Comparison of PRAS and Non-PRAS Media for Anaerobes

Abstract

This study shows that PRAS (pre-reduced anaerobically sterilized) media are beneficial for isolating and growing anaerobes. A PRAS plate, a Non-PRAS plate, and a plate that has been reduced post sterilization were struck with *Bacteroides fragilis*, *Fusobacterium nucleatum*, and *Porphyromonas levii*. A statistical analysis was done on the data. Photos of plates showed additional differences between these media.

Introduction

Our purpose is to illustrate the impact of reduced media on the isolation and growth of anaerobes in a clinical laboratory. Agar plates for anaerobes can be exposed to oxygen during preparation and again after being formed. Microbiologists found that some anaerobes were sensitive to oxidized products formed during preparation. Hence the practice of pre-reducing media before sterilization was developed. Media prepared this way was called PRAS.

Anaerobes make up a diverse group in regard to tolerance or sensitivity to oxygen.[1] Members of the *Bacteroides fragilis* group or *Clostridium perfringens* are known as 'moderate' anaerobes surviving oxygen levels up to 2%-8%.[2] Other anaerobes are sensitive to oxygen at much lower levels.

Three anaerobes were used for this study: *Bacteroides fragilis*, *Fusobacterium nucleatum*, and *Porphyromonas levii*. Each microorganism was taken to a known cell density in which it could be recovered without being too numerous to count and was spread across the entire surface of the petri dish. The base agar used in this experiment is the commercially available Brucella Agar with 5% sterile defibrinated sheep blood post additive. Three different versions of this media were used in this experiment. The first being PRAS where sodium bisulfite was added to reduce the media before sterilization, the second being Non-PRAS where the sodium bisulfite was omitted, and the third being Non-PRAS, no added sodium bisulfite, with a post reduction by being placed into an anaerobic environment after production.

Methods

The commercially available Brucella Agar medium with 5% sterile defibrinated sheep blood post additive was used to make the PRAS plates in this experiment. Non-PRAS media were created by using individual reagents. Sodium bisulfite is included in the commercial Brucella formulation. It was necessary to use individual reagents to omit it. All three versions of this media were supplemented with Vitamin K and Hemin. The PRAS formulation also contained the Oxyrase Enzyme System at a concentration of 300u/L. The Non-PRAS post reduction formulation was bagged individually with an Ageless oxygen absorber and allowed to sit for a minimum of 48 hours to ensure all oxygen was removed prior to plating. The strict Non-PRAS plates used were not reduced before sterilization nor after production. Therefore, they were bright red, oxidized plates.

Using a spectrophotometer at 600nm, an approximate cell density was determined based on known values of the McFarland turbidity standards. All three organisms were taken to an

approximate $1x10^3$ cfu/mL. It was determined in a separate experiment that this cell density was optimum for having quantifiable colonies without it being too numerous to count. This cell density was achieved through a series of 10-fold dilutions using a sterile potassium phosphate buffer that had been reduced with Oxyrase for Broth.

A single drop (~50uL) of inoculum was placed onto each plate and spread across the entire surface. The same inoculum was used for each organism, 30 plates of each type of medium were plated in quick succession. The plates were then incubated anaerobically for 72 hours at 37ºC in an anerobic jar. All colonies on each plate were counted and recorded.

An analysis of the data was performed to determine statistical significance between the three types of media for each organism. This was done using a T test which uses the following equation:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(s^2(\frac{1}{n_1} + \frac{1}{n_2}))}}$$

In this formula, *t* is the *t* value, x_1 and x_2 are the means of the two groups being compared, s_2 is the pooled standard error of the two groups, and n_1 and n_2 are the number of observations in each of the groups. A larger t value shows that the difference between group means is greater than the pooled standard error, indicating a more significant difference between the groups. The T-value can be compared against the values in a critical T-value chart to calculate a P-value. If the P-value is less than .05, there is a statistically significant difference between the two data sets.[3] This calculation was performed on all three data sets, the Post Reduction plates and Non-PRAS plates were compared to the PRAS plates.

Results

Raw plate count data are shown in Figure 3.

Statistical analysis showed there was no significance between the three types of media for *B. fragilis*. However, *F. nucleatum* and *P. levii* performed significantly better on PRAS media compared to the Non-PRAS media. *F. nucleatum* recovery was enhanced on the Non-PRAS media that had been reduced post-production.

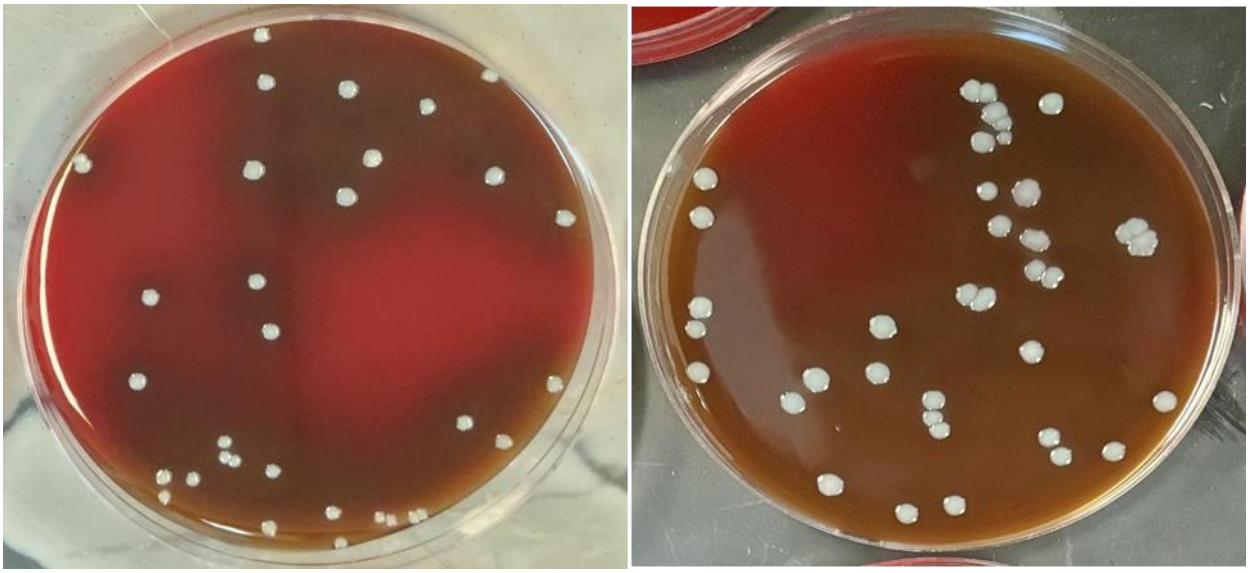
Quantitative analysis shows one part of the importance of reduced media for isolating and growing anaerobes. Photographs of these anaerobes growing on plates shows another. For example, even though there was no significant difference quantitatively between the media types for *B. fragilis*, it grew larger, more luxurious colonies on the PRAS medium compared to the Non-PRAS medium. This was shown photographically.

Photos of *P. levii* showed it grew at lower levels on Non-PRAS plates compared to PRAS plates. It also formed pin-point colonies on Non-PRAS plates too. This anaerobe could be missed on a Non-PRAS plate from a mixed culture specimen.

Fig. 1. Photographs of each organism to demonstrate the differing colony sizes.

Bacteroides fragilis

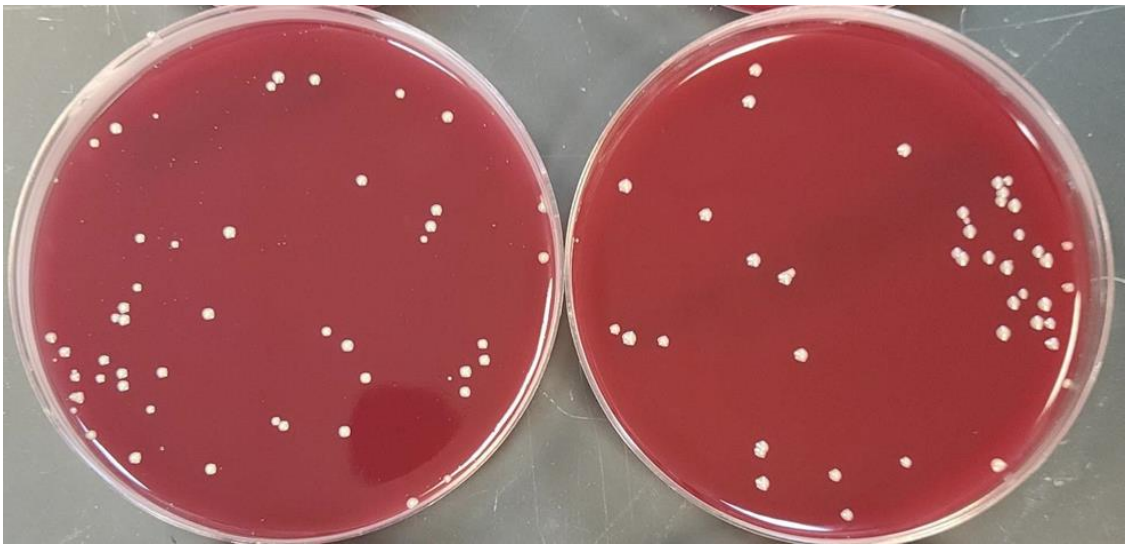
Non-PRAS PRAS



Colony sizes on average were about 2 to 4mm in diameter on the Post Reduced and Non-PRAS plates whereas on the PRAS plate, colonies were 2 to 7mm.

Fusobacterium nucleatum

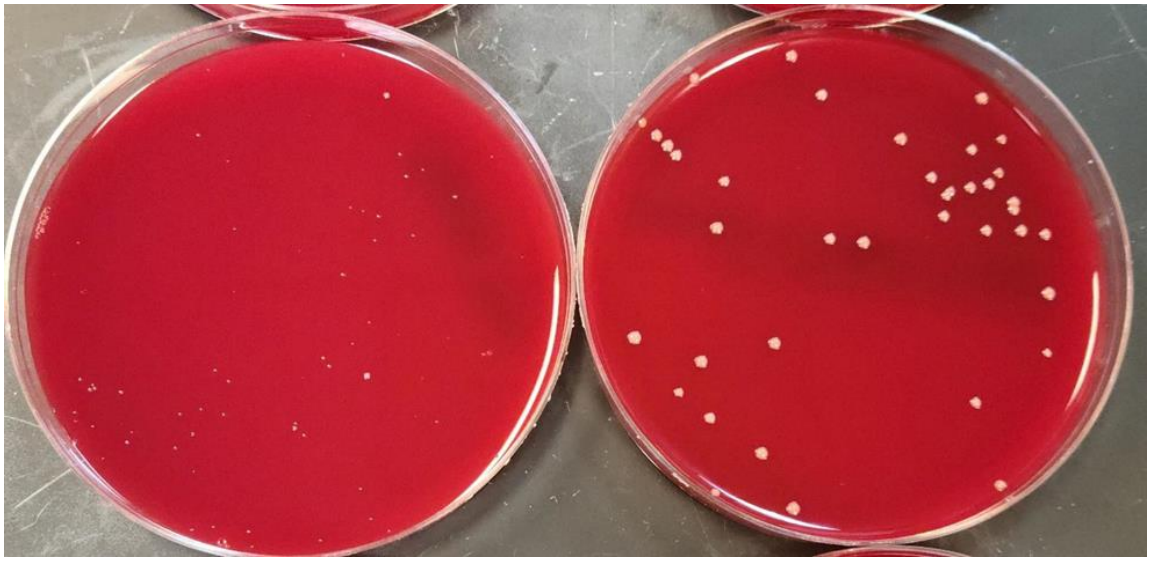
Non-PRAS PRAS



No significant difference in colony size was observed. Colony count significantly greater on PRAS versus Non-PRAS.

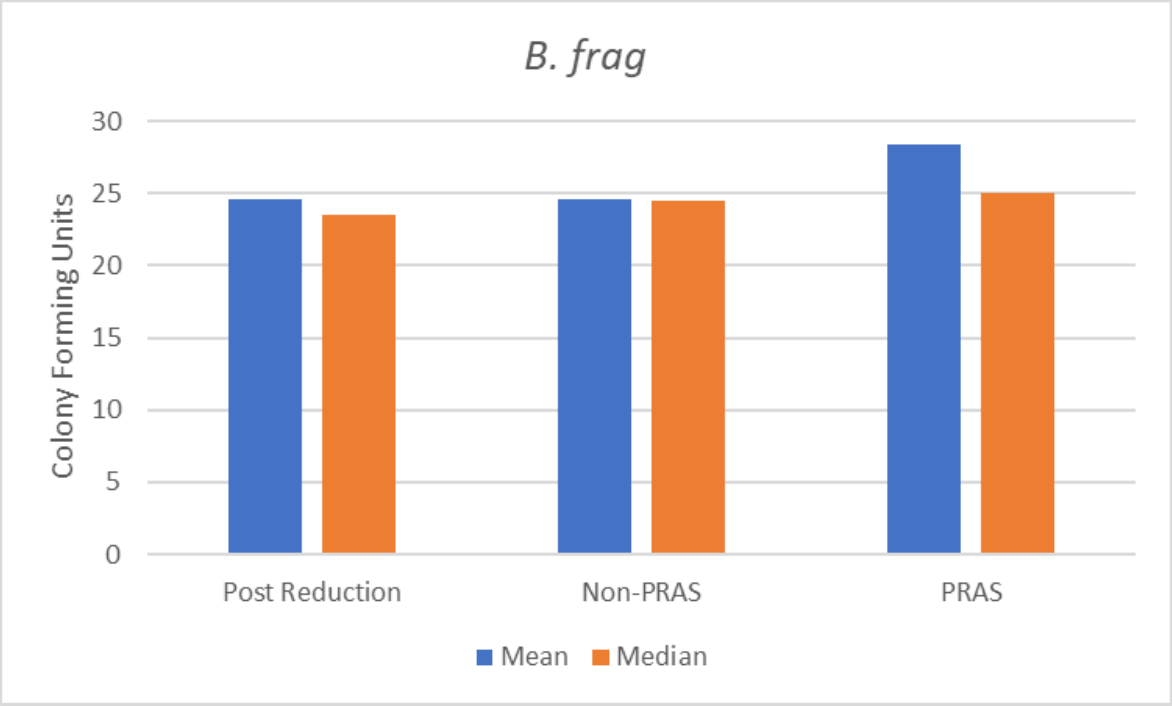
Porphyromonas levii

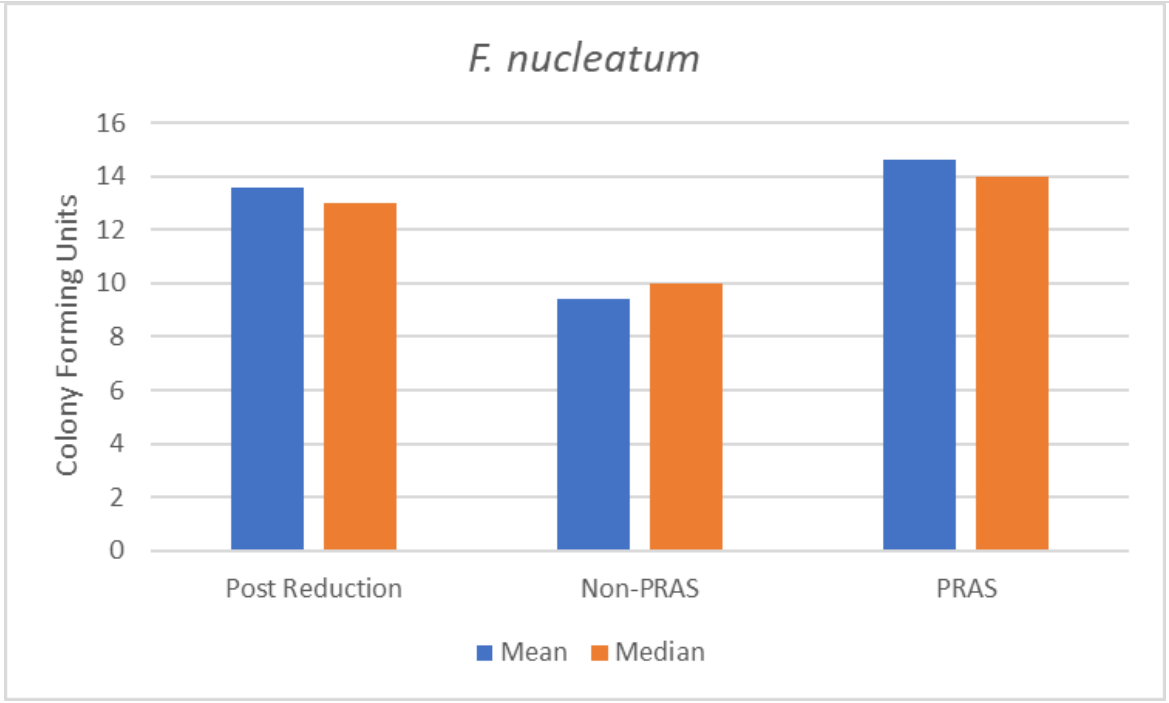
Non-PRAS PRAS



Colony sizes were pinpoint on the Post Reduced and Non-PRAS plates and 1 to 3 mm in diameter on the PRAS plates.

Fig. 2. Comparison of the descriptive statistics for each media type and organism struck.




F. nucleatum
Colony Forming Units
16
14
12
10
8
6
4
2
0
Post Reduction
Non-PRAS
PRAS
Mean
Median

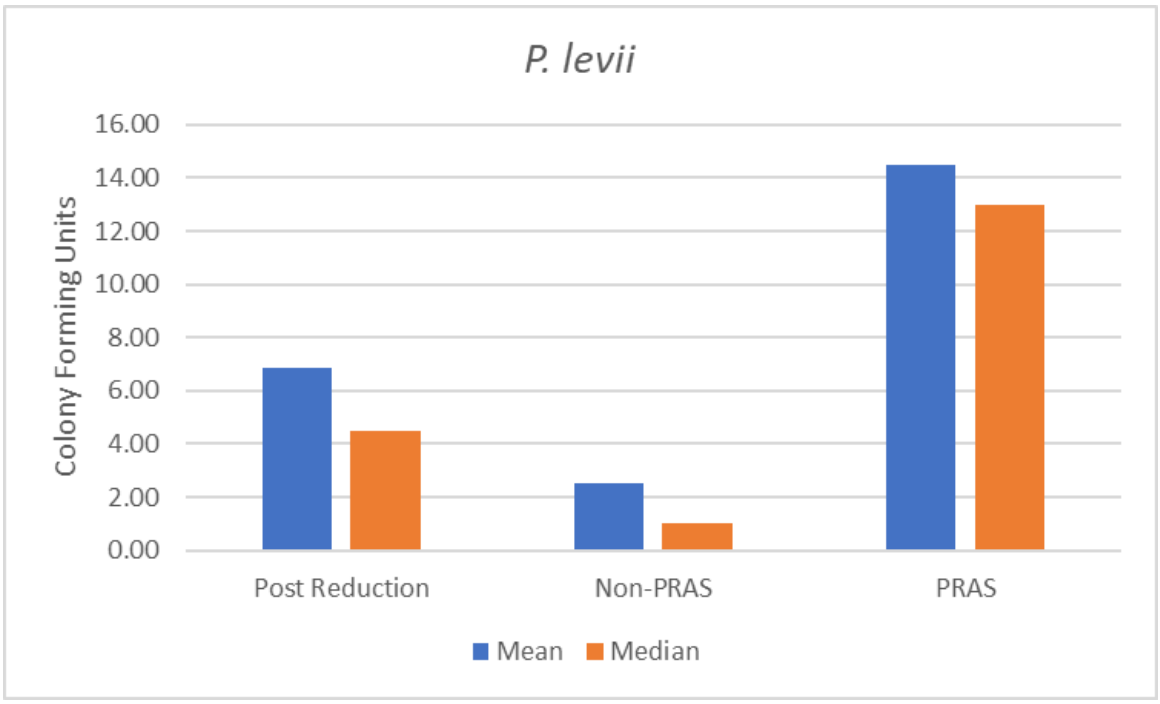

P. levii
Colony Forming Units
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Post Reduction
Non-PRAS
PRAS
Mean
Median

Fig. 3. Actual data points from each experiment

B. fragilis				*F. nucleatum*			
Post Reduction	Non-PRAS	PRAS		Post Reduction	Non-PRAS	PRAS	
13	12	17		4	2	7	
15	17	17		4	3	7	
15	17	18		7	5	7	
17	18	19		9	5	8	
17	19	19		9	6	11	
18	20	19		10	6	12	
19	20	21		11	6	12	
20	20	22		12	6	13	
22	22	23		12	7	13	
22	23	23		12	7	13	
22	23	24		12	8	14	
23	23	24		12	8	14	
23	23	24		13	9	14	
23	24	25		13	9	14	
23	24	25		13	10	14	
24	25	25		13	10	14	
25	25	26		14	10	16	
26	25	27		15	10	16	
27	26	30		15	11	16	
27	26	30		15	11	16	
29	26	31		15	11	16	
29	26	33		16	11	16	
29	26	35		18	11	17	
29	27	36		18	12	17	
31	27	36		18	12	17	
33	30	38		18	14	19	
33	34	42		19	14	19	
34	34	44		19	15	20	
35	37	46		20	16	20	
35	39	54		21	18	26	
24.60	24.60	28.43	Mean	13.57	9.43	14.60	Mean
23.5	24.5	25	Median	13	10	14	Median
13	12	17	Min	4	2	7	Min
35	39	54	Max	21	18	26	Max

P. levii

Post Reduction	Non-PRAS	PRAS	
0	0	9	
0	0	9	
0	0	9	
1	0	9	
1	0	10	
1	0	11	
2	0	11	
2	0	11	
2	1	12	
3	1	12	
3	1	12	
3	1	12	
4	1	13	
4	1	13	
4	1	13	
5	1	13	
5	2	14	
7	2	15	
7	2	15	
7	2	15	
8	2	16	
8	2	17	
9	2	18	
10	2	18	
11	3	19	
12	4	19	
16	9	21	
17	9	21	
22	13	23	
32	14	24	
6.87	2.53	14.47	Mean
4.5	1	13	Median
0	0	9	Min
32	14	24	Max

Conclusion

This study shows that anaerobes are a diverse group when compared by their response to oxygen and oxidized products. They form a spectrum from tolerant to sensitive. Media exposed to oxygen affects subsequent isolation and growth of anaerobes. If media were pre-reduced and anaerobically sterilized (PRAS), most anaerobes grew better. Additional benefits were found for some anaerobes when media were reduced after being made but before use to grow them (Post Reduction). These oxygen effects on media were quantified and statistically analyzed. More was learned by recording observations photographically. This study shows that reduced media preparation and pre-reduction is important to the successful isolation and recovery of anaerobes.

References

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2. Nagy, E., Boyanova, L., & Justesen, U. S. (2018). How to isolate, identify and determine antimicrobial susceptibility of anaerobic bacteria in routine laboratories. *Clinical Microbiology and Infection*, *24*(11), 1139–1148. https://doi.org/10.1016/j.cmi.2018.02.008

3. Bevans, R. (2022, December 19). *An introduction to T tests: Definitions, formula and examples*. Scribbr. Retrieved April 12, 2023, from https://www.scribbr.com/statistics/t-test