Evaluation of machine learning methods for 16S rRNA gene data

Running title: Machine learning methods in microbiome studies

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Abstract

Machine learning (ML) modeling of the human microbiome has the potential to identify the microbial biomarkers and aid in diagnosis of many chronic diseases such as inflammatory bowel disease, diabetes and colorectal cancer. Progress has been made towards developing ML models that predict health outcomes from bacterial abundances, but rigourous ML models are scarce due to the flawed modeling methods that call the validity of developed ML models into question. Furthermore, the use of black box ML models has hindered the validation of microbial biomarkers. To overcome these challenges, we benchmarked seven different ML models that use fecal 16S rRNA sequences to predict the presence/absence of colorectal cancer (CRC) lesions (n=490 patients, 261 controls and 229 cases). To show the effect of model selection, we assessed the predictive performance, interpretability, and computational efficiency of the following models: L2-regularized logistic regression, L1 and L2 support vector machines (SVM) with linear and radial basis function kernels, a decision tree, random forest, and extreme gradient boosting (XGBoost). The random forest model was best at detecting CRC lesions with an AUROC of 0.695 but it was slow to train (83.2 h) and hard to interpret. Despite its simplicity, L2-regularized logistic regression followed random forest in predictive performance with an AUROC of 0.680, and it trained much faster (12 min). This study showed that we should choose ML models based on our expectations of predictive performance, interpretability and our computational resources. It also established standards for modeling pipelines of microbiome-associated ML models.

Importance

Prediction of health outcomes using ML is rapidly being adopted by human-associated microbiome studies. However, the developed ML models so far are overoptimistic in terms of validity and predictive performance. Without rigorous ML pipelines, we cannot trust ML models. Before we can speed up progress, we need to slow down, define and start using good ML practices.

Background

Advances in sequencing technology and decreasing costs of generating 16S rRNA gene sequences have allowed rapid exploration of human associated microbiome and its health implications. Currently, the human microbiome field is growing at an unprecedented rate and as a result, there is an increasing demand for methods that identify associations between members of the microbiome and human health. However, this is difficult as human associated microbial communities are remarkably complex, high-dimensional and uneven within and between individuals with the same disease. It is unlikely that a single species can explain a disease. Instead, subsets of those communities, in relation to one another and to their host, account for differences in health outcomes.

Machine learning (ML) methods are effective at recognizing and highlighting patterns in complex microbial datasets. Therefore, researchers have started to explore the utility of ML models that use microbiota associated biomarkers to predict human health and to understand the microbial ecology of diseases such as liver cirrhosis, colorectal cancer, inflammatory bowel diseases (IBD), obesity, type 2 diabetes and others (1–11). However, currently the field's use of ML lacks clarity and consistency on which methods are used and how these methods are implemented (12, 13). More notably, we commonly see flawed ML practices such as using ML pipelines where there is no seperate held-out test dataset to evaluate model performance or reporting few or only the best outcomes of cross-validation. Even when there are seperate testing sets to evaluate model performance, there are large differences between cross-validation and testing performances that indicate overfitting as well as large confidence intervals for testing performances (4, 14–20). Moreover, there is a lack of discussion on why a particular ML model is utilized. Recently, there is a trend towards using more complex ML models such as random forest, extreme gradient boosting and neural networks without a discussion on if and how much model interpretibility is necessary for the study (11, 21–23). Black box machine learning models require posthoc explanations to determine the feature importances in making a prediction. These explanations can be misleading and at times unreliable when making high-stake decisions about someone's health (24). The lack of transparency on model selection and interpretation as well as flawed modeling methods negatively impact model validity and reproducibiliy. We need to strive toward better machine learning practices

by (1) implementing rigourous machine learning pipelines and (2) selecting ML models that reflect the goal of the study as it will inform our expectations of model accuracy, complexity, interpretibility and computational efficiency.

To showcase a rigorous ML pipeline and to shed light on how much ML model selection can affect modeling results, we performed an empirical analysis comparing several different ML models using the same dataset and the same ML pipeline. We used a previously published colorectal cancer (CRC) study (3) which had fecal 16S rRNA gene sequences from 490 patients. CRC is a type of cancer which the human-associated human microbiome is hypothesized to directly contribute to its development and fecal 16S rRNA gene sequences have been used to detect CRC. We built seven ML models using fecal 16S rRNA gene sequences to predict healthy patients versus patients with colorectal lesions that were identified by colonoscopy as screen relevant neoplasias (SRN). The study had 261 normal and 229 SRN samples. We established modeling pipelines for L2-regularized logistic regression, L1 and L2 support vector machines (SVM) with linear and radial basis function kernels, a decision tree, random forest and XGBoost. Our ML pipeline utilized held-out test data to evaluate predictive performance and generalizability of each ML model. We used the area under the receiver operating characteristic curve (AUROC) as the predictive performance metric. The median test AUROC varied from 0.601 to 0.695. Random forest had the highest median AUROC for detecting SRN. Despite its simplicity, the L2-regularized logistic regression was second best in predictive performance. In terms of computational efficiency, L1 SVM with linear kernel trained the fastest (0.202 hours, std ± 0.028), while XGBoost took the longest (155.104 hours, std ± 0.959). We also found that depending on how the data is split to create a held-out test set, the AUROC values of a ML model can vary up to 0.295 which highlighted the importance of performing many randomized modeling runs. This study established standards for microbiome-associated ML models and underscored the importance of model selection.

Results

Model selection and pipeline construction

We used a cohort of 490 patients with 261 cases of SRN. For each patient, we had 6920 features (fecal bacterial abundances) and a two-class label that defines their colorectal health (having colorectal lesions that are identified as SRN or normal). All the cases were independently labeled through colonoscopies. We established modeling pipelines for a binary prediction task with L2-regularized logistic regression, L1 and L2 support vector machines (SVM) with linear and radial basis function kernels, a decision tree, random forest and extreme gradient boosted decision tree (XGBoost) to emphasize the differences in model accuracy, complexity, interpretibility and computational efficiency due to model selection.

For regularized logistic regression and SVM with linear kernel we used L2 regularization to keep all potentially important features. For comparison, we also trained an L1 regularized SVM model with linear kernel. L1-regularization on microbiome data lead to a sparser solution (i.e., force many coefficients to zero). Finally, to explore the potential for non-linear relationships among features and the outcome of interest, we trained tree based models, decision tree, random forest and XGboost, as well as an SVM with non-linear kernel.

We established a ML pipeline where we train and validate each of the seven models [Figure 1]. We randomly split the data into training/validation and test sets so that the training/validation set consisted of 80% of the full dataset while the test set was composed of the remaining data [Figure 1]. Since the cases are not uniformly represented in the data, the initial data-split was stratified to maintain the overall label distribution in both the training/validation and test sets. Training/validation set consisted of 393 patients (209 SRN), while the test set was composed of 97 patients (52 SRN). The training/validation data was used for training purposes and validation of hyperparameter selection (i.e. model selection), and the test set was used for evaluation purposes. Validation of hyperparameter selection was performed using repeated five-fold cross-validation on the training/validation set [Figure 1]. Similar to the initial data-split, five-fold cross-validation was also stratified to maintain the overall label distribution on the training and validation sets. We validated the cross-validation performances of each hyperparameter setting over 100 randomizations and selected the best performing hyperparameter setting in terms of AUROC metric and trained the full training/validation dataset [Figures S1 and S2]. We then used the held-out test set to evaluate the prediction performance of each ML model. The data-split, hyperparameter selection, training and

testing steps were repeated 100 times to get a reliable and robust reading of model performance [Figure 1].

Discriminative performance and generalizability of the seven models.

We evaluated the prediction performances of seven binary classification models when applied to held-out test data using AUROC metric [Figure 2]. Random forest had significantly higher test AUROC values than the other models for detecting SRNs when AUROC values were compared to the other six by Wilcoxon rank sum test ($p < 0.01$). The median AUROC of the random forest model was 0.695 (IQR 0.044). L2-regularized logistic regression, XGBoost, L2-regularized SVM with linear and radial basis function kernel AUROC values were not significantly different from one another. They had median AUROC values of 0.68 (IQR 0.055), 0.679 (IQR 0.052), 0.678 (IQR 0.056) and 0.668 (IQR 0.056) respectively. L1 SVM with linear kernel and decision tree had significantly lower AUROC values than the other ML models with median AUROC of 0.65 (IQR 0.066) and 0.601 (IQR 0.059), respectively [Figure 2].

For each model, we compared the median cross-validation AUROC to the median testing AUROC. The difference between the two should be low to suggest the model is not overfitting despite the large number of features. The largest difference between the two was 0.021 in L1 SVM with linear kernel, followed by SVM with radial basis function kernel and decision tree with a difference of 0.007 and 0.006, respectively [Figure 2].

We reported the testing AUROC values over 100 randomizations of the initial data-split. The results showed that depending on the data-split, the testing AUROC value showed great variability [Figure 2]. The testing AUROC values within each model varied 0.23 on average across the seven models. For instance, the lowest AUROC value of the random forest model was 0.59 whereas the highest was 0.81.

Interpretation of each ML model.

The ML models we built using L2-regularized logistic regression, L1 and L2 support vector machines (SVM) with linear and radial basis function kernels, a decision tree, random forest and XGBoost decrease in interpretibility as they increase in complexity. We interpreted L1 and L2 SVM with

linear kernel and L2 logistic regression using the feature weights of the trained models. We ranked the absolute weights of all the OTUs for each data-split [Figure 3]. We calculated the median ranks of these features over the 100 data-splits. In the three linear models, OTUs that had the largest median ranks and drove the detection of SRNs belonged to families *Lachnospiraceae*, and *Ruminococcaceae* (OTU01239, OTU00659, OTU00742, OTU00012, OTU00015, OTU00768, OTU00822, OTU00609), genera *Gamella* (OTU00426) and genera *Peptostreptococcus* (OTU00367) [Figure 3]. Some of the OTUs with the highest ranks were shared among the linear models. We explained the feature importances in non-linear models using permutation importance on the held-out test data where we randomly permuted non-correlated features individually and groups of correlated features together (see methods) to calculate the effect of permuted OTUs or group of OTUs on testing AUROC. The top 5 OTUs with the largest negative impact on testing AUROC overlapped in tree-based models [Figure 4]. Specifically, permuting *Peptostreptococcus* (OTU00367) abundances randomly, dropped the predictive performances the most in all tree-based methods [Figure S3]. Decision tree, random forest and XGBoost models' predictive performance dropped from 0.6 base testing AUROC median to 0.52, from 0.69 to 0.68 and from 0.68 to 0.65, respectively [Figure 4].

We also used permutation importance to interpret linear models to show the differences between the two interpretation methods [Figure S3]. L1-regularized SVM with linear kernel picked out the same OTUs as the most important features in methods that use feature rankings [Figure 3] as well as permutation importance [Figure S3]. However, L2-regularized models had different feature importances in the two methods of interpretation due to collinearity in microbial communities.

The computational efficiency of each ML model.

As the complexity of a ML model and the number of tuned hyperparameter settings increased [Table S1], its training times increased as well [Figure 5]. Linear models trained faster than non-linear models. L1 and L2 SVM with linear kernel and L2 logistic regression had training times of 0.2 hours, (std ± 0.03), 0.2 hours, (std ± 0.02), and 0.2 hours, (std ± 0.02), respectively. Whereas, a decision tree, SVM with radial basis function kernel, random forest and xgboost had training times of 4.4 hours, (std ± 0.3), 59.6 hours, (std ± 8.8), 83.2 hours, (std ± 11.3) and 155.1 hours, (std ± 1),

respectively [Figure 5].

Discussion

In this study we established a rigorous ML pipeline to use 16S rRNA sequence counts to predict a binary health outcome. We set-up standards for developing and evaluating ML models for microbiome data. First, we used a held-out test set to illustrate the difference between cross-validation and testing AUROC values. When the differences between cross-validation and test performance is low, this suggets the models are not overfit and that they will perform similar with similar data. In all seven models, the differences in cross-validation and testing AUROC values did not exceed 0.021 which suggests that these models will be able to test similar new data. Second, we performed the initial 80%-20% random datasplit 100 times in our ML pipeline. The randomization of the initial data-split to create a held-out test set is a crucial step in the ML pipeline to develop generalizable ML models and to report reliable performance metrics for a ML model. Depending on the data-split, there is the chance of being overoptimistic about the predictive performance of a model. In our study, we showed that there was variability in AUROC values between different random data-splits in each of the models we tested. Our results showed that the testing AUROC values varied 0.23 on average, depending on the data-split.Third, we used AUROC metric instead of accuracy in our study to evaluate predictive performance of the ML models. AUROC is always random at the value 0.5 and is a robust metric when a dataset is imbalanced. We also performed a full grid search for hyperparameter settings when building a ML model. It is not enough to use the default hyperparameter settings when using previously developed ML packages in programming languages such as R, Python and Matlab. In the example of L1-regularized SVM with linear kernel (Figure S1), the model showed large variability between different regularization coefficients (C) and was susceptible to performing poorly if the wrong regularization coefficient was assigned to the model by default.

Our results showed that we should choose to use ML models based on the goal of the study and our expectations of predictive performance, interpretibility and computational burden. In terms of predictive performance, random forest model had testing AUROC values statistically significantly

larger than others. However the second best model was L2-regularized logistic regression following random forest with a median AUROC difference of only 0.015. In terms of interpretation, random forest was a more complex ML model and it could only be explained using methods such as permutation importance. On the other hand, L2-regularized logistic regression was easy to interpret (i.e. regression coefficients of the trained model). If the goal of a study is to learn the ecology behind a disease and to identify microbial biomarkers of a disease, then easily interpretable models would be better suited. It is also important to consider the computational burden of developing ML models. Random forest model was slow to train 83.2 hours whereas L2-regularized logistic regression trained in 12 minutes. Another criteria when choosing a ML model is generalizability. The generalization performance of ML models depends on size. The more complex the model, the more data it will need. The dataset we used for our study had 490 samples, however microbiome studies that have smaller sample sizes would benefit from using less complex models such as L2-regularized logistic regression.

ML model interpretation methods in the previous studies microbiome studies lacked clarity and transparency. The studies that made the model interpreatation code publicly available, used default functions in previously built packages in R, python and matlab. In this study we used two methods to interpret our models. Feature weights of trained models ad permutation importance. the differences between the two interpretation methods

Materials and Methods

Data collection and study population. The data used for this analysis are stool bacterial abundances and clinical information of the patients recruited by Great Lakes-New England Early Detection Research Network study. These data were obtained from Sze et al (25). The stool samples were provided by recruited adult participants who were undergoing scheduled screening or surveillance colonoscopy. Colonoscopies were performed and fecal samples were collected from participants in four locations: Toronto (ON, Canada), Boston (MA, USA), Houston (TX, USA), and Ann Arbor (MI, USA). Patients' colonic health was labeled by colonoscopy with adequate preparation and tissue histopathology of all resected lesions. Patients with an adenoma greater

than 1 cm, more than three adenomas of any size, or an adenoma with villous histology were classified as advanced adenoma. Study had 172 patients with normal colonoscopies, 198 with adenomas and 120 with carcinomas. Of the 198 adenomas, 109 were identified as advanced adenomas. Stool provided by the patients was used for 16S rRNA gene sequencing to measure bacterial population abundances. The bacterial abundance data was generated by Sze et al, by processing 16S rRNA sequences in Mothur (v1.39.3) using the default quality filtering methods, identifying and removing chimeric sequences using VSEARCH and assigning to OTUs at 97% similarity using the OptiClust algorithm (26–28).

Data definitions and pre-processing.

The colorectal health of the patient was defined as two encompassing classes; Normal or Screen Relevant Neoplasias (SRNs). Normal class includes patients with non-advanced adenomas or normal colons whereas SRN class includes patients with advanced adenomas or carcinomas. The bacterial abundances are the features used to predict colonic health of the patients. Bacterial abundances are discrete data in the form of Operational Taxonomic Unit (OTU) counts. OTU counts were set to the size of our smallest sample and were subsampled at the same distances. They were then transformd by scaling to a [0-1] range.

Model training and evaluation.

Models were trained using the machine learning wrapper caret package (v.6.0.81) in R (v.3.5.0). Within the caret package, we have made modifications to L2-regularized SVM with linear kernel function **svmLinear3** and developed a L1-regularized SVM with linear kernel function **svmLinear4** to calculate decision values instead of predicted probabilities. These changes are available at https://github.com/SchlossLab/Topcuoglu_ML_XXXX_2019/.

For L2-regularized logistic regression, L1 and L2 support vector machines (SVM) with linear and radial basis function kernels we tuned the **cost** hyperparameter which determines the regularization strength where smaller values specify stronger regularization. For SVM with radial basis function kernel we also tuned **sigma** hyperparameter which determines the reach of a single training instance where for a high value of sigma, the SVM decision boundary will be dependent on the

points that are closest to the decision boundary. For the decision tree model, we tuned the **depth of the tree** where deeper the tree, the more splits it has. For random forest, we tuned the **number of features** to consider when looking for the best tree split. For XGBoost, we tuned for **learning rate** and the **fraction of samples** to be used for fitting the individual base learners.For hyperparameter selection, we started with a granular grid search. Then we narrowed and fine-tuned the range of each hyperparameter. The range of the grid depends on the ML task and ML model. A full grid search needs to be performed to avoid variability in testing performance. We can use hyper-band to help us with our hyperparameter selection (29).

The computational burden during model training due to model complexity was reduced by parallelizing segments of the ML pipeline. In this study we have parallelized each data-split which allowed 100 data-splits to be processed through the ML pipeline at the same time for each model. We can further parallelize the cross-validation step for each hyperparameter setting.

Permutation importance workflow. We created a Spearman's rank-order correlation matrix, corrected for multiple pairwise comparisons. We then defined correlated OTUs as having perfect correlation (correlation coef=1 and $p<0.01$).Other OTUs were permuted individually to get permutation importance but the correlated ones are grouped together and permuted at the same time. The reported OTUs that have impact on testing AUROC are reported in Figures xxx. If we want we can decrease the correlation coefficient to consider OTUs that are correlated with ecological consequences but this was out of the scope of this study but will be followed up in further analyses.

Statistical analysis workflow. Data summaries, statistical analysis, and data visualizations were performed using R (v.3.5.0) with the tidyverse package (v.1.2.1). We compared the AUROC values of the seven ML models by Wilcoxon rank sum tests to determine the best discriminative performance.

Code availability. The code for all sequence curation and analysis steps including an Rmarkdown version of this manuscript is available at https://github.com/SchlossLab/Topcuoglu_ML_XXXX_2019/.

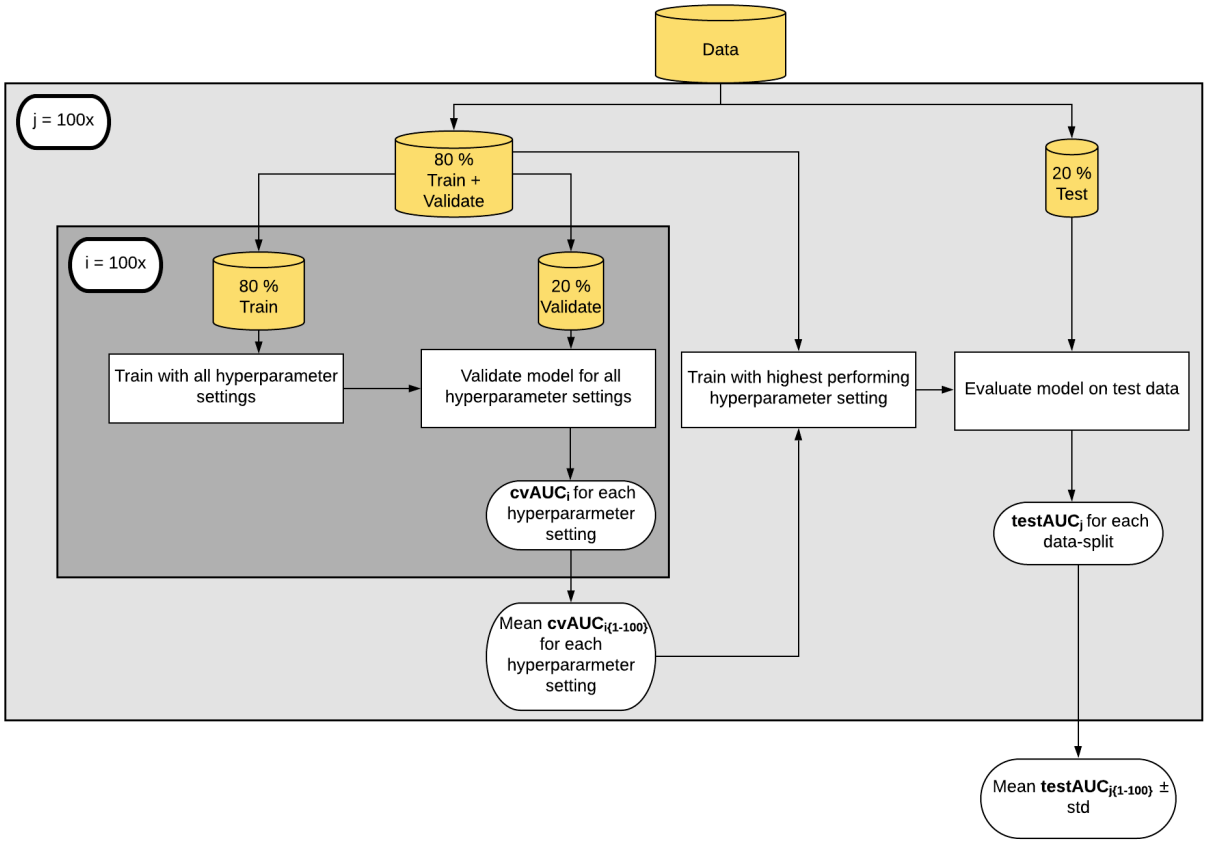


Figure 1. Machine learning pipeline showing predictive model training and evaluation flowchart. We split the data 80%/20% stratified to maintain the overall label distribution, performed five-fold cross-validation on the training data to select the best hyperparameter setting and then using these hyperparameters to train all of the training data. The model was evaluated on a held-out set of data (not used in selecting the model). Abbreviations: AUROC, area under the receiver operating characteristic curve

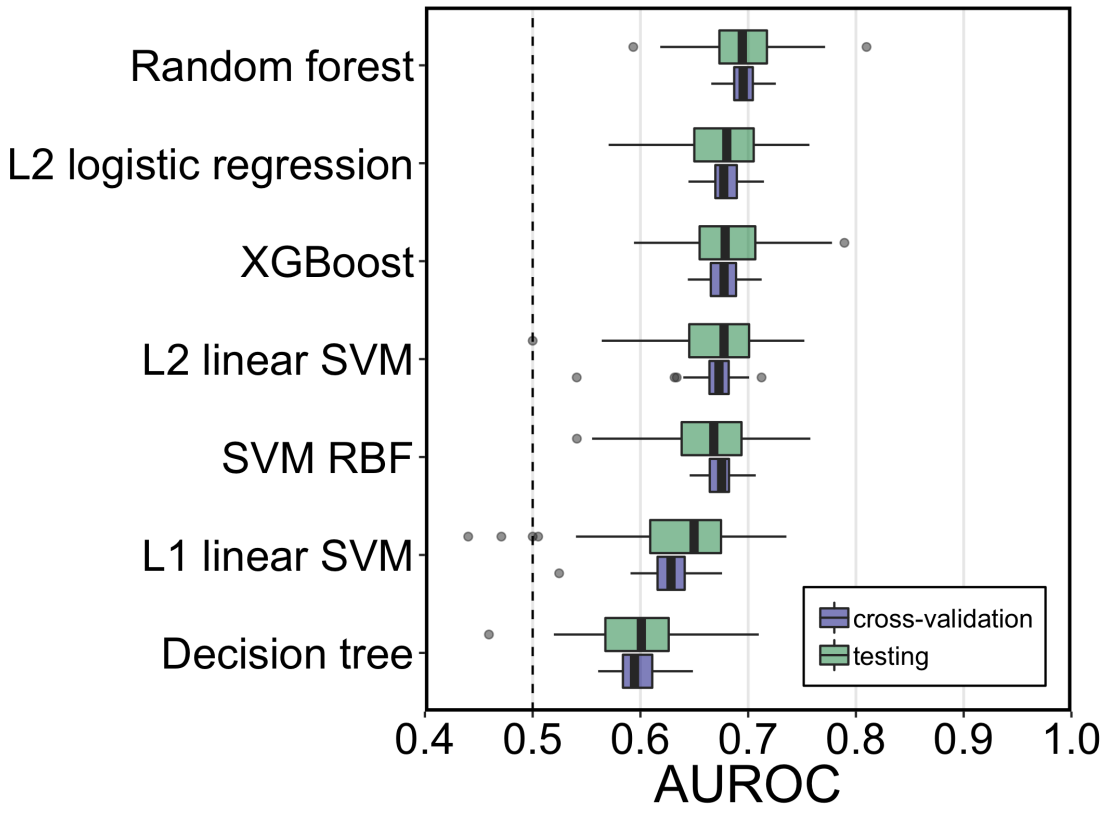
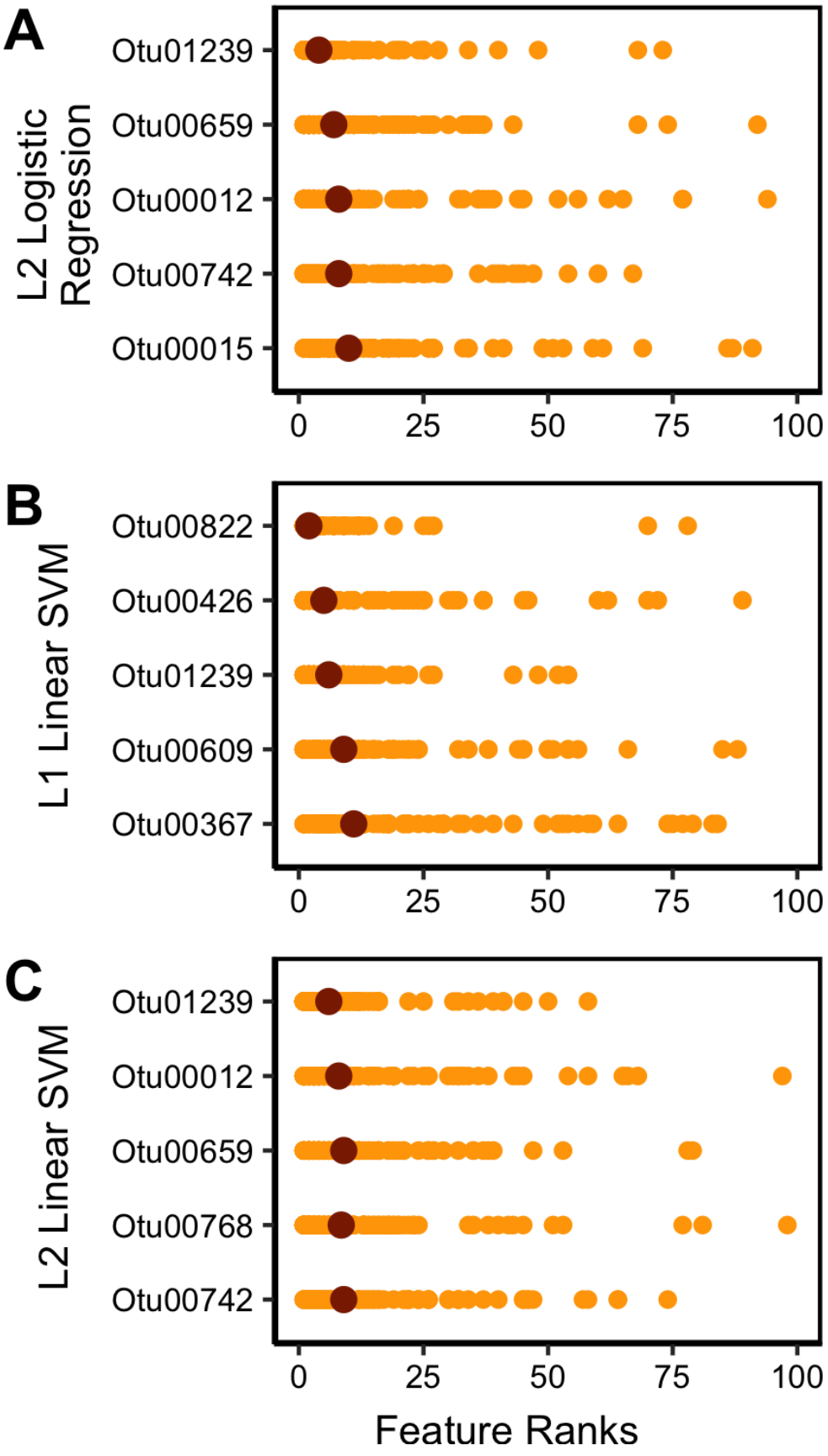


Figure 2. Generalization and classification performance of ML models using AUROC values of all cross validation and testing performances. The median AUROC for diagnosing individuals with SRN using bacterial abundances was higher than chance (depicted by horizontal line at 0.50) for all the ML models. Discriminative performance of random forest model was higher than other ML models. The boxplot shows quartiles at the box ends and the statistical median as the horizontal line in the box. The whiskers show the farthest points that are not outliers. Outliers are data points that are not within 3/2 times the interquartile ranges. Abbreviations: SRN, screen-relevant neoplasias; AUROC, area under the receiver operating characteristic curve; SVM, support vector machine; XGBoost, extreme gradient boosting


A
L2 Logistic Regression
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Otu00426
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Otu00609
Otu00367
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C
L2 Linear SVM
Otu01239
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Feature Ranks

288 **Figure 3.**

Interpretation of the linear ML models. The absolute feature weights of (A) L2 logistic regression coefficients (B) L1 SVM with linear kernel (C) L2 SVM with linear kernel were ranked from highest rank 1 to 100 for each data-split. The feature ranks of the highest ranked five OTUs based on their median ranks are shown here. Similar OTUs had the largest impact on the predictive performance of L2 logistic regression and L2 SVM with linear kernel. Abbreviations: SVM, support vector machine; OTU, Operational Taxonomic Unit.

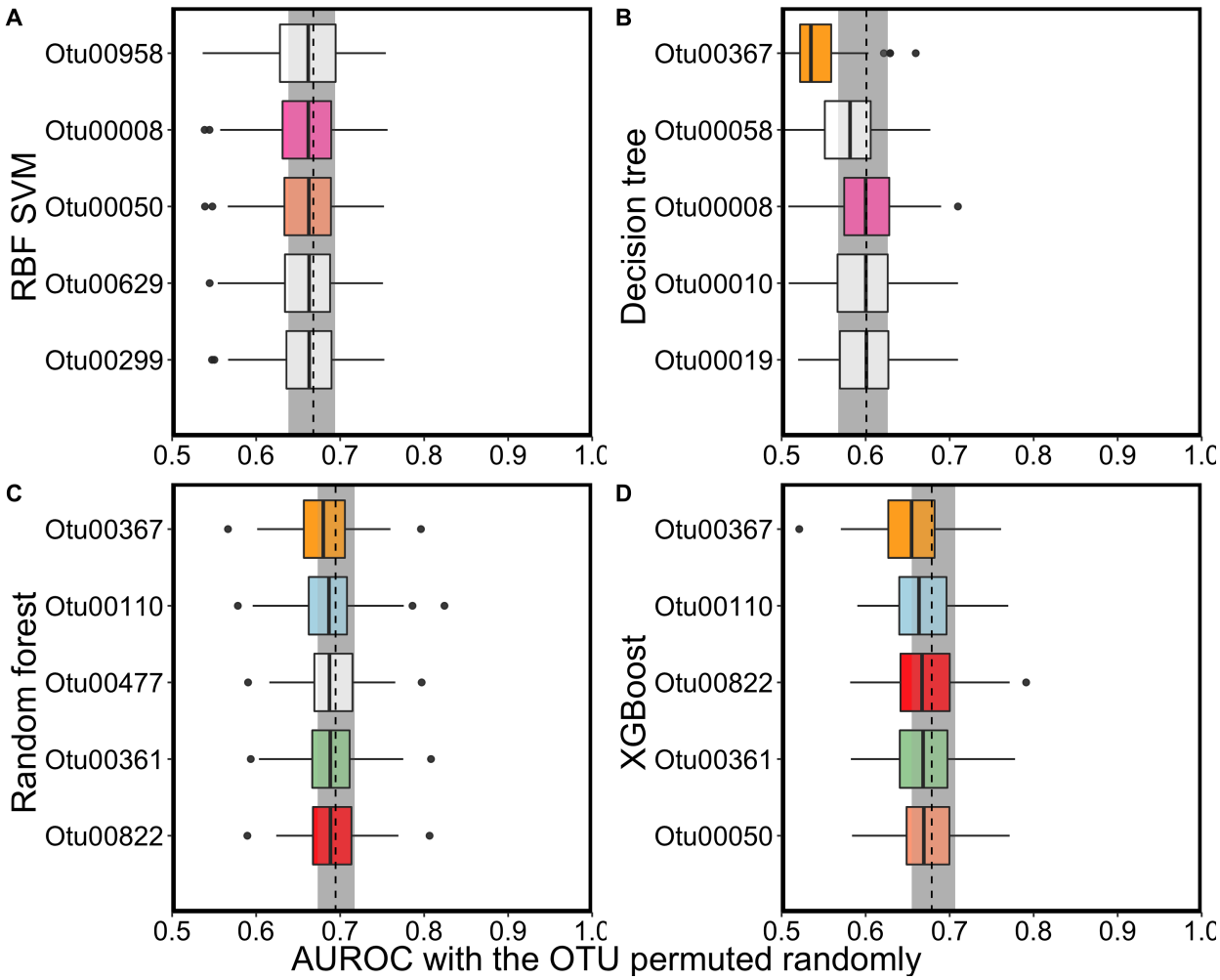
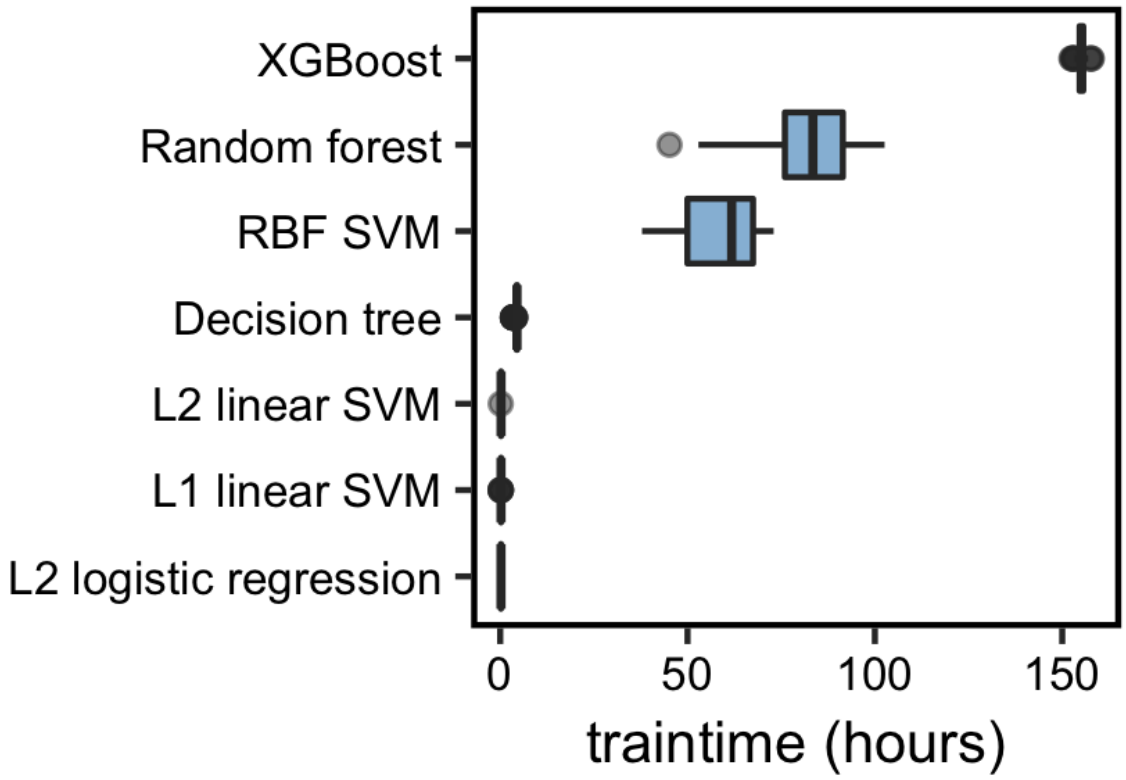


Figure 4. Explanation of the non-linear ML models. (A) SVM with radial basis kernel (B) decision tree (C) random forest (D) XGboost feaure importances were explanied using permutation importance using held-out test set. The gray rectangle and the dashed line show the IQR range and median of the base testing AUROC without any permutation performed. The colors of the boxplots stand for the unique OTUs that are shared among the different models; pink for OTU0008, salmon for OTU0050, yellow for OTU00367, blue for OTU00110, green for OTU00361 and red for OTU00882. For all the tree-based models, a *Peptostreptococcus* species (OTU00367) had the largest impact on predictive performance of the model. Abbreviations: SVM, support vector machine; OTU, Operational Taxonomic Unit; RBF, radial basis kernel; OTU, Operational Taxonomic Unit.



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307 **Figure 5. Computational efficiency of seven ML models.** The training times for of each
308 data-split showed the differences in computational efficieny of the seven models. The median
309 training time in hours was the highest for XGBoost and shortest for L1-regularized SVM with linear
310 kernel. The boxplot shows quartiles at the box ends and the statistical median as the horizontal line
311 in the box. The whiskers show the farthest points that are not outliers. Outliers are data points that
312 are not within 3/2 times the interquartile ranges. Abbreviations: AUROC, area under the receiver
313 operating characteristic curve; SVM, support vector machine; XGBoost, extreme gradient boosting.

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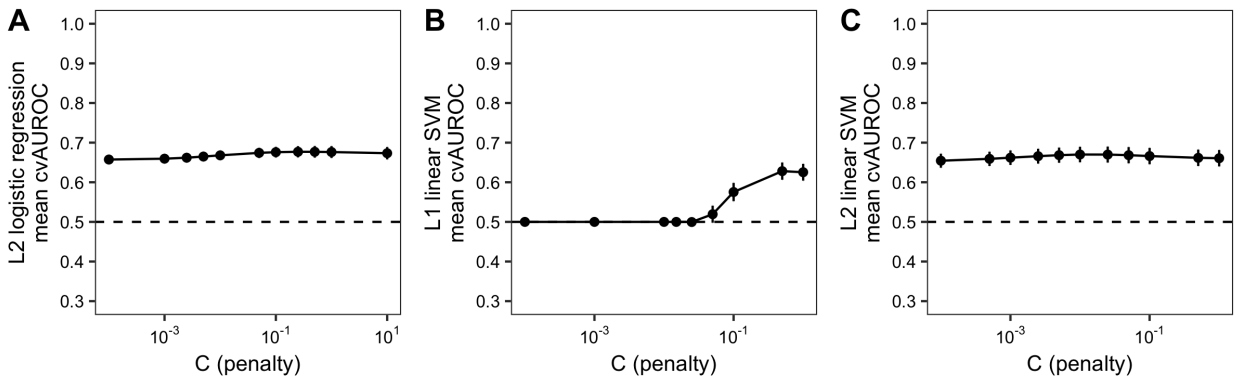
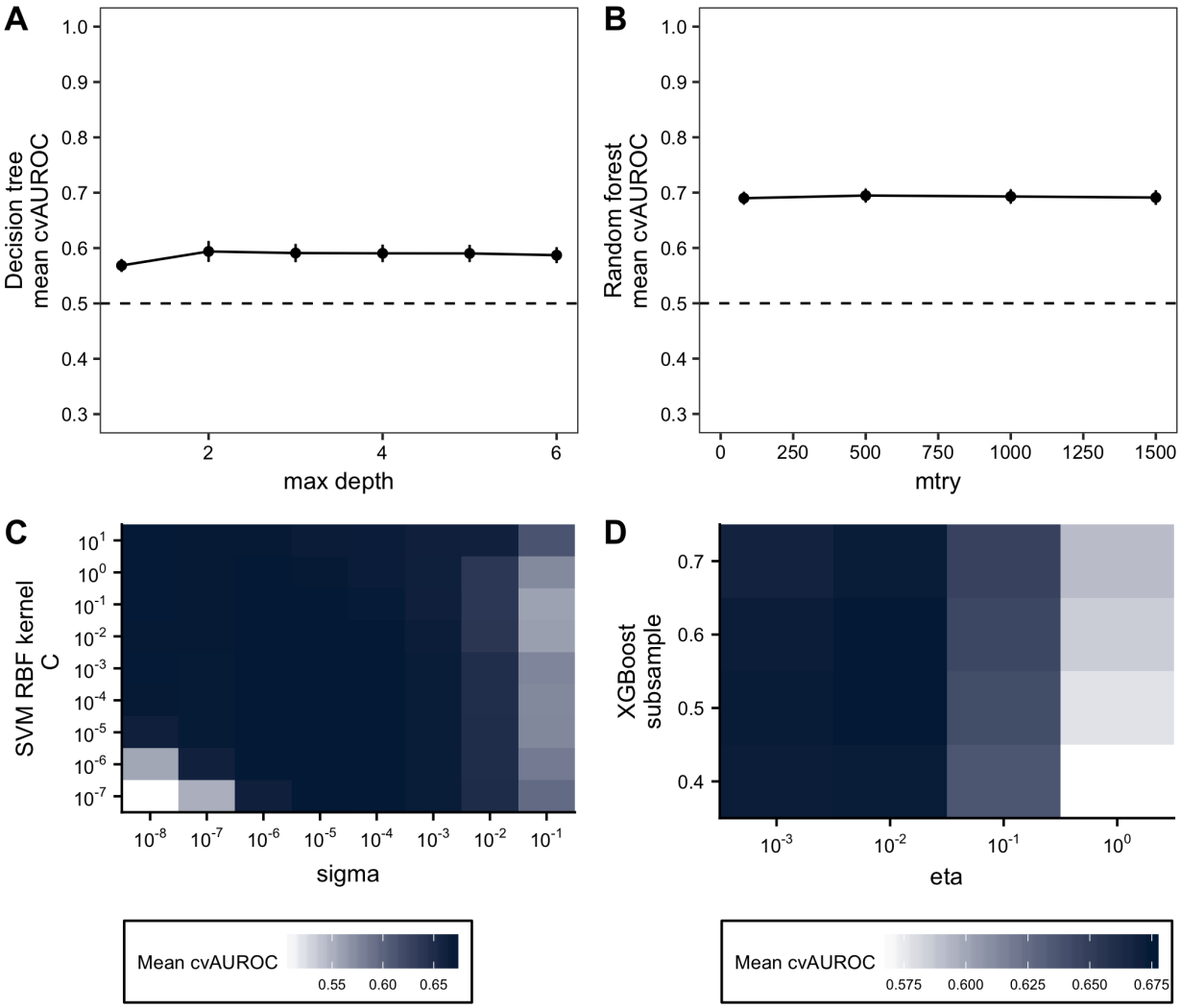


315

316 **Figure S1. Hyperparameter setting performances for linear models.** (A) L2 logistic regression
317 (B) L1 SVM with linear kernel (C) L2 SVM with linear kernel mean cross-validation AUROC values
318 when different hyperparameters are used in training the model. The differences in AUROC values
319 when hyperparameters change show that hyperparameter tuning is a crucial step in building a ML
320 model.



321

322 **Figure S2. Hyperparameter setting performances for non-linear models.** (A) Decision tree
323 (B) Random forest (C) SVM with radial basis kernel (D) XGoost mean cross-validation AUROC
324 values when different hyperparameters are used in training the model. The differences in AUROC
325 values when hyperparameters change show that hyperparameter tuning is a crucial step in building
326 a ML model.

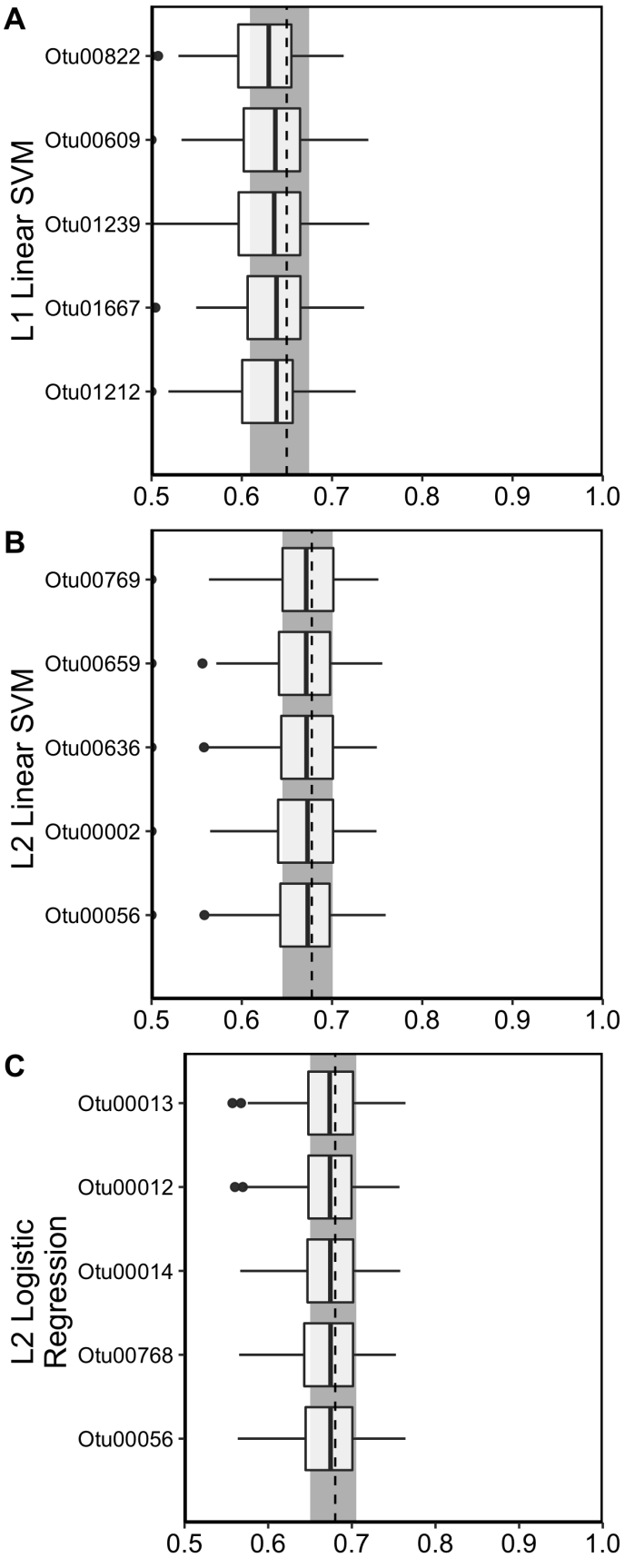


Figure S3. Interpretation of the linear

ML models with permutation importance. (A) L1-regularized SVM with linear kernel (B) L2-regularized SVM with linear kernel and (C) L2-regularized logistic regression were interpreted using permutation importance using held-out test set. The gray rectangle and the dashed line show the IQR range and median of the base testing AUROC without any permutation performed. Abbreviations: SVM, support vector machine; OTU, Operational Taxonomic Unit; RBF, radial basis kernel; OTU, Operational Taxonomic Unit.

Table 1: Model complexity: n is the number of training samples, f the number of features, n_{trees} is the number of trees.

Machine learning model	**Training complexity**	**Hyperparameters**
Logistic regression	$O(nf)$	C
SVM with linear kernel	$O(nf)$	C
SVM with radial basis kernel	$O(n^2 f)$	C, σ
Decision tree	$O(n^2 f)$	tree depth
Random forest	$O(n^2 f n_{trees})$	number of features
XGBoost	$O(nf n_{trees})$?	

1

Table S1. ML model complexity and hyperparameter range.

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