti-inflammatory agents) that can down-modulate distinct
5 components of the host immune response has been proposed as an adjunct for treating periodontal
6 disease.(Preshaw, 2018; Ren, 2023) Such “host modulatory therapy” can enhance the outcomes of
7 conventional periodontal therapy by inhibiting the host response induced by bacterial pathogens.
8 A potential alternative to the use of drugs is PBMT which involves exposing diseased or damaged
9 tissues to low-intensity light that triggers photochemical changes within cells.(Dompe, 2020; Karu,
10 2013) PBMT has been used to manage many medical conditions, demonstrating its effectiveness
11 in offering pain relief, reducing inflammation, and facilitating wound healing.(De Oliveira, 2022;
12 Dompe, 2020; Kim, 2020) Thus, PBMT could have the potential as a non-pharmacologic form of
13 host modulatory therapy.

14 In this study, we employed an *in vitro* model that simulates direct interactions between host
15 cells and intact pathogens. We hypothesized that PBMT on HGKs will alter their response to
16 bacterial challenges from one that is dominated by the production of pro-inflammatory molecules
17 to one that is anti-microbial. Our data demonstrated that PBMT ameliorates the pro-inflammatory
18 response of HGKs challenged with pathogenic oral microbes while enhancing the ability of the
19 cells to mount a direct antibacterial response. These alterations in the HGKs' response to microbial
20 challenges could enhance the overall barrier function of gingival epithelium while minimizing
21 inflammation-mediated breakdown of the underlying structures.

22 23 **Results**

The challenge with oral microbes reduces the viability and enhances the pro-inflammatory activity of HGKs

Before evaluating the impact of PBMT on the anti-microbial response of HGKs, we characterized the cellular response when co-cultured with increasing numbers of oral pathogens (Fig.1A). The employed organisms were those commonly detected in human periodontal lesions, *Streptococcus oralis* J22 (So) and *Candida albicans* 529L (Ca) (Cavalcanti, 2017; Hwang, 2022) or *Staphylococcus aureus* ATCC6538 (Sa) from sites adjacent to dental implants with periimplantitis.(Lafaurie, 2017) We chose those oxygen-tolerant microorganisms for co-culturing with HGKs. Microscopic analysis revealed a significant decrease in the number of adherent HGKs in response to co-culture with each microbe, with differing dose-response relationships (Fig.1B). Particularly, Ca induced the largest loss of adherent cells over the entire range of added CFUs, starting from $\sim 1 \times 10^2$ CFU/mL. A similar impact was observed from bacterial infections (So or Sa) at higher populations, starting from $\sim 1 \times 10^3$ CFU/mL. In addition to causing the most substantial loss of adherent cells, co-culture with a high number of CFUs of Ca also induced a dramatic change in the morphology of the remaining HGKs (Fig.1A).

Subsequently, HGKs were exposed to various combinations of microbes to better mimic the situation *in situ* where epithelial cells encounter multispecies biofilms. Using the lowest CFUs of each microbe that induced a significant decrease in the number of HGKs after 24h of co-culture (i.e., $\sim 1 \times 10^2$ CFU/mL of Ca and $\sim 1 \times 10^3$ CFU/mL of So (or Sa)), mixed-species challenges caused a more significant reduction in adherent HGKs (Fig.1C). Co-culture with all three microbes resulted in a cell loss equivalent to that observed with the highest individual bacterial populations ($\sim 1 \times 10^6$ CFU/mL).

Western blotting was then performed to determine the expression of molecules involved in microbial recognition, induction of an HGK pro-inflammatory response, and participation in the effector phase of this response (Fig.1D). Challenge with either bacterial species alone increased the synthesis of TLR2, and co-culture with Ca resulted in elevated levels of both TLR2 and TLR4. Combinations of multiple-species challenges induced enhanced production of TLR2 and TLR4. Interestingly, in the absence of Ca, the combination of So and Sa caused an increase in the level of TLR4 synthesis, but to a lesser extent than observed in the presence of the fungus. All combinations of microbes led to enhanced levels of cytosolic proteins involved in TLR-mediated signaling, including Myd88, TRAF6, p-IKK α/β , p-I κ B α and p-p65.

Finally, all co-culture of HGKs with any of the microbes alone or in combinations resulted in increased levels of Nox2, a critical player in the generation of ROS. Collectively, these findings indicate that the co-culture of viable oral microbes with HGKs primarily induces a pro-inflammatory response by the epithelial cells.

PBMT enhances the viability and inhibits the pro-inflammatory activity of microbe-challenged HGKs

To investigate the effects of pre-treatment with red (R-LED, 615nm) and near-infrared (NIR-LED, 880nm) light on HGKs, we initially determined how the light pre-treatment affects the epithelial cells. The data revealed a significant increase in cell number (>130% vs. control; Fig.2A) in response to PBMT, with the 90-min treatment duration showing the maximum efficacy. While 120-min exposure reduced efficacy (vs. 90-min), it remained higher than 60-min exposures. Additionally, R-LED pre-treatment promoted HGK growth slightly higher (vs NIR-LED) at the same durations (Fig.2A). Consequently, we chose the 90-min duration for further investigation

1 into the efficacy of PBMT on HGK viability against microbial challenges. While 24h exposure of
2 HGKs to single or combinations of the microbes without PBMT decreased cell numbers by up to
3 50% (vs. control), pre-treated HGKs numbers, in all cases, exhibited significantly higher cell
4 numbers than non-treated HGKs (Fig.2B). Notably, both R-LED and NIR-LED pre-treatment
5 maintained viable HGKs number similar to control HGKs (no PBMT, no microbial challenges),
6 even under triple-species challenges. Intriguingly, NIR-LED pre-treatment exhibited better
7 efficacy in most microbial challenges.

8 HGKs co-cultured with or without a triple-species challenge were also visualized using a
9 confocal microscope (Fig.2C). In the absence of microbes, confluent monolayers with marked cell-
10 cell contacts were observed. Conversely, under triple-species challenges, the tissue surface was
11 predominantly covered by bacteria and fungus, disrupting HGK confluence and cell-cell contacts.
12 Excitingly, Red-LED or NIR-LED pre-treatment almost completely restored HGK confluence and
13 cell-cell contact features, with significantly lower colonized bacteria and fungus, particularly with
14 NIR-LED pre-treatment.

15 The effect of PBMT on the pro-inflammatory behavior of microbe-challenged HGKs was
16 then evaluated using western blotting. Co-culture with individual microbes elicited enhanced
17 expression of TLR2, TLR4, and other proteins involved in TLR signaling (Fig.2D). However,
18 PBMT significantly reduced the expression levels of Myd88 and TRAF6 without affecting TLR2
19 and TLR4 levels. PBMT also markedly reduced Nox2 expression. Importantly, NIR-LED
20 outperformed Red-LED pre-treatment in reducing those inflammatory cytokines. Quantified
21 protein levels of TLR2, Nox2, TLR4, and Nox4 in the presence/absence of microbes and PBMT
22 are shown in Supplementary Fig.S1.

Finally, the mechanisms through which PBMT down-modulates pro-inflammatory behavior were investigated by evaluating the expression of CXCL11. Co-culture of pre-treated HGKs with the triple-species challenges significantly increased CXCL11 expression (Fig.2E). Time-dependent experiments exhibited a stagnant expression up to 8h, followed by an exponential increase after 12h (Supplementary Fig.S2). While increased expression levels of other CXCLs (i.e., CXCL9, 10, and 20) were observed, particularly for triple-species challenges, these were not as obvious as CXCL11 (Supplementary Fig.S3).

Altogether, these results suggest that PBMT enhances HGKs' ability to resist the cytotoxic effects of co-culture with oral microbes while simultaneously downmodulating the pro-inflammatory behavior of the epithelial cells.

PBMT enhances the antimicrobial behavior of HGKs co-cultured with viable oral microbes

Next, we further investigated the antimicrobial response exhibited by HGKs due to PBMT and its impact on the viability of co-cultured microbes. Initially, we determined the number of adherent and planktonic microbial cells after 24h of co-culture. Under all microbial challenge conditions, significantly fewer microbial cells were recovered from Red-LED or NIR-LED pre-treated co-cultures (vs. non-treated cultures; Fig.3A-C). The reduction of microbes adhered to HGKs was more pronounced than those found in the planktonic state. Notably, direct LED exposures (either Red-LED or NIR-LED) did not affect the viability of microbial cells (Supplementary Fig.S4), suggesting that PBMT enhanced the antimicrobial activity of HGKs.

Mucosal epithelial cells, including HGKs, participate in innate antimicrobial immunity via the secretion of AMPs such as human β -defensins (hBD) and lysozyme (Lyz).(Lokken-Toyli, 2021; Mathews, 1999) To investigate whether PBMT enhances the expression of these AMPs, allowing

cells to resist the effects of co-culture with the oral pathogens, we conducted qRT-PCR to quantify the expression levels of hBDs and Lyz (Fig.3D-G) using relevant primers (Supplementary Table.S1). In the absence of microbial stimulation without LED pre-treatment, there was extremely low-level expression of the evaluated genes. Co-culture of non-treated HGKs resulted in at least a three-fold increase in the expression levels of hBD-1 and -2, whereas genes encoding hBD-3 and Lyz increased less than three-fold (vs. control; Fig.3D-G). Pre-treated HGKs with Red-LED or NIR-LED exhibited further increases in all genes except for hBD-3 (vs. control; Fig.3D-G). The most dramatic increases were observed in pre-treated HGKs co-cultured with triple-species challenge (up to 25, 16, and 8-fold for hBD-1, -2, and Lyz, respectively). In general, NIR-LED pre-treatment induced larger increases in gene expression (vs. Red-LED). Time-dependent analyses for hBD-1 revealed increasing expressions over time while the expressions of Lyz peaked at 16h of co-culturing and then decreased (Fig.3H,I). Western blotting analyses of protein expression levels of hBD-1 and Lyz corroborated these trends (Supplementary Fig.S5). These findings indicate that PBMT results in enhanced productions of hBD-1, -2, and Lyz by HGKs co-cultured with oral microbes, contributing to the enhanced survival of the epithelial cells under these conditions.

PBMT induces a reduction in intracellular ROS levels and enhanced expression of antioxidant-related genes in microbe-challenged HGKs

Microbial challenge to HGKs led to an enhanced expression of Nox2, contributing to an increase in intracellular ROS levels associated with oxidative stress and eventual cell death. To investigate whether PBMT enhances HGK viability following exposure to oral microbes by inhibiting the accumulation of intracellular ROS, we conducted an analysis using DCF-DA. All types of

1 microbial challenges induced significant increases in intracellular ROS levels (Fig.4A). Single-
2 species challenges increased the ROS level ~4-fold (vs. control with no PBMT). This increase was
3 further enhanced to ~6 and ~8-fold under double- and triple-species challenges, respectively. In
4 contrast, ROS levels in all conditions were significantly reduced when HGKs were pre-treated
5 with Red-LED or NIR-LED (vs. non-treated control). Notably, NIR-LED pre-treatment appeared
6 to further reduce intracellular ROS production than Red-LED.

7 To better understand the ROS reduction mechanism by PBMT, we assessed the expression
8 levels of antioxidant-related genes. Most of the antioxidant-related genes showed upregulation in
9 the presence of PBMT under all microbial challenge conditions (Supplementary Fig.S6). Among
10 them, the expression levels of Nrf2 (Fig.4B) and Gpx3 (Fig.4C) were significantly increased.
11 Particularly, the level of Gpx3 was significantly upregulated by NIR-LED pre-treatment (vs. Red-
12 LED; Fig.4C). These findings indicate that PBMT effectively reduces intracellular ROS in
13 microbe-challenged HGKs by increasing ROS scavenging activity.

15 **PBMT enhances barrier function of microbe-challenge HGKs**

16 To investigate the effectiveness of pre-treated HGKs in enhancing the barrier function of
17 keratinocytes to protect underlying HGFs against noxious external stimuli, we constructed a three-
18 dimensional co-culture system using transmembrane inserts (Fig.5A). Either direct contact of
19 HGFs with triple-species challenge (without a transmembrane insert) or indirect contact (microbial
20 cells existed only in the transmembrane phase) induced morphological changes in HGFs, making
21 them bipolar and elongated (Fig.5B). Additionally, there was a decrease in the number of microbe-
22 challenged HGF, particularly under conditions of direct contact with microbial cells (Fig.5C).
23 When HGKs and HGFs were cultured, both Red-LED and NIR-LED pre-treatment of HGKs

significantly improved the ability of the microbe-challenged epithelial cells to protect the underlying HGFs from the deleterious effects of the microbes (Fig.5B,C).

Discussion

PBMT has emerged as a potential alternative to chemical agents (including antibiotics) for treating diseases with microbial and/or inflammatory origins.(de Freitas, 2016). Concerning periodontitis, studies have shown that GECs exposed to PBMT, when challenged with lipopolysaccharide (LPS) or heat-killed periodontal pathogens, enhance their viability and proliferation rate, and alter the production of pro-inflammatory molecules.(Pansani, 2018) While the host cell response induced by a single type of PAMP was widely studied,(Dickson, 2019; Maldonado, 2016) this model does not address the cellular response to intact microbes, presenting multiple PAMPs and other molecules that might modulate the response. Furthermore, the response of epithelial cells to PBMT could vary depending on the types of stimulants (e.g., microbes and their metabolic products). This study aimed to evaluate the effect of PBMT on HGKs in response to challenges with viable oral microbes at the cellular/molecular levels to better understand the mechanisms through which the technology might enhance the overall barrier function of gingival epithelium while minimizing inflammation-mediated breakdown of the underlying structures.

Direct contact between HGKs and the microorganisms led to elevated ROS generation and the induction of a pro-inflammatory response via the TLR/Nox/NFκB pathway (Fig.1D). Particularly, mixed-species infection increased the expression of TLR2/4, Nox2/4, and Toll/IL-1R domain-containing adaptor proteins such as MyD88 and TRAF6. This observation is consistent with previous studies demonstrating that TLR2/4 recruits MyD88 upon contact with microbial products (LPS or heat-killed bacteria), linking it to IL-1R-associated kinase via TRAF6 which

triggers the activation of NF κ B and the production of pro-inflammatory cytokines and chemokines that can dictate the outcome of innate immune responses.(Kawai, 2010; Sameer, 2021) Our data demonstrated that the HGK response is not solely dependent on LPS exposure as we observed the pro-inflammatory response with Gram-positive bacteria and a fungal organism.

The mitochondrial cytochrome c oxidase is capable of absorbing red/near-infrared lights that facilitate ATP production, thereby inducing transcription factors and modulating ROS production.(Sun, 2018) As a consequence, photobiomodulation increases cell proliferation/migration, modulates the levels of cytokines, growth factors, and inflammatory mediators, and increases the amount of oxygen in the tissues. Similarly, we found that the number of HGKs in pre-treated cultures was significantly higher (vs. non-treated cultures; Fig.2A). Furthermore, PBMT moderated the host inflammatory response to microbial challenges by downregulating Nox2/4, MyD88, and TRAF6, which resulted in a decrease in NF κ B levels. Additionally, CXCL11 is known to recruit CD4-positive T cells to sites of inflammation and under certain conditions will induce their differentiation into regulatory T cells that can down-modulate the inflammatory reaction (Tregs).(Zohar, 2018) Marked reduction in Nox2 and dramatic increase in CXCL11 expression by pre-treated HGKs represent potential explanations for the inhibition of their pro-inflammatory response to microbial challenge. Notably, CXCL11 has been shown to exhibit anti-bacterial activity (Cole, 2001). Thus, it is feasible that in addition to recruiting and inducing the differentiation of Tregs, the antimicrobial activity of CXCL11 might further protect the HGKs from the effects of the oral microbes. Further investigations are necessary to identify the mechanism underlying this observation.

Beyond cell proliferation and the pro-inflammatory response, PBMT directly mediated antimicrobial immunity via the production of AMPs and extracellular ROS. Among many, hBDs

are expressed in response to bacterial stimuli or inflammation,(Chung, 2004) exhibiting antimicrobial effects.(Dale, 2001) In addition, Lysozyme (Lyz), one of the host defense peptides, can kill bacteria through the catalytic hydrolysis of cells.(Gill, 2011) Interestingly, Lyz can operate synergistically with hBD-2 and hBD-3;(Lippross, 2012) Lyz can crack the bacteria cell wall polysaccharide chain, enhancing the entry of hBD into the cell.(Travis, 2001) In this study, we observed significant increases in hBD-1, hBD-2, and Lyz expression levels (Fig.3), particularly under triple-species challenges. Also, PBMT effectively reduced intracellular ROS in microbe-challenged HGKs by increasing ROS scavenging activity. This mechanism could be involved in the down-modulation of the pro-inflammatory activity of microbe-challenged HGKs by interfering with ROS-mediated activation of the NF κ B signaling pathway and assembly of the NLRP3 inflammasome.(Liu, 2017; Palazon-Riquelme, 2018). These findings indicate that PBMT of HGKs prior to culture with oral microbes causes an alteration in the response of the epithelial cells from one that is predominantly pro-inflammatory to the antimicrobial response.

Interestingly, Red-LED pre-treatment was more effective than NIR-LED in stimulating HGK proliferation, while NIR-LED appeared to more effectively activate the cell's antimicrobial activity (Fig.2). The antioxidant enzyme Gpx, crucial for detoxifying ROS and shielding epithelial cells from oxidative damage,(Ottaviano, 2009) and the transcription factor Nrf2, involved in the antioxidant response,(Huang, 2015) were upregulated by PBMT. Particularly, Gpx3 exhibited a remarkable upregulation under NIR-LED pre-treatment (vs. Red-LED; Fig.4), potentially explaining the higher antimicrobial activity and reduction of cellular inflammation in HGKs with NIR-LED.

Finally, keratinocytes are known to act as a protective barrier against microbial invasion of underlying tissues as well as to activate fibroblasts to produce a variety of soluble mediators.(Ren,

2019; Shephard, 2004) Thus, a 3-dimensional co-culture system was developed to assess the capacity of HGKs to protect underlying HGFs from microbial challenge. In the absence of HGKs, HGFs were severely damaged by either direct contact with microbial cells or indirectly by their products (Fig.5). In contrast, the viability of HGFs significantly improved even under triple-species challenges when co-cultured with HGKs. The numbers and confluence of HGFs were dramatically increased when the HGKs were pre-treated with LEDs. The findings suggest that PBMT enhances the overall barrier function of gingival epithelium while minimizing inflammation-mediated breakdown of the underlying structures.

In summary, we comprehensively evaluated the effect of PBMT on the response of HGKs to viable oral microbes challenge at the cellular/molecular levels. The data demonstrate that light pre-treatment of microbially-challenged HGKs induced the production of various AMPs in addition to well-known PBMT effects on cell viability/proliferation, ROS scavenging, and down-modulation of pro-inflammatory activity (Fig.5D). Our data also suggest a potential explanation regarding the superior efficacy of NIR-LED compared with Red-LED in enhancing antimicrobial activity and reducing cellular inflammation of HGKs. While the findings of this study exhibit the potent benefit of utilizing phototherapy, further studies are warranted to enhance its clinical efficacy. Since bacteria and fungi coexist in the oral cavity and reciprocally enhance one another's pathogenic potential, for example, it will be useful if irradiation conditions can be identified that stimulate HGKs to produce both anti-bacterial and anti-fungal proteins such as histatins.(Buda, 2020) In addition, it is certainly of interest to evaluate the efficacy of post-challenge or intermittent treatment during challenges. Furthermore, determining the time course of effect with an elongated experimental period using an organoid model would be necessary to better evaluate the efficacy. Finally, the cellular and molecular responses can be altered by varying the PBMT parameters (e.g.,

1 power (intensity), energy (density), spot area, operation mode) and/or the type of pathogens.
2 Further understanding of the mechanism and the efficacy of PBMT addressing those perspectives
3 may broaden the applicability for oral and extraoral microbially-induced inflammatory conditions.

4

5 **Materials and Methods**

6 The detailed materials and methods are presented in the Supplementary Material.

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Acknowledgments

The authors would like to thank Dr. Dana T. Graves at the School of Dental Medicine, University of Pennsylvania for providing HGK cells.

Funding

This work was financially supported by the National Institutes of Health, National Institute of Dental and Craniofacial Research grant DE027970 (GH) and DE032162 (GH and AK), the National Science Foundation grant ECCS-2225697 (GH) and ECCS-2300985 (AK), and the Schoenleber OMFS Research Fund (GH and JK).

Author contributions

G.H. and H-E.K. designed the research. H-E.K. performed experiments. G.H., J.K., J.T., and H-E.K. analyzed the data. J.T. and H-E.K. drafted the paper. G.H., J.K., and J.T. revised the paper. S-M.L. assisted with the tissue collection and separation. A.K. assisted with experiments. All authors read and approved the final manuscript.

Competing interests

G.H. and A.K. are co-founders of LEDEN LLC.

G.H., A.K., J.K., and H.-E.K. are inventors on US Patent Application No. 18/064,747 (filed on December 12th, 2022).

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Figure Caption

Figure 1. Effects of microbial challenges on human gingival keratinocytes. (A) Optical microscopic images of human gingival keratinocytes (HGKs) under single-species microbial challenges depending on their populations. The scale bar represents 100 μm . (B) Normalized number of adhered HGKs under single-species microbial challenges, depending on the microbial CFU. (C) A normalized number of adhered HGKs under single-, double-, and triple species microbial challenges. Inoculum size; *Streptococcus oralis* J22 and *Staphylococcus aureus* ATCC 6548 at $\sim 1 \times 10^3$ CFU/mL, and *Candida albicans* 529L at $\sim 1 \times 10^2$ CFU/mL. (D) Western blotting analyses of TLR2, Nox2, TLR4, Nox4, MyD88, TRAF6, p-IKK α/β , p-I κ B α , p-p65, and p65. GAPDH was used as a loading control. *p*-value was determined using a two-tailed t-test. *p*-value (vs. Con): **p*<0.05, ***p*<0.01, ****p*<0.001.

Figure 2. Effects of red or near-infrared pre-irradiation on human gingival keratinocytes against microbial challenges. (A) Effect of light irradiation (red:615 nm; R, near-infrared:880 nm; NIR) on the viability of HGKs without microbial challenges. (B) Normalized number of HGKs with or without light pre-irradiation (90 min) under various microbial challenges. Inoculum size; *S. oralis* J22 and *S. aureus* ATCC 6548 at $\sim 1 \times 10^3$ CFU/mL, and *C. albicans* 529L at $\sim 1 \times 10^2$ CFU/mL. Con: control group without light pre-irradiation. (C) Representative confocal images of phalloidin-red/DAPI-blue stained HGKs, exhibiting blue-fluorescent nuclei and red-fluorescent

cytoskeleton Syto 9 was used to stain bacteria and Tdtomato was used to label *C. albicans*. The scale bar indicates 100 μ m. (D) Western blotting analyses of expression levels of inflammatory-related proteins under triple-species challenges with or without light pre-irradiation: GAPDH was used as a loading control. (E) Expression levels of mRNA of CXCL11 after 24 h of single or multiple-species microbial challenges in the presence/absence of PBMT. * p <0.05, ** p <0.01, *** p <0.001.

Figure 3. Changes in CFU and expression levels of antimicrobial peptides in human gingival keratinocytes (HGKs) in the presence and/or absence of photobiomodulation therapy (PBMT) and microbial challenges. (A-C) Microbial populations (CFU/mL) in the planktonic phase and on the HGKs after 24 h incubation. F: free-floating supernatant, I: interface at HGKs. p-value was determined using a two-tailed t-test. *,[#] p <0.05, **,^{##} p <0.01, ***,^{###} p <0.001. Expression levels of mRNA of (D) hBD-1, (E) hBD-2, (F) hBD-3, and (G) Lyz after 24 h of microbial challenges in the presence/absence of PBMT; HGKs were pre-irradiated for 90 min ahead of microbial challenges. p-value was determined using a two-tailed t-test. * p <0.05, ** p <0.01, *** p <0.001. Changes in expression levels of mRNA of (H) hBD-1 and (I) Lyz in each condition in a time-dependent manner. p-value was determined using a two-tailed t-test. * p <0.05, ** p <0.01, *** p <0.001 (vs 0 hr).

Figure 4. Effect of PBMT on intracellular levels of ROS and antioxidant-related gene expressions. (A) Fold changes of the amount of intracellular ROS produced from HGKs with microbial challenges for 24 h. Fold changes of mRNA expression levels for (B) Nrf2 and (C) GPx3 genes. *P*-value was determined using a two-tailed t-test. **p*<0.05, ***p*<0.01, ****p*<0.001.

Figure 5. Interaction between HGKs and HGFs in the absence/presence of polymicrobial challenges and PBMT. (A) A schematic diagram describing the co-culture system employing insert transmembrane. (B) Images of HGFs with (bottom lane) or without (upper lane) HGKs. No challenge; HGFs without microbial challenges, Direct; HGFs directly exposed to microbial suspension without insert transmembrane, Indirect; HGFs indirectly exposed to microbial suspension only on insert transmembrane, Nontreated; HGFs (below insert transmembrane) with non-treated HGKs exposed to microbial suspension (on insert transmembrane), Red-LED; HGFs (below insert transmembrane) with pre-treated HGKs by Red-LED exposed to microbial challenges (on insert transmembrane), NIR-LED; HGFs (below insert transmembrane) with pre-treated HGKs by NIR-LED exposed to microbial challenges (on insert transmembrane). The scale bar represents 100 μ m. (C) The number of HGFs in each condition. ***p*<0.01. (D) A schematic diagram describing the role of photobiomodulation therapy on the interactions between bacteria and gingival epithelium cells.