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March 30, 2017

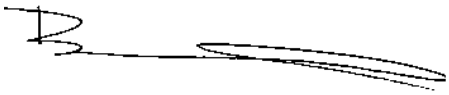
RE: Decision on your PeerJ submission: "Impact of enzymatic digestion on bacterial community composition in CF airway samples" (#2016:12:15267:0:0:REVIEW)

Dear Dr. Tulkens,

Thank you for the opportunity to address the concerns regarding our manuscript entitled, “Impact of enzymatic digestion on bacterial community composition in CF airway samples.” We are pleased to submit our revised manuscript for your review and are hopeful that the current revision is satisfactory for publication in PeerJ.

We appreciate the careful review of our manuscript and would like to thank the reviewers for providing suggestions that have led to significant improvements in our paper. We have addressed the reviewers’ comments and have documented our responses in detail as follows:

Sincerely,



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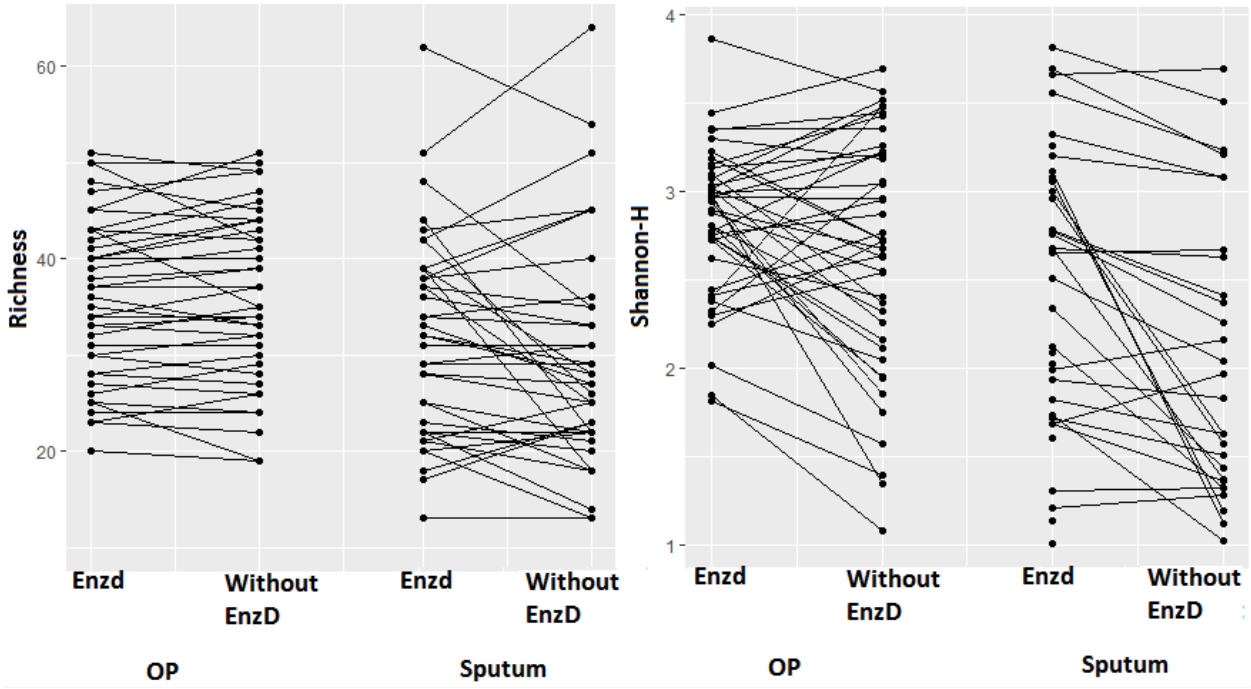
Reviewer 1:

Comment #1: I find myself distracted by the large range in patient age and wondering whether the initial alpha diversity of the samples [e.g. younger patients have higher diversity in sputum (Cox et al. 2010)] contribute to the overall effect of the enzD. In Fig. 3B, a subset of patients clustered tightly together – the authors briefly mention this group as primarily represented by Veillonella, but not whether these are adult patients with low initial diversity or whether other Gram- taxa were dominant.

RESPONSE: We completely agree with the reviewer that the initial alpha diversity of the samples used in this study could be affected by the differences in patient ages, however, the use of duplicate aliquots of the sample for comparison, result in each patient being used as their own control. In addition, the age range of subjects with sputum samples corresponding to Figure 3B is more narrow (9-24 years) than the OP sample type (1.5-23 years), this information has been added to the paper. To further address this question though, we have included an additional figure (Figure S3 copied below). From this figure, we did not see any indication that samples with lower initial alpha diversity reacted differently to EnzD compared to those with higher initial alpha diversity. We also feel that the use of samples from subjects with a large range in age make the results generalizable to a larger population since we didn't specifically focus on a narrow age range.

Figure S3.



Comment #2: The authors do not mention correcting p values for multiple comparisons following Wilcoxon Signed rank testing of the relative abundances for genera and phyla. This should be performed and corrected p values reported.

RESPONSE: Thank you for the opportunity to address this issue. We have performed the Benjamini-Hochberg correction for multiple testing on the Wilcoxon p-values, the results were robust after adjusting for multiple comparisons. We have updated the statistical analysis methods section to reflect the change as follows:

> **"***Differences in phyla and genera between EnzD samples versus non-EnzD samples with a median RA of at least 1% were evaluated using Wilcoxon Signed rank test for paired samples. Benjamini-Hochberg corrections were used to account for multiple comparisons* (Benjamini & Yekutieli, 2001)(Benjamini & Yekutieli, 2001)*."*

Furthermore, Table 2 and Figure 1 have been revised to include the corrected p-values.

Comment #3: The authors mention that there are three distinct clusters in the sputum samples. I think it would be worthwhile to report the age distribution and average alpha-diversity of the patients within each cluster and to validate these clusters statistically (or remove mention of the clusters).

RESPONSE: We agree with the reviewer that the clusters were not well described and have removed mention of them since it detracts from the main point of these figures. The beta diversity section has been updated to the following:

> *"As indicated in Figure 3B, sputum samples showed less impact from EnzD, with a large group of samples having limited changes in MH. A subset of the sputum samples demonstrated large changes in MH with EnzD, but with no consistent direction of change as was seen in the OP samples (Figure 3B). Sputum appears to be more heterogeneous compared to OP samples. Sputum was primarily influenced by enhanced extraction of Staphylococcus, Veillonella, and Streptococcus (Figure 3B). This result is consistent with Table 2 where Staphylococcus and Veillonella are shown to have a larger change than Streptococcus."*

Comment #4: Shannon diversity was reduced with enzD in sputum samples (and, to some extent, the OP samples). I agree that this is likely due to increased relative abundance of Gram+ and a less even community, but it would be helpful to untangle the richness and evenness and calculate these metrics separately (richness and Pielou's Evenness). I'd hypothesize that richness would increase in enzD-treated samples while evenness would decrease.

RESPONSE: The reviewer makes an excellent point, and we have now added specific analyses for richness and evenness to address which component of alpha diversity might be driving the differences. These results have been added to the text and are copied below.

> *"The Shannon-H alpha Diversity Index was significantly lower ($p < 0.01$) with EnzD in sputum samples (Figure S3) due to higher RA of Staphylococcus resulting in a*

significantly less even community ($p < 0.01$). For OP samples, the Shannon-H alpha Diversity Index was also lower likely due to higher RA of Streptococcus with EnzD, although this result did not reach statistical significance ($p = 0.06$). Richness and evenness were also not significantly affected by EnzD in OP samples. Changes in alpha diversity due to use of EnzD do not necessarily correspond to changes in microbial community composition as measured using beta diversity (Figure S4)."

Comment #5: "Standard homogenization" procedures were used to process sputum samples. What does this refer to? Did they use sputolysin to homogenize the samples?

RESPONSE: Thanks for pointing out that this was unclear, sputum homogenization followed CF Foundation guidelines and utilized sputalysin. We have modified the manuscript to specify sputalysin was used.

"Standard sputum processing protocol was performed for sample homogenization utilizing sputalysin and standard CF culture was performed following CF Foundation guidelines(Burns et al., 1998)*."*

Comment #6: The legends for Figures 2 and 4 could use more detail.

RESPONSE: We thank the reviewers for their critique. The legends for Figures 2 and 4 have been updated to the following:

"Figure 2. Boxplots showing distribution of Morisita-Horn (MH) similarity metric for paired samples in OP and sputum. Wilcoxon rank sum tests show the distributions between the two sample types were statistically different ($p<0.01$). The MH metric ranges from 0-1 with a value of 1 indicating identical communities, and 0 indicating no overlap between communities. The line indicates the median value and the box ranges from the 25th and 75th percentiles, the whiskers extend to 1.5times the interquartile range."

"Figure 4: A) Scatterplot of relative abundance of Gram-positive taxa in non-EnzD samples versus Morisita-Horn (MH) similarity metric of paired samples. MH metric ranges from 0-1 with a value of 1 indicating identical communities, and 0 indicating no overlap between communities. OP samples are shown in red and sputum in blue. B) Scatterplot of the difference in relative abundance of Gram-positive taxa versus MH of paired samples OP samples are shown in red and sputum in blue."

Comment #7: Figure 2 would benefit from an arrow along the y-axis to indicate that values approaching 0 are less similar and values approaching 1 are more similar. The MH index is opposite some of the other commonly used beta-diversity indices used in microbiome studies where 0=identical and 1=no overlap. The authors should also include the p value on this figure/within the legend.

RESPONSE: Thank you for the helpful suggestion, we agree it is important to address the representation of MH as a similarity metric rather than a dissimilarity matrix in this paper. The legend for Figure 2 has been updated to include this information as indicated above.

Comment #8: The authors discuss in the Materials and Methods that these samples were also sent for standard culture. Did enzD increase molecular detection of Gram positive taxa in specimens that also cultured G+ bacteria?

RESPONSE: This is a great question, the only gram positive bacteria detected by culture was S. aureus. Our first paper (Johnson et al., 2016) addressed this question specifically by investigating whether increased sensitivity for *Staphylococcus* was obtained after EnzD compared to the clinical culture and qPCR results. This current paper builds upon that work by investigating changes in the entire community rather than simply focusing on *Staphylococcus*. To better clarify this point, we have expanded the discussion of our first paper in the Introduction.

> *"Cystic Fibrosis (CF) is an autosomal recessive disease, characterized by chronic airway infection, whose predominant pathogens include Staphylococcus aureus, Pseudomonas aeruginosa and other Gram-negative bacteria*(Heijerman, 2005; LiPuma, 2010)*. OP swabs are commonly used when children are unable to expectorate* (Zemanick et al., 2015)*. This is particularly true for pediatric, non-expectorating subjects with CF in whom oropharyngeal (OP) cultures are used as a surrogate for lower airway bacteria* (Zemanick et al., 2015)*. In the pediatric studies, OP swabs have ranged from 30% to 68% of samples collected* (Armstrong et al., 1996; Zemanick et al., 2010; Wolter et al., 2013; Hoppe et al., 2015) *and therefore represent an important sample type in early CF.*
>
> *Sequencing is becoming more widely used to evaluate bacterial communities in CF airway samples* (Cummings et al., 2016)*. DNA preparation prior to sequencing, including cell lysis, can have a profound effect on microbial community composition especially in detecting certain organisms such as S. aureus* (Yuan et al., 2012; Zhao et al., 2012; Willner et al., 2012; Lozupone et al., 2013; Pérez-losada, Crandall & Freishtat, 2016)*. S. aureus in particular has a rigid cell wall that can be hard to rupture, hindering the ability to efficiently extract DNA for molecular detection*(Zhao et al., 2012)*.*
>
> *Previous studies have indicated that use of enzymatic digestion (EnzD) may enhance the ability to detect Staphylococcus*(Schindler & Schuhardt, 1964; Browder et al., 1965; Yuan et al., 2012; Zhao et al., 2012; Johnson et al., 2016)*. Specifically, Yuan et al (2012) performed a comprehensive experiment evaluating multiple DNA extraction methods utilizing human associated bacterial species as well as a mock community. Zhao et al. (2012) found that EnzD increased the yield of Staphylococcus in sputum samples. Similarly, Johnson et al (2016) determined EnzD improved the sensitivity of sequencing in OP swabs while no differences were observed in sputum samples. Increased sensitivity with EnzD was robust when compared to clinical culture results and qPCR. Furthermore, the work showed that the majority of Staphylococcus was S. aureus. It remains unclear, however, how the use of EnzD effects the remaining bacterial community in clinical CF samples. In this work, we build on these two previous CF studies to evaluate the effect of DNA extraction using enzymatic digestion (EnzD) on bacterial communities detected from oropharyngeal swab (OP) and sputum samples collected from patients with CF."*

Comment #9: Mechanical lysis (bead-beating) is another commonly used modification to DNA extraction protocols and can help break apart fungal cell walls. There is no mention of alternative lysis methods throughout the manuscript, and I wonder whether mechanical lysis in the presence or absence of enzD can also increase abundance of

gram positive taxa within a sample. The authors should discuss mechanical lysis methods in the context of their results in the discussion.

RESPONSE: The reviewer makes an excellent point. Mechanical lysis is commonly employed in microbiome studies. We have aligned our methods with the clinical laboratory to facilitate translation of these approaches to patient care. The clinical lab utilizes the Qiagen EZ1, which does not use a mechanical lysis step. We therefore did not utilize mechanical lysis, and unfortunately cannot provide any insight on this point. The goal of this project was to evaluate the enzymatic digest proposed by Zhao et al (2012) in pediatric samples and we did not perform a systematic evaluation of lysis methods.

Comment #10: Line 79: 16S rRNA gene Amplicon Library Construction.

RESPONSE: We thank the reviewers for the correction and have updated the section header to reflect the correction.

Reviewer 2:

Comment #1: Adequate, except does not do a good job citing prior work, explaining EnzD, or even in discussing why it would be important to look at an OP sample in a CF patient.

RESPONSE: We thank the reviewer for the opportunity to address this issue. We have included both the use of EnzD and the importance of OP samples in CF patients in the revised paper. The following sections have been added:

> *"EnzD was performed on one replicate of each sample by mixing with lysostaphin (final concentration 0.18 mg/mL) and lysozyme (3.6 mg/mL) and incubated at 37°C for 30 minutes. Samples were then digested with proteinase K (1.4 mg/mL) and incubated at 65°C for ten minutes, then incubated at 95°C for 10 minutes.*(Zhao et al., 2012; Johnson et al., 2016). *Lysozyme and lysostaphin target degradation of the bacterial cell wall by targeting peptidoglycan (lysozyme) and pentaglycine bridges (lysostaphin)* (Salazar & Asenjo, 2007). *Lysostaphin is specific to the subset of staphylococci that contain pentaglycine bridges including the human pathogen S. aureus* (Trayer & Buckley, 1970). *Proteinase K is a broad specificity endopeptidase that is used to digest proteins* (Gradisar et al., 2005)*."*

> *"Cystic Fibrosis (CF) is an autosomal recessive disease, characterized by chronic airway infection, whose predominant pathogens include Staphylococcus aureus, Pseudomonas aeruginosa and other Gram-negative bacteria*(Heijerman, 2005; LiPuma, 2010). *OP swabs are commonly used when children are unable to expectorate* (Zemanick et al., 2015). *This is particularly true for pediatric, non-expectorating subjects with CF in whom oropharyngeal (OP) cultures are used as a surrogate for lower airway bacteria* (Zemanick et al., 2015). *In the pediatric studies, OP swabs have ranged from 29.6% to 67.5% of the samples* (Armstrong et al., 1996; Zemanick et al., 2010; Wolter et al., 2013; Hoppe et al., 2015) *and therefore represent an important sample type in early CF."*

Comment #2: Lacks validation of the role of EnzD. Maybe this is due to lack of literature cited, but would be important to know if the enhancement of gram positive organisms after EnzD digestion reflects true relative abundances or something else.

RESPONSE: The reviewer makes an excellent point, obtaining community composition that is reflective of the actual community is important particularly in clinical samples with diverse community composition. Any variation in the methodology is likely to impact the results to some extent. Given EnzD specifically targets Gram-positive organisms it is likely it provides a better representation of the communities. This is supported by our previous work in which the total amount of DNA increased after EnzD. The fundamental issue however is the dependence of the community composition on the degree of change. This was true for communities dominated by both Gram-positive and Gram-negative communities. If the community primarily consisted of Gram-positive organisms then digestion had very little impact. Likewise, for some communities dominated by Gram-negatives there was little change, which presumably represents patients with limited amounts of Gram-positive organisms targeted by the digestion. There were cases where significant changes were observed with the enhanced extraction of Gram-positives shifting the community results. This reflects the personalized nature of the communities in the patient population, and is part of the justification for the conclusion that digestion is methodology required to obtain reliable calls for detection of Gram-positive bacteria. Previous studies have been performed looking at mock communities with various cell lysis methodologies. We have included references for these studies but given their reduced complexity related to clinical samples, it is unknown how generalizable these results are to clinical samples.

Comment #3: The S. aureus vs other staphylococci question is important to distinguish if possible.

RESPONSE: We agree that the speciation of taxa in the context of clinical setting is an important distinction. Calling species with short sequences is not routinely performed, and we have not attempted that in this analysis. This standard is not apparent in the literature where species level calls based on short reads is not normally reported. However, we did look specifically at *Staphylococci* species in our previous paper (Johnson et al 2016) utilizing qPCR assays and did find that that majority of *Staphylococcus* was *S. aureus* based on the high correlation between *S. aureus* specific qPCR and *Staphylococcus* relative abundance. We have expanded the introduction to include the findings of previous studies that have focused on *Staphylococcus* specifically.

> *"Similarly, Johnson et al (2016) determined EnzD improved the sensitivity of sequencing in OP swabs while no differences were observed in sputum samples. Increased sensitivity with EnzD was robust when compared to clinical culture results and qPCR. Furthermore, the work showed that the majority of Staphylococcus was S. aureus."*

Comment #4: What this study is missing is some form of validation. For example, it is noted that S. aureus detection is enhanced by the use of EnzD. Is it really that we are seeing it's true relative abundance because of the use of EnzD, or is it because other bacteria relatively speaking are of lower abundance after EnzD digestion? Would be

nice for the authors to perhaps spike some samples with known concentrations of some mock communities to decipher whether EnzD digestion gets us closer to the true relative abundances of these bacteria, or whether it simply enhances the relative abundance of some singular bacteria while not truly reflecting their relative abundances. Perhaps this data already exists, and if it does, the authors should cite those articles and describe in the introduction.

RESPONSE: The reviewer suggests an interesting experiment, however, these data were collected in 2012, and it isn't clear that contemporaneous spike experiments would provide additional insight into interpretation of this data set. The motivation for this experiment was to investigate the sensitivity of *S. aureus* detection using molecular methods (mainly qPCR) in CF airway samples. This was due to the observation that we routinely identified *Staphylococcus* sequences in samples that were not culture positive for *S. aureus*. Our first paper evaluated the comparison for *Staphylococcus* specifically. This evaluation included culture results, qPCR assays and quantification of total DNA, all of which help to address this reviewer's question. Our current analysis was meant to examine taxa beyond *Staphylococcus*. As stated above (comment #2) the community composition has a strong influence on the amount of change observed. The observed changes are consistent with digestion resulting in community composition that better reflects the community present. Performing adequate replicates (both technical and with variation of the community spikes) would require a substantial project that is not feasible at this time.

We have expanded the introduction to include more of the findings from the original paper and to clarify the contribution of this paper, refer to comment #3 for added paragraph.

Comment #5: In the introduction, the authors don't really state what EnzD does? Is it digesting the cell wall carbohydrates, etc...?

RESPONSE: The reviewer is correct that we did not restate this information in the current manuscript, and have corrected this oversight in the revision. The Introduction of the manuscript now states,

> *"EnzD was performed on one replicate of each sample by mixing with lysostaphin (final concentration 0.18 mg/mL) and lysozyme (3.6 mg/mL) and incubated at 37°C for 30 minutes. Samples were then digested with proteinase K (1.4 mg/mL) and incubated at 65°C for ten minutes, then incubated at 95°C for 10 minutes.* (Zhao et al., 2012; Johnson et al., 2016)*. Lysozyme and lysostaphin target degradation of the bacterial cell wall by targeting peptidoglycan (lysozyme) and pentaglycine bridges (lysostaphin)* (Salazar & Asenjo, 2007)*. Lysostaphin is specific to the subset of staphylococci that contain pentaglycine bridges including the human pathogen S. aureus* (Trayer & Buckley, 1970)*. Proteinase K is a broad specificity endopeptidase that is used to digest proteins* (Gradisar et al., 2005)*."*

Comment #6: The methods section does not cover how the EnzD digestion was performed.

RESPONSE: The enzymatic digestion was performed as specified in Zhao et al (2012) and Johnson et al (2016). We have added the following statement to the manuscript.

> *"EnzD was performed on one replicate of each sample by mixing with lysostaphin (final concentration 0.18 mg/mL) and lysozyme (3.6 mg/mL) and incubated at 37°C for 30 minutes. Samples were then digested with proteinase K (1.4 mg/mL) and incubated at 65°C for ten minutes, then incubated at 95°C for 10 minutes."*

Comment #7: I don't fully understand the paragraph from lines 140-151. It states that Prevotella, Fusobacterium, etc... may not be clinically meaningful, which is quite a vague statement. Later, it points to an increase in the RA for Staphylococcus with the suggestion that the Staphylococcus is clinically meaningful. Based on the species of Staphylococcus present, I'm not sure it would be any more clinically meaningful than the other examples. Aren't we really asking is this S. aureus or other staphylococcal species? I'm not sure if the V1-V2 region is sufficient to know this answer, but is hard to read about an increase in the RA of Staphylococcus without wondering whether this specifically refers to a pathogen like S. aureus or mostly nonpathogens such as S. epidermidis.

RESPONSE: The reviewer makes an excellent point. We did not adequately describe the organisms generally accepted as CF pathogens. This information has been added to the revision. It is also correct that we do not have adequate information to make species calls. However, our previous study that focused specifically on *Staphylococcus* did indicate that the majority of *Staphylococcus* was *S. aureus*. A description of these previous findings has been added.

> *"Genera traditionally associated with CF include Pseudomonas, Staphylococcus, Haemophilus, Stenotrophomonas, and Achromobacter (Zemanick et al, 2015)."*

> *"Similarly, Johnson et al (2016) determined EnzD improved the sensitivity of sequencing in OP swabs while no differences were observed in sputum samples. Increased sensitivity with EnzD was robust when compared to clinical culture results and qPCR. Furthermore, the work showed that the majority of Staphylococcus was S. aureus."*

Comment #8: The Shannon Index I don't believe is a good measure of diversity in these situations. It does not penalize for the relatively low abundant species present, and thus, you will have great shifts in diversity when using the EnzD and some species become much more abundant. Probably better to use several different diversity measures to show they all show the same trend, or to switch to a different measure that penalizes for a higher number of lower abundance species.

RESPONSE: The reviewer is correct that Shannon does not overly weight rare (or dominant) taxa. This is one reason that we utilize this metric. However, we agree that the increase in rare taxa with EnzD might be the driver in the difference in alpha diversity. We have included analyses evaluating richness and evenness to the paper. Richness can also be thought of as an alpha diversity measure that provides more weight to rare taxa (Jost, 2006). We did not observe shifts in richness with EnzD.

"The Shannon-H alpha Diversity Index was significantly lower ($p < 0.01$) with EnzD in sputum samples (Figure S3) due to higher RA of Staphylococcus resulting in a significantly less even community ($p <0.01$). For OP samples, the Shannon-H alpha Diversity Index was also lower likely due to higher RA of Streptococcus with EnzD, although this result did not reach statistical significance ($p = 0.06$). Richness and evenness were also not significantly affected by EnzD in OP samples. Changes in alpha diversity due to use of EnzD do not necessarily correspond to changes in microbial community composition as measured using beta diversity (Figure S4)."

Comment #9: With regards to the sputum samples, was there any quality control to ensure the sputum samples were good specimens? Most of the bacteria identified in this study are oral microbes. The authors should probably explain why so many oral microbes are identified in sputum, and whether this is a common feature of the CF lung compared to healthy individuals.

RESPONSE: The reviewer asks an important question. These samples were collected as part of standard clinical bacterial surveillance and excess specimen was frozen for molecular studies. Adequacy of sample is an important point, but these are the samples that are employed to make clinical decisions. Mucus plugs are selected during processing for sputum, and the OP swabs reflect the oropharynx. The rationale for using these samples is that they are reflective of clinical practice, and thus realistic for how these assays would be required to perform clinically. Further, most airway samples are dominated by "oral" taxa in all sample types and across many conditions. This is primarily due to the large source of exposure to the lower airways from the upper airway and oral cavity. In late stage disease (or during exacerbation) CF patients typically have lower levels of these organisms, but it is not that surprising to see high levels of these taxa in sputum or OP samples. The lower age range from our pediatric population includes subjects with less severe lung disease which increases the prevalence of these taxa relative to adult CF populations. We have added a brief discussion of these points to the paper.

"The samples used in this study were collected for clinical evaluation of airway infection, although separate evaluation for sample adequacy was not performed, these samples are reflective of what is being used to make clinical decisions. The patients that were not capable of expectorating sputum tend to be younger with presumably less lung disease (i.e., fewer pathogens). There are also differences between upper and lower airways (Zemanick et al., 2015) *that could also impact the community composition observed."*

Comment #10: Any explanation for the paradoxical values observed with Granulicatella in response to EnzD?

RESPONSE: The reviewer poses an important question regarding paradoxical values observed for Granulicatella in response to EnzD. *Granulicatella* has been shown to have pleomorphic and variable Gram stain characteristics (Bottone et al., 1995; Christensen & Facklam, 2001). Given the variable Gram stain characteristics of Granulicatella we would suggest this particular taxon might not conform to expectations of EnzD given cell wall structure. Discussion has been added to the manuscript to address this issue.

"Granulicatella, a member of the Firmicutes had decreased RA with EnzD, which was not consistent with other Gram positive taxa. *Granulicatella* is reported to have pleomorphic and variable Gram stain characteristics, which may explain this observation *(JJ. Christensen et al 2001, Bottone EJ et al. 1995)."*

Reviewer 3:

Comment #1: The authors show that with their protocol of enzymatic digestion there were shifts in community composition. However, they did not report changes in the relative abundance of important CF-pathogens such as Pseudomonas aeruginosa, Stenotrophomonas maltophilia, Burkholderia cepacia complex. The authors should comment on this.

RESPONSE: We agree that a discussion specific to important CF pathogens would be helpful. The following has been added to the paper:

"Genera traditionally associated with CF include Pseudomonas, Staphylococcus, Haemophilus, Stenotrophomonas, and Achromobacter (Zemanick 2015). All of the samples have low RA for Achromobacter and all of the OP samples had low RA for Pseudomonas and Stenotrophomonas. In the sputum samples, 11 had low amounts of all CF pathogens, 11 were dominated by Staphylococcus, all of which increased with EnzD, and 3 were dominated mostly by Haemophilus. Of the remaining 14 sputum samples, 3 samples were dominated by Stenotrophomonas, 1 increased, 1 decreased and 1 remained unchanged with EnzD. Eleven sputum samples had > 5% RA Pseudomonas prior to EnzD, the RA increased after EnzD in 6 of these samples (median 18.7 range 1.1% to 68.4%), in those where RA decreased, the median and range was -6.1%, -1.2% to 42.0% (Table 3)."

Comment #2: Since S. aureus was the only species with higher relative abundance in sputa as the authors reported the conclusion that enzymatic digestion should be especially used for oropharyngeal swabs in order to optimize detection of S. aureus is not justified.

RESPONSE: Thank you for the opportunity to address this issue. The authors acknowledge the concern related to the justification for use of EnzD in OP swabs to optimize detection of *S. aureus*. Conclusions made in this paper draw on the conclusions found by Zhao et al. 2012 and Johnson et al. 2016, as well. Findings in this paper support the claim that EnzD may be of the most importance in OP swabs as seen by the enhanced detection of organisms with a Gram-positive cell wall structure. Johnson et al 2016 found increased detection specifically in *S. aureus* with the use of EnzD. Zhao et al 2012 found EnzD resulted in greater RA of *Staphylococcus.* Thus, the authors conclude that given previous findings of increased RA following the use of EnzD, this method is appropriate for improved detection of Gram-positive taxa which may, in part, be attributed to higher detection of *S. aureus*. The authors have modified the discussion to more clearly indicate that this recommendation was based on several studies and not simply the results presented in this paper.

"There is a compelling argument to utilize EnzD in studies that rely on OP swabs as previous studies have shown increased sensitivity for detection of Staphylococcus. While the argument is less compelling for sputum, EnzD should be applied consistently."

Comment #3: The authors report about 7 samples that were negative for "standard" CF pathogens. The authors should define what "standard" CF pathogens are. Furthermore, they should show the results from sequencing for these specimens because these data are especially interesting. How is the clinical course in these patients? Are these specimens from routine visits? Or from exacerbations?

RESPONSE: The reviewer identifies an interesting set of samples, and we have added a supplemental figure (Figure S2) to show the community composition for these specimens. The lack of known CF pathogens identified by culture is not uncommon (Zemanick et al., 2010) and this specific analysis was targeted at samples with culture identified *S. aureus*, which makes the culture negative proportion small relative to typical culture negative rates. We have added a brief description of organisms typically considered as CF pathogens. Only basic subject demographics were pulled for these samples, so the clinical status at the time of sample collection is unknown. However, the use of paired samples from duplicate aliquots lead to each subject being used as their own control, so the comparison of with and without EnzD is not confounded by clinical status. The following paragraph has been added to the limitations section of the paper.

"The patient's clinical status at the time of sample collection is unknown, this hampers our ability to determine whether these samples are reflective of both clinical stability or during a pulmonary exacerbation. However, because paired samples were used to assess the effect of EnzD, the difference for each pair is not confounded by clinical status."

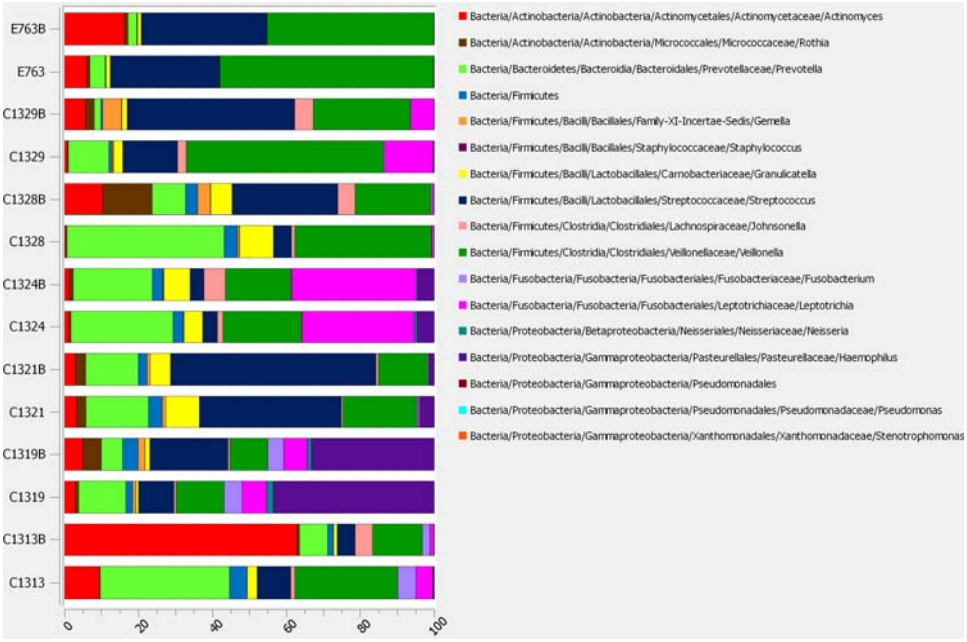


Figure S2: bacterial community composition for 7 culture negative samples. Samples ending with 'B' correspond to those with EnzD.

Comment #4: Line 143: The authors report about less abundance of some bacterial species. Here, they also report about less abundance of Haemophilus, which is especially in young patients considered as a real CF pathogen. The authors should stress this finding.

RESPONSE: On line 144 we state that the particular genera mentioned in line 143 is not necessarily clinically meaningful given shifts in RA of less than 5%. The shift in RA for Haemophilus is -0.91 and only found to be significant in OP swabs. We have revised this sentence to better clarify that the changes in relative abundance are small and may therefore not be clinically meaningful and not that the genera listed are not clinically meaningful. In addition, we have added discussion to specifically address CF pathogens.

"For these genera, while the changes are statistically significant, the changes may not be clinically meaningful."

"Genera traditionally associated with CF include Pseudomonas, Staphylococcus, Haemophilus, Stenotrophomonas, and Achromobacter (Zemanick 2015). All of the samples have low RA for Achromobacter and all of the OP samples had low RA for Pseudomonas and Stenotrophomonas. In the sputum samples, 11 had low amounts of all CF pathogens, 11 were dominated by Staphylococcus, all of which increased with EnzD, and 3 were dominated mostly by Haemophilus. Of the remaining 14 sputum samples, 3 samples were dominated by Stenotrophomonas, 1 increased, 1 decreased and 1 remained unchanged with EnzD. Eleven sputum samples had > 5% RA Pseudomonas prior to EnzD, the RA increased after EnzD in 6 of these samples (median 18.7 range 1.1% to 68.4%), in those where RA decreased, the median and range was -6.1%, -1.2% to 42.0% (Table 3)."

Comment #5: Once "Staphylococcus aureus" has been introduced, the authors should only write "S. aureus". This also should be changed for all bacteria.

RESPONSE: We appreciate the reviewer identifying this issue and have changed all taxa names as suggested.

Comment #6: References: There are unusual terms in the reference list, which do not belong there, e.g.:
LiPuma, J. J. 2010. "The Changing Microbial Epidemiology in Cystic Fibrosis." Clinical 279 Microbiology Reviews 23(2):299–323. Retrieved August 23, 2016 280 (http://cmr.asm.org/cgi/doi/10.1128/CMR.00068-09).

RESPONSE: We thank the reviewer for this critique. References have been formatted using Mendeley PeerJ format, and we will work with editorial staff to make any further modifications required.

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