Research Article

Effects of Fruit and Vegetable Low Molecular Mass Fractions on Gene Expression in Gingival Cells Challenged with Prevotella intermedia and Actinomyces naeslundii

Laura Canesi,1 Cristina Borghi,1 Monica Stauder,1 Peter Lingström,2 Adele Papetti,3 Jonathan Pratten,4 Caterina Signoretto,5 David A. Spratt,4 Mike Wilson,4 Egija Zaura,6 and Carla Pruzzo1

1 DIPTERIS, University of Genova, Corso Europa 26, 16132 Genova, Italy
2 Department of Cariology, Institute of Odontology at Sahlgrenska Academy, University of Gothenburg, P.O. Box 450, 405 30 Gothenburg, Sweden
3 Department of Drug Sciences, University of Pavia, Viale Taramelli 12, 27100 Pavia, Italy
4 Department of Microbial Diseases, UCL Eastman Dental Institute, 256 Gray’s Inn Road, London WC1X 8LD, UK
5 Microbiology Section, Department of Pathology and Diagnostics, University of Verona, Strada Le Grazie 8, 37134 Verona, Italy
6 Department of Preventive Dentistry, Academic Centre for Dentistry Amsterdam (ACTA), Gustav Mahlerlaan 3004, 1081 LA Amsterdam, The Netherlands

Correspondence should be addressed to Laura Canesi, laura.canesi@unige.it

Received 14 June 2011; Accepted 12 July 2011

Academic Editor: Itzhak Ofek

Copyright © 2011 Laura Canesi et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Low molecular mass (LMM) fractions obtained from extracts of raspberry, red chicory, and Shiitake mushrooms have been shown to be an useful source of specific antibacterial, antiadhesion/coaggregation, and antibiofilm agent(s) that might be used for protection towards caries and gingivitis. In this paper, the effects of such LMM fractions on human gingival KB cells exposed to the periodontal pathogens Prevotella intermedia and Actinomyces naeslundii were evaluated. Expression of cytokeratin 18 (CK18) and β4 integrin (β4INT) genes, that are involved in cell proliferation/differentiation and adhesion, and of the antimicrobial peptide β2 defensin (HβD2) in KB cells was increased upon exposure to either live or heat-killed bacteria. All LMM fractions tested prevented or reduced the induction of gene expression by P. intermedia and A. naeslundii depending on the experimental conditions. Overall, the results suggested that LMM fractions could modulate the effects of bacteria associated with periodontal disease in gingival cells.

1. Introduction

Periodontal diseases are a heterogeneous group of inflammatory conditions that involve the supporting tissues of the teeth and include gingivitis, in which only the gingivae are involved, and the various forms of periodontitis, chronic inflammatory conditions initiated by a polymicrobial infection that leads to gingival tissue destruction and alveolar bone resorption [1]. Gingivitis is the most prevalent form of periodontal disease that can be defined as “a nonspecific inflammatory process of the gingivae (gums) without destruction of the supporting tissues”. This is a reversible condition as a return to meticulous dental hygiene practices will restore gingival health [2]. Several bacterial species have been implicated as aetiological agents of this disease: these include Actinomyces israelii, Actinomyces naeslundii, Actinomyces odontolyticus, Lactobacillus spp., Prevotella intermedia, Treponema spp., and Fusobacterium nucleatum [3].

Bacteria and their products can directly damage periodontal tissues and/or initiate inflammation locally. The clinical outcomes of these events are determined by the host response to the infections [1]. Different experimental systems can be utilized to evaluate cellular responses to different bacteria or antibacterial agents, from fibroblasts...
derived from human periodontal ligaments to epithelial cells and fibroblasts derived from human gingivae [4].

Foodstuffs as a source to obtain agents/fractions that can improve oral health have been the focus of intensive research because such natural agents are likely to be nontoxic and edible; for example, they can be used to supplement various oral hygiene products. In studies described elsewhere in this issue, it has been shown that low molecular mass (LMM) fractions obtained from extracts of raspberry, chicory, and mushrooms inhibit coaggregation, biofilm formation, and adhesion to hydroxyapatite and/or cultured gingival cells of oral bacteria involved in caries and/or gingivitis [5]. To further evaluate the beneficiary effect on oral health of these dietary fractions, the present study was designed to determine the effect of the raspberry, chicory, and mushroom LMM fractions on the ability of gingivitis-associated bacteria to induce deleterious gene expression in the gingival KB cell line.

2. Materials and Methods

2.1. Bacterial Cultures. A. naeslundii ATCC 19039 and P. intermedia ATCC 25611 were employed. Bacteria were grown in Brain Heart Infusion Broth (BHIB, Difco Laboratories, Detroit, Mich.) supplemented with haemin (final concentration, 5 mg mL$^{-1}$) and vitamin K (final concentration, 1 mg mL$^{-1}$) and incubated at 37$^\circ$C under anaerobic conditions. Cells were harvested at stationary phase by centrifugation (5,000 × g for 10 min at 4$^\circ$C) and washed twice with 10 mM phosphate buffered saline, pH 7.0. Bacterial suspensions (final concentration, 2 × 10$^8$ cfu mL$^{-1}$) were prepared in PBS (0.1 M Na$_2$HPO$_4$, 0.1 M KH$_2$PO$_4$, 0.15 M NaCl, pH 7.2 to 7.4) alone or suspensions containing different concentrations of test LMM fractions (pH adjusted to 7). Aliquots (10–100 μL) of the bacterial suspensions were suitably added to KB cell monolayers in order to reach a nominal bacteria: KB cell ratio of 50 and incubated at 37$^\circ$C for different periods of time in 5% CO$_2$ atmosphere with gentle shaking. For each strain, untreated controls were included. In experiments with killed bacteria, bacteria were heat inactivated at 70$^\circ$C for 15 min, centrifuged at 10,000 × g, and washed twice in PBS, before being adjusted to the appropriate density in the same buffer.

2.2. LMM Fraction Preparation. Food and vegetable extracts and fractions were prepared as described elsewhere in this issue [6]. Briefly, aliquots of frozen mushroom (Lentinus edodes) (400 g) and fresh chicory (Cichorium intybus var. silvestre) (500 g) and fresh raspberry fruit (Rubus idaeus L. var. tulameen) (200 g) were homogenized and centrifuged (10 min, 8000 rpm). The juice, after separation from the solid part, was filtered on paper filter. Mushroom and chicory extracts were then fractionated into low and high molecular weight (LMM and HMM) fractions using an ultradialfiltration system. The obtained LMM and HMM fractions contained all the compounds with molecular mass lower and higher than 5000 Da, respectively. Raspberry extract was fractionated by dialysis with cellulose ester membrane (Spectrum Europe B.V.) with a 3500 Da MWCO. The obtained LMM and HMM fractions contained all the compounds with molecular mass lower and higher than 3500 Da, respectively. All LMM fractions were subjected to sterile ultrafiltration and freeze dried.

2.3. Gingival Cell Culture and Treatments. The gingival fibroblast KB cell line (accession number ICLC HTL96014) obtained from Cell bank Interlab Cell Line Collection (ICLC) of IST-Istituto dei Tumori di Genova (Genoa, Italy) was cultured in a complete medium consisting of Dulbecco’s Modified Eagle’s Medium (DMEM)-high glucose, with 4.5 g L$^{-1}$ glucose and sodium bicarbonate supplemented with 10% fetal calf serum, penicillin (100 U mL$^{-1}$), streptomycin (100 μg mL$^{-1}$), and 2 mM L-glutamine. Cells were incubated at 37$^\circ$C in a 5% CO$_2$ atmosphere to about 90% confluence and used after 5–10 passages.

For MTT assay, cells were seeded in flat-bottom 96-well plates, at a density of 2 × 10$^4$ cells per well, in 0.2 mL of complete medium without antibiotics in triplicate and incubated for 24 h at 37$^\circ$C.

For RT-Q-PCR analysis cells were plated (3 × 10$^5$ cells) in to 25 cm$^2$ flasks in 5 mL of complete medium and incubated for 48 hrs at 37$^\circ$C.

After 24 hrs of starvation in DMEM without serum, cell monolayers were washed twice with PBS and then exposed to bacterial suspensions alone or suspensions containing LMM test fractions at the indicated concentrations. A parallel set of untreated cells in triplicate was utilised as a control. All experiments were performed in quadruplicate.

2.4. Viability Assay. KB cell viability was evaluated by the MTT assay [7]. After each treatment MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide] solution in phosphate buffered saline (PBS, pH 7.4) (final concentration of 1 mg/mL) was added to each well and incubated for 3 h at 37$^\circ$C. Following incubation, 100 μL of 0.04 N HCl isopropanol was added to each sample and the plates incubated for 10 minutes at room temperature. The absorbance at 550 nm was determined by a Varian Cary 50-bio UV-visible spectrophotometer. Data are expressed as percent of control values (mean ± SD).

2.5. RT-Q-PCR. The bacteria-induced changes in mRNA expression of keratinocyte growth factor receptor (KFGFR), cytokeratin 18 (CK18), β4 integrin (B4ITG), and β defensin 2 (HBD2) were evaluated by quantitative RT-PCR analysis. After harvesting, total cellular RNA was isolated by the procedure of Chomczynski and Sacchi [8] using TriReagent (SIGMA, Milan, Italy). RNA purity was evaluated by measuring the 260/280 nm absorbance ratio, and only samples with OD$_{260/280} > 1.8$ were processed, resolved on a 1.5% agarose gel, and stained with ethidium bromide, to check for purity. cDNA synthesis was performed from 1.5 μg of total DNasel (Fermentas, M-Medical, Italy) treated RNA using 200 units RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas Italy, M-Medical, Milan) in presence of 201 pmoles oligo (dT)$_{18}$ (TIB MOLBIOL,
Table 1: Oligonucleotide primers used for quantitative RT-PCR analysis.

| Gene  | Primer forward (5′–3′)                                                                 | Primer reverse (5′–3′)                                                                 | GenBank   |
|-------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|-----------|
| KGFR  | GATTACAGCTTCCCCCAGACTACC                                                             | GAAGAGGCGGTGTTGTATCC                                                               | M806534   |
| CK18  | TCAAGACCTGAGCCATTTGTCG                                                               | CAGTCGTGATATTGTTGTCA                                                                | NM199187  |
| ITGB4 | GCCGCTACAGGGGGTCAGG                                                                  | TCCATTACAGTGGCCTTACC                                                               | NM000213  |
| HBD2  | GGGTCTTTAGATCTTCTTCTTCG                                                             | ACAGGTGCAAATTGTGTATAC                                                                | NM004942  |
| GAPDH | GTCAGTGGTGGACCTGACCT                                                                | AGGGGTCTACATGGCAACT                                                                | M17851    |

Genova, Italy), 40 units Ribolock (ribonuclease inhibitor), and 1 mM dNTPs (Fermentas Italy, M-Medical, Milan) at 42°C for 60 min in a reaction volume of 20 μL according to the manufacturer’s protocol. 5 μL of cDNA was then used to amplify the genes of interest using a Chromo 4 System real-time PCR apparatus (Biorad Italy, Segrè, Milan) in a final volume of 20 μL containing 1x iTaq SYBR Green Supermix with Rox (Biorad, Milan, Italy) and 0.25 μM of each primer (TIB MolBiol, Genoa, Italy). The primer pairs were used, and their accession numbers are shown in Table 1. The primers for KGFR, CK18, HBD2, and GAPDH were designed with the Primer3 (v.0.4.0) software [9], those for B4ITG were from Wang et al. [10].

The thermal protocol consisted of 3 min initial denaturation at 95°C, followed by 40 cycles: 15 s at 95°C; 30 s at 54°C; 20 s at 72°C. A melting curve of PCR products 55–94°C was also performed to ensure the absence of artefacts. Gene expression was determined relative to the expression of the gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by the comparative C_T threshold method [11] using the Biorad software tool Genex-Gene Expression Macro [12]. The normalized expression obtained was expressed as relative quantity of mRNA with respect to control samples.

2.6. Data Analysis. Data representing the mean ± SD of at least 4 experiments in triplicate were analysed by the Mann-Whitney U test (P ≤ 0.05).

3. Results

3.1. Effects of Live Bacteria and LMM Fractions, Alone and in Combination, on KB Cell Viability and Gene Expression. The effects of the gingivitis-associated *P. intermedia* and *A. naeslundii* and LMM fractions (Raspberry, Chicory, Mushroom) alone on KB cell viability were first evaluated by the MTT assay. LMMs were tested at different concentrations (0.2x, 0.5x, 1x) and times of incubation with monolayers (4 and 6 h).

The viability of KB cells treated with live bacteria alone (at the nominal bacteria: KB cell ratio of 50) was not affected at 4 h; the number of viable bacteria remained the same during the assay as evaluated by cfu counting. KB cell incubation for longer than 4 h or in the presence of 0.5x and 1x concentrations of LMM fractions alone decreased by 20–30% (data not shown). Therefore, all subsequent experiments with live bacteria were performed by treating KB cells for 4 h with bacteria and 0.2x LMM fractions, both alone and in combination. In these conditions, no effects on KB cell viability were observed (Figure 1).

![](image)

**Figure 1:** Effects of live *P. intermedia* and *A. naeslundii*, and of Raspberry, Chicory, and Mushroom LMM fractions on KB cell viability. Cells were exposed to bacteria (at the nominal bacteria: KB cell ratio of 50) and fractions (0.2x), alone and in combination, for 4 h. Cell viability was evaluated by the MTT assay as described in Methods. Data, expressed as percent cell viability with respect to controls, represent the mean ± SD of four experiments in triplicate.

Pi: *P. intermedia*; A.n.: *A. naeslundii*; R: Raspberry; Ch: Chicory; M: Mushroom; Pi./R: *P. intermedia*/R; Pi./Ch: *P. intermedia*/Ch; Pi./M: *P. intermedia*/M; A.n./R: *A. naeslundii*/R; A.n./Ch: *A. naeslundii*/Ch; A.n./M: *A. naeslundii*/M.

The viability of KB cells treated with live bacteria alone (at the nominal bacteria: KB cell ratio of 50) was not affected at 4 h; the number of viable bacteria remained the same during the assay as evaluated by cfu counting. KB cell incubation for longer than 4 h or in the presence of 0.5x and 1x concentrations of LMM fractions alone decreased by 20–30% (data not shown). Therefore, all subsequent experiments with live bacteria were performed by treating KB cells for 4 h with bacteria and 0.2x LMM fractions, both alone and in combination. In these conditions, no effects on KB cell viability were observed (Figure 1).

The effects of KB cell treatment for 4 h with *P. intermedia* and *A. naeslundii* and LMM fractions, alone and in combination, on the expression of different genes (KGFR, CK18, β4INT and HBD2) were evaluated. No appreciable changes could be detected in the expression of KGFR by KB cells in response to different treatments (not shown). In contrast, *P. intermedia* and *A. naeslundii* alone decreased significantly the transcription of CK18 by 40% and 18%, respectively (P ≤ 0.05) (Figure 2(a)). No significant changes in CK18 expression were noted in KB cells exposed to LMM fractions. Nevertheless, raspberry and chicory LMM fractions prevented the downregulation of CK18 induced by both bacteria, whereas the LMM mushroom fraction did not.

All bacteria and LMM fractions, when tested alone, induced a decrease in transcription of β4ITG with respect to controls (Figure 2(b)). The effect was significant for all treatments, with decreases in β4ITG mRNA level ranging from about ~60% (*P. intermedia*) to ~40% (mushroom fraction) (P ≤ 0.05). However, when KB cells were exposed to a mixture of bacteria and LMM fractions, raspberry,
P. intermedia

KB cells exposed for 6 h to subsequent experiments. In Figure 3, a significant loss in cell viability, it was no longer utilized for experiments; however, since raspberry LMM still induced viability was observed in all experimental conditions, and raspberry LMM extracts in particular. At 6 hr, stable cell conditions with both bacteria and test substances, with incubation and lower concentrations of LMM extracts (0.1x) and in combination with LMM extracts. Longer times of treatment versus controls;

§ = P ≤ 0.05: all treatment versus controls; $ = P ≤ 0.05$: bacteria/LMM fractions versus bacteria alone. Mann-Whitney U test.

the decrease in β4ITG expression was attenuated; moreover, mushroom LMM fraction prevented the effect induced by A. naeslundii. On the other hand, no effect was observed with chicory fractions.

3.2. Effects of Heat-Killed Bacteria and LMM Fractions, Alone and in Combination, on KB Cell Viability and Gene Expression. The same experiments as above were carried out with heat-killed P. intermedia and A. naeslundii alone and in combination with LMM extracts. Longer times of incubation and lower concentrations of LMM extracts (0.1x) were tested; however, from 8 to 20 hr incubation, decreases in cell viability were observed in different experimental conditions with both bacteria and test substances, with raspberry LMM extracts in particular. At 6 hr, stable cell viability was observed in all experimental conditions, and therefore this time of exposure was chosen for subsequent experiments; however, since raspberry LMM still induced significant loss in cell viability, it was no longer utilized for subsequent experiments. In Figure 3, data on viability of KB cells exposed for 6 h to P. intermedia and A. naeslundii, alone and in combination with chicory and mushroom LMM fractions, are reported.

Possible changes in gene expression induced by KB cell exposure for 6 h to inactivated bacteria and LMM fractions, alone and in combination, were evaluated, and the results are reported in Figure 4. Heat-killed P. intermedia and A. naeslundii alone did not affect CK18 expression. On the other hand, in these experimental conditions, chicory fraction caused a significant decrease (−27%) and shiitake mushroom fraction an increase (+43%) in CK18 expression (P ≤ 0.05) (Figure 4(a)). In cells incubated with bacteria and LMM fractions, upregulations of CK18 were observed: in the presence of P. intermedia, both chicory and mushroom fractions induced an increase in CK18 mRNA levels with respect to control cells (+58% and +133%, resp.; P ≤ 0.05); a larger increase was observed in the presence of A. naeslundii which was induced by both chicory and mushroom (+269% and +247%, resp., P ≤ 0.05) (Figure 4(a)).

Changes in β4ITG expression were observed with heat-killed bacteria alone, with P. intermedia inducing an increase (+119%; P ≤ 0.05), and A. naeslundii a decrease (−30%; P ≤ 0.05) in the level of β4ITG mRNA (Figure 4(b)). Both chicory and mushroom LMM fractions alone caused a decrease in β4ITG expression (−39% and −54%, resp.; P ≤ 0.05) (Figure 4(b)). However, both fractions prevented the upregulation of β4ITG induced by P. intermedia and the downregulation of β4ITG induced by A. naeslundii.
In these experimental conditions, changes in expression of the defensin gene HβD2 were also observed (Figure 4(c)). *P. intermedia* alone induced a 2-fold increase in the level of HβD2 mRNA; however, the effect was not significant due to large sample variability. In contrast, *A. naeslundii* alone induced a decrease in transcription of HβD2 (−52% with respect to controls; *P* ≤ 0.05). Even larger decreases were observed with chicory and shiitake mushroom LMM fractions alone (−80% and −70%; *P* ≤ 0.05). When tested in combination with *P. intermedia*, both fractions prevented the increase in HβD2 expression induced by bacteria. Moreover, chicory fractions prevented the decrease in HβD2 expression induced by *A. naeslundii*, whereas mushroom was ineffective.

### 4. Discussion

In this work, the effects of different fruit and vegetable LMM fractions, alone or in combination with the gingivitis-associated bacteria *P. intermedia* and *A. naeslundii*, on cell viability and gene expression by human KB gingival cells were evaluated. In preliminary experiments, different concentrations of LMM fractions and times of exposure with bacteria and fractions were tested; however, since loss in cell viability was observed in different experimental conditions, assays with live bacteria were carried out at 4 h of exposure and at a concentration of LMM fractions of 0.2x, whereas assays with heat-killed bacteria were carried out for longer times (6 h) in the presence of lower concentrations of LMM fractions (0.1x). In these conditions, no effects on cell viability were observed, as evaluated by the MTT assays, that measures the number of live and metabolically active cells.

The possible effects of bacteria and LMM fractions, alone and in combination, on the expression of selected genes associated with cell differentiation, proliferation, and adhesion, and the production of antimicrobial peptides were evaluated. Significant changes in expression of Cyt18, β4ITG, and HβD2 were observed in different experimental conditions. When expression of other genes such as KGFR (keratinocyte growth factor receptor), β1 integrin, or β3 defensin (HβD3) was evaluated, no changes were induced by different bacteria and LMM fractions, alone and in
Cytokeratins are connected through transmembrane cell-the noncovalent association of are heterodimeric transmembrane glycoproteins formed by as a marker for healthy gingival cells in culture [4, 14].

polypeptides. Their expression varies with proliferation, transformation [13]; Cyt 18 in particular has been utilized signaling, induced by live P. intermedia induced an increase in expression of 4β

and LMM fractions abolished the downregulation induced by individual treatments. Again, the results indicate a comparable effect of Chicory and Mushroom LMM fractions on the overall changes in gene expression induced by heat-killed gingivitis-associated bacteria.

5. Conclusions

On the basis of the results of various assays, reported elsewhere in this issue, raspberry, chicory, and mushroom LMM fractions show that individual treatments induced changes in the level of HβD2 mRNA similar to those observed for that 4βINT, with upregulation by P. intermedia and downregulation by A. naeslundii and LMM fractions. Both chicory and mushroom prevented the upregulation of HβD2 induced by P. intermedia and combined exposure to A. naeslundii and LMM fractions abolished the downregulation induced by individual treatments. Again, the results indicate a synergistic effect of Chicory and Mushroom LMM fractions on the overall changes in gene expression induced by heat-killed gingivitis-associated bacteria.

indicating a synergistic effect between LMM fractions and heat-killed bacteria.

In these experimental conditions, changes in expression of the antimicrobial peptide HβD2 were also observed. Antimicrobial peptides (AMPs) are components of the host innate immune defence system, exerting broad-spectrum antimicrobial activity via the binding and perforation of cell membranes [20, 21], as well as exerting neutralising effects on the LPS activity of Gram negative bacteria, including periodontopathogens such as P. intermedia [22]. Human β-defensins are small, cationic AMPs: β-defensin 1 (HβD1) is expressed constitutively in epithelial tissues, whereas HβD2 and HβD3 are expressed in response to bacterial stimuli or inflammation [23–26]. Defensins, including HβD2, may also participate in epithelial differentiation and tissue damage repair in periodontal disease and contribute to the host defense by recruiting neutrophils to the site of inflammation and modulate the expression of cytokines, thus playing an important role in the control of oral health [22, 26–31]. Gingival epithelial cells and tissue express HβD2 mRNA and peptide in response to inflammatory mediators and challenge from commensal bacteria naturally present in the oral cavity [32]. In gingival epithelial cells, HβD2 mRNA was induced in response to the supernatant from Porphyromonas gingivalis, and this expression might be associated with periodontal health and disease [33]. Cell wall components of periodontal pathogens induce HβD2 expression through activation MAP kinases [34]; such effect can be mediated by activation of fibronectin–integrin components [35].

The results here obtained with heat-killed P. intermedia and A. naeslundii and with chicory and mushroom LMM fractions show that individual treatments induced changes in the level of HβD2 mRNA similar to those observed for that 4βINT, with upregulation by P. intermedia and downregulation by A. naeslundii and LMM fractions. Both chicory and mushroom prevented the upregulation of HβD2 induced by P. intermedia and combined exposure to A. naeslundii and LMM fractions abolished the downregulation induced by individual treatments. Again, the results indicate a comparable effect of Chicory and Mushroom LMM fractions on the overall changes in gene expression induced by heat-killed gingivitis-associated bacteria.
A. naeslundii. This supports the hypothesis that chicory and mushroom LMM fractions can modulate the responses of gingival cells to periodontopathogens and can be used as source for obtaining agents to be included in toothpaste, mouthwashes, and other oral health care products.

Acknowledgments

The research leading to these results has received funding from the European Union’s Sixth Framework Programme (FP6) under the contract FOOD-CT-2006-036210 (project NUTRIDENT).

References

[1] W. J. Loesche and N. S. Grossman, “Periodontal disease as a specific, albeit chronic, infection: diagnosis and treatment,” Clinical Microbiology Reviews, vol. 14, no. 4, pp. 727–752, 2001.

[2] R. C. Page, “Gingivitis,” Journal of Clinical Periodontology, vol. 13, no. 5, pp. 345–359, 1986.

[3] A. Tanner, M. F. J. Maiden, P. J. Macuch, L. L. Murray, and R. L. Kent, “Microbiota of health, gingivitis, and initial periodontitis,” Journal of Clinical Periodontology, vol. 25, no. 2, pp. 85–98, 1998.

[4] A. Suzuki, J. Yagisawa, S. I. Kumakura, and T. Tsutsui, “Effects of minocycline and doxycycline on cell survival and gene expression in human gingival and periodontal ligament cells,” Journal of Periodontal Research, vol. 41, no. 2, pp. 124–131, 2006.

[5] D. Spratt et al., “Evaluation of the beneficial oral health properties of plant and fungal extracts,” Journal of Biomedicine and Biotechnology. In press.

[6] M. Daglia, A. Papetti, D. Mascherpa et al., “Rapid calorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays,” Journal of Biomedicine and Biotechnology. In press.

[7] T. Mosmann, “Rapid calorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays,” Journal of Immunological Methods, vol. 65, no. 1–2, pp. 55–63, 1983.

[8] P. Chomczynski and N. Sacchi, “Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction,” Analytical Biochemistry, vol. 162, no. 1, pp. 156–159, 1987.

[9] S. Rozen and H. J. Skaltsky, “Primer3 on the WWW for general users and for biologist programmers,” in Bioinformatics Methods and Protocols: Methods in Molecular Biology, S. Krawetz and S. Misener, Eds., pp. 365–386, Humana Press, Totowa, NJ, USA, 2000.

[10] T. W. Wang, J. S. Sun, H. C. Wu, Y. H. Tsuang, W. H. Wang, and F. H. Lin, “The effect of gelatin-chondroitin sulfate-hyaluronic acid skin substitute on wound healing in SCID mice,” Biomaterials, vol. 27, no. 33, pp. 5689–5697, 2006.

[11] M. W. Pfaff, “A new mathematical model for relative quantification in real-time RT-PCR,” Nucleic Acids Research, vol. 29, no. 9, article e45, p. e45, 2001.

[12] J. Vandesompele, K. De Preter, F. Pattyn et al., “Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes,” Genome Biology, vol. 3, no. 7, RESEARCH0034, 2002.

[13] A. Hansson, B. K. Bloor, Y. Haig, P. R. Morgan, J. Ekstrand, and R. C. Grafström, “Expression of keratins in normal, immortalized and malignant oral epithelia in organotypic culture,” Oral Oncology, vol. 37, no. 5, pp. 419–430, 2001.

[14] K. Inoue, S. I. Kumakura, M. Uchida, and T. Tsutsui, “Effects of eight antibacterial agents on cell survival and expression of epithelial-cell-or cell-adhesion-related genes in human gingival epithelial cells,” Journal of Periodontal Research, vol. 39, no. 1, pp. 50–58, 2004.

[15] G. A. Rezniczek, J. M. de Pereda, S. Reipert, and G. Wiche, “Linking integrin α6β4-based cell adhesion to the intermediate filament cytoskeleton: direct interaction between the β4 subunit and plectin at multiple molecular sites,” The Journal of Cell Biology, vol. 141, no. 1, pp. 209–225, 1999.

[16] H. G. Gräber, G. Conradts, J. Wihlborg, and F. Lampert, “Role of interactions between integrins and extracellular matrix components in healthy epithelial tissue and establishment of a long junctional epithelium during periodontal wound healing: a review,” Journal of Periodontology, vol. 70, no. 12, pp. 1511–1522, 1999.

[17] N. Gürses, A. K. Thorup, J. Reibel, W. G. Carter, and P. Holmstrup, “Expression of VLA-integrins and their related basement membrane ligands in gingiva from patients of various periodontitis categories,” Journal of Clinical Periodontology, vol. 26, no. 4, pp. 217–224, 1999.

[18] M. Tanno, S. Hashimoto, T. Muramatsu, M. Matsuki, S. Yamada, and M. Shimono, “Differential localization of laminin γ2 and integrin β4 in primary cultures of the rat gingival epithelium,” Journal of Periodontal Research, vol. 41, no. 1, pp. 15–22, 2006.

[19] T. Kinumatsu, S. Hashimoto, T. Muramatsu et al., “Involvement of laminin and integrins in adhesion and migration of junctional epithelial cells,” Journal of Periodontal Research, vol. 44, no. 1, pp. 13–20, 2009.

[20] K. A. Brodgen, “Antimicrobial peptides: pore formers or metabolic inhibitors in bacterial,” Nature Reviews Microbiology, vol. 3, no. 3, pp. 238–250, 2005.

[21] O. Sørensen, N. Borregaard, and A. Cole, “Antimicrobial peptides in innate immune responses,” Contributions to Microbiology, vol. 15, pp. 61–77, 2008.

[22] S. H. Lee, H. K. Jun, H. R. Lee, C. P. Chung, and B. K. Choi, “Antibacterial and lipopolysaccharide (LPS)-neutralising activity of human cationic antimicrobial peptides against periodontopathogens,” International Journal of Antimicrobial Agents, vol. 35, no. 2, pp. 138–145, 2010.

[23] S. Krisanaprapornkit, A. Weinberg, C. N. Perez, and B. A. Dale, “Expression of the peptide antibiotic human β-defensin 1 in cultured gingival epithelial cells and gingival tissue,” Infection and Immunity, vol. 66, no. 9, pp. 4222–4228, 1998.

[24] J. M. Schröder and J. Harder, “Human beta-defensin-2,” The International Journal of Biochemistry and Cell Biology, vol. 31, no. 6, pp. 645–651, 1999.

[25] J. Harder, J. Bartels, E. Christophers, and J. M. Schröder, “Isolation and characterization of human β-defensin-3, a novel human inducible peptide antibiotic,” The Journal of Biological Chemistry, vol. 276, no. 8, pp. 5707–5713, 2001.

[26] J. Bissell, S. Joly, G. K. Johnson et al., “Expression of β-defensins in gingival health and in periodontal disease,” Journal of Oral Pathology and Medicine, vol. 33, no. 5, pp. 278–285, 2004.

[27] T. Bircher, R. Seibl, K. Bchnert et al., “Human Toll-like receptor 2 mediates induction of the antimicrobial peptide human beta-defensin 2 in response to bacterial lipoprotein,” European Journal of Immunology, vol. 31, no. 11, pp. 3131–3137, 2001.
[28] M. N. Becker, G. Diamond, M. W. Verghese, and S. H. Randell, "CD14-dependent lipopolysaccharide-induced β-defensin-2 expression in human tracheobronchial epithelium," *The Journal of Biological Chemistry*, vol. 275, no. 38, pp. 29731–29736, 2000.

[29] A. Dunsche, Y. Açıl, H. Dommsch, R. Siebert, J. M. Schröder, and S. Jepsen, “The novel human beta-defensin-3 is widely expressed in oral tissues,” *European Journal of Oral Sciences*, vol. 110, no. 2, pp. 121–124, 2002.

[30] M. Boniotto, W. J. Jordan, J. Eskdale et al., “Human β-defensin 2 induces a vigorous cytokine response in peripheral blood mononuclear cells,” *Antimicrobial Agents and Chemotherapy*, vol. 50, no. 4, pp. 1433–1441, 2006.

[31] A. Rizzo, R. Paolillo, E. Buommino et al., “Modulation of cytokine and β-defensin 2 expressions in human gingival fibroblasts infected with Chlamydia pneumoniae,” *International Immunopharmacology*, vol. 8, no. 9, pp. 1239–1247, 2008.

[32] S. Krisanaprakornkit, J. R. Kimball, A. Weinberg, R. P. Darveau, B. W. Bainbridge, and B. A. Dale, “Inducible expression of human β-defensin 2 by Fusobacterium nucleatum in oral epithelial cells: multiple signaling pathways and role of commensal bacteria in innate immunity and the epithelial barrier,” *Infection and Immunity*, vol. 68, no. 5, pp. 2907–2915, 2000.

[33] H. Dommsch, W. O. Chung, M. G. Rohani et al., “Protease-activated receptor 2 mediates human beta-defensin 2 and CC chemokine ligand 20 mRNA expression in response to proteases secreted by Porphyromonas gingivalis,” *Infection and Immunity*, vol. 75, no. 9, pp. 4326–4333, 2007.

[34] S. Krisanaprakornkit, J. R. Kimball, and B. A. Dale, “Regulation of human β-defensin-2 in gingival epithelial cells: the involvement of mitogen-activated protein kinase pathways, but not the NF-κB transcription factor family,” *The Journal of Immunology*, vol. 168, no. 1, pp. 316–324, 2002.

[35] K. Ouhara, H. Komatsuzawa, H. Shiiba et al., "Actinobacillus actinomycetemcomitans outer membrane protein 100 triggers innate immunity and production of β-defensin and the 18-kilodalton cationic antimicrobial protein through the fibronectin-integrin pathway in human gingival epithelial cells," *Infection and Immunity*, vol. 74, no. 9, pp. 5211–5220, 2006.