Batch Effect Management

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WIFI

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Objectives of this workshop

- To recognise the importance of addressing batch effects for research reproducibility.
- To understand assumptions, applications and limitations of existing methods handling batch effects.
- To gain practical skills in managing batch effects and evaluating correction effectiveness.

Batch effects

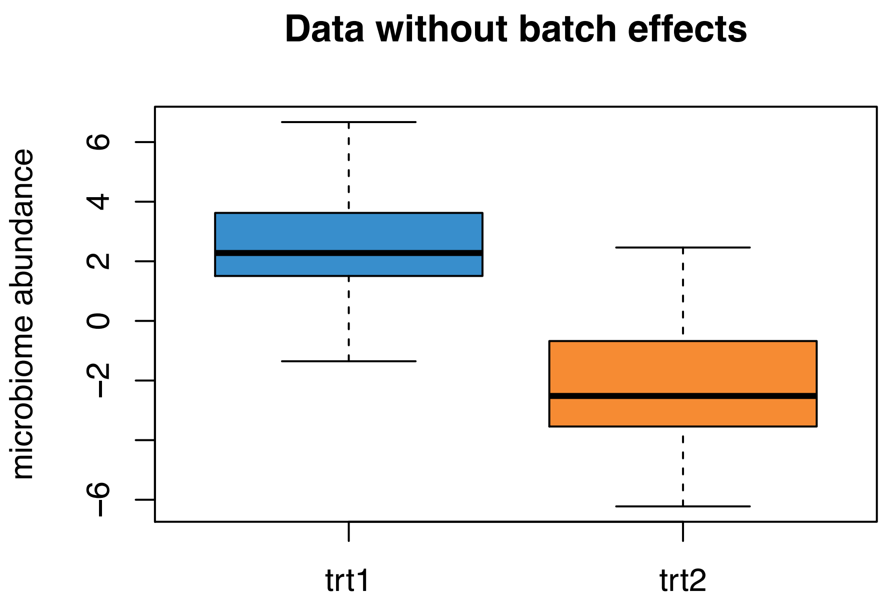
Definition: unwanted variation unrelated to but obscuring the biological factors of interest (e.g., treatments).

- Batch effects are associated with the outcome independently of treatments
- If batch effects correlate with treatment effects → confounders
- If batch effects don‘t correlate with treatment effects → prognostic variables
- Batches are usually categorical variables

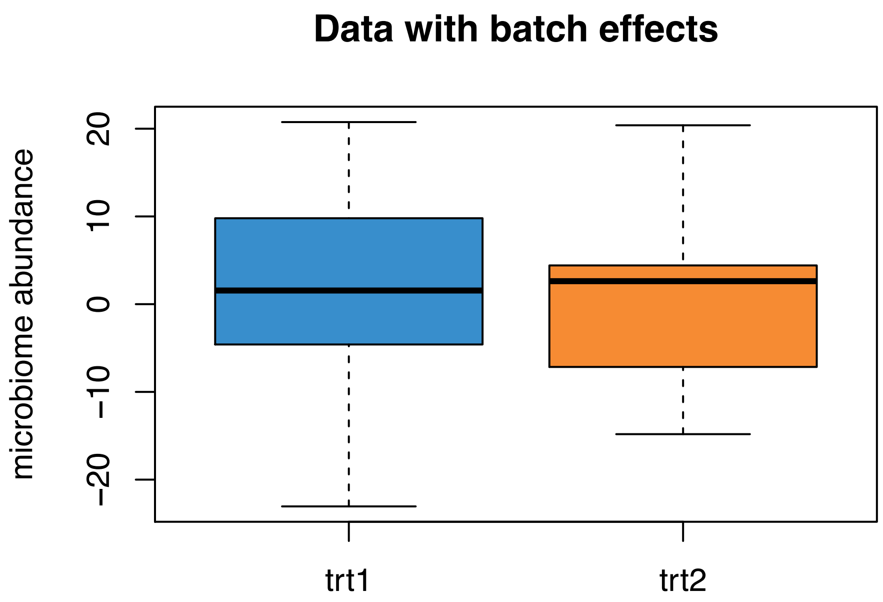
Wang & Lê Cao (2020). Managing batch effects in microbiome data. *Briefings in Bioinformatics*.

Batch effects

Consequence:



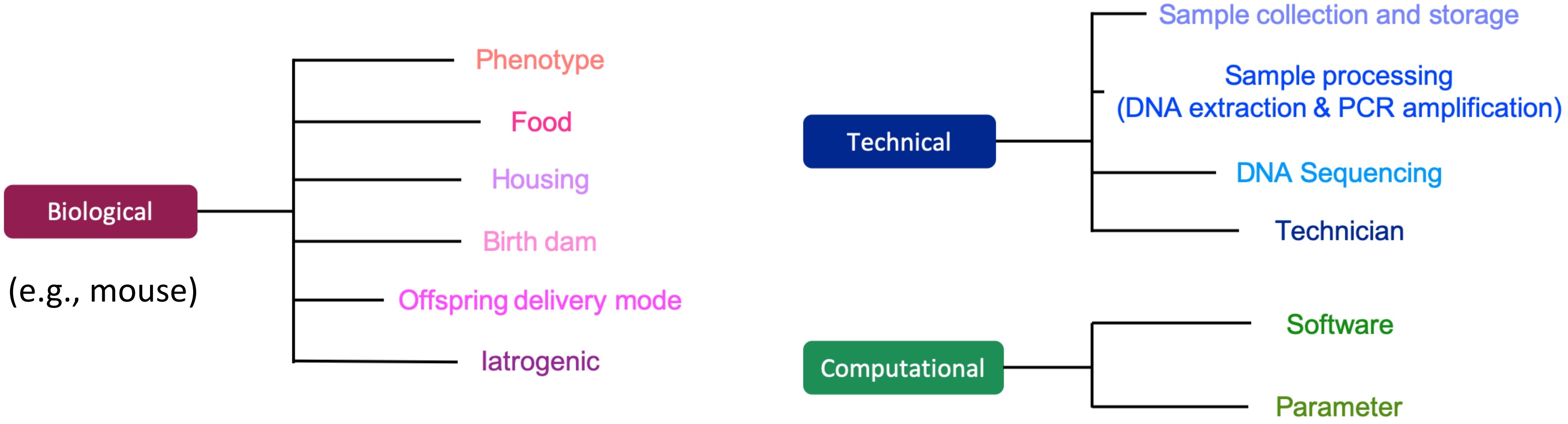
P < 0.001 of the treatment effect in T-test



P > 0.05 of the treatment effect in T-test

Potential batch sources

Batch effects may happen in any step of experiments.



Wang & Lê Cao (2020). Managing batch effects in microbiome data. *Briefings in Bioinformatics*.

Assumptions about batch effects

Most statistical methods that correct for batch effects assume balanced batch x treatment designs, which means batch effects are independent of the effects of interest.

Balanced (prognostic)

	Treat 1	Treat 2
Batch 1	10	10
Batch 2	10	10

Unbalanced (confounding)

	Treat 1	Treat 2
Batch 1	4	16
Batch 2	16	4

Nested (confounding)

	Treat 1	Treat 2
Batch 1	10	0
Batch 2	0	10
Batch 3	10	0
Batch 4	0	10

Nested ✗

	Treat 1	Treat 2
Batch 1	0	20
Batch 2	20	0

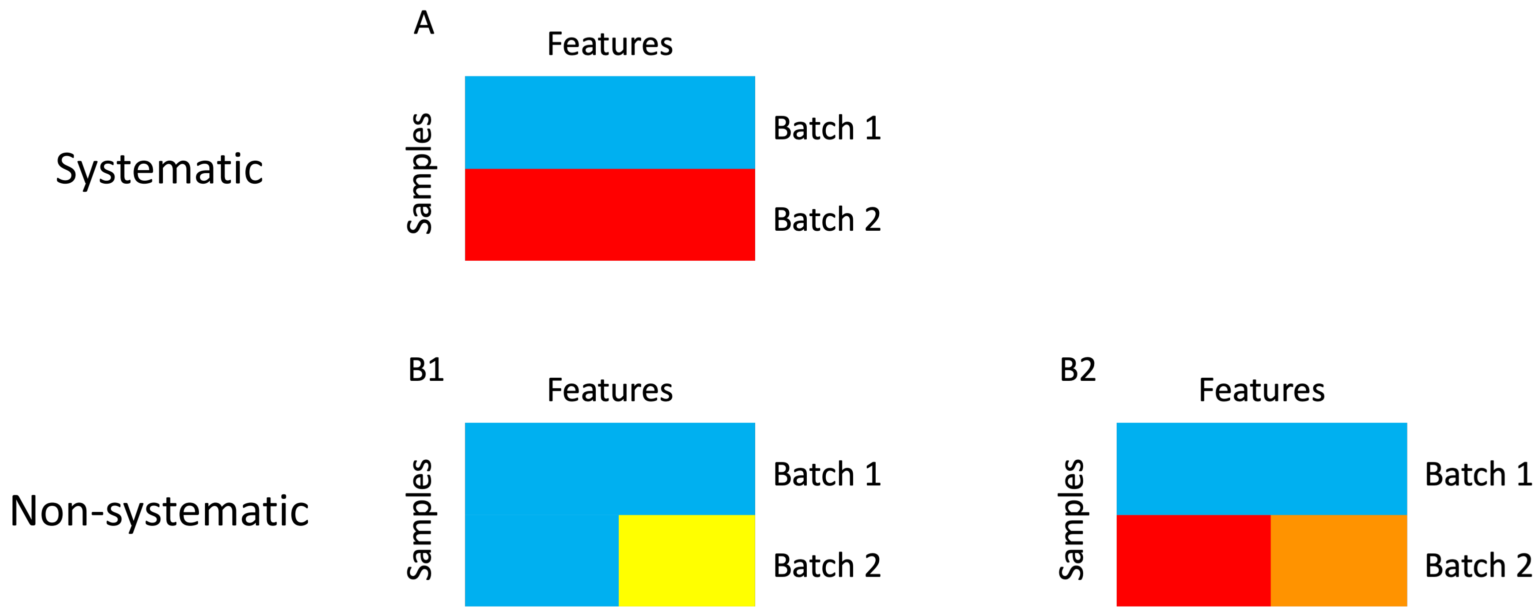
Wang & Lê Cao (2020). Managing batch effects in microbiome data. *Briefings in Bioinformatics*.

Assumptions about batch effects

Batch effects have different scale of influence on variables:

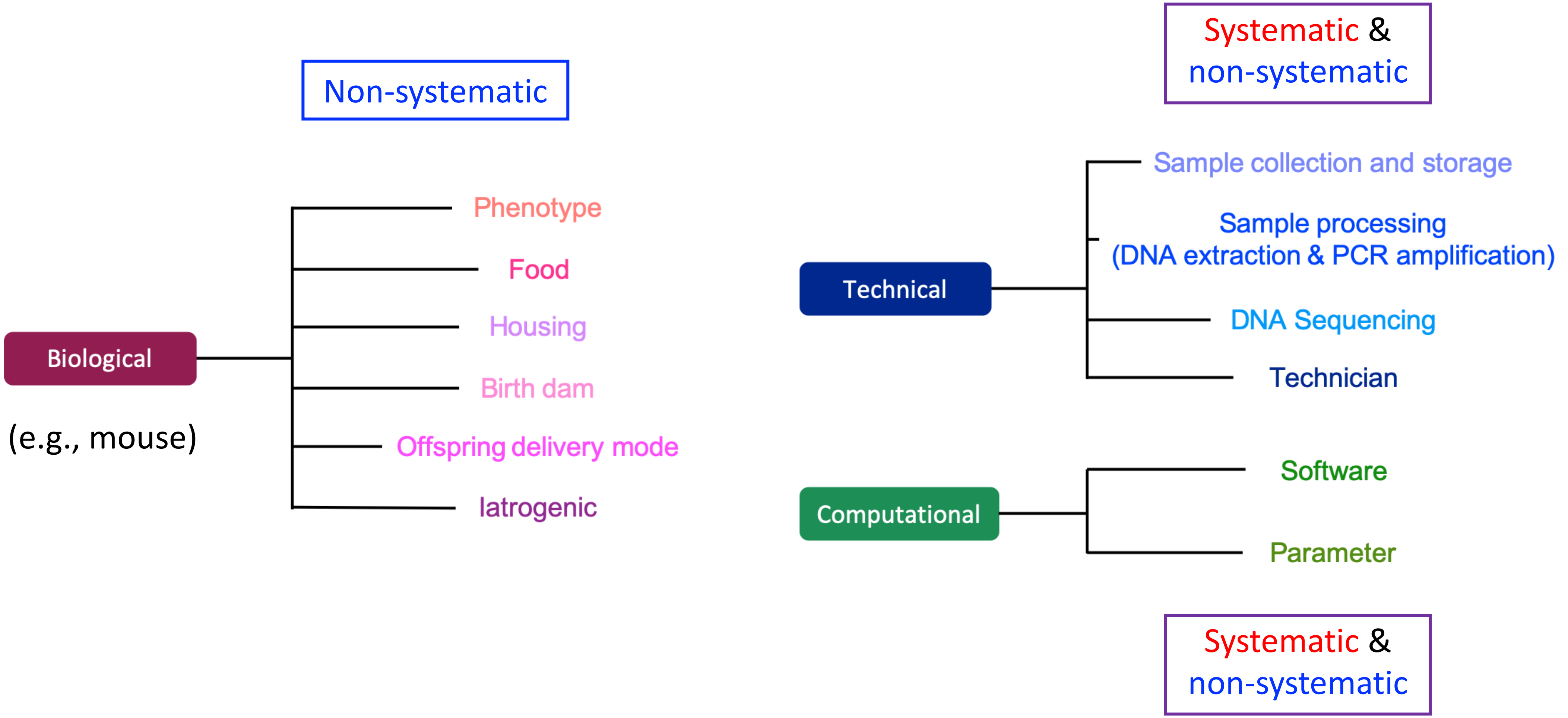
- Systematic batch effects have a homogeneous influence on all variables, e.g., microbial growth rates follow the same distribution.
- Non-systematic batch effects have a heterogeneous influence on different variables.

Many methods assume systematic batch effects.



Wang & Lê Cao (2020). Managing batch effects in microbiome data. *Briefings in Bioinformatics*.

Assumptions about batch effects

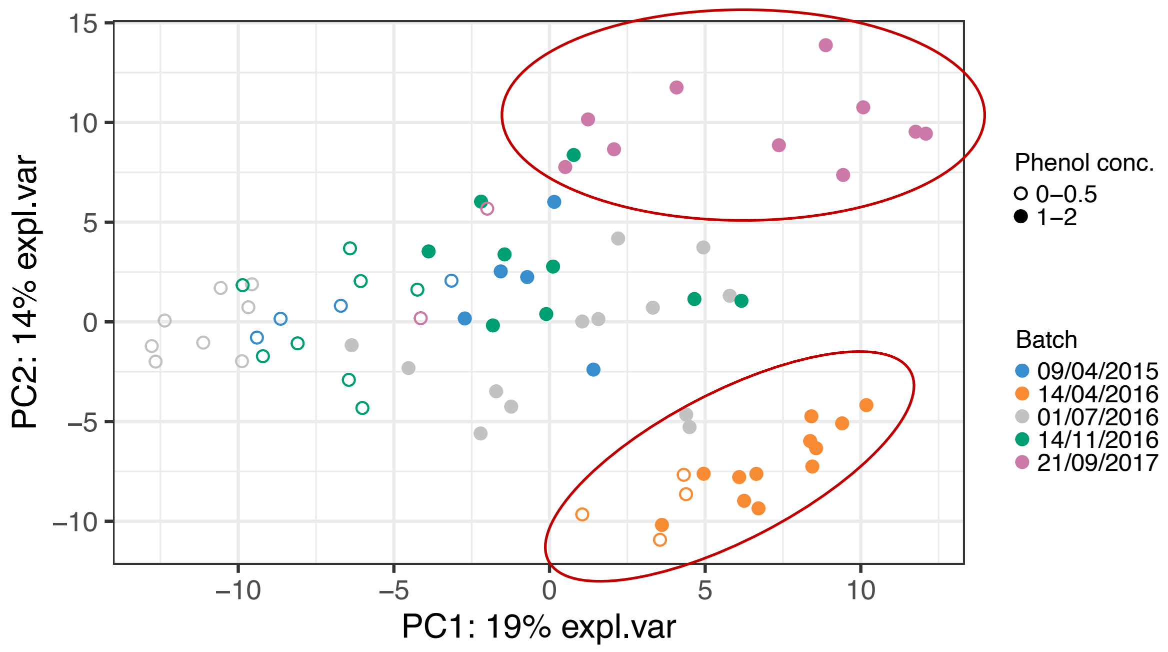


Wang & Lê Cao (2020). Managing batch effects in microbiome data. *Briefings in Bioinformatics*.

Case studies

Anaerobic Digestion (AD data): ★

- Bioreactor experiment: aimed at improving biowaste digestion
- 567 microbial variables & 75 samples
- Treatment effect: 2 levels of phenol concentrations
- (Technical) batch effect: samples processed on 5 different dates



Chapleur, et al (2016). Increasing concentrations of phenol progressively affect anaerobic digestion of cellulose and associated microbial communities. *Biodegradation*.

Case studies

Sponge data:

- Investigating differences in microbial composition between specific sponge tissues
- 24 microbial variables & 32 samples
- Effect of interest: 2 different tissues
- (Technical) batch effect: sample processed on 2 different experimental gels
- Data characteristic: completely balanced design

	Tissue 1	Tissue 2
Batch 1	8	8
Batch 2	8	8

Sacristan-Soriano, et al (2011). Exploring the links between natural products and bacterial assemblages in the sponge aplysina aerophoba. *Appl. Environ. Microbiol.*

Case studies

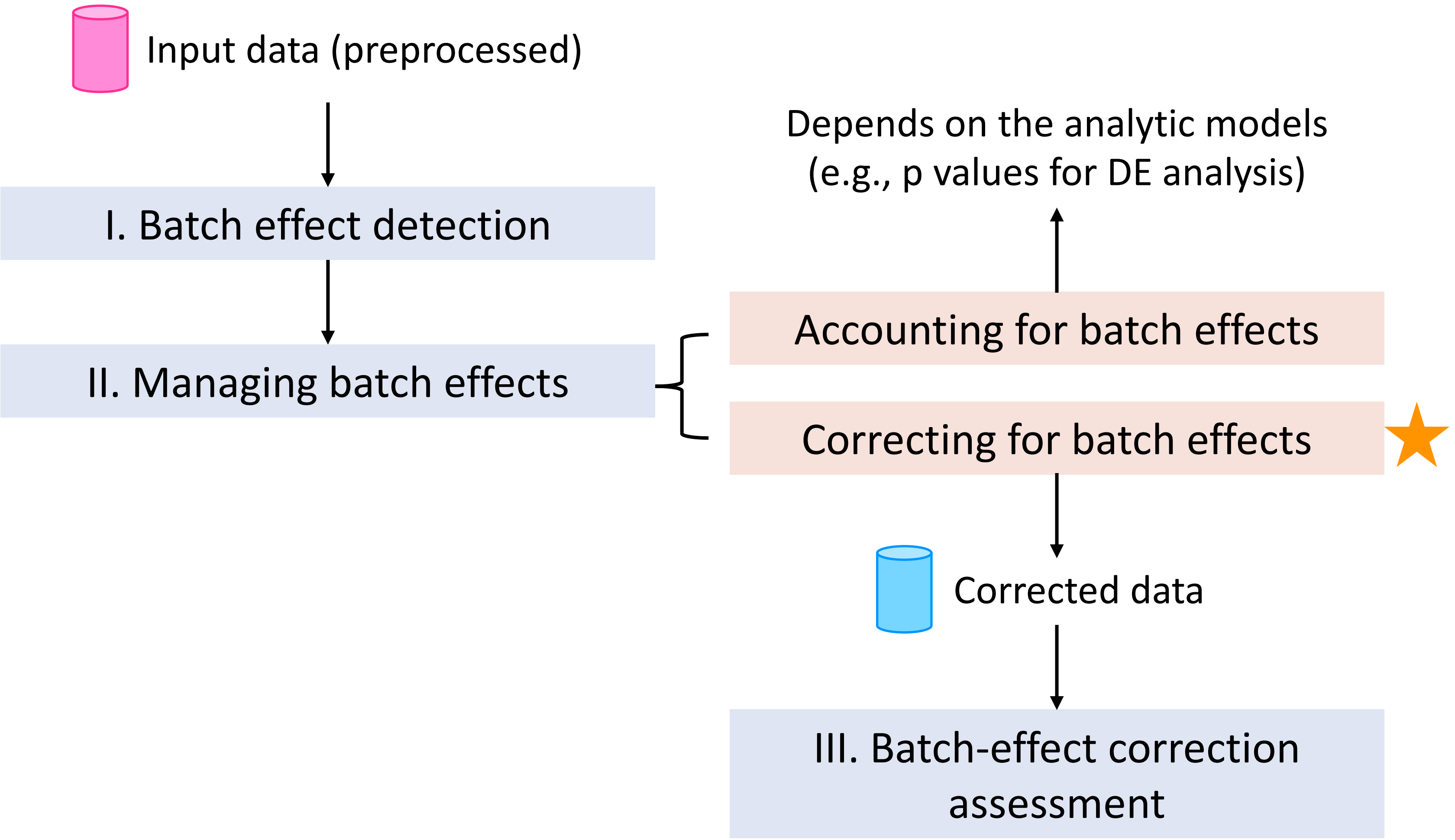
Mice models with Huntington's disease (HD data):

- Exploring differences in microbial composition between Huntington's disease and wild-type mice
- 368 microbial variables & 30 samples
- Effect of interest: 2 different genotypes
- (Biological) batch effect: samples from 10 different mouse cages

Cages\Genotypes	HD	WT
Cage A	2	0
Cage B	3	0
Cage C	2	0
Cage D	0	4
Cage E	0	4
Cage F	0	3
Cage G	3	0
Cage H	3	0
Cage I	2	0
Cage J	0	4

Kong, et al (2016). Microbiome profiling reveals gut dysbiosis in a transgenic mouse model of Huntington's disease. *Neurobiol Dis*.

Workflow for batch effect management



Wang & Lê Cao (2020). Managing batch effects in microbiome data. *Briefings in Bioinformatics*.

I. Batch effect detection

Purpose: to detect batch effects and determine if the batch effect management is required.

A. Visual approaches: limited for very weak batch effects

- Principal component analysis (PCA)
- Boxplots and density plots
- Heatmap

B. Quantitative methods: very sensitive to batch effects

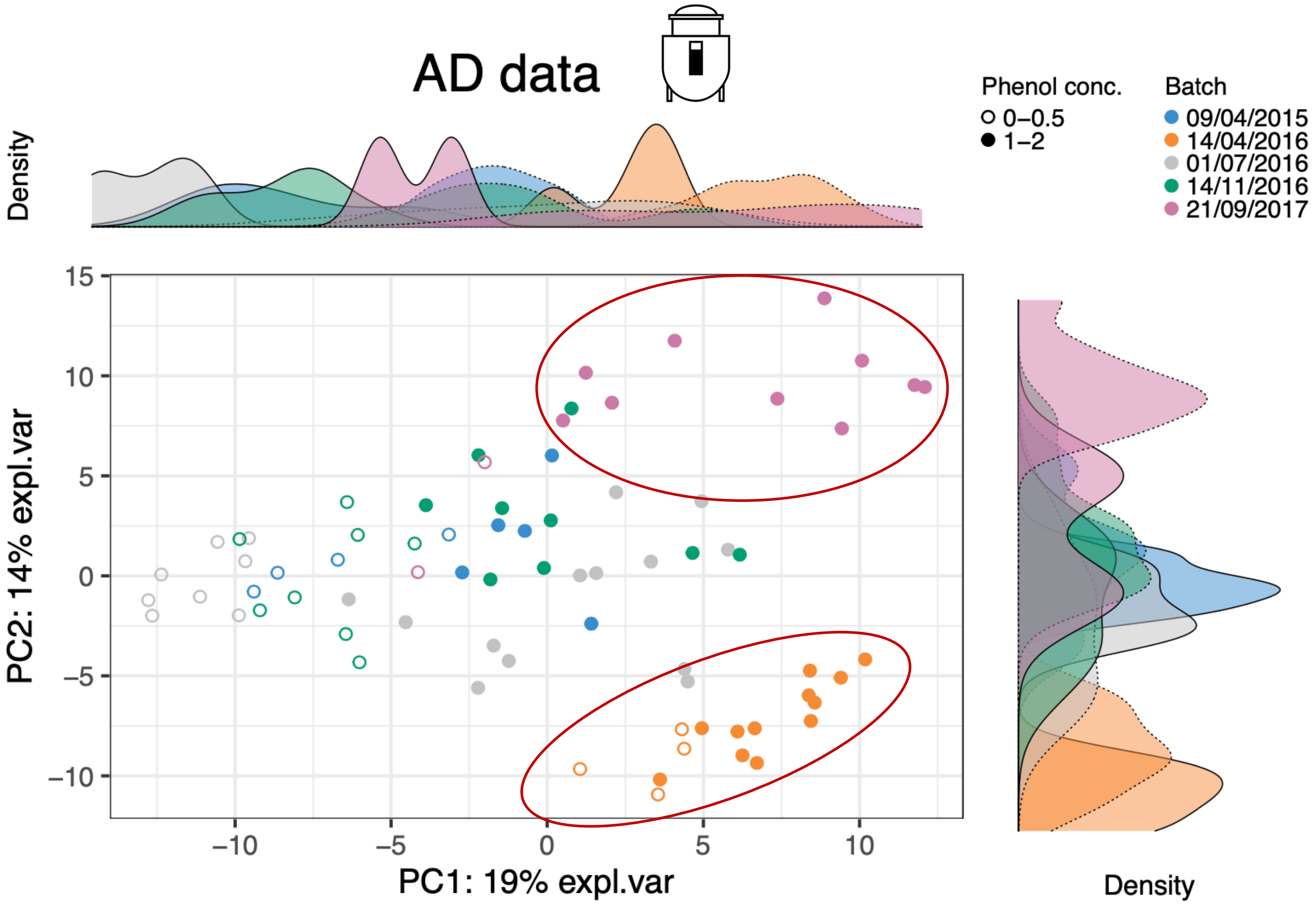
- Partial redundancy analysis (pRDA)

Wang & Lê Cao (2020). Managing batch effects in microbiome data. *Briefings in Bioinformatics*.

I. Batch effect detection

PCA plots with density per component

=> Multivariate: combination of all taxa



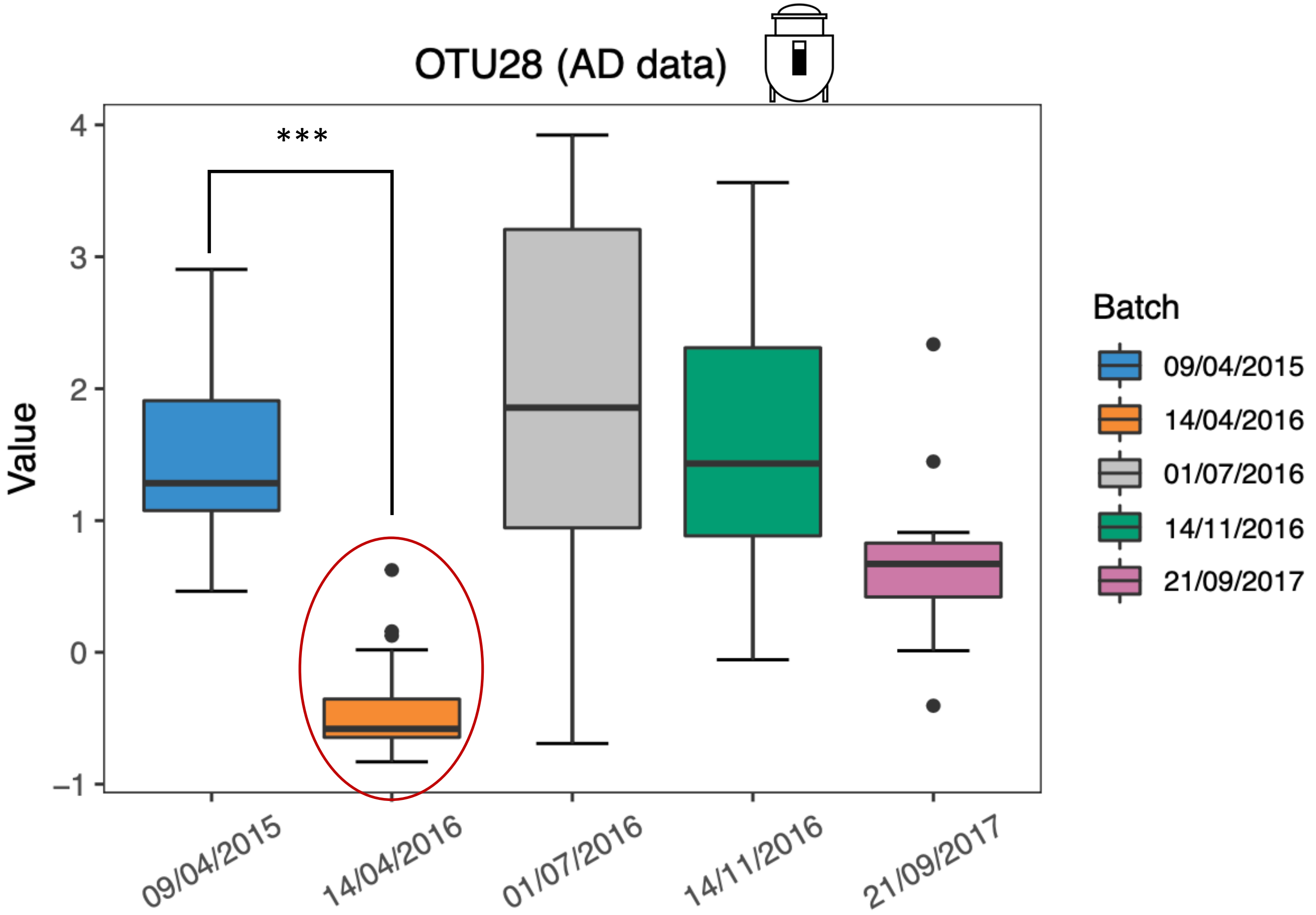
Batch effect variation on the second PC.

Wang & Lê Cao (2020). Managing batch effects in microbiome data. *Briefings in Bioinformatics*.

I. Batch effect detection

Boxplots

=> Univariate: single taxon



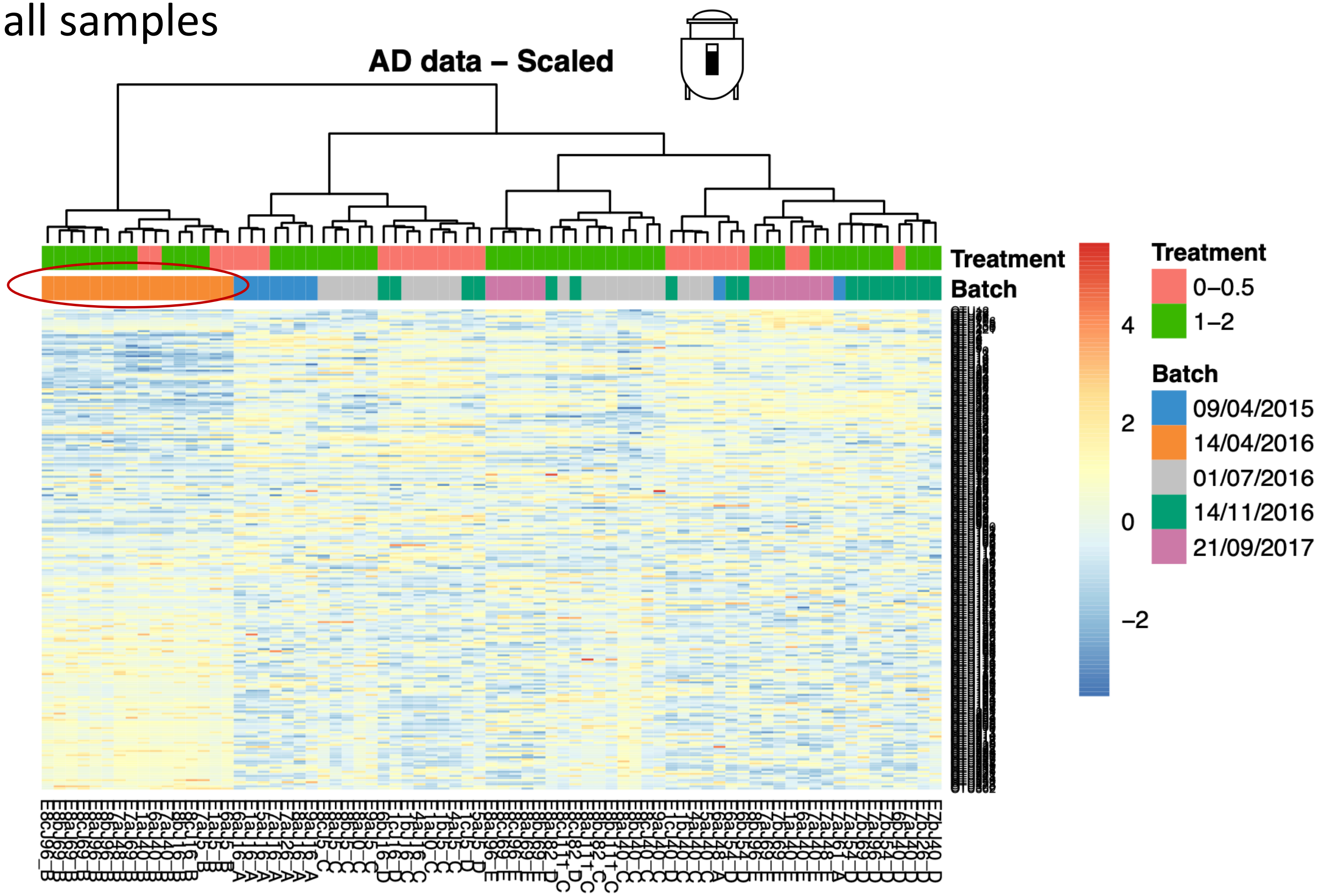
Test for the difference between batches (here P-value < 0.001, t-test).

Wang & Lê Cao (2020). Managing batch effects in microbiome data. *Briefings in Bioinformatics*.

I. Batch effect detection

Heatmap

=> All taxa and all samples



Samples from the batch dated "14/04/2016" clustered together.

Wang & Lê Cao (2020). Managing batch effects in microbiome data. *Briefings in Bioinformatics*.

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Wang & Lê Cao (2020). Managing batch effects in microbiome data. *Briefings in Bioinformatics*.

I. Batch effect detection

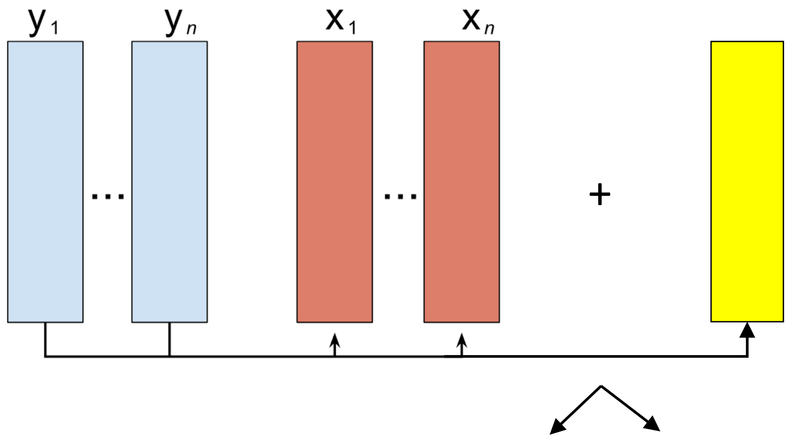
Partial redundancy analysis (pRDA): multivariate

OTUs

Controlled variables

Condition

Multiple linear regression



Output: the proportional explained variance of batch and treatment effects (fitted as controlled variables) across all microbial variables at once.

Fitted values

Residuals

Constrained PCA

The variance explained by fitted values

The variance explained by residuals

Unconstrained PCA

Borcard, et al (1992). Partialling out the spatial component of ecological variation. *Ecology*.

I. Batch effect detection

pRDA: can indicate if batch x treatment design is balanced

Approx. balanced batch x treatment design (**AD data** )

Dates\Phenol conc.	0-0.5	1-2
09/04/2015	4	5
14/04/2016	4	12
01/07/2016	8	13
14/11/2016	8	9
21/09/2017	2	10

The intersection variance indicates how unbalanced the design is:

=> batch and treatment effects are correlated.

##	Df	R.squared	Adj.R.squared	Testable
Treat only = Treat | Batch	1	NA	0.08943682	TRUE
Intersection	0	NA	0.01296248	FALSE
Batch only = Batch | Treat	4	NA	0.26604420	TRUE
## [d] = Residuals	NA	NA	0.63155651	FALSE

I. Batch effect detection

pRDA:

Completely balanced batch x treatment design (sponge data)

	Tissue 1	Tissue 2
Batch 1	8	8
Batch 2	8	8

##		Df	R.squared	Adj.R.squared	Testable
Treat only	= Treat | Batch	1	NA	0.16572246	TRUE
Intersection		0	NA	-0.01063501	FALSE
Batch only	= Batch | Treat	1	NA	0.16396277	TRUE
## [d]	= Residuals	NA	NA	0.68094977	FALSE

No intersection variance:

=> batch and treatment effects are independent.

I. Batch effect detection

pRDA:

Nested batch x treatment design (**HD data**)

Cages\Genotypes	HD	WT
Cage A	2	0
Cage B	3	0
Cage C	2	0
Cage D	0	4
Cage E	0	4
Cage F	0	3
Cage G	3	0
Cage H	3	0
Cage I	2	0
Cage J	0	4

##			Df	R.squared	Adj.R.squared	Testable
Treat only	=	Treat | Batch	0	NA	-2.220446e-16	FALSE
Intersection			0	NA	9.730583e-02	FALSE
Batch only	=	Batch | Treat	8	NA	1.608205e-01	TRUE
## [d]	=	Residuals	NA	NA	7.418737e-01	FALSE

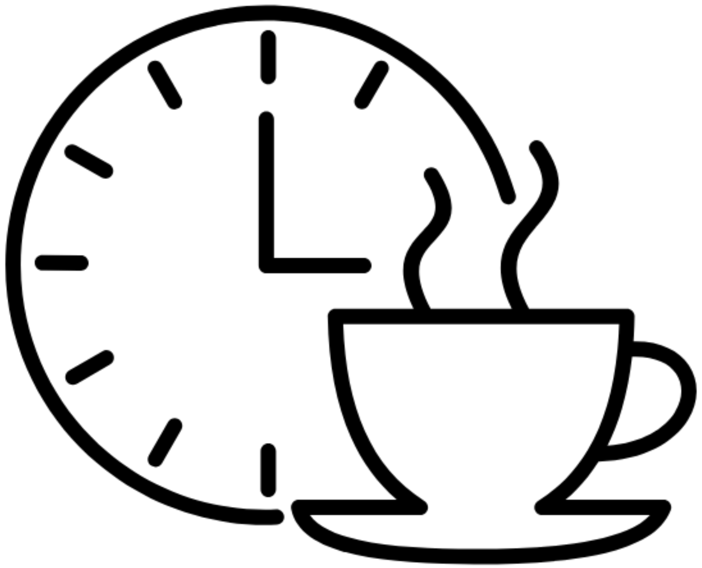
No treatment variance & considerable intersection variance:

=> batch and treatment effects are collinear.

Your turn!

Practice detecting batch effects in the AD data by following the steps in the "Batch effect detection" section. (30 mins)

Break



Created by Mukholifah
from Noun Project

~ 15 mins

II. Managing batch effects

- Accounting for batch effects:

  include batch effects as covariates in statistical models

- Correcting for batch effects:

  remove batch effects from the original data

- Univariate vs. multivariate:
  - Univariate: process each variable individually.

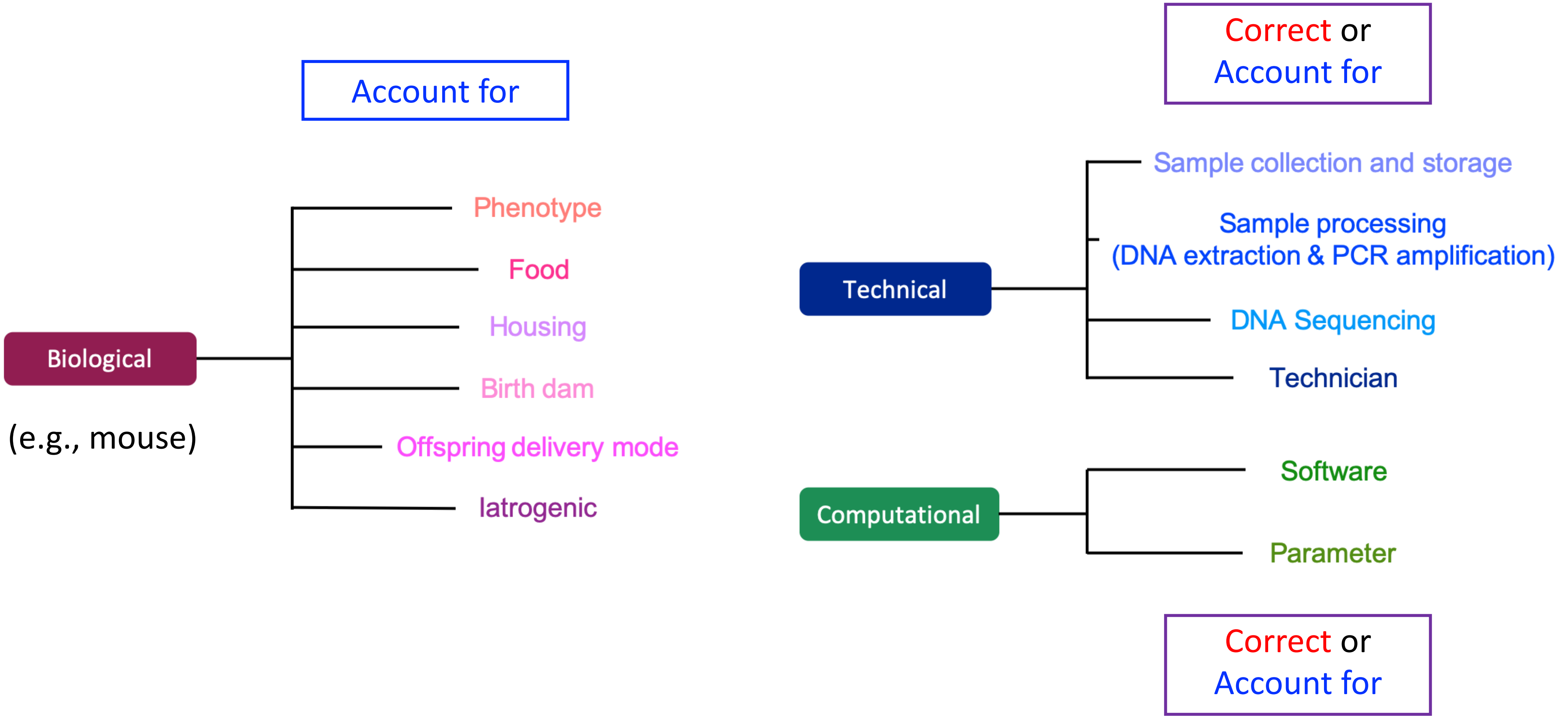
    E.g., Differential expression analysis. in *DESeq2*, *edgeR*
  - Multivariate: process all variables together.

    E.g., PCA, CCA, etc. in *phyloseq*

- Multivariate methods allow to consider variables as multivariate, rather than independent. E.g., better for microbiome data.

Wang & Lê Cao (2020). Managing batch effects in microbiome data. *Briefings in Bioinformatics*.

III. Managing batch effects



Wang & Lê Cao (2020). Managing batch effects in microbiome data. *Briefings in Bioinformatics*.

III. Managing batch effects

Methods accounting for batch effects:

- Pros: can consider the data characteristics and correlation between batch and treatment effects.

- Cons:
  - Limited to specific analyses according to the model (e.g., differential abundance analysis: taxa with p values)
  - Difficult to be assessed explicitly

e.g., Linear regression

Wang & Lê Cao (2020). Managing batch effects in microbiome data. *Briefings in Bioinformatics*.

III. Managing batch effects

Methods accounting for batch effects:

A. Designed for microbiome data (applied to count data):

- Cumulative-Sum Scaling normalisation + Zero-inflated Gaussian mixture model (CSS+ZIG):
  - Differential abundance analysis
  - Handle data characteristics, including uneven library sizes, compositional structure and undersampling zeroes

B. Adapted for microbiome data (after preprocessing):

- ★ Linear regression: handle nested batch x treatment design (**HD data** ⚖)
- Surrogate variable analysis (SVA): estimate unknown batch effects without extra information
- ★ Remove unwanted variation in 4 steps (RUV4): estimate unknown batch effects but require negative control variables or sample replicates that capture the batch variation

Wang & Lê Cao (2020). Managing batch effects in microbiome data. *Briefings in Bioinformatics*.

III. Managing batch effects

Methods correcting for batch effects (main focus of this workshop)**:**

- Applied to preprocessed data, e.g., CLR transformed microbiome data
- Pros: corrected data can be input in any downstream analyses
  - Dimension reduction; Visualisation; Clustering; Variable selection
- Cons:
  - cannot account for specific data characteristics within models; these need to be addressed in advance, e.g., CLR transformation
  - cannot handle correlations between batch and treatment effects within models; require additional processing to consider these correlations

e.g., ComBat

Wang & Lê Cao (2020). Managing batch effects in microbiome data. *Briefings in Bioinformatics*.

III. Managing batch effects

Methods correcting for batch effects:

- removeBatchEffect (rBE):
  - Linear regression (removeBatchEffect(), *limma*)
  - Univariate
- ★ ComBat:
  - Empirical Bayesian method
  - Assumes all variables are affected by batch effects in a systematic manner
  - Mixture of univariate and multivariate
- Percentile normalization (PN):
  - Each case sample's feature values are converted into percentiles of the control distribution within each batch
  - Require sufficient control samples within each batch
  - Univariate

Wang & Lê Cao (2020). Managing batch effects in microbiome data. *Briefings in Bioinformatics*.

III. Managing batch effects

Methods correcting for batch effects:

★ • Remove Unwanted Variation-III (RUVIII):
  – Requires negative control variables and sample replicates that capture the batch variation
  – Multivariate

★ • PLSDA-batch:
  – Non-parametric: can handle non-Gaussian distributions
  – No assumption of systematic batch effects
  – Include two variants: sparse PLSDA-batch (avoid overfitting); weighted PLSDA-batch (for unbalanced batch x treatment design)
  – Multivariate

Wang & Lê Cao (2023). PLSDA-batch: a multivariate framework to correct for batch effects in microbiome data. *Briefings in Bioinformatics*.

Your turn!

Practice accounting for and correcting batch effects in the AD data by following the steps in the "Managing batch effects" section. (40 mins)

IV. Assessing batch effect correction

➢ **Methods that detect batch effects:**

- Visualisation: PCA, boxplots, density plots, heatmap
- pRDA: proportion of explained variance across all variables

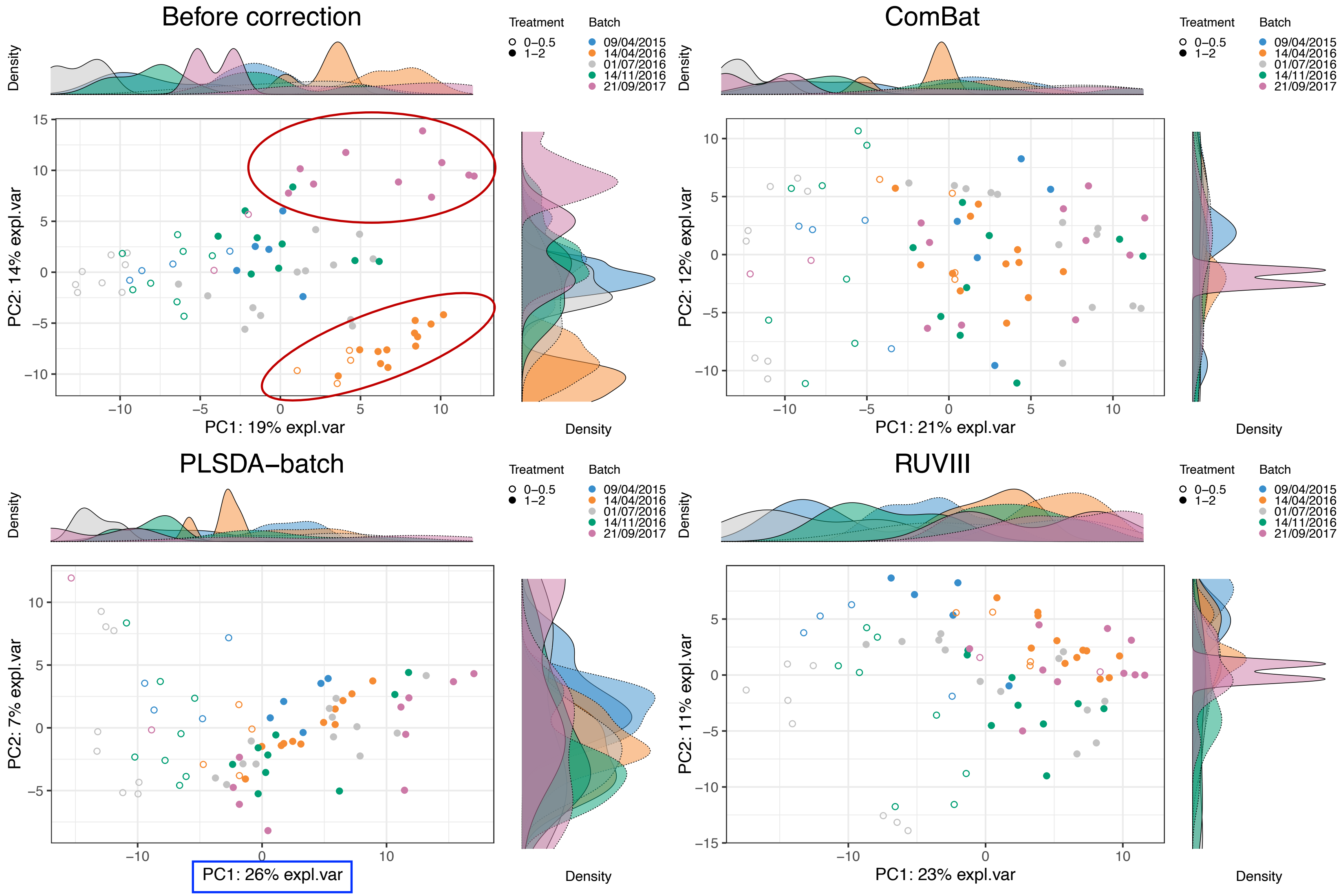
➢ **Other methods:**

- R^2 from one- way ANOVA: proportion of explained variance for each variable
- Alignment scores: [0,1], poor to excellent mixing samples among the different batches

Wang & Lê Cao (2023). PLSDA-batch: a multivariate framework to correct for batch effects in microbiome data. *Briefings in Bioinformatics*.

IV. Assessing batch effect correction

PCA:

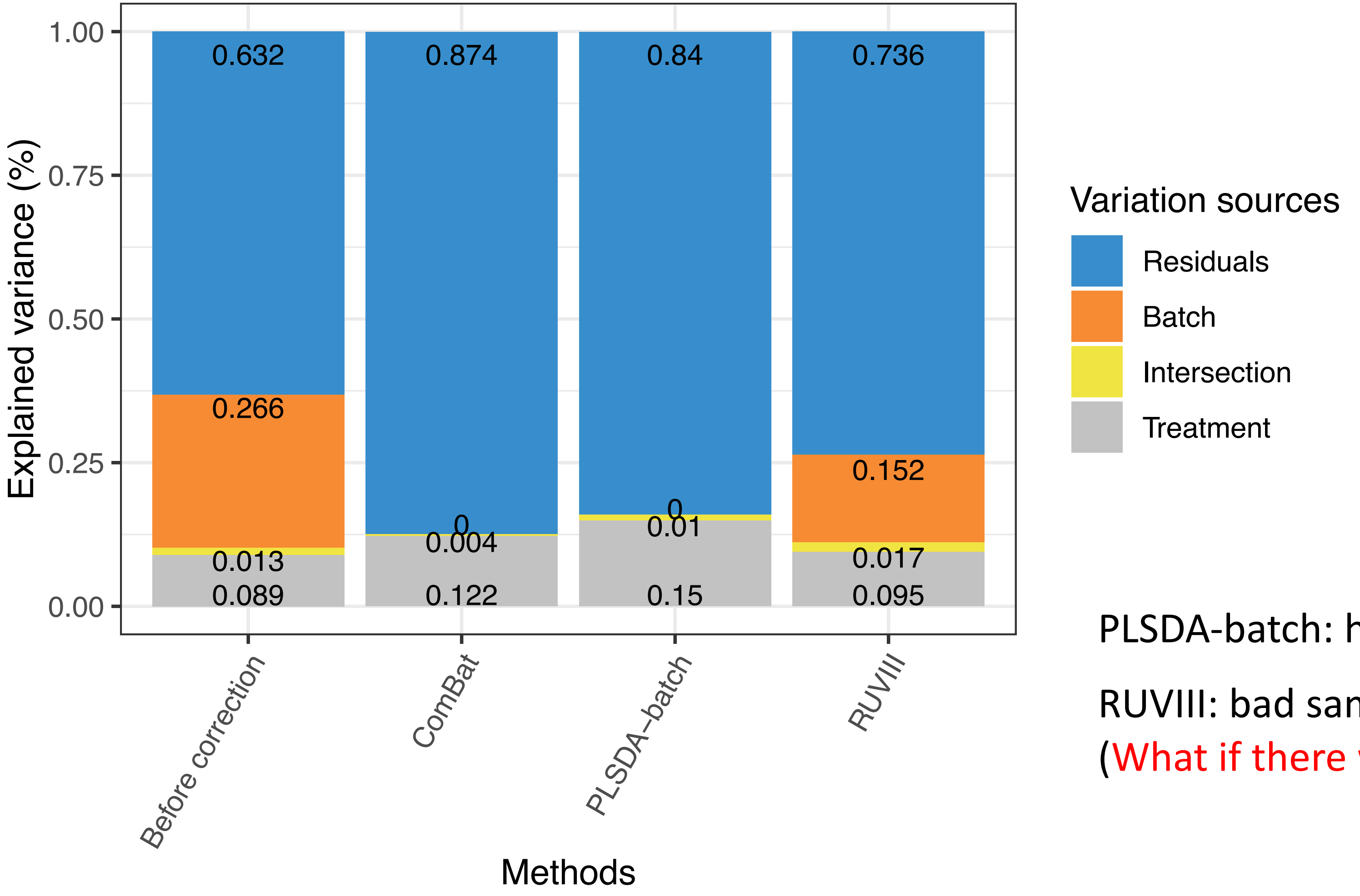


Batch effect variation is removed.

Wang & Lê Cao (2023). PLSDA-batch: a multivariate framework to correct for batch effects in microbiome data. *Briefings in Bioinformatics*.

IV. Assessing batch effect correction

pRDA:



PLSDA-batch: higher treatment variance

RUVIII: bad sample replicates
(What if there were better replicates?)

Wang & Lê Cao (2023). PLSDA-batch: a multivariate framework to correct for batch effects in microbiome data. *Briefings in Bioinformatics*.

IV. Assessing batch effect correction

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IV. Assessing batch effect correction

Based on the sample dissimilarity matrix calculated from the PCA projection:

$$\text{Alignment scores} = 1 - \frac{\bar{x} - \frac{k}{n}}{k - \frac{k}{n}},$$

k: the number of nearest neighbours

n: the sample size

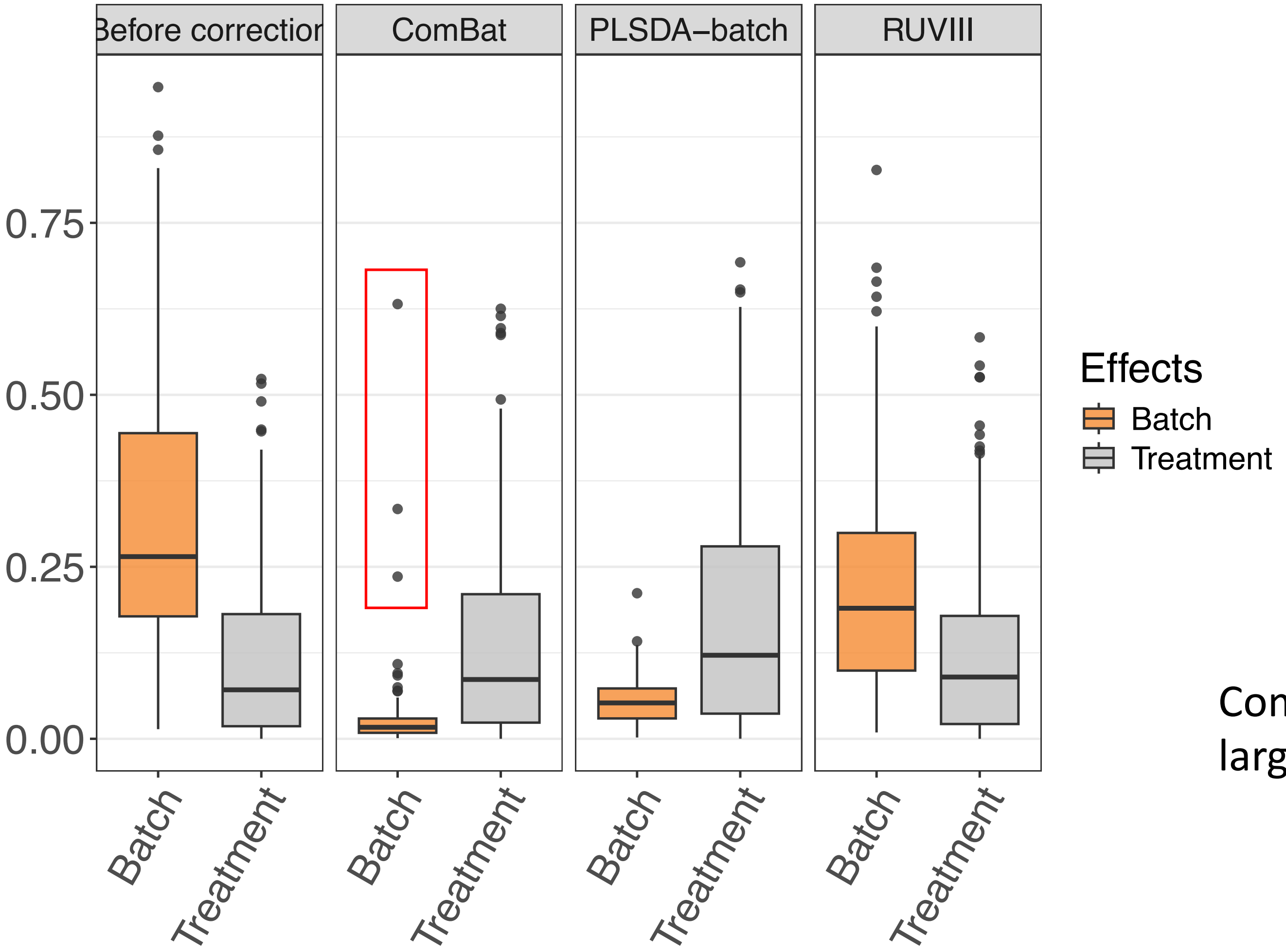
x: the number of each sample's nearest neighbours that belong to the same batch

$\bar{x}$: the average of all x

Wang & Lê Cao (2023). PLSDA-batch: a multivariate framework to correct for batch effects in microbiome data. *Briefings in Bioinformatics*.

IV. Assessing batch effect correction

R^2 from one- way ANOVA:

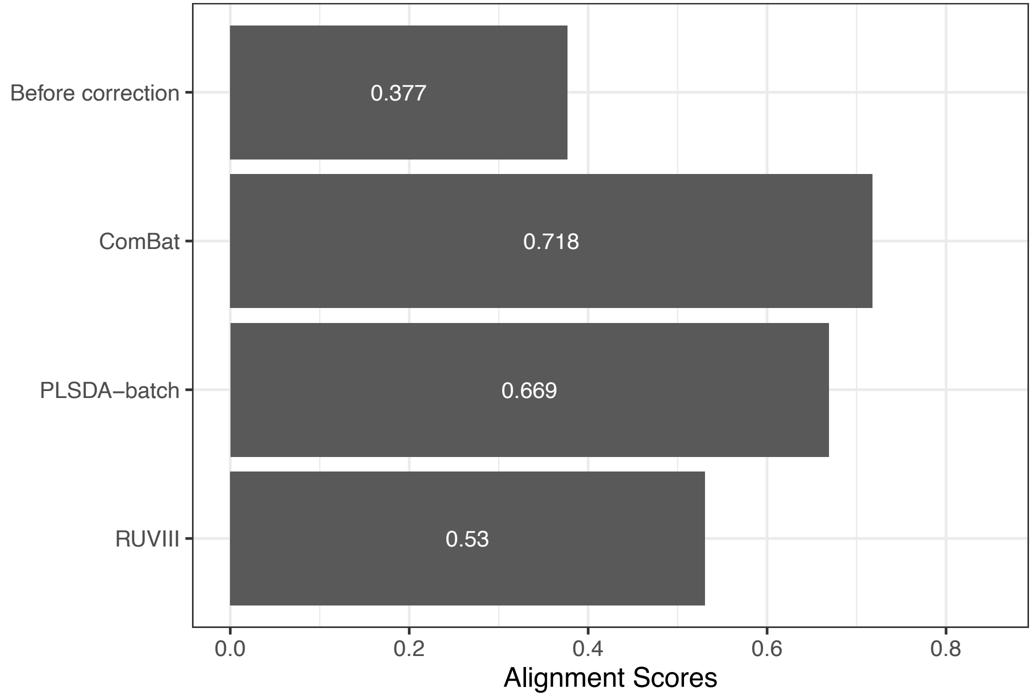


ComBat: a few variables with a large batch variance

Wang & Lê Cao (2023). PLSDA-batch: a multivariate framework to correct for batch effects in microbiome data. *Briefings in Bioinformatics*.

IV. Assessing batch effect correction

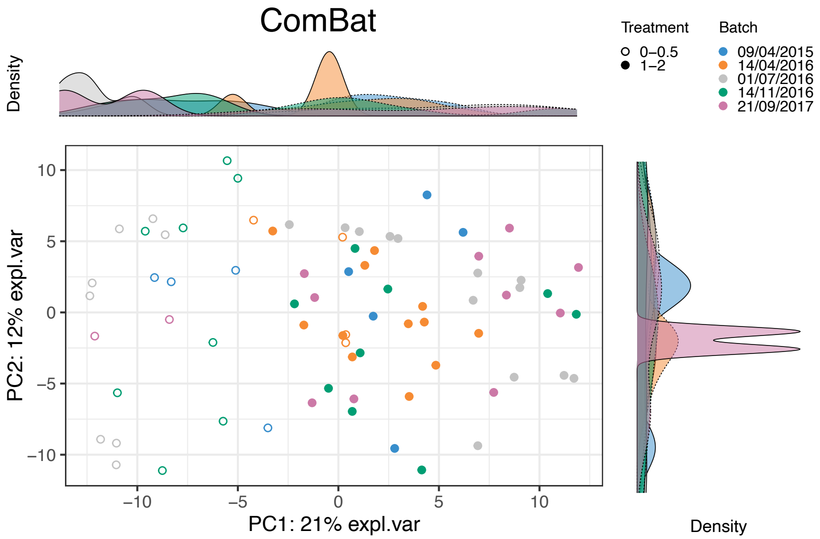
Alignment scores:

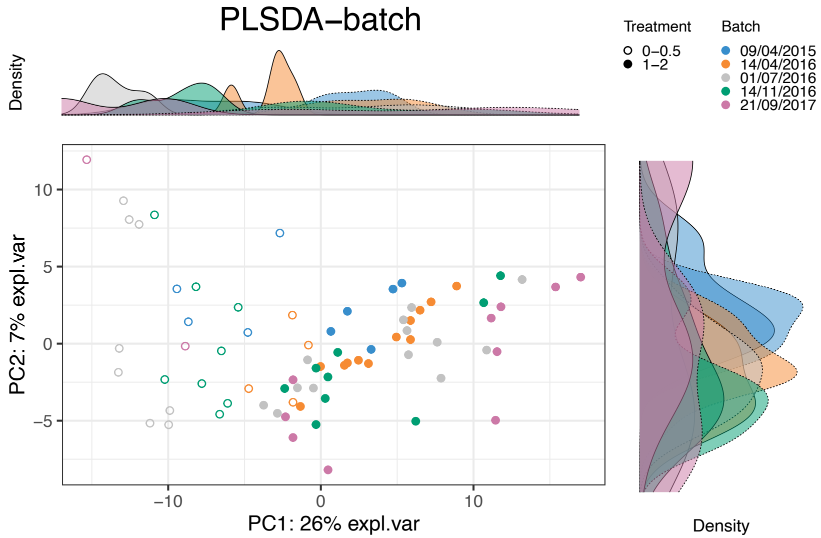


ComBat vs. PLSDA-batch :

Better mixing of batches?

→ Greater variance in PCA projection





Wang & Lê Cao (2023). PLSDA-batch: a multivariate framework to correct for batch effects in microbiome data. *Briefings in Bioinformatics*.

Your turn!

Practice using the AD data by following the steps in the "Assessing batch effect correction" section. (30 mins)

Conclusions

- Batch effects are ubiquitous, can arise from biological, technical and computational sources, and are sometimes unavoidable.

- Managing batch effects should consider corresponding data characteristics (preprocessing beforehand or inclusion in the model), batch sources (account for or correct), batch x treatment designs and the scale of influence (method assumptions).

Conclusions

Input data (preprocessed)

I. Batch effect detection

Visual

PCA, boxplots, density plots, heatmap (+ statistical tests)

No clear batch-driven clusters or non-significate p values

Quantitative

pRDA

Minimal batch variance

Batch effects are negligible

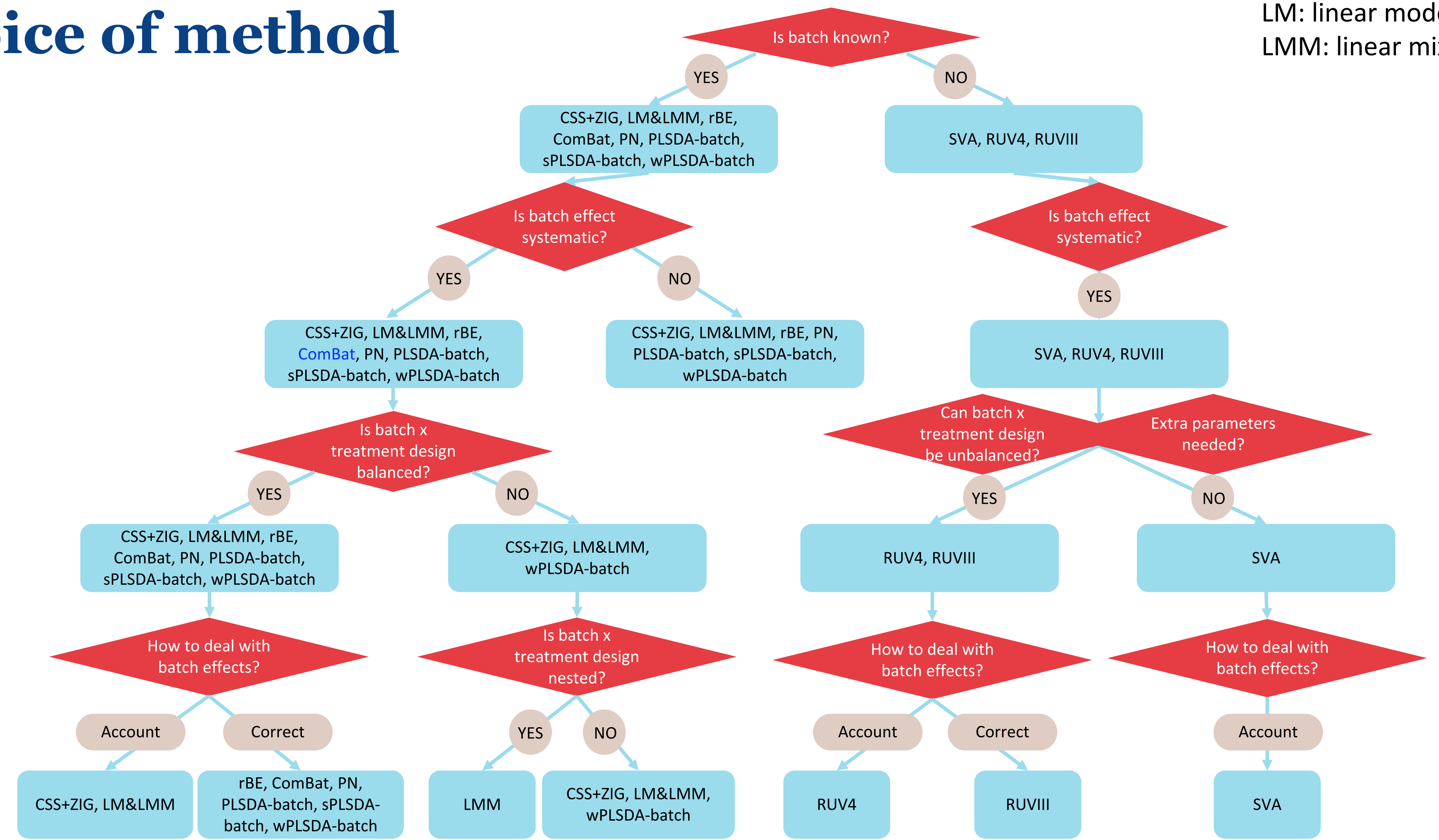
Batch effects are substantial

II. Managing batch effects

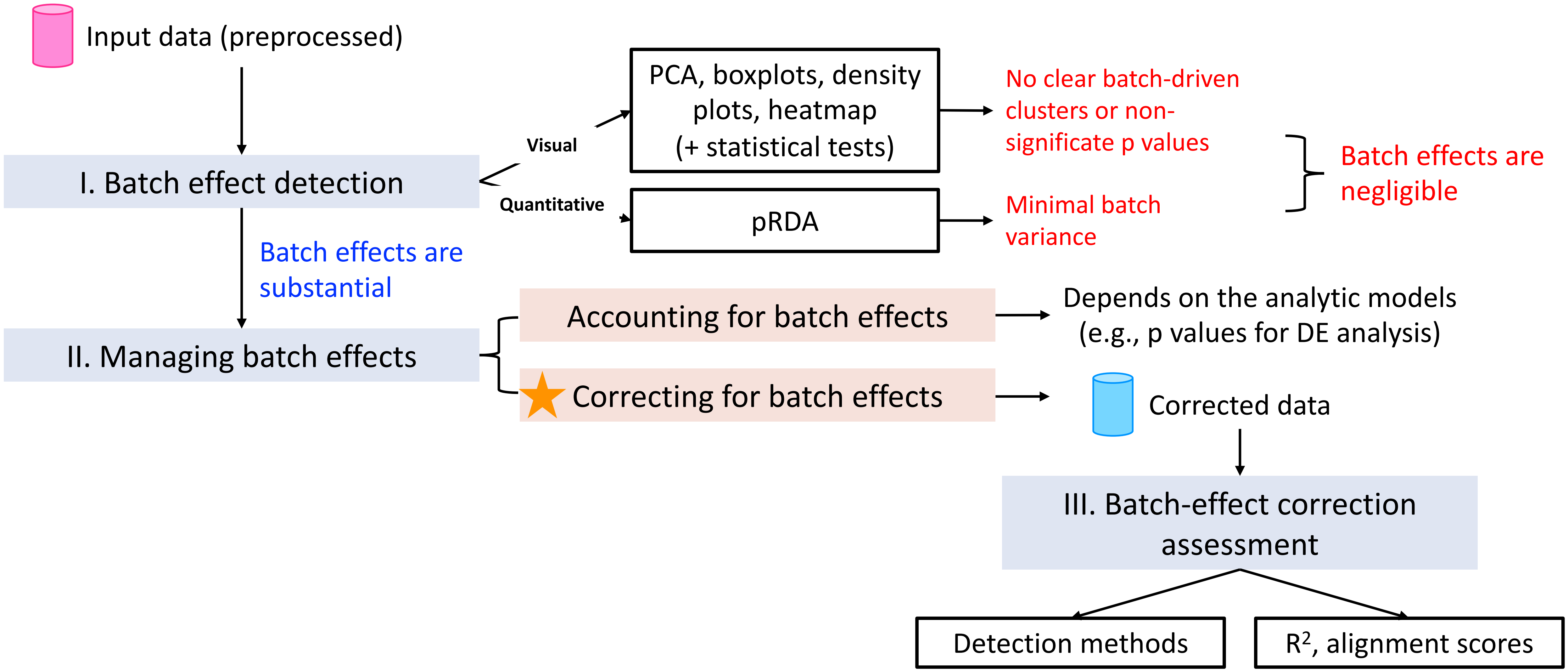
- Accounting for batch effects
- ★ Correcting for batch effects

Choice of method

LM: linear model
LMM: linear mixed model



Conclusions



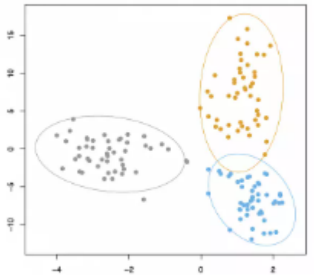
Next workshop

MIG Workshop: Multivariate analysis for omics data integration (bulk)

Training or Workshop

Tuesday 8 July 2025 Add to my calendar

Book Now



Kenneth Myer Building (144), Education Room, Ground Floor

Contact email

mig-ea@unimelb.edu.au

Date: Tuesday 8 July 2025

Time: 9:30am - 12:30pm

Host: Melbourne Integrative Genomics

Location: Kenneth Myer Building (144), Education Room, Ground Floor

Cost: $25

Lead instructors: Prof Kim-Anh Lê Cao (MIG)

Technological improvements have allowed for the collection of data from different molecular compartments (e.g. gene expression, protein abundance) resulting in multiple 'omics data from the same set of biospecimens or individuals (e.g. transcriptomics, proteomics). This workshop will introduce multivariate analysis to explore and integrate omics data using the R package mixOmics. We will present a few methods implemented in the package and define statistical

Time to reflect and give feedback!

Please fill in the 3-question form before you leave!

It’s really important for us to receive feedback so that we can continue delivering these workshops!

Appendix

Further information is available upon interest.

Data pre-processing for omics data

RNA-seq data:

Characteristics:

Count data (discrete, non-negative), zero inflation, overdispersion, uneven library sizes

Transformation:

- Trimmed Mean of M-values (TMM, *edgeR*)
- Median of Ratios (*DESeq2*)

Microbiome data:

Characteristics:

Count data, zero inflation (severe than RNA-seq data), overdispersion, uneven library sizes, compositional structure and multivariate nature

Transformation:

- Centered Log-Ratio (CLR, *mixOmics*)
- Cumulative Sum Scaling (CSS, *metagenomeSeq*)

Data pre-processing for omics data

Metabolomic and proteomic data :

Characteristics:

Continuous and right-skewed data, high intra-group (replicate) variability, missing values

Transformation:

- Imputation of missing values
- Log transformation to reduce skewness
- Median or quantile normalisation to match distributions across samples
- Normalisation to internal standards / housekeeping variables to control for sort of technical variation (systematic)