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Identification and characterisation of virome and bacteriome in calves suffering from infectious bronchopneumonia



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Abstract

Infectious bronchopneumonia represents, with diarrhea, 30% to 90% of calf breeding mortality but there is almost no information in biology literature on these infections. The purpose of this project was to establish links between bacteria found from calf noses and those found in calf lungs, and also to explain the presence of a list of viruses using the abundance of these bacteria.

Hence, the data set under analysis gives information about infectious agents: some samples were taken from 23 calf breeding farms where the calves were suffering from infectious bronchopneumonia. We implemented statistical methods in order to analyse this data set, such as principal component analysis, sparse partial least squares discriminant analysis, partial least squares in regression mode and random forest. Then, healthy calf breeding data was compared with these results.

[...]

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Nathalie Villa-Vialaneix, [...]

Elias Salem, [...]

Introduction

1 Context and description of the dataset

1.1 Context

The dataset provided comes from biological studies performed on calf breeding farms during the acute phases of the respiratory disease complex (BRD). Bovine respiratory disease complex is considered one of the most common and economically important diseases in calves, it is displayed as pneumonia with different states of severity and respiratory signs depending on the causative agents and stressors (host immune reaction, environment...). Furthermore, a control group of healthy calves was sampled as well.

The dataset is divided in three files containing microbiota data; we call microbiota all the microbes that are found in a particular niche or habitat. In our case, the niche is the respiratory tract of the calves, especially in the nasal cavities (upper respiratory tract) and in the lungs (lower respiratory tract).

First part: Samples originating from 23 breeding farms taken from calves suffering from BRD, the calves were sampled during the acute phase (less than 3 days since the onset of symptoms) and were not vaccinated nor treated with antibiotics (in order not to intervene with the present microbiota). In each breeding farm, a group of 4-5 calves were studied, from which 2 kinds of samples were taken:

- A nasal swab, taken from the nasal cavity and representing the upper respiratory tract (called "EN");
- Bronchoalveolar lavage fluid, taken from the lungs and representing the lower respiratory tract (called "LBA").

For every pool of calves in the breeding farms and each of the sample types, we have the abundance of each of the detected bacteria i.e how many times the sequence of specific bacteria was detected.

The set contains also data indicating the presence/absence of seven major pathogens known to cause the respiratory disease; these data were generated by RT-PCR (specific and direct test).

Second part: The same kind of samples was taken from 6 breeding farms where the calves did not have any sign of respiratory disease.

In the next section will be seen the details of each one of these data.

1.2 Dataset description

As explained previously, three text files are available for the study:

- **abundances:** bacteria counts where calves are suffering from infectious bronchopneumonia;
- **pathogenes:** presence/absence of seven pathogens targets;
- **abundances_ctrl:** bacteria counts where calves are healthy.

They are all under *Comma-Separated Values* (CSV) format. In this chapter will be seen what they contain and what type of transformations have been performed, in order to make them suitable for further study.

The abundances file

In this file, there is information (counts) about every bacteria found in every samples.

At the begining, the file contained 99 columns and 406 rows. A certain number of pretreatments have been performed in response to some findings on the dataset:

- **First nine columns removed.** They contained the name of each bacteria, its type, its family, its "blast taxonomy" and technical information that we will not use. We are interested in the name of each bacteria (an identifier) and the measurements made in each sample;
- **Columns A and B summed.** For each sample, there were two columns, identified by the letters *A* and *B*. It corresponds to the two technical replicates of all the farms involved in the study. These columns will be merged (simple sums as the counts have already been normalized to identical library sizes), which leads to 45 columns;
- **Duplicates of bacteria merged.** The list of bacteria names is not unique. We could have used the blast identifier but the problem remains the same. First, let us keep only the species name. It is the last one on each row. But sometimes the species is unknown, as we can see above. In this case, the name that appears before will be kept. Then, the replicates are merged by adding together each one of them, which lead us to a list of 270 unique bacteria.
- **Name of one bacteria corrected.** One name of the bacteria was "&". It is now replaced by the correct one.
- **29th column removed** (only for paired studies). The number of columns was odd (45), but on each calf was supposed to be taken two samples: one in the nose and the other in the lungs. It will be removed from the study when a paired analysis will be made.

These pretreatments lead us to a data frame with 270 rows and 45 columns. A part of this data frame can be seen in the Figure 1.1 below.

| | 10_EN | 11_EN | 15_EN | 16_EN | 17_EN | 18_EN | 19_EN | 01_EN | 20_EN | 21_EN | 23_EN | 24_EN | 25_EN | 26_EN | 28_EN |
|---------------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| & | 39 | 0 | 6 | 15 | 7 | 9 | 26 | 16 | 39 | 8 | 0 | 0 | 112 | 178 | |
| [Eubacterium] coprostanoligenes group | 18 | 0 | 1 | 2 | 70 | 28 | 52 | 54 | 38 | 74 | 27 | 27 | 0 | 11 | |
| [Ruminococcus] gauvreuii group | 9 | 14 | 0 | 1 | 0 | 27 | 39 | 32 | 0 | 0 | 0 | 0 | 27 | 87 | 5 |
| Acetobacteraceae bacterium SAP1007.2 | 15 | 0 | 0 | 1 | 3 | 0 | 6 | 9 | 0 | 0 | 0 | 0 | 0 | 0 | |
| Acholeplasma laidlawii PG-8A | 0 | 0 | 2 | 2 | 15 | 16 | 14 | 31 | 61 | 21 | 0 | 0 | 182 | 84 | |
| Achromobacter sp. | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| Achromobacter spanius | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | |
| Actinoalloteichus cyanogriseus | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| Aerococcaceae | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| Aerococcus sp. | 25 | 0 | 2 | 0 | 0 | 4 | 0 | 0 | 96 | 102 | 10 | 5 | 30 | 44 | |
| Aeromicrobium | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 0 | 0 | 0 | |
| Agreia | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 7 | 0 | |
| Agrobacterium tumefaciens | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 2 | |
| AKAU3644 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 1 | 0 | 0 | 1 | |
| Alcaligenes faecalis | 1 | 44 | 0 | 0 | 0 | 2 | 16 | 8 | 23 | 38 | 5 | 14 | 10 | 21 | |
| Alcaligenes sp. | 88 | 67 | 0 | 0 | 23 | 13 | 19 | 17 | 24 | 23 | 43 | 43 | 67 | 60 | |
| Alloprevotella | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 14 | 32 | 0 | 3 | 0 | 0 | 1 | 228 |
| Alysiella crassa | 0 | 5 | 0 | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 0 | 0 | 0 | |
| Anaerosporbacter | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| Aquamicrobium | 0 | 0 | 43 | 46 | 14 | 31 | 71 | 96 | 48 | 20 | 0 | 24 | 33 | 85 | 1 |
| Arcobacter | 12 | 21 | 1 | 2 | 0 | 0 | 0 | 0 | 79 | 109 | 11 | 0 | 11 | 59 | 2 |
| Arcobacter cryaerophilus | 0 | 0 | 7 | 3 | 9 | 9 | 35 | 0 | 22 | 9 | 0 | 0 | 0 | 9 | 1 |
| Arenimonas | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 2 | 0 | |
| Arthrobacter | 183 | 154 | 4719 | 4920 | 6 | 25 | 188 | 261 | 456 | 531 | 9 | 112 | 273 | 398 | 2 |
| Arthrobacter arilaitensis | 0 | 0 | 8 | 2 | 30 | 7 | 19 | 6 | 12 | 0 | 0 | 0 | 0 | 11 | |

Figure 1.1: *Abundances data*

The pathogenes file

The second file is composed of 9 columns and 46 rows. This file contains the presence or not of seven viruses for each farm in the two condition. Some pretreatments have been performed:

- **Identifier and condition merged.** The first and the last columns stand for the identifier of each farm and the sampling condition. A unique identifier is needed, hence these two columns are merged.
- **Binary code for presence/absence of virus.** The presence of a virus was coded with a letter, and the absence with 0. In order to simplify the analysis, it will be replaced as 1 and 0 respectively.

Unlike the previous file, there is no missing calf: there is 23 paired individuals. A part of the data set is displayed in the Figure 1.2.

The viruses observed are the following:

- "Ct.RSV" for respiratory syncytial virus
- "Ct.PI.3" for parainfluenza virus
- "Ct.Coronavirus" for coronavirus
- "Ct.P.multocida" for pasteurella multocida virus
- "Ct.M.haemolytica" for mannheimia haemolytica virus

- "Ct.M.bovis" for mycobacterium bovis virus
- "Ct.H.somni" for histophilus somni virus

| | Ct.RSV ⁺ | Ct.PI.3 ⁺ | Ct.Coronavirus ⁺ | Ct.P.multocida ⁺ | Ct.M.haemolytica ⁺ | Ct.M.bovis ⁺ | Ct.H.somni ⁺ |
|--------|---------------------|----------------------|-----------------------------|-----------------------------|-------------------------------|-------------------------|-------------------------|
| 10_EN | 0 | 0 | 1 | 1 | 0 | 0 | 1 |
| 11_EN | 0 | 1 | 1 | 1 | 0 | 0 | 0 |
| 15_EN | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| 16_EN | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| 17_EN | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 18_EN | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| 19_EN | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| 01_EN | 0 | 0 | 1 | 1 | 0 | 0 | 1 |
| 20_EN | 0 | 0 | 1 | 1 | 0 | 0 | 1 |
| 21_EN | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| 23_EN | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| 24_EN | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| 25_EN | 0 | 0 | 0 | 1 | 1 | 1 | 1 |
| 26_EN | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| 28_EN | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| 30_EN | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| 31_EN | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| 03_EN | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| 04_EN | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| 06_EN | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 07_EN | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| 09_EN | 0 | 0 | 1 | 1 | 0 | 0 | 1 |
| 10_LBA | 0 | 0 | 1 | 1 | 0 | 0 | 1 |
| 11_LBA | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| 15_LBA | 0 | 0 | 0 | 1 | 0 | 1 | 1 |
| 16_LBA | 0 | 0 | 1 | 0 | 0 | 0 | 1 |

Figure 1.2: *Pathogenes data*

In addition to the first file, this one will allow us to study links between virus and bacteria.

The abundances_ctrl file

This file has the same design as the **abundances** file; it will be treaded the same way. The first difference is that this file corresponds to the samples taken from healthy calves in 6 breeding farms, which gives us 12 samples.

The other difference concern the list of bacteria. The bacteria found in healthy individual are not the same as sick individuals. This list is shorter (293 rows) and the bacteria are not the same.

This list is still not composed by unique names of bacteria. It will be fixed by taking the last name of each row as explained before.

Now that this list is composed by unique bacteria names, let us see how much they are different from the abundances file in the Figure 1.3.

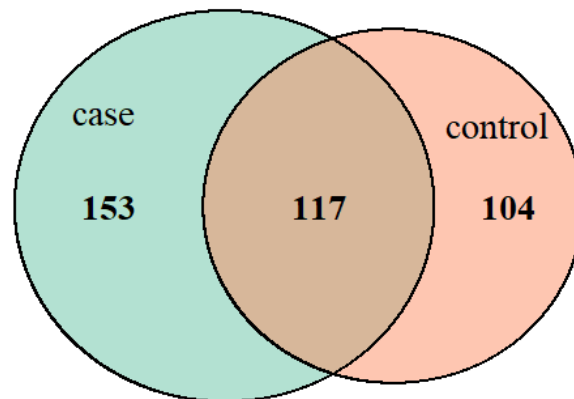


Figure 1.3: *Venn diagram comparing two lists of bacteria: for sick calves and for healthy calves*

2 Methods

2.1 Normalisation

The purpose of the normalisation is to make variable distributions more symmetrical. Furthermore, some statistical methods are sensitive to non-normalised data: most of the classification methods compute the distance between two points using eucliden distance. If one of the variable has high values comparing to the others, it will have a greather influence on the final distance, and consequently will lead to a higher variance on prediction quality.

Let us have a look at the distribution of the first simple, with a log scale for the frequencies.

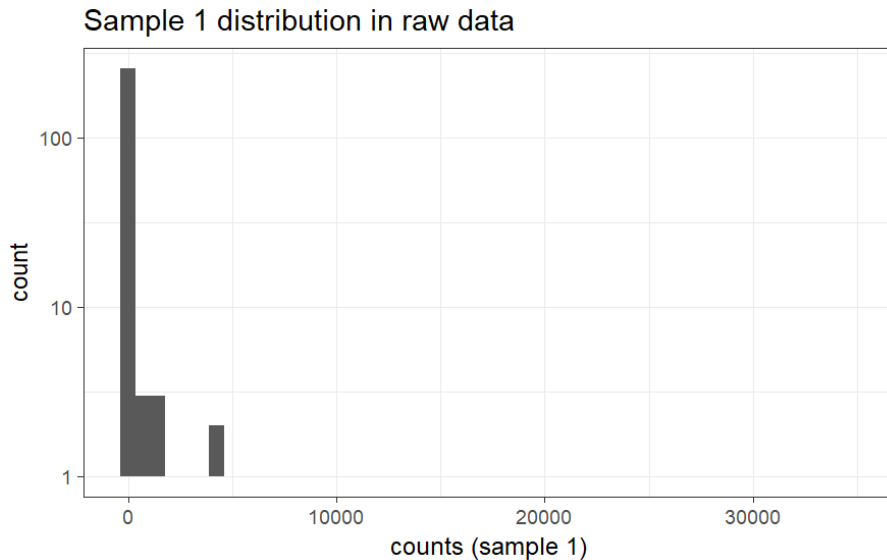


Figure 2.1: *Sample 1 distribution in raw data*

What can be noticed in the Figure 2.1 is that there is a lot of very low values, hence the distribution is highly skewed. It was expected because the observations are counts. In order to analyse this dataset, we will try several transformations known for microbiote datasets [3].

Let $y_{i,j}$ be the counts of a bacteria i found in the sample j , where $i \in [1, \dots, n]$ and $j \in [1, \dots, p]$, n and p standing for the number of bacteria and the number of samples respectively. Let \hat{y} be the data after transformation.

Log transformation

$$\tilde{y}_{ij} = \log(y_{ij} + 1)$$

TSS transformation

It is common in metagenomic datasets to perform TSS (*Total Sum Scaling*) before further normalization. TSS transformation computes relative abundances:

$$y_{ij} = \frac{n_{ij}}{\sum_{k=1}^p n_{ik}}$$

for n_{ij} the counts of species j in sample i , p the number of species and n the number of individuals.

TSS+CLR transformation

CLR (*Centered Log Ratio*) transformation:

$$\tilde{y}_{ij} = \log \frac{y_{ij}}{\sqrt[p]{\prod_{k=1}^p y_{ik}}}.$$

TSS+ILR transformation

ILR (*Isometric Log Ratio*) transformation:

$$\tilde{\mathbf{Y}}' = \tilde{\mathbf{Y}} \times \mathbf{V}$$

for $\tilde{\mathbf{Y}}$ the matrix of CLR transformed data and a given matrix \mathbf{V} with p rows and $p - 1$ columns such that $\mathbf{V}\mathbf{V}^\top = \mathbb{I}_{p-1}$ and $\mathbf{V}^\top\mathbf{V} = \mathbb{I} + a\mathbf{1}$, a being any positive number and $\mathbf{1}$ a vector full of 1.

CSS transformation

The CSS (*Cumulative Sum Scaling*) normalisation is an adaptive extension for metagenomic data of the quantile normalisation used in microarray expression datasets. It is designed so as to account for technical differences between samples.

2.2 Principal Component Analysis

Principal Component Analysis (PCA) is a multidimensional descriptive method that allows to explore the links between variables and the similarities between individuals.

The objective of the PCA is to identify the largest sources of variation and return to a reduced size space (for example 2 or 3) by maximizing the variability of the projection. In other words, PCA reduces the number of variables, in this way the information becomes less redundant.

From a mathematical point of view, the PCA corresponds to the approximation of a matrix (n, p) by a matrix of the same dimensions but of rank $q < p$. In fact, an orthogonal linear transformation is applied on the data to convert a set of observations of possibly correlated variables into uncorrelated principal components. q is often of small value 2, 3 and contributes to the construction of easily understandable graphs. The interpretation of these graphs helps to understand the structure of the analyzed data.

2.3 Partial least squares regression

Partial least squares regression (PLS) is a fast, efficient and optimal statistical method that is widely used to deal with situations with high multicollinearity and when big data are to be taken into account. Its use is recommended in the case where the number of variables p is much greater than the number of individuals n : $p \ll n$, which is relevant for our dataset.

There are different versions of PLS regression depending on the objective:

- **PLS1**: A quantitative target variable Y is to be explained, model, predict by p quantitative explanatory variables X^j
- **PLS2**: Canonical version. Relates a set of q quantitative variables Y^k and a set of p quantitative variables X^j .
- **PLS2**: Regression version. Tries to explain, model a set of q variables Y^k by a set of p quantitative explanatory variables X^j .
- **PLS-DA**: Discriminant version. Special case of the previous case. The qualitative variable Y with q classes is replaced by q dummy variables of these classes.

During this project, only the PLS-regression mode and PLS-DA versions were used because they are adapted to our dataset and the objective pursued.

2.4 Random Forest

Random Forest is a machine learning method for classification and regression generally. This algorithm is based on the aggregation of decision, regression or classification binary trees, depending on the type of the target variable Y . It consists of drawing $n_estimators$ bootstrap samples. A tree is estimated for each bootstrap sample and the prediction is obtained by averaging (regression) or vote (classification) the individual predictions of each tree. During the construction of each node of each tree in the learning process, it is selected a random subset of the features that allows to build less correlated trees and make aggregation more efficient. It is generally a effective method, which could give satisfying predictions for presence/absence of viruses.

Algorithm:

Let Y be the predictable variable and $z = (x_1, y_1), \dots, (x_n, y_n)$ a sample.

For $b = 1$ to B do

 Draw a bootstrap sample z_b^*

 Estimate a tree on this sample with a random subset of the features

Calculate the average estimate $\hat{f}_B(Y) = \frac{1}{B} \sum_{b=1}^B \hat{f}_{z_b}(Y)$ or the result of the vote.

3 Results

3.1 Exploratory analysis

3.1.1 Normalisation

Previously, we saw that the first sample distribution was highly asymmetrical due to the fact that it is from counts data.

If we look at the graphs in the Figure 3.1, the less asymmetric distribution seems to be the one obtained with the CSS transformation and the log-transformation, but there is still a lot of very low values. In the following will be used the log-transformation.

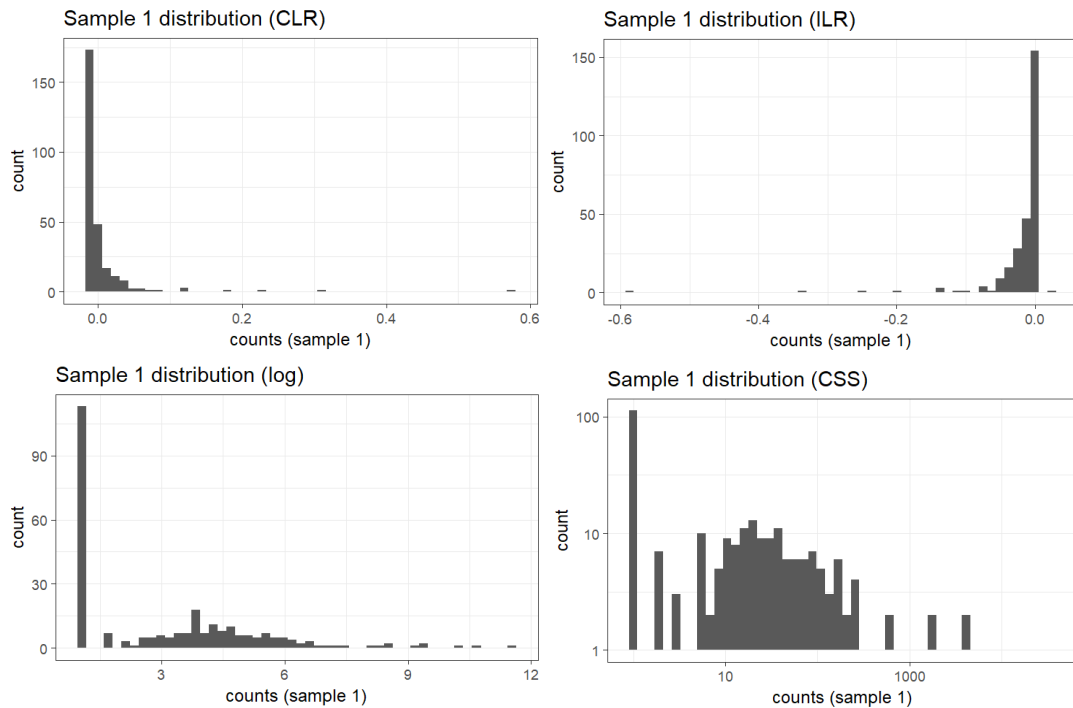


Figure 3.1: *Sample 1 distribution according to the type of transformation*

Let us have a look at the distribution by boxplots of all the samples displayed in the Figure 3.2. It confirms that the best transformations are CSS and logarithmic. It seems that the CSS normalisation treats outliers more efficiently than the log one.

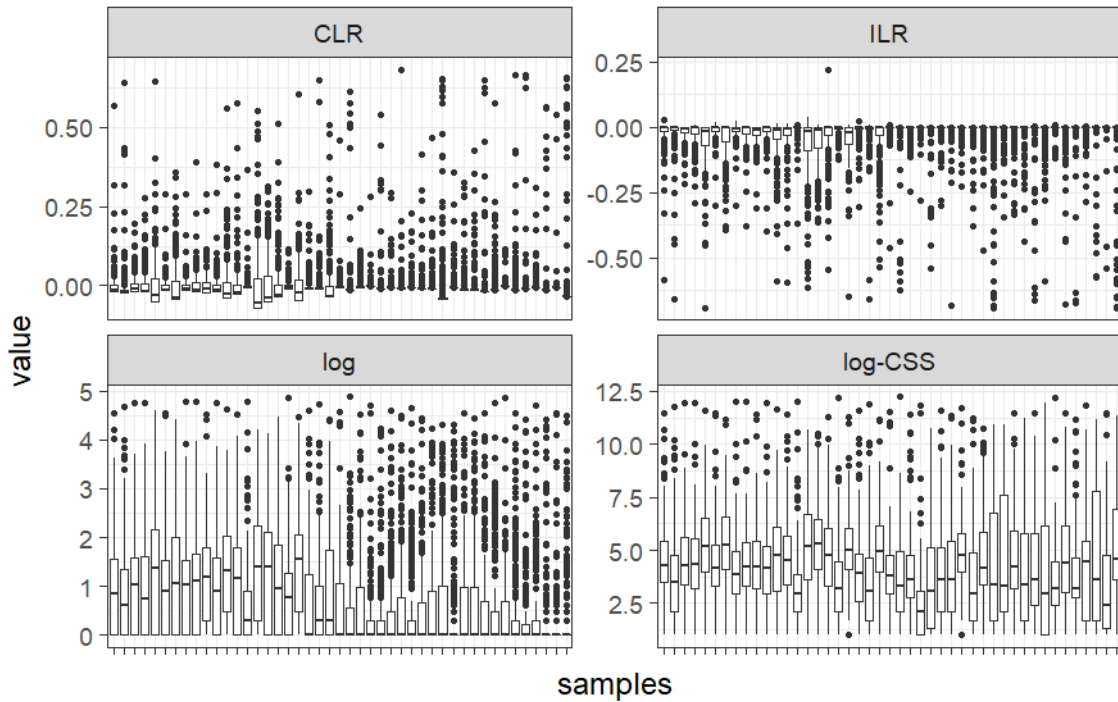


Figure 3.2: *Distributions of all samples according to the type of transformation and the sample*

3.1.2 Principal Component Analysis

It is important to have a clear idea of the data structure. A principal component analysis is adapted to this objective. In the following, we will analyze the results of the PCA on the Log and CSS transformations made on the observation matrix because the two others show very similar results. It will be performed in R, using the `mixOmics` library [1].

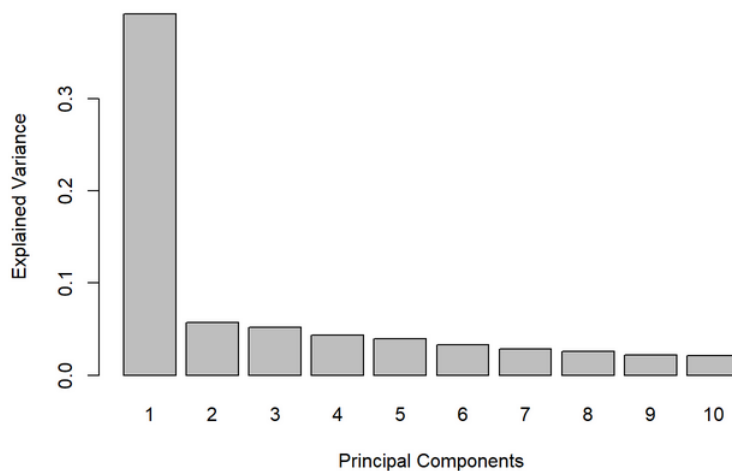


Figure 3.3: *Sample 1: The variance explained by the first 10 components*

The Figure 3.3 represents the decay of the eigenvalues associated to the principal components. It seems that the first component on 406 is sufficient to reconstitute

the data. This is explained by the rapid decay of the firsts ten eigenvalues, which are not important beyond that. The first axis, showing the projection of the data on the first principal component seems to illustrate its stability in the construction of the representation subspace.

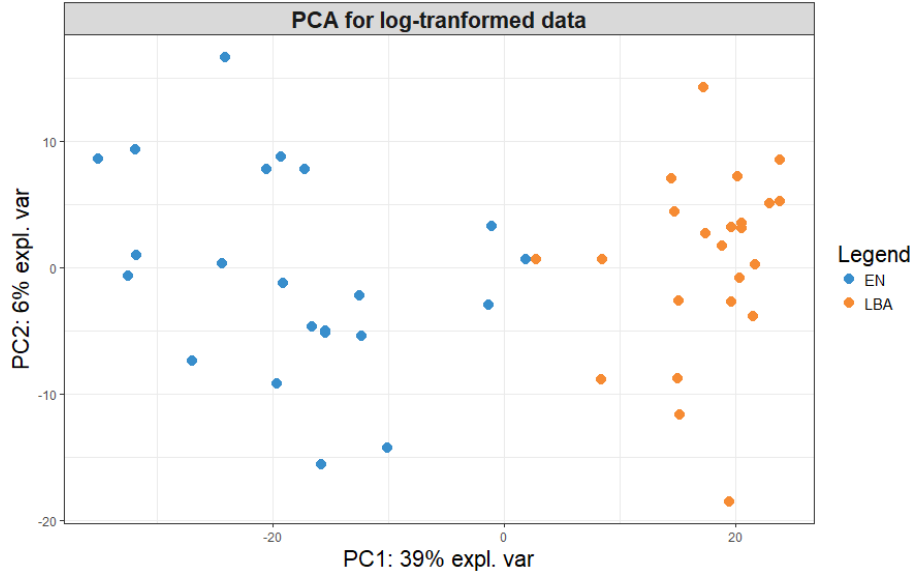


Figure 3.4: *Sample 1: Projection of individuals in the first factorial plane*

The projection of individuals in the first factorial plan displayed in the Figure 3.4 plan shows that the separation of "EN" and "LBA" classes is marked, and can even be divided linearly. Also, it can be seen that the first axis associated to the first principal component explains 39% of variance and with the second, they contribute mainly to the reconstitution of the data.

3.2 Differences between EN and LBA samples

The purpose of this part is to find bacteria which are an impact on the EN/LBA samples. The methods used are PLS-DA, and more specifically sparse PLS-DA. They will be performed using paired or unpaired analysis, which means indicating the sparse PLS-DA that our samples are paired or not.

3.2.1 PLS-DA - paired samples

A first PLS-DA is computed (with 10-fold CV) to check the efficiency of the method and which type of distance to use in its computation.

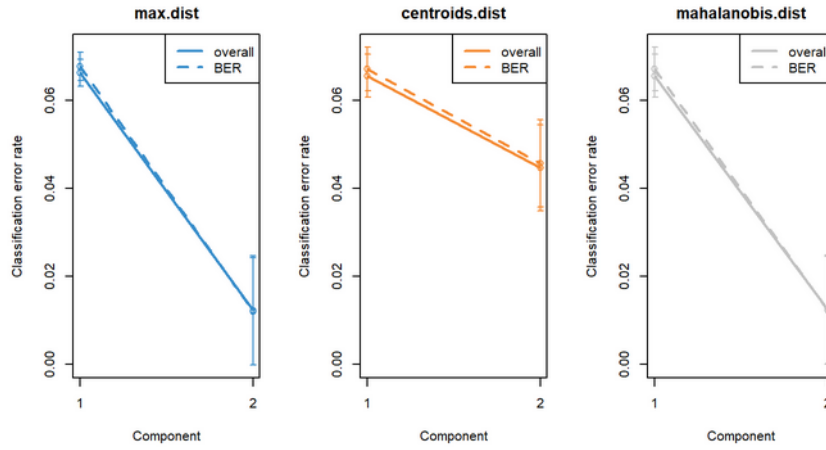


Figure 3.5: *Distances for the first two components*

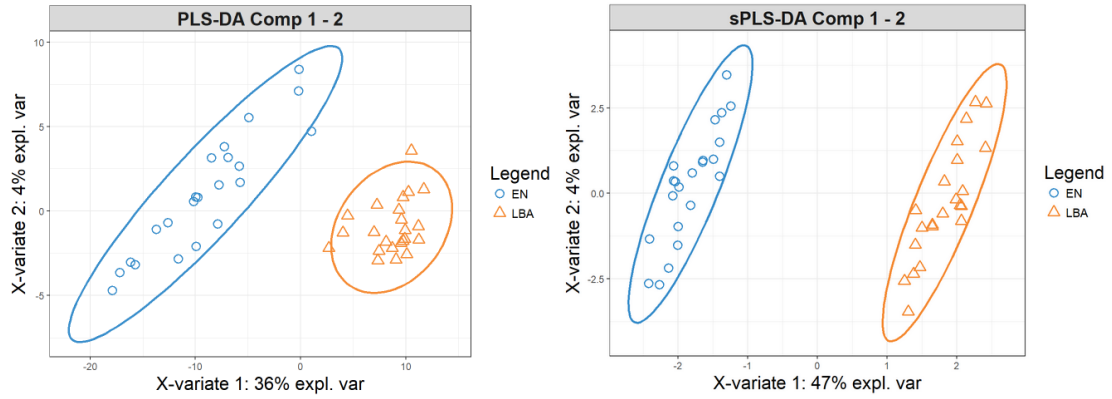


Figure 3.6: *PLS-DA and sPLS-DA first two components*

The ellipse are 0.95 confidence interval ellipse for each condition type.

3.2.2 PLS-DA - unpaired samples

3.3 Predicting the presence of viruses

3.3.1 In the nasal cavity (EN)

PLS in regression mode

Random Forest

3.3.2 In the lungs (LBA)

PLS in regression mode

Random Forest

Conclusion

Bibliography

- [1] Kim-Anh Le Cao, Florian Rohart, Sebastien Dejean with key contributors Benoit Gautier Ignacio Gonzalez, Francois Bartolo, contributions from Pierre Monget, Jeff Coquery, FangZou Yao, and Benoit Liquet. *Package mixOmics: Omics Data Integration Project*. 2016.
<https://CRAN.R-project.org/package=mixOmics>.
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