Microbiome Analysis

Microbiome Toolbox: Methodological approaches to derive and visualize microbiome trajectories

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Abstract

Summary: The gut microbiome changes rapidly under the influence of different factors such as age, dietary changes or medications to name just a few. To analyze and understand such changes we present a microbiome analysis toolbox. We implemented several methods for analysis and exploration to provide interactive visualizations for easy comprehension and reporting of longitudinal microbiome data. Based on abundance of microbiome features such as taxa as well as functional capacity modules, and with the corresponding metadata per sample, the toolbox includes methods for 1) data analysis and exploration, 2) data preparation including dataset-specific preprocessing and transformation, 3) best feature selection for log-ratio denominators, 4) two-group analysis, 5) microbiome trajectory prediction with feature importance over time, 6) spline and linear regression statistical analysis for testing universality across different groups and differentiation of two trajectories, 7) longitudinal anomaly detection on the microbiome trajectory, and 8) simulated intervention to return anomaly back to a reference trajectory.

Availability and implementation: The software tools are open source and implemented in Python. The link to the interactive dashboard is https://microbiome-toolbox.herokuapp.com/. For developers interested in additional functionality of the toolbox, the Python package can be downloaded from https://pypi.org/project/microbiome-toolbox/. The toolbox is modular allowing for further extension with custom methods and analysis. The code is available on Github https://github.com/JelenaBanjac/microbiome-toolbox.

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Supplementary Information: Supplementary data are available at Bioinformatics online.

1 Introduction

Microbiome as a concept usually refers to the composition and function of myriads of bacteria in an ecosystem, such as the gut or other body sites of humans or animals (Dogra SK, et al. Front Microbiol. 2020). The gut microbiome is particularly dynamic during early life development yet is still susceptible to change and malleable once it reaches a stable state at around 2-3 years of age (Cher A & Yassour M, 2020). Diet is a major factor affecting the microbiome throughout life. For example, the adult microbiome was shown to change in response to drastic changes in diet such as high-fat or ketogenic diet (David LA, et al. Nature. 2014; Mardinoglu A, et al. Cell Metab. 2018). Not surprisingly, as a response to antibiotics the gut microbiome is also drastically impacted but recovers subsequently to a large extent (Palleja A, et al. Nature Microbiol. 2018).

Exploring and understanding changes in the microbiome in relation to different factors such as time or age, changes in the environment and diet, as well as medications, are of great interest especially as they relate to health conditions. In early life, an age appropriate microbiome is understood to be critically important for appropriate immune competence development (Dogra S, et al. Gut Microbes. 2015). Equally, numerous examples relate adult gut microbiome features to health conditions (Dogra SK, et al. Front Microbiol. 2020). Hence, the importance of a toolbox that allows identification of the key microbiome features characterizing an appropriate microbiome versus one that deviates.

Here, we present a microbiome toolbox to facilitate such explorations and understanding that can be employed as is for efficient dataset analysis or customized for further data exploitation. Beyond the customary data visualizations and explorations, we implement some specific methods towards exploring the microbiome trajectories. Besides working with relative abundances, a key aspect of the calculations is using log-ratios for which we integrate relevant algorithms. We take a machine-learning based approach to derive a microbiome trajectory. We define on- and off- the trajectory based on various criteria, and we identify key determining features that place a sample on- or off- the trajectory. Furthermore, we suggest what changes can bring a sample back on to the trajectory.

2 Materials and Methods

2.1 Data Analysis and Exploration: (Figure 1a) To get an overview and understanding of the data, the microbiome toolbox has a plot to visualize the sampling statistics. The example data used here, briefly described in legend of Figure 1, is from the R-package – metagenomeSeq (Paulson JN, et al. Nat Methods. 2013) and its source pointed to in Supplementary Table 1 under "mouseData". We can visualize the microbiome data in an ultra-dense manner. Feature abundances, such as taxa, can be visualized over time as heatmap or as median or mean with error bars. Diversity indices such as Shannon or Simpson can be calculated. Embedding plots can be used to visualize the multi-dimensional data in a low-dimensional latent space. Outlier clusters can be identified and further analyzed to identify discriminating features distinguishing these outliers from the rest of the group.

2.2 Data Preparation: (Figure 1b) Besides having feature abundances, one can perform a log-ratio transformation. For the purpose of calculating this log-ratio, we can identify a suitable bacterium to go in the denominator by using methods such as least number of crossings, bacteria with very low or very high differential rankings, or choosing the bacteria with the best model performance when used as a denominator. We can also make a 'hybrid model' by applying the domain knowledge to select features in addition to the ones identified as important by the model. Next, we can define a reference or control group to be used as a basis for setting the trajectory. We can then classify all other samples into a non-reference group or use an unsupervised novelty detection algorithm to decide whether the observed sample belongs to the reference or not. Before deriving microbiome trajectories, we first need to prepare the data by removing correlated or low variance features. We can also choose the subset of taxa the model will be trained on and still achieve the optimal performance.

2.3 Microbiome Trajectory: (Figure 1c) Using machine learning algorithms, we predict a microbiome maturation index (MMI) as a function of time. A smooth fit is then used to obtain the trajectory. To interpret the predictions of the model we use SHapley Additive exPlanation (SHAP) values analysis (Lundberg SM, et al. Nat Mach Intell. 2020). Confidence interval or prediction interval can be utilized to check the nature of the spread of points on the trajectory. Comparison between groups or references can be done by fitting lines specific to each group and then running statistical tests to determine if these are significantly different. Additionally, we can evaluate trajectory performance using more than forty different models including a custom-made deep learning model for the dataset - different models can be chosen individually for different datasets.

2.4 Outlier Discovery: (Figure 1d) Outliers can be identified by various methods such as being outside the prediction interval or by longitudinal anomaly detection algorithms such as low-pass filter or

isolation forest (Liu FT, et al. Data Mining. 2008) implemented using a rolling average window. We can then use a machine learning ensemble model between the outliers and the (subsampled) rest of the dataset to classify these outliers, and interpret the predictions using SHAP analysis to identify the bacteria or metadata factors that differentiate the outliers from the (subsampled) rest.

2.5 Important Features and Intervention Simulation: (Figure 1e) Lastly, we can identify the key bacteria that are important per user-defined time-window within the trajectory. For outliers, some of these key features are out of the normal range causing the deviation from the trajectory. In a simulation setting, by restoring these numbers to be the same as for the key features in the reference group, we can make the outliers move closer to the trajectory. Additionally, we can see what features are shared between the detected outlier samples and compare whether they can be differentiated from the non-outlier samples. This then provides insights on how external factors affect our samples and what could be possible key interventions, in real life, to reposition these outliers back to normal.

3 Conclusions

We present here a microbiome toolbox to derive microbiome change as trajectories under different conditions such as time, diet changes or perturbations. While the toolbox has implemented intricate methods and complex algorithms, it also provides a rich variety of plots for easy visual comprehension and reporting. We hope that microbiome researchers will find this toolbox particularly useful for examination of their data and deriving meaningful insights.

Acknowledgements

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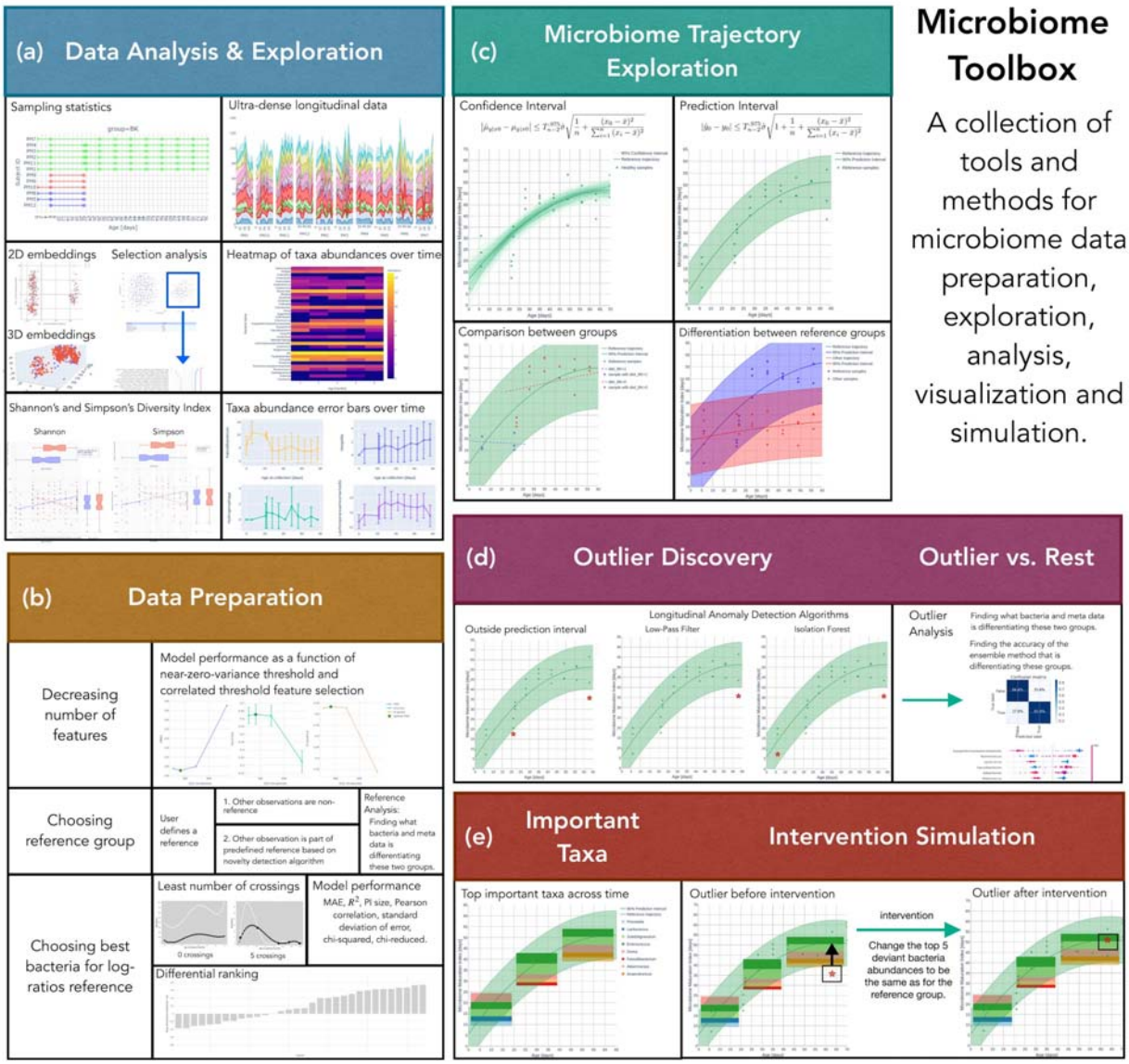

Figure 1: This microbiome toolbox has multiple components for (a) exploration of microbiome data, (b) preparing data for subsequent analyses, (c) constructing microbiome trajectories, (d) determining who is and outlier or within a trajectory and (e) identifying important features and possibilities to return an outlier sample back on to the trajectory. The data used to illustrate the possibilities of the toolbox comes from the R-package – metagenomeSeq (Paulson JN, et al. Nat Methods. 2013). Briefly, twelve germ-free mice fed a low-fat, plant polysaccharide–rich diet, were inoculated with adult human fecal material by gavage. Mice remained on the same diet for four weeks before a subset of 6 mice were switched to a high-fat, high-sugar diet for an additional eight weeks.

[SUPPLEMENTARY]

Microbiome Toolbox: Methodological approaches to derive and visualize microbiome trajectories

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Supplementary Table 1: methods and algorithms in the microbiome toolbox incorporated from different sources

Methods	Source	Purpose	Remarks
Machine Learning Algorithms	https://scikit-learn.org/stable/	To learn models to predict Microbiome Maturation Index (MMI)	
SHAP (Lundberg SM, et al. Nat Mach Intell. 2020)	https://github.com/slundberg/shap	To interpret the predictions of the model using the features it was trained on	
songbird (Morton JT, et al. Nat Commun. 2019)	https://github.com/biocore/songbird	To describe log-fold change of taxa compared to the reference taxa for getting the differential ranking	
CLOUD (Montassier E, et al. Microbiome. 2018)	Supplementary material of the paper.	Non-parametric detection test for microbiome outliers	Translated and modified for Python
mouseData	https://www.rdocumentation.org/packages/metageno	To test the methods we used mouse data from	We extract only the metadata and taxa

	meSeq/versions/1.14.0/topics/mouseData	RDocumentation	information
splinectomeR (Shields-Cutler RR, et al. Front Microbiol. 2018.)	https://github.com/RRShieldsCutler/splinectomeR	Longitudinal statistical analysis tool to compare two non-linear lines	Translated and modified for Python
xgboost	https://github.com/dmlc/xgboost	An optimized distributed gradient boosting library	
scipy	https://www.scipy.org/	Tools for mathematics, science, and engineering	
plotly	https://plotly.com/dash/	Building an interface for the application developed in Python	
catboost	https://github.com/catboost	Gradient boosting on decision trees library	
statsmodels	https://www.statsmodels.org/stable/index.html	To conduct statistical tests and statistical data exploration	

Input data:

Different types of microbiome data such as compositional and functional data tables, generated from different technologies such as 16s or shotgun metagenomics can be very well used in this toolbox.

Microbiome Maturation Index (MMI):

Although, the toolbox can be used to analyse any kind of microbiome data that is changing with time, or even any other data tables that essentially follow the same longitudinal structure of features changing over time, we have oriented this toolbox more towards analysis of Early Life Microbiome in infants. Thus, some of the terminology follows the convention used in the field such as "microbiome maturation" (Dogra SK, et al. Microorganisms. 2021).

Briefly, Microbiome Maturation Index (MMI) is defined using the approach of Subramanian, et al. Nature. 2014. Similar approach has also been followed by others in the field (Wan Y, et al. Gut. 2021; Depner M, et al. Nat Med. 2020; Galazzo G, et al. Gastroenterology. 2020; Stewart CJ, et al. Nature. 2018; Ho NT, et al. Nat. Commun. 2018; Blanton LV, et al. Science. 2016; Bäckhed F, et al. Cell Host Microbe. 2015; Vangay P, et al. Cell Host Microbe. 2015). Thus, it is well-used method in the microbiome field particularly for studying Early Life Microbiome progression in infant cohorts.

Here, the actual/chronological age of subject (infant) is predicted from gut microbiome data (obtained from a fecal sample) using machine learning based approaches. The term "microbiota age" may encompass both a subject's "microbiota compositional age" and "microbiota functional age". A "microbiota compositional age" may refer to the age which is determined using microbial composition data such as at genus level or species level. A "microbiota functional age" may refer to the age which is determined using functional data such as pathway modules/submodules or metabolites data. Combined, these can also be termed as the "Microbiome Maturation Index" for easier understanding.

Statistical methods:

The following formulae were used for defining the fit of the trajectory and determining whether a sample is on or off the ELM trajectory.

Least Squares Polynomial Fit

$$\{\hat{\beta}_j\}_{j=0}^{2} = \underset{\{\beta_j \in \mathbb{R}\}}{\arg\min}\, E \quad where \quad E = \sum_{i=0}^{n} |y(x|\beta_0, \beta_1, \beta_2) - y_i|$$

- n is the number of samples (x_i, y_i), $i \in [0, n]$ in the dataset.
- x_i is independent variable (i.e. age at data collection).
- y_i is dependent variable to be fitted by model function y with 3 parameters and degree 2 (i.e., Microbiome Maturation Index (MMI)).
- $\beta_0, \beta_1, \beta_2$ are parameters of the model to be optimised to minimise E.

Chi-Square

First, consider y_i has uncertainty described by standard deviation σ_i. Second, consider that residuals $r = y(x|\beta_0, \beta_1, \beta_2) - y_i \sim N$. Then the function:

$$\sum_{i=1}^{n} \left[\frac{y_i - y(x|\beta_1 \dots \beta_m)}{\sigma_i}\right]^2 \sim \chi^2$$

Once we find the best parameters for optimal fit, the probability distribution of χ^2 will be χ^2 distribution for $n - 2$ degrees of freedom. If $y1$ has no uncertainty, $\sigma_i = 1$. This is only used to measure the goodness of a fit. Moreover, we use reduced chi-square χ_r^2 where a good fit should have $\chi_r^2 = 1$.

Confidence Interval

The 95% probability interval around a fit line contains the mean of new values at a specific age of collection value. Iterative resampling of residuals is done 500 times.

$$\left|\hat{\mu}_{y|x0} - \mu_{y|x0}\right| \leq T_{n-2}^{.975}\hat{\sigma}\sqrt{\frac{1}{n} + \frac{(x_0 - \bar{x})^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2}}$$

- $\hat{\mu}_{y|x0} = \sum_{i=0}^{2} a_i x_i$ is polynomial fit with degree of the polynomial equal to 2.
- $\mu_{y|x0}$ is the mean response of new values.
- x_0 is specific value.
- $T_{n-2}^{.975}$ is 97.5th percentile of the Student's t-distribution with n-2 degrees of freedom.
- n is number of samples in the dataset.
- $\hat{\sigma}$ is the standard deviation of the error term in the fit:

$$\hat{\sigma} = \sqrt{\sum_{i=1}^{n}\frac{(y_i - \hat{y})^2}{n-2}}$$

Prediction interval

The 95% probability that this interval around a fit line contains a new future observation at specific age at collection value.

$$|\hat{y}_0 - y_0| \leq T_{n-2}^{.975}\hat{\sigma}\sqrt{1 + \frac{1}{n} + \frac{(x_0 - \bar{x})^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2}}$$

- $\hat{y}_0 = \sum_{i=0}^{2} a_i x_0$ is polynomial fit with degree of the polynomial equal to 2.
- y_0 is the new observation.
- x_0 is specific value.
- $T_{n-2}^{.975}$ is 97.5th percentile of the Student's t-distribution with n-2 degrees of freedom.

- n is number of samples in the dataset.
- $\hat{\sigma}$ is the standard deviation of the error term in the fit:

$$\hat{\sigma} = \sqrt{\sum_{i=1}^{n} \frac{(y_i - \hat{y})^2}{n-2}}$$

Comparing Trajectories:

We used two different statistical analysis tools that are used to compare the significant difference between two trajectories:

1. Splinectomy longitudinal statistical analysis tools - The methods used are translated from R to Python and accommodated for our project. The original package is called splinectomeR, implemented in R. For more details, please refere to Shields-Cutler RR, et al. Front Microbiol. 2018. Briefly, the test compares whether the area between two polynomial lines is significantly different. Our trajectory lines are the polynomial lines with degrees 2 or 3.
2. Linear regression statistical analysis tools: A statistical method based on comparing the two linear lines (line y=k*x+n). To compare two linear lines, we compare the significant difference in the two coefficients that represent the line k (slope) and n (y-axis intersection).

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Case-studies

The Microbiome Toolbox implements methods that can be used for microbiome dataset analysis and microbiome trajectory calculation. The dashboard offers a wide variety of interactive visualizations. Although, we believe that python code is the one to be used for detailed analysis, we have still made a web-based tool for (i) quick overview and understanding of the capabilities and functions of this toolbox (ii) easy use by non-specialized users who don't necessary use python-code based analysis for their work.

We have dataset checks executed on dataset upload. If the dataset does not follow the formatting rules, the upload error will be reported to the user for suitable modifications of data format. We provide two demo datasets from studies on mouse and human infants. Both datasets can be downloaded (exported) and can be immediately uploaded for testing as "Custom data". Thus, these datasets can be used as example references for the users to understand how to format their own data.

Options to change the plots dynamically have been provided now such as change of axis-ticks, number of figures in a panel, height and width of the plot and many other options as relevant for the figure being displayed. (Some of the control over axes, labels names, and automatically generated colors are fixed by the framework). For more appearance tuning, we suggest SVG export option, so the user can further modify the plot in image editing software and save it as higher resolution.

For easy understanding, users can take demo datasets and look at the analysis section by section, where many useful technical details and references have been provided as relevant for that analytical section. This is illustrated below by two case studies using the mouse and human infants datasets.

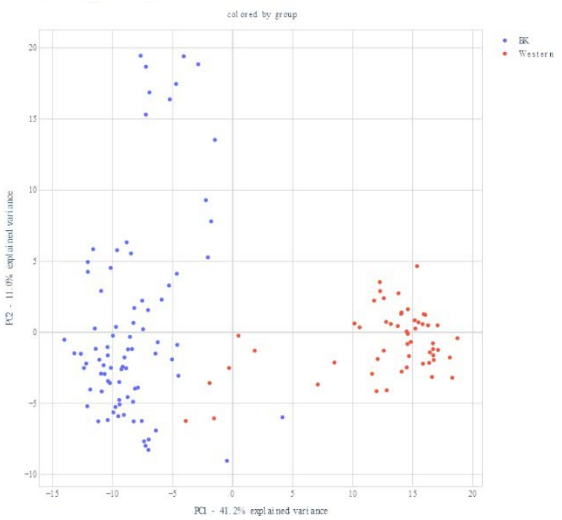
Case Study 1:

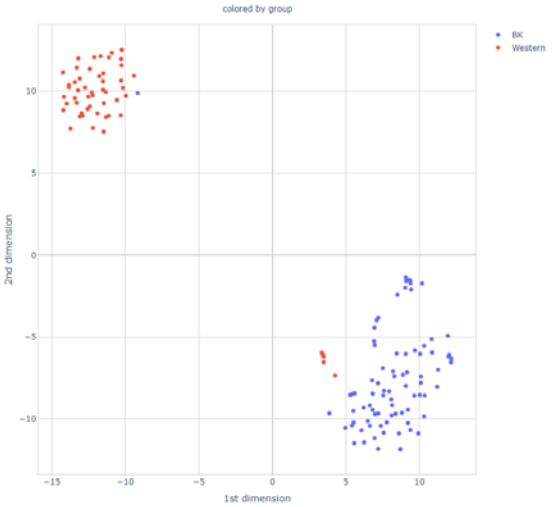
We use a mouse study where all mice were fed the same low-fat, plant polysaccharide–rich diet for the first 21 days of the study. At this point 6 of the mice were then switched to a high-fat, high-sugar "Western" diet. We label these groups as "BK" and "Western". The subsequent changes in the microbial community were then observed over a follow-up of roughly 60 days.

The main thing we examine is the changes in microbiome in response to dietary differences.

Dimensionality-reduction plots:

In order to visualize samples with lots of feature columns, we use embedding methods like PCA to embed the high-dimensional sample to a lower dimension (2-dim or 3-dim). With this type of visualization, we hope to catch patterns or clusters that cannot be seen otherwise. If dataset has groups column, we will be able to distinguish samples based on the group they belong to. By multiple different methods here, we see clear differences in the microbiota profiles of samples distinguished by diet group. From these plots, it also seems that some samples have either been mislabeled or had some other issue, such as didn't respond to dietary intervention at all, since they cluster with the original diet "BK" group.





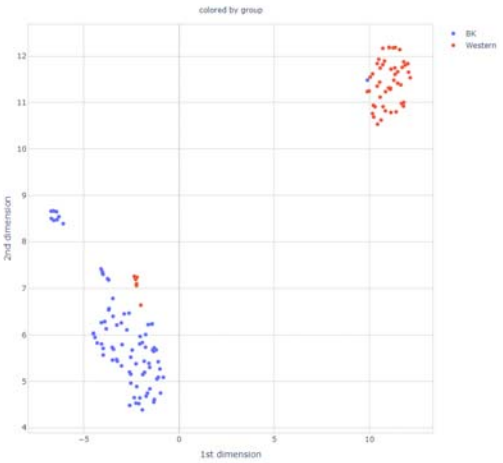
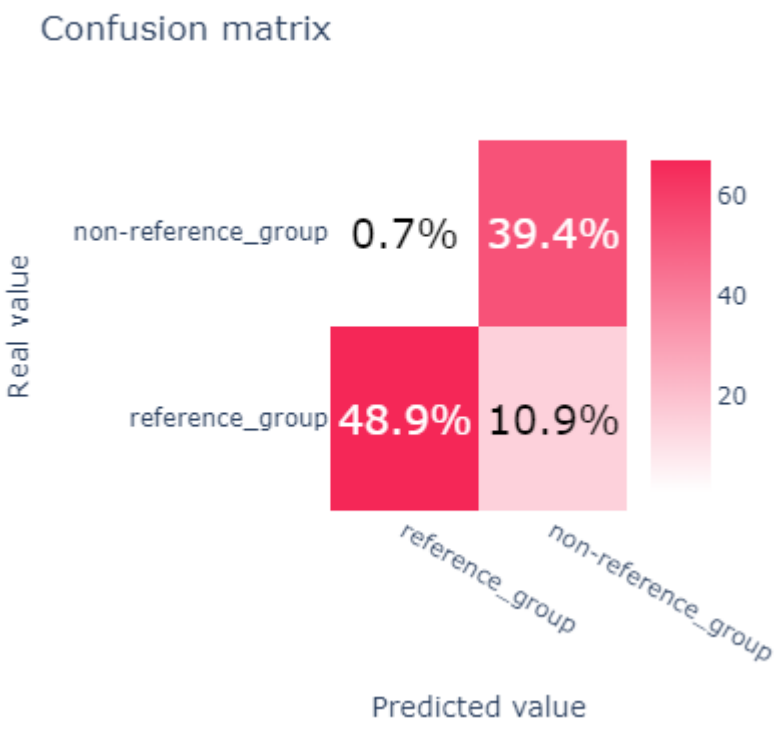
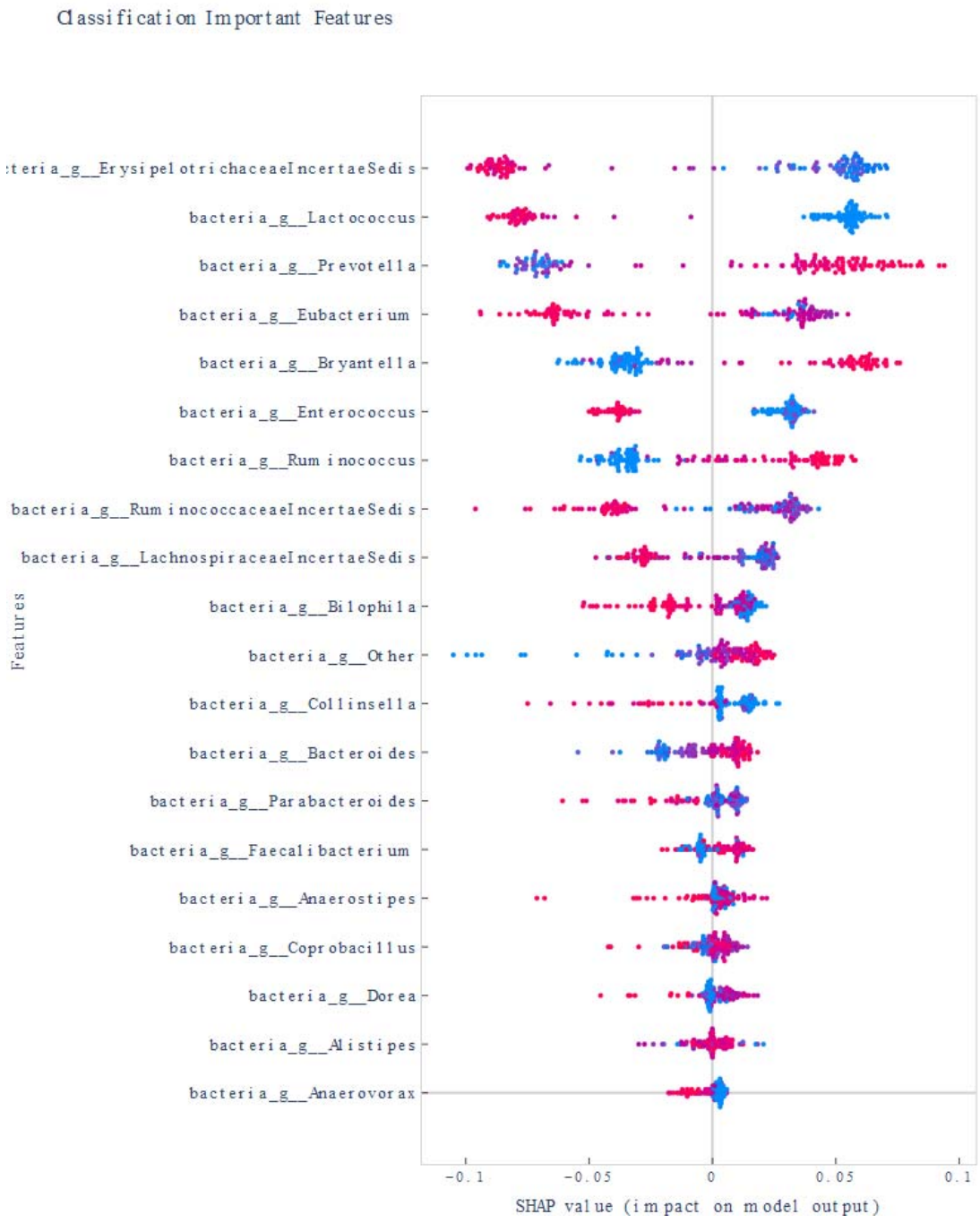


Fig 1. multidimensionality reduction methods show clear differences in microbiota patterns by dietary groups (top-left) PCA (top-right) TSNE (bottom-left) ISOMAP.

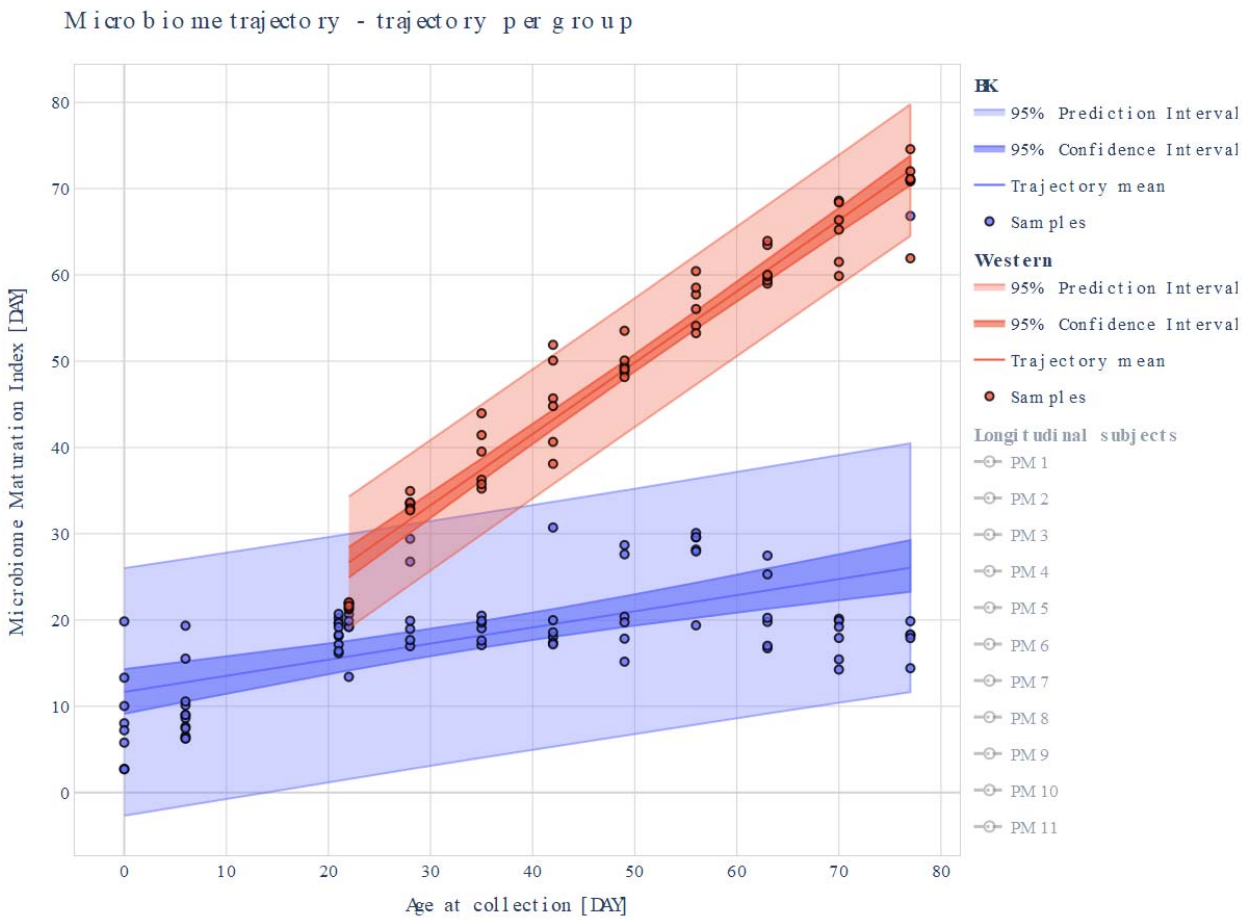
Using RandomForest based machine learning model, we can check if we can distinguish the two dietary groups successfully. The ideal separation between two groups (reference vs. non-reference) will have 100% of values detected on the second diagonal. This would mean that the two groups can be easily separated knowing their taxa abundances and metadata information. Here, Accuracy: 88.54 and F1-score: 0.79 indicates a very good separation.



Using a SHAP based analysis, we can identify the microbial signature, i.e., the bacteria taxa driving these differences under two dietary groups.



Differences in microbiome changes over time under the influence of external dietary changes here, also called as succession in ecology, can be simply visualized as follows



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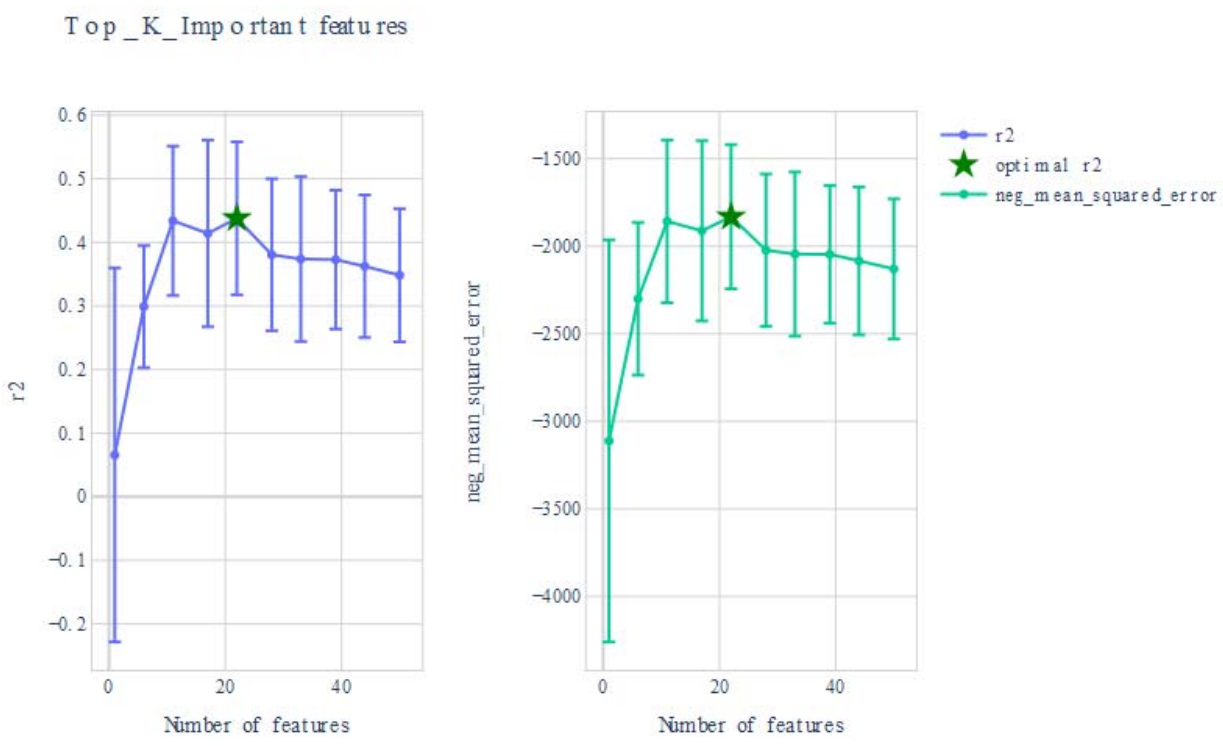
Description of deign of human microbiota transplant experiments here at: https://pubmed.ncbi.nlm.nih.gov/20368178/#&gid=article-figures&pid=fig-1-uid-0

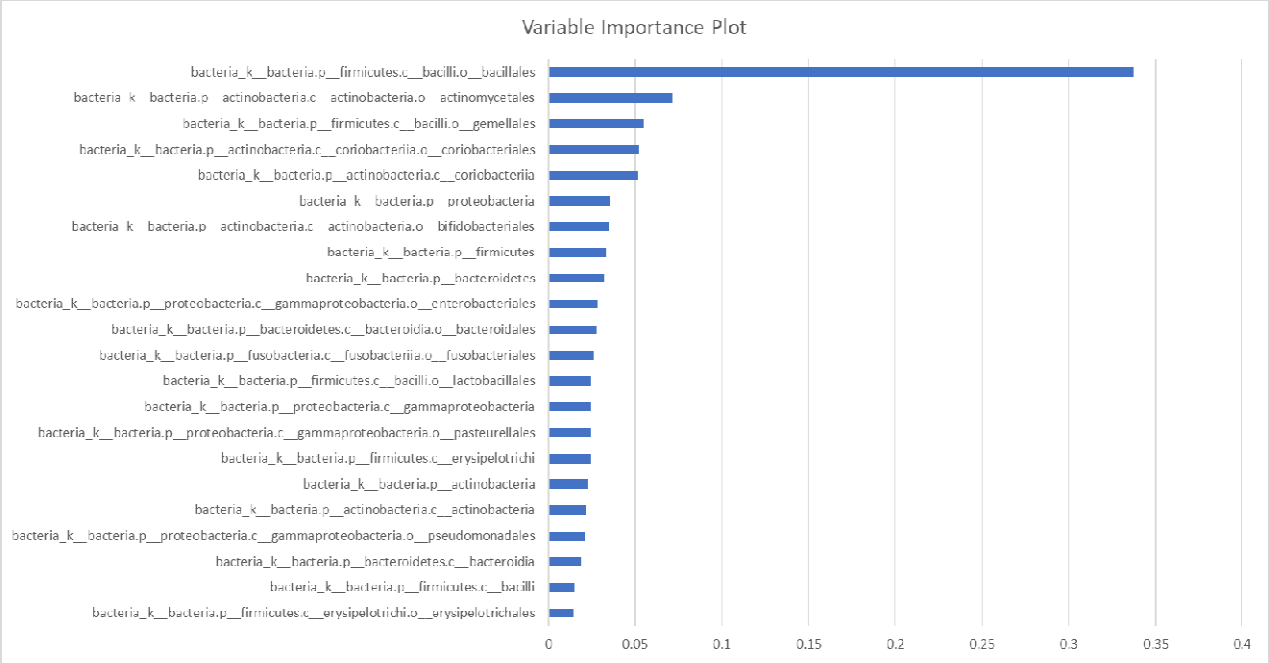
Case Study 2:

Here, we examine evolution of microbiota of healthy singletons, twins and triplets from a cohort in Bangladesh. While the study is larger, for the purpose of demonstration on the dashboard, we take a small snippet of the data for 9 subjects from about 0-6 months of infant age (66 samples).

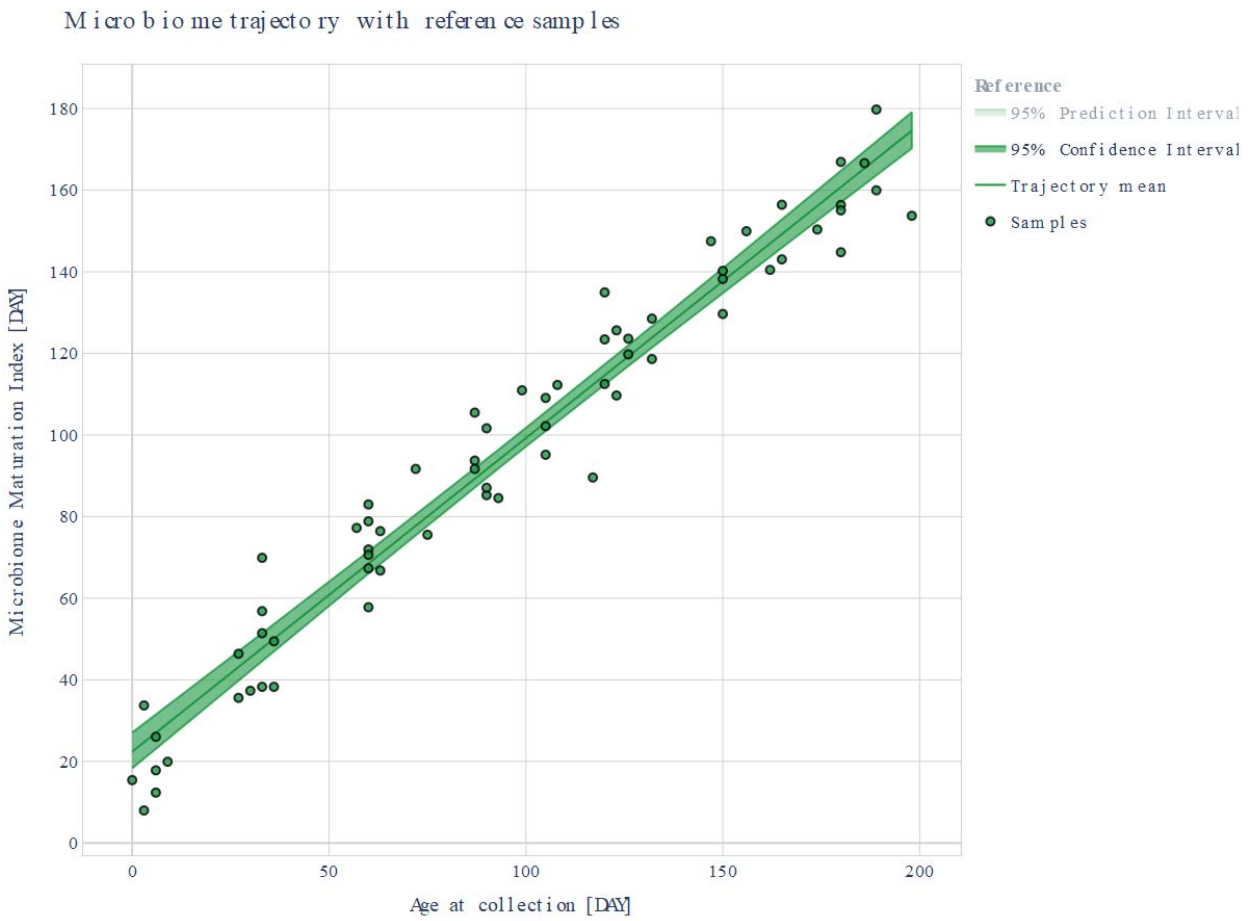
Feature extraction plots show how does trajectory performance look like when only working with the top 15 or top 20 bacteria used for the model (when TOP_K_IMPORTANT option is selected). Equivalent thinking goes for the other options (NEAR_ZERO_VARIANCE and CORRELATION). Metrics used to evaluate the performances of different model sizes are – mean_squared_error and R-squared. The x-axis shows the number of features used to train the model, y-axis shows the performance value. Below we see the feature importance for the microbiome trajectory (sorted from the most important to the least important feature).



The importance of the 22 bacteria features used to train the model above can be examined as below with the most important bacteria at the top and the least one at the bottom:



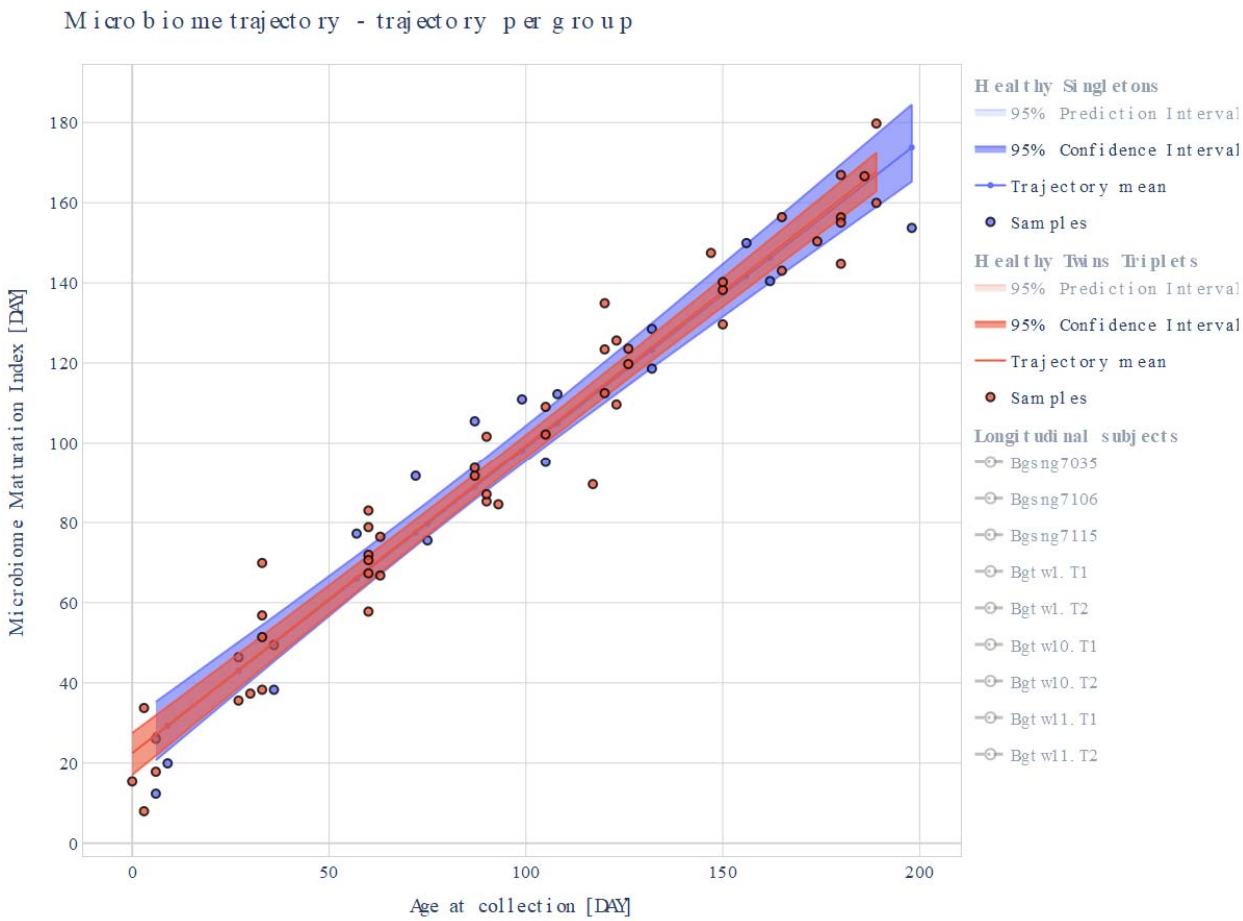
Next, the microbiome trajectory is built on the reference samples. The plot below shows the reference samples, and confidence intervals with mean. The aim is to capture the relation between x- and y- the best, ideal falling on the diagonal. Here, R^2 obtained is 0.918 with a MAE of 13.266



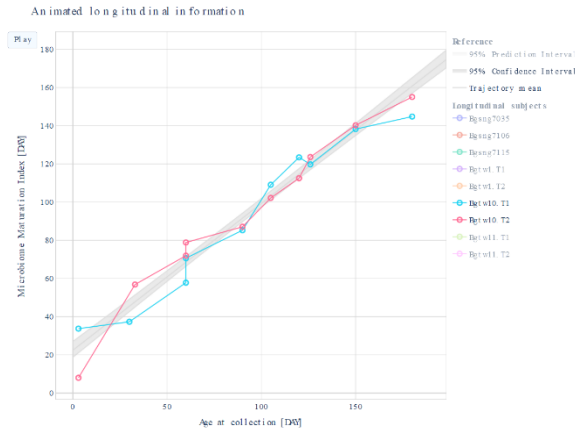
This dataset can be further split into singletons (18 samples) and twins, triplets (48 samples) and separate trajectories derived for each group. Here, for singletons we had R^2 of 0.909, MAE of 13.673 days. For, twins and triplets, we had R^2 of 0.920, MAE of 13.114 days

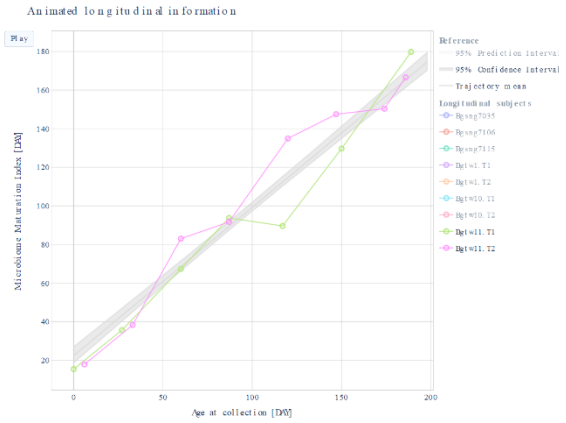
Further, these can be examined separately to test if these groups of infants follow similar trajectories or not. This can be done both visually as well as by statistical tests (explained in Supplementary Text and in the dashboard of toolbox at: http://microbiome-toolbox.herokuapp.com/page-3). There was no statistically significant difference in trajectories of singletons vs. twins and triplets. Linear p-value (k, n) between Healthy Singletons vs. Healthy Twins Triplets was deemed insignificant as it was >0.05(0.924, 0.987).

We can

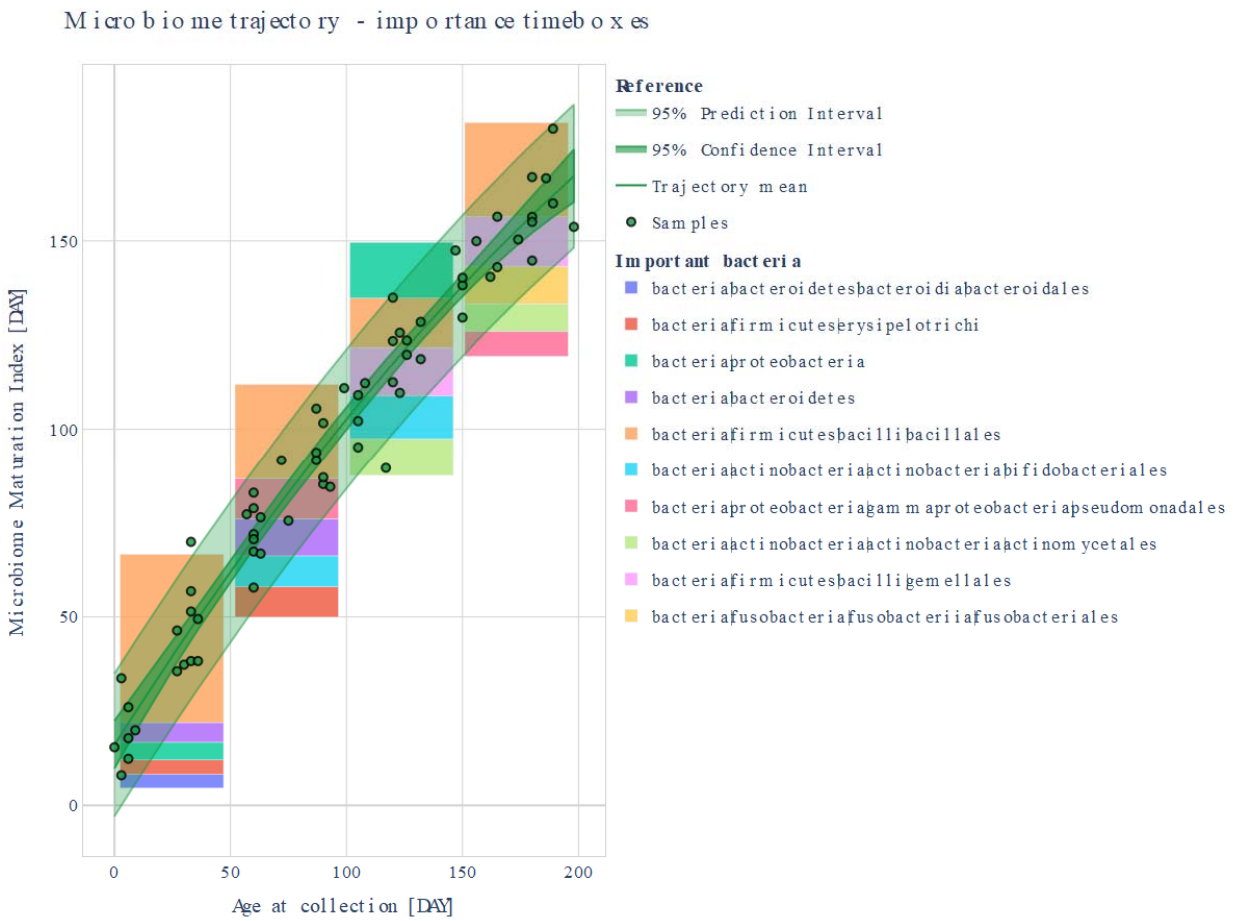


We can also longitudinally trace for example, if twins are following a similar path in and around the reference trajectory (left figure) or if they are deviant from each other (right figure).

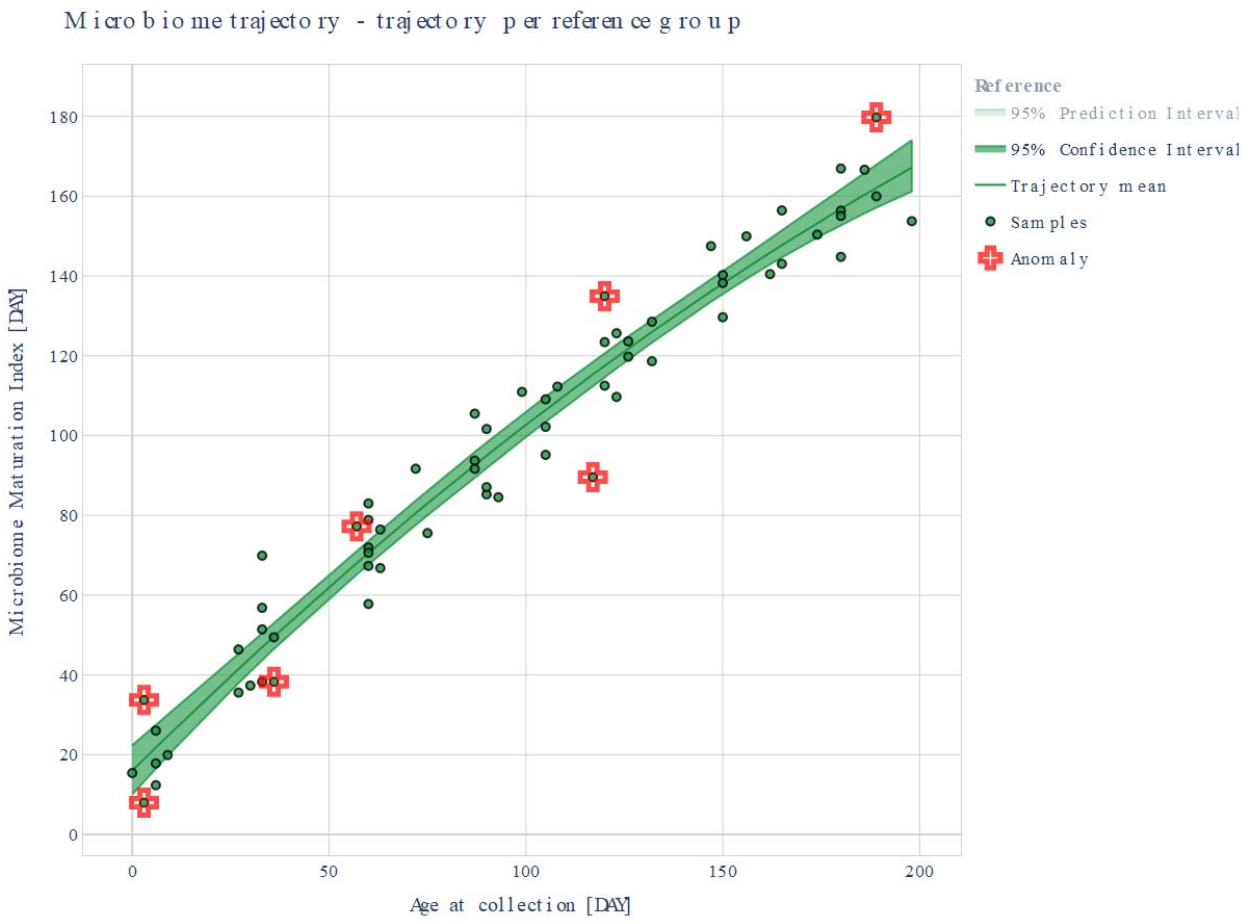




Further, cross-sectional analyses to examine which are the important bacteria to determine the trajectory as per the time-window can be performed (customizable to change number of top important bacteria and definition of time windows).



Outliers can be defined by different statistical measures such as by prediction interval, low pass filter or isolation forest (plot below) and examined to identify which values are out of range that make them outliers. Some of the methodological details are explained in the corresponding section of the dashboard at: http://microbiome-toolbox.herokuapp.com/page-5



References:

(1) Subramanian S, et al. Nature 2014 Persistent Gut Microbiota Immaturity in Malnourished Bangladeshi Children, raw data.
https://gordonlab.wustl.edu/supplemental-data/supplemental-data-portal/subramanian-et-al-2014/

(2) The effects of exclusive breastfeeding on infant gut microbiota: a meta-analysis across populations, some processing included on raw data. https://zenodo.org/record/1304367#.Ygo9zvnMKUm

(3) Meta-analysis of effects of exclusive breastfeeding on infant gut microbiota across populations dataset
https://github.com/nhanhocu/metamicrobiome_breastfeeding

(4) Ho NT, et al. Nat. Commun. 2018 Meta-analysis of effects of exclusive breastfeeding on infant gut microbiota across populations. https://www.nature.com/articles/s41467-018-06473-x