Reviewer #1 (Comments for the Author):  
  
The manuscript "Multi-omic 1 Signatures of Host Response Associated with Presence, Type, and Outcome of Enterococcal Bacteremia" addresses an important scientific question. It presents several interesting results. However, there are some flaws in analytical methodology which have to be addressed to ensure which results and more importantly which conclusions are well supported.

We thank the reviewer for finding that our study addresses an important scientific question and presents several interesting results. We are happy to adress reviewer comments addressing the analytical methodology below and incorporate them into a revised version of our manuscript.

**Major comments**

1. Presently, individual features are identified as predictors of health vs. infection, E. Faecalis vs. E. Faecium, and survivorship vs. mortality. However, greater power could be achieved through a supervised machine learning approach, i.e., by constructing predictors that incorporate multiple factors, and the importance of individual factors could be determined from predictor composition. Moreover, training and evaluating a predictor on the same set of data can result in overfitting and an inaccurate estimate of predictor performance on new data. We therefore suggest the following:

1. For each of these comparisons, create one predictor or an ensemble of multiple predictors that utilize all markers passing some feature selection threshold.
2. Perform cross-validation on the training set after training to evaluate predictor performance within the training data.
3. Divide the samples into a training set and a test set. A commonly accepted ratio is 70% of samples in the training set and 30% of samples in the test set. Evaluate the predictor(s) on the test set to estimate predictor performance on new data. (Note: The test set would ideally be an entirely separate dataset. However, in the absence of such a dataset, this method allows predictor performance to still be estimated.)

We thank the reviewer for suggesting an alternative analysis strategy through which we could analyze our proteomics and metabolomics data.

We agree that machine learning could be an interesting alternative approach used in this data set. However, we also believe that our original analysis is appropriate and informative as we are describing how well each of the features identified in our study are different between healthy and infected, faecalis and faecium, and mortality and survival. Furthermore, we are interested in single features, as they are more interpretable than a predictor or ensemble of multiple predictors that utilize all markers passing some feature selection threshold. We believe that as an initial assessment of the differences between our comparisons, this is a reasonable, interpretable approach.

We agree that training and evaluating a predictor on the same set of data can result in an inflated estimate of predictor performance on new data. This is why we suggest “ “ .

We believe there are problems with utilizing a machine learning approach to this data set, as it is plagued by the low n large p problem (p >> n) that is inherent to data analysis challenges where a large numbers of predictors are used to classify a relatively low numbers of samples.

Ideally, we would be able to overcome this problem by incorporating more samples into our study- but this is not realistic given the resources required to obtain state of the art proteomics and metabolomics data from > 100 subjects.

There are two potential solutions to this problem.

1. Use a regularized algorithm.
2. Perform feature selection prior to machine learning.
   1. As the reviewer suggests.

To incorporate the machine learning approach highlighted by the reviewer above into our study.

2. It seems there is a design problem with comparison between infection and healthy individual. Control samples consist of plasma collected from blood bank volunteers, while EcB samples were collected from patients at UW Health. This discrepancy in patient and sample collection circumstances may be a significant source of confounding. Although steps are taken to identify confounding variables from patient metadata, it is nonetheless probable that further unidentified confounding variables remain.

We thank the reviewer for bringing up this point.

We agree that the logistical constraints of sample collection when comparing healthy samples obtained via blood bank volunteers and EcB samples are not ideal. However, on a practical level these are the only samples from healthy individuals that we were able to obtain for this study, and even though there were slight differences in sample collection methodology between the two sites to be we believe it is reasonable to expect the differences in the underlying biological material to be the major source of variation between these groups. We believe that the benefits of including a healthy cohort for comparison far outweigh the downsides inflicted by having slight differences in sample collection.

First, it is worth considering what the specific differences between the two collection methods were.

For the UW health samples:

1. Blood was collected from EcB patients
2. Centrifuged
3. Stored at -80C until

For the volunteer blood bank samples:

1. Blood was collected from
2. Centrifuged using the blood banks process
3. Transferred to … tubes/
4. Stored at p

The major differences in the way the two groups of samples were collected was the centrifugation process and the plastic that the samples were collected in.

We can discount the centrifugation process as a minor concern because the differences in the centrifugal forces used are not sufficient to result in differences in protein or metabolites.

Thus, the type of plastic the samples were stored in would be expected to be the major driver of differences caused by sample collection. Type of plastic used is unlikely to have a large effect on proteomics data, given .

It is much more likely that this would lead to differences in the metabolomics data, as plastic molecules from the container could leech into our samples and be detected via our approach.

Results of our proteomics data are consistent with the logic above. We observed that the primary differences in the proteome , which is exactly what one would expect when comparing healthy samples to bacteremic.

1. **inflammatory response**
2. **neutrophil chemotaxis**

Furthermore, the most different proteins had been reported by other studies to be associated with bacteremia/sepsis in other contexts.

These findings combined with and the lack of obvious mechanism one would expect proteins to be altered between the two cases of sample processing suggests that the proteomics data was not affected significantly by the differences in sample processing.

To further confirm this point, we performed the thoughtful analysis suggested by the reviewer, finding that ….. proteomic features had the same direction of change. We think that this analysis (which could is limited by the assumption that health and infection share a molecular basis with survivorship and mortality – this may not be the case for all proteomic and metaboloic features that we detect) combined with the rationale highlighted above, makes the case that the sample processing effects on the proteomics data were indeed limited.

When considering the metabolomics data, we agree with the reviewer that this is a very important consideration. Given the differences in the sample processing methods, it is reasonable to suspect there would be differences in metabolites between the two conditions. Indeed, when we analyzed the data, we saw that several of the most different metabolites between healthy and EcB samples were molecules with roles as plasticizers. These molecules were almost certainly due to differences between the plastics that the samples were collected in.

To examine the data as the reviewer suggested, we perfomed the following analysis.

1. Created a contingency table by taking the mean of for all samples

There is potential solution at least partially overcome this problem. If we assume that the transition between health and infection shares a molecular basis with that between survivorship and mortality, then we would expect features truly caused by EcB to be shared between the two comparisons and to have the same direction (up or down) in both. We therefore suggest the following:

1. Create a contingency table which sets the two comparisons against each other.
2. If most features share a direction of change, use these features for the construction of predictors.
3. Otherwise, it is more likely that confounding variables will play a significant role in the infected/healthy analysis, and so it may be wiser to place the greater focus on the E. Faecalis vs. E. Faecium and survivorship vs. mortality analyses performed within the hospital dataset.

**Minor comments**

1. The sizes of the mortality and survival groups in Figure 1 do not sum to the same value as the E. Faecalis vs. E. Faecium groups (32 E. Faecalis + 44 E. Faecium = 76, while 17 mortality + 57 survival = 74). We suggest that this disparity be corrected if it is an error, or explained if it is not.

We thank the reviewer for bringing this disparity to our attention, as it was a consequence of an unintended mistake in our clinical metadata that affected our analysis. As explained below, this ultimately ended up having a limited effect on the results, but it was a very important oversight to correct.

For 2 of the 74 EcB patients, designated S49 (patient id # 78 ) and S76 (patient id # 79 ) = faecium we noticed that the mortality values were NA in our metadata, causing the discrepancy in the patient #s. Further manual inspection showed that other clinical metadata fields were NA from these samples as well. This was unexpected, as clinical data for all specimens had been collected as part of our study.

We suspected that this was a result of manual manipulation of excel files that occurred somewhere in the data cleaning process, and as such reverted to the initial version of the clinical metadata.

Comparing these versions of the metadata, we noticed that there was a mistake in the excel file holding the patient metadata for patient id S49 (ID 78) and S76 ( ID 79). In both of these cases the type of EcB bacteremia had been correctly assigned (78 = faecalis, 79 = faecium), but the remaining metadata fields had been deleted. As such, we added the missing clinical metadata.

We then took this opportunity to confirm that the rest of the clinical metadata was correct, which it was. The analysis was then rerun using this updated, complete, metadata. Figures and text were updated accordingly.

1. The subsection "Human Plasma Samples" under the section "Materials and Methods" states that data was collected from 32 patients with E. faecium bacteremia and 44 patients with E. faecalis bacteremia, totaling 76 patients (line 128). However, the following subsection "Clinical Data Collection", states that there were 83 patients with enterococcal bacteremia (line 137). We suggest that this disparity be corrected if it is an error, or explained if it is not.

We thank the reviewer for noticing this disparity. The number 83 was written in error, it was intended to be 76. The updated version of the text had been modified to reflect this.

1. In figure 1B, we suggest either placing the text in the title of each plot beneath its x-axis or removing the x-axis label to avoid duplication. Further, the title of the plot "Sensitivity to Vancomycin" is cut off.

We thank the reviewer for this suggestion to improve the aesthetics of the figure. These recommendations have been added to the new version of the figure.

1. On line 46, we suggest that the URL be made a hyperlink.

Done.

1. On line 46, the sentence is missing a period.

Corrected.

1. On line 134, a comma is missing after the closing paren.

Corrected.

1. On line 138, we suggest removing the comma after the word "identified".

Corrected.

1. On line 191, please check whether "cystines" should instead be "cysteines". It's possible that there may have been a misunderstanding on our end.

Corrected.

1. On line 199, we believe that the word "Analysis" should not be capitalized.

fixed

1. On line 208, The text "20oC" contains the character "o" rather than a degree symbol".

fixed

1. On line 211, the text "-80{degree sign}C{degree sign}C" contains a duplicate instance of "{degree sign}C".

fixed

1. Lines 218-221 use "minute" where they should say "minutes". We also suggest that semicolons are used to break up the list, such as "...following gradient: 0 to 1 minutes, 5% B; 1 to 7 minutes, a linear increase from 5% to 100% B; 7 to..."

fixed

1. On lines 233 and 234, the spacing around the equals signs is inconsistent.

fixed

1. On line 244, we suggest changing "GNPS FBMN option" to "the GNPS FBMN option".

fixed

1. On line 274, we believe "across any" should instead be either "for any" or "in any".

fixed

1. On line 281, a comma is missing after the word "randomforest".

fixed

1. On line 282, "R-package" should instead be "R package".

fixed

1. On lines 314 and 316, the temperatures "37C" and "50C" lack degree symbols.

fixed

1. On line 348, "GEForce" should instead be "GeForce".

fixed

1. On line 369, we suggest using "EcB" instead of "EB" for consistency.

fixed

1. On line 380, "Enterococcous" should instead be "Enterococcus".

fixed

1. On line 401, "1x1025" should instead be "1x10-25".

fixed

1. On line 417, "(30" should be "(30)".

fixed

1. On line 420, we believe the word "one" should be removed.

fixed

1. On lines 430 and 431, "gene ontology" should be capitalized to "Gene Ontology".

fixed

1. On line 461, a period is missing after the second close paren.

fixed

1. On line 484, there is an extra space before the em dash.

fixed

1. On line 512, the text "are leveraged" should be deleted.

fixed

1. On line 589, the word "of" should be inserted between the words "analysis" and "unsupervised".

fixed

1. On line 595, a period is missing after the close paren.

fixed

1. On line 662, a comma is missing between the words "biospecimen" and "and".

fixed

1. On line 758, a space is missing between the period and the word "Interestingly".

fixed  
  
  
  
Reviewer #2 (Comments for the Author):  
  
The authors of this manuscript performed a comprehensive comparative analysis of plasma samples from 3 cohorts: 29 healthy volunteers, 32 patients with E. faecium bacteremia and 44 patients with E. faecalis bacteremia, with respect to the content of proteins and metabolites. Their main goals were to characterize the host response to Enterococcus bacteremia (EcB), to determine the systemic differences between E. faecalis and E. faecium bacteremia, and to identify the systemic response markers associated with increased mortality. They used an unbiased approach based on a comparison of proteoms and metaboloms of the collected plasma samples, supported by results of characterization of Enterococcus isolates from the patients and the selected case history parameters of each patient (including gender, the Charleston comorbidity index, duration of bacteremia, day of blood draw, and mortality during admission). Based on the used methodologies they could capture from among hundreds of proteins and metabolites analyzed, those that are increased or decreased in all or in particular types of bacteremias.   
They also compared their data concerning EcB with their earlier data concerning Staphylococcus aureus bacteremia. Based on the results of these comparisons they identified among detected plasma proteins and metabolites, the proteins and metabolized increased or decreased in bacteremia patients, proteins or metabolites common for all types of bacteremia, and proteins or metabolites with changed levels specific for E. faecalis, E. faecium or S. aureus bacteremia. Gene ontology (GO) enrichment analysis of proteins differentiating all or particular bacteremias allowed the authors to identify biological processes characteristically altered in all or particular kinds of studied bacteremias. Although the authors confirm the value of two previously approved markers for the detection of infection, most of their results are novel.   
The introduction section provides sufficient background and motivation to perform the studies described by the authors. The methods are described in sufficient detail. The results are mostly presented in the form of graphs that allows one to see the differences in plasma proteomes and metaboloms observed by the authors in infected versus healthy individuals. Statistical analysis is satisfactory. Additionally, the authors discuss the differences of insufficient statistical significance that can be potentially useful if further analyzed. The manuscript is a valuable contribution to the knowledge concerning common processes in the host response to all three kinds of bacteremias studied by the authors and processes in the host response characteristic for particular kinds of bacteremias. Additionally, it has a practical value as a guide to developing plasma analysis-based diagnostic methods that could be helpful in the fast detection of EcB cases, identification of the infecting Enterococcus species and the prediction of infection fatal outcome. An important observation of the authors was that most of the E. faecalis isolates were resistant to vancomycin, while vancomycin resistance among E. faecium isolates was rare, which may help in the fast decision concerning antibiotic therapy. In general, the manuscript is interesting to read**. Only the Discussion section seems to be too wordy, and could be shortened.**

We are happy that the reviewer found our study to be a valuable contribution to the field with practical value, as well as an interesting read.

We also thank the reviewer for suggesting that reducing the length of the discussion section could benefit the paper, we have edited to be more concise.

My additional specific comments are below.  
  
L. 41. Please explain the abbreviations at their first use in the text

Thank you for bringing this to our attention, it has been fixed in the updated version.   
  
L. 311-312. Please provide a reference.

See below comment  
  
L. 312. "10"? What do you mean by that? Please explain.

We apologize, this was intended to be a citation but got disconnected from our reference manager. This has been corrected in the updated version.  
  
L. 317. Please provide the catalog number.

This has added as requested in the updated version.  
  
L. 370. In the classification of patients with bacteremia, the authors took into account the duration of bacteremia. Could the beginning of persistent bacteremia be precisely determined by the authors? Please explain.

Thank you for bringing this important consideration to our attention.

We took the day of admission to be the start of bacteremia in the previous version of the manuscript.

We agree that this is not the same as the start of bacteremia since there is likely some degree of variation in the amount of time that it takes a patient to seek ER treatment after onset.

While we cannot confidently determine the exact start of bacteremia we were able to detect the end of bacteremia and still believe this to be a relevant metric. We have renamed it to be post admission duration of bacteremia to eliminate confusion and better reflect what this metric is actually measuring.

L. 380. Could the authors expect the detection of enterococcal proteins in blood samples? What was the range of titers of enterococci in patients' blood samples?

The titer of bacteria in bacteremic blood is expected to be very low. **Include citation here.**

In our study, the exact levels were not attempted to be quantified. Instead, we used standard clinical microbiology methods that amplify bacteria via propagation for subsequent identification of the bacterial species.

Due to their expected low abundance, it was unsurprising to us that these proteins were not detected using our DDA proteomic methods, as their stochastic nature is more likely to detect highly abundant proteins.

The text “Notably, no proteins were found to map to the *Enterococcus* proteomes” has been modified to “No proteins were found to map to the *Enterococcus* proteomes” in an attempt to make this clearer.

L. 561. With "E" ? Please provide a complete abbreviation.

We apologize for this oversight. The text has been corrected to *E. faecium*  
  
L. 589-595. What was the reason of the differences between the initial and later findings?

Thank you for bringing this to our attention. The previous version did not read as intended. We intended to express that while no high-level patterns observable through unsupervised hierarchical clustering were able to differentiate mortality by overall proteomic or metabolomic profiles, individual proteins were significantly associated with mortality outcomes.

In order to reflect this, the text has been changed from:

to:  
  
“Our unsupervised hierarchical clustering analyses of proteomics (**Figure 1C**) or metabolomics data (**Figure 1D**) revealed no high-level associations of mortality with overall proteomic or metabolomic profiles. However, when conducting feature-level analysis, we identified specific proteins that were significantly associated with mortality outcomes.”  
  
Figure 3A. What do exactly the authors mean by "spectral match" and "no spectral match" in this Figure? This can be guessed intuitively. However, clear explanations by the author would be helpful for the readers.

We have added text to the figure legend to better explain what the spectral match vs no spectral match legend means.

“No spectral match (red points) indicates features that did not produce a spectral match to any of the metabolites in the GNPS database, while spectral match (blue points) indicates features that produced a spectral match to metabolites in the GNPS database.”

Figure S2. Fonts in this Figure are too small to be readable. They should be at least slightly increased.

Due to the amount of information contained in this figure, this will be submitted as a high-resolution image that will allow the reader to zoom in on the network of interest. Fonts have also been increased to make more readable as suggested.   
  
The authors compared their results with the previously described results obtained with similar methods for S. aureus bacteremia. In my opinion, the conclusions based on this kind of comparison should be made with care and used as suggestive rather than indicating something. The manuscript concerning staphylococcal bacteremia was published by the authors a few years ago. Thus, in the discussion section, the authors should at least briefly, compare the methodology of studies performed for that manuscript and the current manuscript.

We thank the reviewer for bringing up this important point. We had intended our conclusions comparing the S. aureus results to the EcB results to be suggestive, rather than definitive in nature and had intended to highlight the limitations of comparing these two studies.

“Technical considerations as to the experimental design of our *S. aureus* and EcB study prevent a direct comparison of these two datasets, but the differences we observe relative to healthy patients **suggest** the existence of features of host response that could be exploited to distinguish these types of bacteremia. A larger study designed to directly compare these, and other types of bacteremia, is warranted to uncover these differences with greater confidence through direct comparisons.”

In an attempt to make our intentions clearer to the reader we have revised the text to the following:

The list of references requires extensive editing and supplementation with missing details concerning quite many listed publications.

L. 1022: Please provide the details of the cited publication

We apologize for this oversight. It appears that our reference manager was not functioning as intended. This reference has been fixed.

L. 1056. Does the reference cited apply to host-microbe interaction as suggested in the manuscript text? Please verify.

We believe that this citation is fitting.

The text in question is:

Host factors are widely recognized as critical determinants of the outcome of host-microbe interactions15 and have been used as prognostic biomarkers to predict patient outcomes in a variety of diseases, ranging from COVID-19 to cancer 16,17

The citation on line 1056 is:

17. Dhanasekaran, S. M. et al. Delineation of prognostic biomarkers in prostate cancer. Nature 1057 412, 822–826 (2001).

We believe that this citation is appropriate as we are claiming that host factors have been used as prognostic biomarkers in a variety of diseases ranging from COVID to cancer and the reference is from a paper where prognostic biomarkers in prostate cancer are reported.

L. 1058, and elsewhere in the text: Please italicize genus and species names

This has been corrected.

L. 1090, 1092, and elsewhere in the text. Please supplement the list of references with multiple missing details.

We apologize for this oversight. Most of these malformed citations are for github repositories containing software that were used in the manuscript. Our reference manager did not cite them as we intended.

This has been corrected in the updated version.

Fonts in Figure S2 are too small and should be increased for better visibility.

This has been addressed in the modified version.

Figures S8 and S9 are hardly readable. They should be provided in a better resolution.

Thank you for bringing this to our attention- it has been addressed by increasing the DPI to 300 for both of these figures.