Report on the faculty Development grant, Summer 2024:

In vitro Investigation of Hydrogen Peroxide Production and Susceptibility of Oral Streptococci Species and Respiratory Commensal Bacteria in Simulated Respiratory Lining Fluid "

Ray K. Saunders, Jr, Ph.D, Assistant Professor, Biology Department. January 2025

The goals of the faculty development were:

[1] Ascertain H_2O_2 production levels for Streptococci species in sRTLF

[2] Ascertain H_2O_2 production levels for lung commensal bacteria in sRTLF

[3] Ascertain H_2O_2 susceptibility for Streptococci species in sRTLF

[4] Ascertain H_2O_2 susceptibility for lung commensal bacteria in sRTLF

[5] Ascertain H_2O_2 production levels for Streptococci species in co-cultures with lung commensal bacteria

[6] Ascertain H_2O_2 production levels for lung commensal bacteria in co-cultures with Streptococci species

[7] Ascertain bacterial percentage of individual bacteria from co-cultures

[8] Collect and store protein samples

[9] Present data via faculty development grant presentation series or biology department meeting

These goals were met as described below:

[1]

Could not perform these experiments as *S. sanguinis* could not be revived from glycerol stocks. *S. mutans* (SM) was meant to be a negative control as it does not produce hydrogen peroxide (H_2O_2) under aerobic conditions.

[2]

To ascertain H_2O_2 production of lung commensal bacterial stains in simulated respiratory tract lining fluid (sRTLF), mid-log phase cultures of *S. pneumoniae* (SP) were inoculated in 100% Brain Heart Infusion (BHI) and 50% sRTLF – 50% BHI for 30 min before phenol red-horseradish peroxidase assay was performed. Concentration of H_2O_2 produced was determined by measuring absorbance at 610nm and comparing to H_2O_2 standards. As can be seen in Figure 1, growth in the sRTLF substantially reduced the concentration of H_2O_2 produced by SP in comparison to bacterial media (BHI).

[3]

To ascertain H_2O_2 susceptibility of streptococci species in sRTLF, mid-log phase SM cultures were inoculated in 100% BHI and 50% sRTLF, and H_2O_2 was added to both media at different final concentrations (30% H_2O_2 was diluted 1:10 in BHI before use.). The bacterial cultures were incubated overnight (24 h) before cell density of the inoculated media was determined by measuring absorbance at 600nm. As can be seen in Figure 2, incubation in 50% sRTLF reduced absorbance in comparison to BHI; however, cell density decrease followed the same overall trend in both media.

[4]

To ascertain H_2O_2 susceptibility of lung commensal bacterial stains in sRTLF, mid-log phase cultures of SP, *H. influenzae* (HP), and *M. cattarhalis* (MC) were inoculated in 100% BHI and 50% sRTLF – 50% BHI, and H_2O_2 was added to both media at different final concentrations. The bacterial cultures were incubated for 1 day (HI) or 2 days (SP, MC). Cell density of the inoculated sRTLF dilutions was determined by measuring absorbance at 600nm (OD600). As can be seen in Figure 3, for the tested lung commensals, incubation in 50% sRTLF reduced absorbance in comparison to BHI. In BHI, SP exhibited the most resistance to H_2O_2 stress, followed by MC and HI; this trend is replicated in the sRTLF. Also, noteworthy is that SP are displaying a high degree of resistance to a H_2O_2 concentration range that has been reported to be produced by Streptococcal species.

[5] [6]

Could not perform these experiments as intended as *S. sanguinis* could not be revived from glycerol stocks. To ascertain H_2O_2 production levels for co-cultures, mid-log phase cultures of SM and SP were incubated together in a 1:1 ratio in 100% BHI and 50% sRTLF for 30 min before phenol red-horseradish peroxidase assay was performed. Concentration of H_2O_2 produced was determined by measuring absorbance at 610nm and comparing to H_2O_2 standards. As can be seen in Figure 4, co-culturing SP and SM did not lead to a change in H_2O_2 production in either media.

[7]

Due to the autoclave being inoperable for a portion of the summer, the media needed to perform these experiments could not be sterilized.

[8]

To determine the impact of sRTLF on H_2O_2 susceptibility and production of bacterial species, protein samples were collected. In the above-mentioned experiments, at least one technical replicate was utilized for protein collection. One set of each experimental condition was centrifuged and re-suspended in lysis buffer, and placed at -80°C.

[9]

The above-mentioned data will be prepared as an oral presentation to be included in the Faculty Research Symposium. Moreover, a portion of the data may be presented by a student at the Louis Strokes Alliance for Minority Participation (LSAMP) research symposium held in the Spring.

Conclusions:

The faculty development grant permitted me to explore the connection between hydrogen peroxide production and susceptibility among microorganisms that would be interacting within the upper respiratory tract. Some of the experiments could not be performed due to one of the principal microbes not reviving from long-term stock; issues with the autoclave; and, to a lesser extent, delays in receiving materials. However, my results provide preliminary insights that I can build upon in the future. The microbes in this study exist primarily in an aerobic environment and would encounter the various reactive oxygen species, notably H_2O_2, either through their metabolic processes or as a released compound from other bacteria. Experiments from a previous faculty development grant demonstrated that growth of lung commensal bacteria in sRTLF is mitigated in comparison to bacterial media. To observe SP generating H_2O_2 in the sRTLF indicates that this is a means for the microbe to inhibit and/or kill other bacterial species in the upper respiratory tract. Conversely, to observe lung commensal bacteria (SP, MC) display a substantial degree of resistance to H_2O_2 stress, while in sRTLF, would support the notion that these microbes possess adaptive/counter mechanisms for this agent. Further supporting a role for H_2O_2 in shaping the respiratory microbiome is the observation that SM, a principal microbe in the oral cavity not found in the respiratory tract, displays a high susceptibility to H_2O_2. While there was no change in H_2O_2 production observed in the SP-SM co-culture experiments, this could be attributed to the co-culture incubation period being short. Moreover, viability of the individual bacterial species would have to be determined (impacted by autoclave issue).

There are several future experiments that could be conducted. These include ascertaining growth kinetics of the bacteria exposed to H_2O_2; inhibitory studies with catalase to demonstrate the extent of antibacterial effect of H_2O_2; and additional co-culture experiments to study changes in H_2O_2 production. Moreover, this project has already resulted in a research opportunity for a Lincoln student. Those supplemental experiments focused on the growth of oral and lung commensal bacteria in media reflecting a transition from the oral cavity to the upper respiratory tract. Along with some repeats of experiments in this study, that data will be presented at an upcoming LSAMP research symposium. This faculty development grant has also aided BIO4001L and BIO4002L; materials to evaluate H_2O_2 production will be used to supplement lab exercises on biochemical tests.

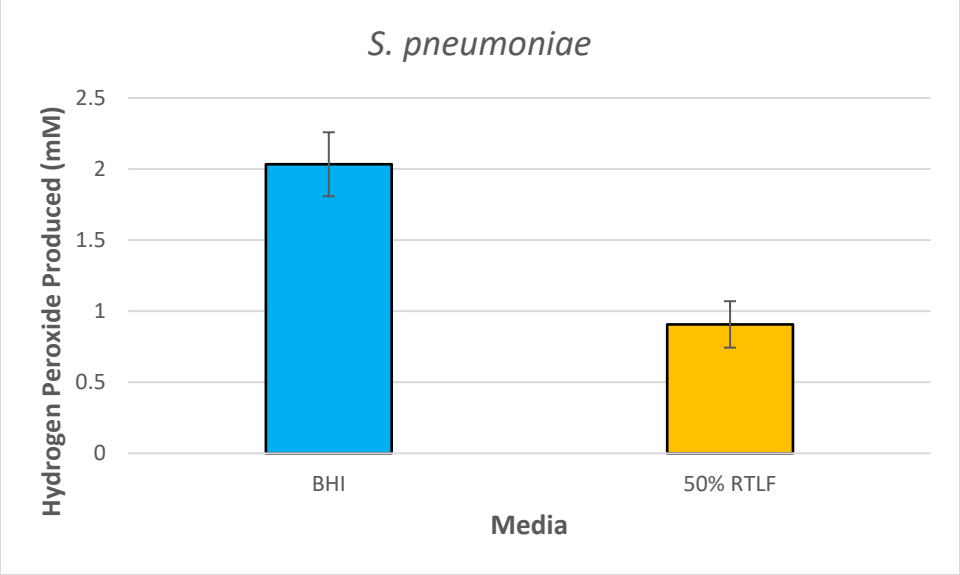


Figure 1: Incubation of *S. pneumoniae* in sRTLF decreases H_2O_2 production. Planktonic *S. pneumoniae* were incubated in BHI and 50% sRTLF before a phenol red-horseradish peroxidase assay was performed and absorbance measured at OD610. Absorbance measurements were correlated with those of H_2O_2 standards to determine H_2O_2 concentration. Results are mean ± SD; representative graph of two experiments with three technical replicates.

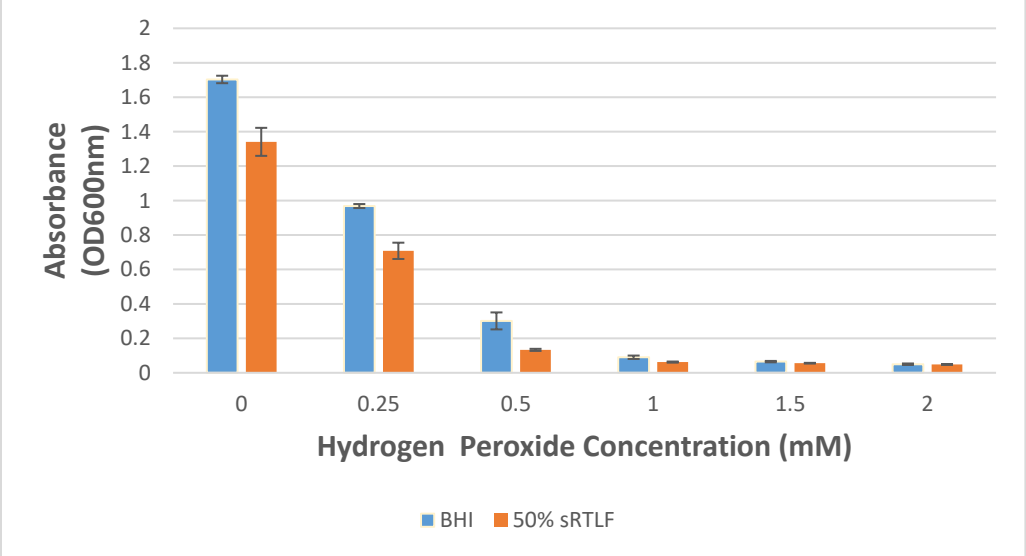
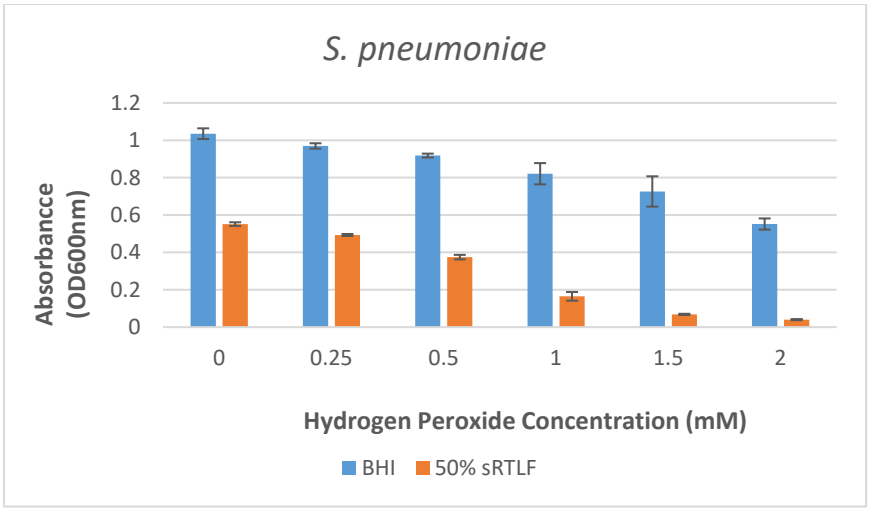
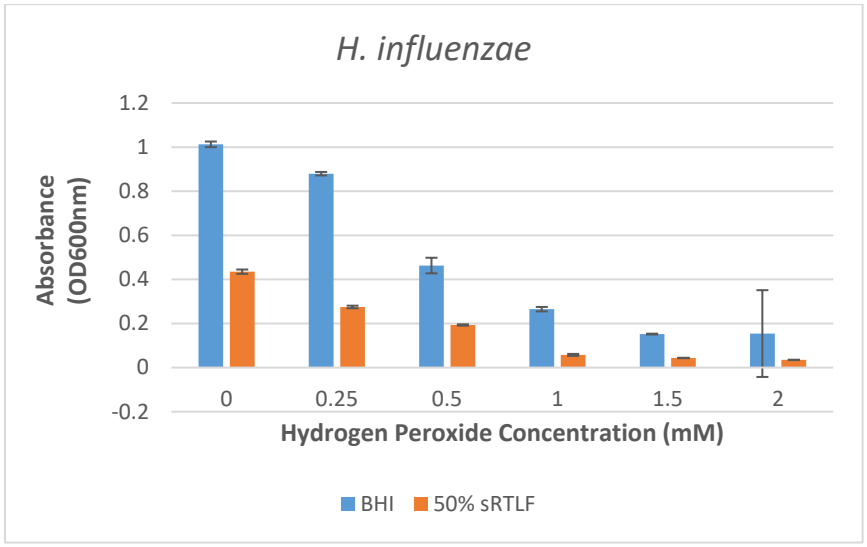


Figure 2: Exposure of *S. mutans* to increasing concentrations of H_2O_2 reduces cell density. Planktonic *S. mutans* were incubated in BHI and 50% sRTLF supplemented with different concentrations of H_2O_2. Concentration range utilized mimics the concentration range reported for Streptococci species that produce and release H_2O_2. After 24 h incubation, absorbance measured at OD600. Results are mean ± SD; representative graph of three experiments.


S. pneumoniae
Absorbancce (OD600nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
0.25
0.5
1
1.5
2
Hydrogen Peroxide Concentration (mM)
BHI
50% sRTLF


H. influenzae
Absorbance (OD600nm)
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
0.25
0.5
1
1.5
2
Hydrogen Peroxide Concentration (mM)
BHI
50% sRTLF

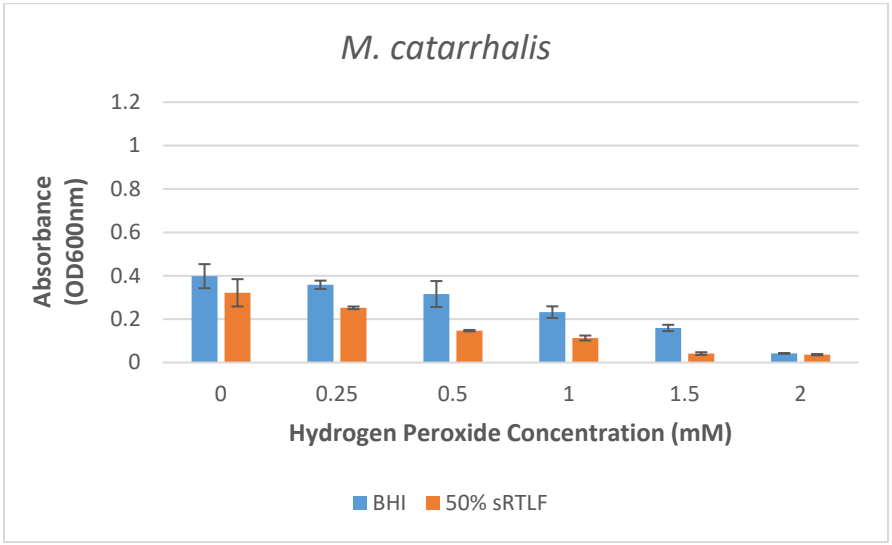

M. catarrhalis
Absorbance (OD600nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
0.25
0.5
1
1.5
2
Hydrogen Peroxide Concentration (mM)
BHI
50% sRTLF

Figure 3: Exposure of lung commensals to increasing concentrations of H_2O_2 reduces cell density. Planktonic *S. pneumoniae, H. Influenzae,* and *M. cattarhalis* were incubated in BHI and 50% sRTLF supplemented with different concentrations of H_2O_2. Concentration range utilized mimics the concentration range reported for Streptococci species that produce and release H_2O_2. After incubation, absorbance measured at OD600. Results are mean ± SD; representative graph of three experiments.

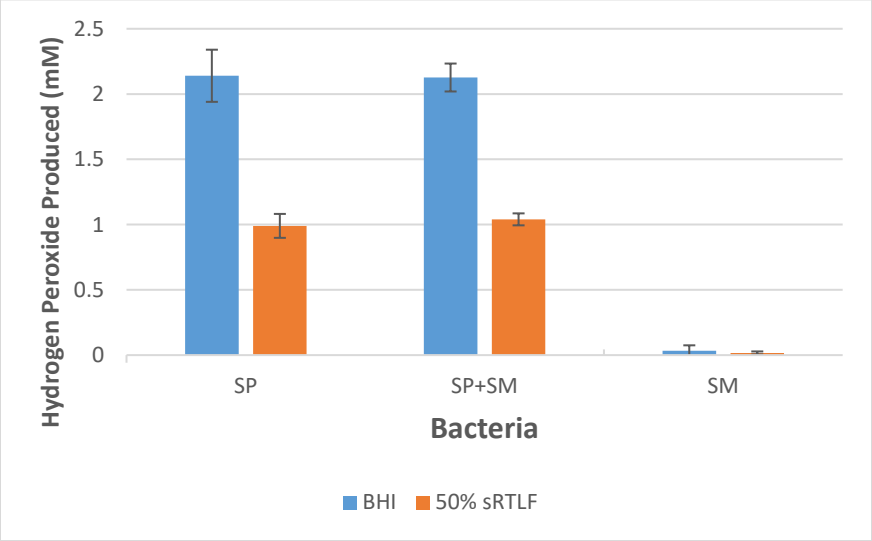


Figure 4: *S. pneumoniae* – *S. mutans* co-culture did not alter H_2O_2 production. Planktonic *S. pneumoniae* and *S. mutans* were incubated, together in a 1:1 ratio, in BHI and 50% sRTLF before a phenol red-horseradish peroxidase assay was performed and absorbance measured at OD610. Absorbance measurements were correlated with those of H_2O_2 standards to determine H_2O_2 concentration. Results are mean ± SD; representative graph of one experiment with three technical replicates.