Streptococcus salivarius K12 and M18 Probiotics Reduce Periodontal Pathogen-induced Inflammation

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BACKGROUND AND RATIONALE

- The use of probiotics, beneficial bacteria, as a therapeutic option has gained significant popularity in western societies recently
- ***Streptococcus salivarius*** **K12 and M18 are commensal strains of the oral cavity that have been found to have probiotic activity against oral pathogens causing throat infections and halitosis; K12 has also been shown to have anti-inflammatory effects on bronchial epithelial cells[1]**
- **Both K12 and M18 were developed by BLIS Technologies and are currently sold in numerous probiotic products worldwide**
- **Periodontal disease, also an inflammation-related disease, is the leading cause of tooth loss in the developed world and is associated with various systemic diseases such as cardiovascular disease and diabetes[2]; it is one of the two leading dental diseases affecting populations worldwide. Gingivitis is highly prevalent all over the world, while advanced periodontitis with deep pockets affects 10%-15%[3]**
- **Numerous pathogenic bacteria have been found to be strongly associated with periodontal disease, specifically periodontitis, including *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, and Fusobacterium nucleatum*[4]**
- **Various beneficial bacterial species have been previously found to influence the inflammatory responses of human gingival fibroblast triggered by *A. actinomycetemcomitans* infection as measured by IL-8 production[5]**
- **Gingival fibroblasts are one of the first cells that encounter periodontal infections by anaerobic bacteria, like *P. gingivalis*, and have been shown to be involved in the immune response in periodontitis[6]**
- **Since inflammation is a key factor in periodontal disease, and probiotics have been shown to have beneficial effects in other inflammatory diseases, modifying the oral microflora with anti-inflammatory probiotics may be beneficial for the treatment and prevention of periodontal disease**

PRELIMINARY WORK

- **Multiple media and growth conditions were tested for all five bacterial strains used; *Streptococcus salivarius* (SS) K12 and M18, *Porphyromonas gingivalis* 33277 (PG), *Aggregatibacter actinomycetemcomitans* Y4 (AA) and *Fusobacterium nucleatum* 10593 (FN) to optimize growth and study conditions**
- **Overnight cultures of all strains were quantitated using spot plating and bacterial CFUs correlated with spectrophotometric measurements (OD 600nm)**
- **Gingival fibroblasts (GFs) were cultured from explanted tissue obtained from healthy volunteers undergoing periodontal procedures in the Oral Surgery Clinic at UWO in accordance with the guidelines of the University's Research Ethics Board with informed patient consent**
- **GFs were cultured in RPMI 1640 with 10% FBS and 100mM L-glutamine. Antibiotics (100U/ml penicillin, 100 ug/ml streptomycin, 10 ug/ml gentamicin) were added during routine cultivation but were not used for infection studies (passages 4-9 were used)**
- **An initial panel of nine cytokines/growth factors was used to generate an expression profile for the GFs: The selected panel involved IL-1b, IL-1ra, IL-6, IL-8, IL-10, FGF-2, TNF-α, and PDGF-AB/BB. Preliminary experiments showed only significant IL-6 and IL-8 expression; as a result, only these two factors were analyzed in the study**

OBJECTIVE

- **The objective of this study was to determine the effects of two probiotics, *S. salivarius* K12 and M18, on periodontal-pathogen-induced pro-inflammatory cytokine expression in human primary gingival fibroblast cells**

HYPOTHESIS

- **We hypothesized that K12 and M18 would inhibit pro-inflammatory cytokine production by gingival fibroblast cells in response to AA, FN, and PG**

METHODS

Day 1 – Prepared monolayers of primary gingival fibroblast cells (5 x 10^4 cells/ well in 24-well culture plate) and cultured them for 48 hours
(37˚C, 5% CO_2, humidified conditions)
Started overnight bacterial cultures in respective bacterial media and incubated at 37˚C with 5%CO_2 for AA, SS K12 and M18; and at 37˚C in anaerobic chamber/ jar for FN and PG

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Day 2 – Bacteria subcultured at 1: 100 dilution in fresh media: 50% bacterial media 50% cell culture media (RPMI 1640 with 10% FBS) or 100% cell culture media, and they were incubated overnight

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Day 3 – Bacterial cultures' turbidity was measured with spectrophotometer at OD 600 nm and concentrations were calculated using results from spot plating
Cell monolayers were washed twice with RPMI-1640 (supplemented) w/o antibiotics, and 500 ul media was added per well
Bacterial cultures were diluted to 10 ul containing MOI of 25:1 for infection and 10 ul aliquots were added to the corresponding wells
Plates were centrifuged at 3000 *g* and incubated at 37˚C and 5% CO_2

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Supernatants were harvested at various timepoints, pooled from two wells, cleared of debris at 10,000 *g*, and 750 ul of each was placed into -80˚C storage

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Supernatants were analyzed using Multiplex immunoassay kits for expression levels of IL-6 and IL-8, and the data were statistically analyzed using Analysis of Variance with Bonferroni's post hoc test

RESULTS

IL-6 Expression 4 Hours

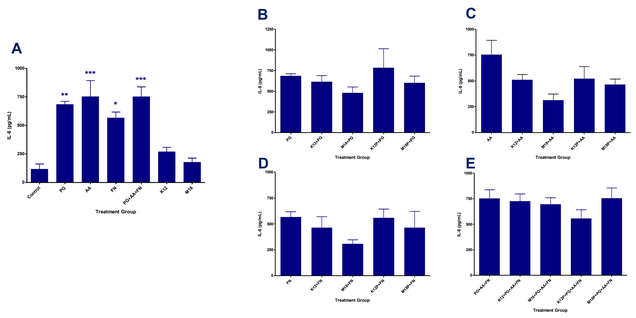

Fig. 1. IL-6 expression at 4 hours. All pathogenic bacteria induced significantly more IL-6 production at 4 hours than K12 and M18, or no bacteria. No significant differences were found in the amount of IL-6 produced in the various probiotic and pathogenic bacteria-treated conditions. Statistical significance: * *p<0.05*, ** *p<0.01*, *** *p<0.001 (N=3)*.

IL-6 Expression 8 Hours

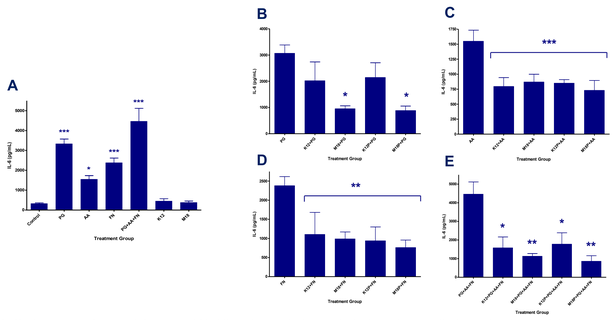

Fig. 2. IL-6 expression at 8 hours. (A) All pathogenic bacteria induced more IL-6 production at 8 hours than K12 and M18, or no bacteria. **(B)** M18 probiotic treatment significantly reduced IL-6 production in PG-treated cells with no difference between pre-treatment and simultaneous treatment. Both probiotics significantly reduced IL-6 production in cells challenged with either AA alone **(C)** , FN alone **(D)** or all three pathogens simultaneously **(E)**.
Statistical significance: * *p<0.05, ** p<0.01, *** p<0.001 (N=3)*.

RESULTS

IL-8 Expression 4 Hours

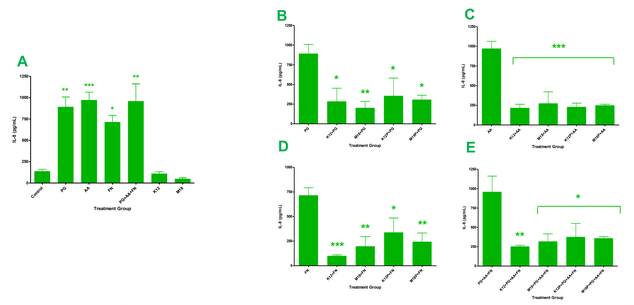

Fig. 3. IL-8 expression at 4 hours. (A) All pathogenic bacteria induced more IL-8 production at 4 hours than K12 and M18, or no bacteria. Probiotics significantly reduced IL-8 production in cells challenged with PG **(B),** AA **(C)**, FN **(D)**, and all three pathogens simultaneously **(E).** Statistical significance: * *p<0.05, ** p<0.01, *** p<0.001 (N=3)*.

IL-8 Expression 8 Hours

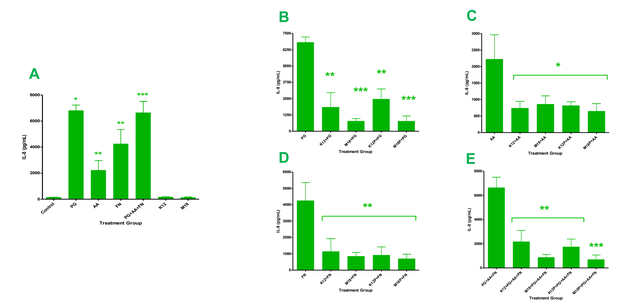

Fig. 4. IL-8 expression at 8 hours. (A) All pathogenic bacteria induced more IL-8 production at 8 hours than K12 and M18, or no bacteria. Probiotics significantly reduced IL-8 production in cells challenged with PG **(B),** AA **(C)**, FN **(D)**, and all three pathogens simultaneously **(E).** Statistical significance: * *p<0.05, ** p<0.01, *** p<0.001 (N=3)*.

SUMMARY & CONCLUSIONS

- **All three pathogens induced significantly increased levels of IL-6 and IL-8 at both 4 and 8 hours compared to the probiotic strains and the control *(Fig. 1-4A)***
- **Probiotic treatment resulted in no effects in IL-6 levels at 4 hrs, but at 8 hrs the reduction in IL-6 production was significant for all pathogens (*Fig. 1 & 2*)**
- **Similarly, probiotic treatment significantly reduced IL-8 production in all pathogenic challenge groups at both timepoints (*Fig. 3 & 4*)**
- **No differences were found between the two probiotics and between the pre-incubation and co-incubation conditions (*Fig. 1-4*)**
- **<u>CONCLUSION:</u> Commercially-available oral probiotic strains *S. salivarius* K12 and M18 were both able to downregulate the expression of cytokines associated with periodontal pathogen-induced inflammation**
- **<u>SIGNIFICANCE:</u> These probiotic strains may offer novel preventive and therapeutic options for patients who suffer from periodontal disease**

FUTURE DIRECTIONS

- **Investigating the effects of *S. salivarius* K12 and M18 on chronic inflammatory cytokine expression in various oral cell types**
- **Test the anti-inflammatory effects of the strains in periodontal patients**

ACKNOWLEDGEMENTS

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