Beyond Normalization: Incorporating Scale Uncertainty in ALDEx2

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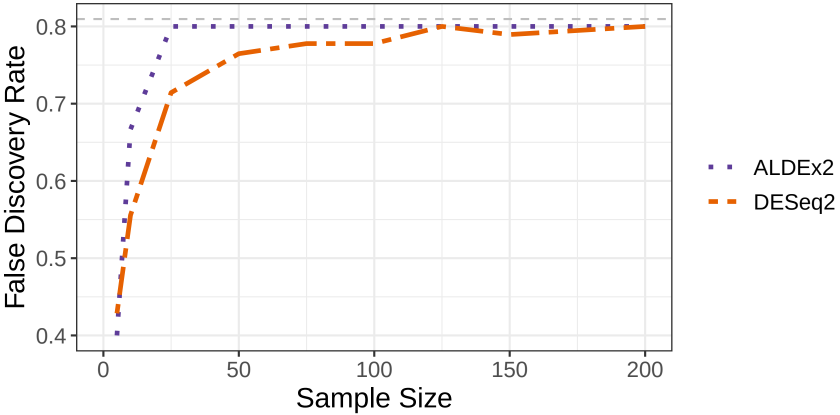
Recap: Sequencing depth can confound conclusions.

Observed data (Y)	Sample 1	Sample 2	Sample 3	
Condition	Pre	Pre	Post	Conclusion
Entity 1	5	10	100	Increase
Entity 2	10	25	3	Decrease
Entity 3	0	1	8	Increase
Entity 4	0	0	19	Increase
Sequencing Depth	15	36	130	

This can mislead analyses.

System data (W)	Sample 1	Sample 2	Sample 3	
Condition	Pre	Pre	Post	Conclusion
Entity 1	227	351	154	Decrease
Entity 2	684	891	3	Decrease
Entity 3	48	32	15	Decrease
Entity 4	43	39	27	Decrease
Scale ($W^{\perp}$)	1,002	1,313	200	

. . . and lead to unacknowledged bias.

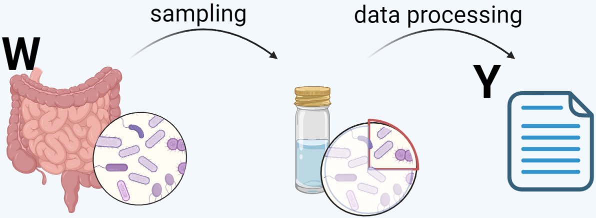


Section 1

Problem Set-Up

Observed Data as a Sample from the System

Information Loss from System to Data:



sampling

data processing

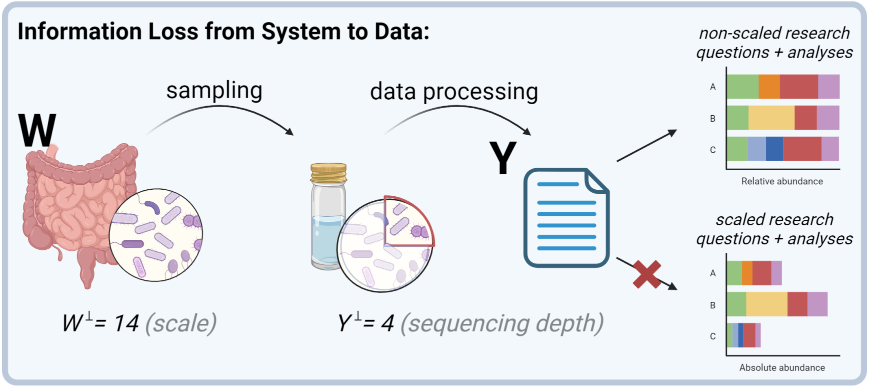
W

Y

$W^{\perp}$= 14 *(scale)*

$Y^{\perp}$= 4 *(sequencing depth)*

Observed Data as a Sample from the System


Information Loss from System to Data:
W
sampling
data processing
Y
non-scaled research questions + analyses
A
B
C
Relative abundance
scaled research questions + analyses
A
B
C
Absolute abundance
$W^{\perp}$= 14 (scale)
$Y^{\perp}$= 4 (sequencing depth)

Notation

- $\mathbf{Y}$: a measurement of the underlying system $\mathbf{W}$.

$$\mathbf{W}_{dn} = \underbrace{\mathbf{W}^{\parallel}_{dn}}_{\text{composition}} \times \underbrace{W^{\perp}_{n}}_{\text{scale}}$$

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- $\mathbf{Y}$: a measurement of the underlying system $\mathbf{W}$.

$$\mathbf{W}_{dn} = \underbrace{\mathbf{W}^{\parallel}_{dn}}_{\text{composition}} \times \underbrace{W^{\perp}_{n}}_{\text{scale}}$$

- **Composition:** $\mathbf{W}^{\parallel}_{dn} = \frac{\mathbf{W}_{dn}}{\sum_{d=1}^{D} \mathbf{W}_{dn}}$
- **Scale:** $W^{\perp}_{n} = \sum_{d=1}^{D} \mathbf{W}_{dn}$

Example: Notation

System data ($W^{\parallel}$)	Sample 1	Sample 2	Sample 3
Condition	Pre	Pre	Post
Entity 1	0.27	0.27	0.77
Entity 2	0.68	0.68	0.02
Entity 3	0.05	0.02	0.08
Entity 4	0.04	0.03	0.13

	Sample 1	Sample 2	Sample 3
Condition	Pre	Pre	Post
Scale ($W^{\perp}$)	1,002	1,313	200

Differential Abundance/Expression Analysis

- **Research Question:** How do entities (e.g., taxa or genes) change between conditions?
- $\boldsymbol{\theta}$: what we want to estimate.

$$\theta_d = \text{mean}_{\text{case}}(\log \mathbf{W}_{dn}) - \text{mean}_{\text{control}}(\log \mathbf{W}_{dn})$$

The Original ALDEx2 Model

Step 1: Model Sampling Uncertainty

$$\begin{aligned} \mathbf{Y}_{\cdot n} &\sim \text{Multinomial}(\mathbf{W}^{\parallel}_{\cdot n}) \\ \mathbf{W}^{\parallel}_{\cdot n} &\sim \text{Dirichlet}(\alpha) \end{aligned}$$

Step 2: Centered Log-Ratio Transformation

$$\log \mathbf{W}_{\cdot n} = \left[\log \mathbf{W}^{\parallel}_{1n} - \text{mean}(\log \mathbf{W}^{\parallel}_{\cdot n}), \ldots, \log \mathbf{W}^{\parallel}_{Dn} - \text{mean}(\log \mathbf{W}^{\parallel}_{\cdot n})\right]$$

Step 3: Calculate LFCs and Test if Different from Zero.

$$\theta_d = \text{mean}_{\text{case}}(\log \mathbf{W}_{dn}) - \text{mean}_{\text{control}}(\log \mathbf{W}_{dn})$$

Implied Assumptions about Scale

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Step 3: Calculate LFCs and Test if Different from Zero.

$$\theta_d = \text{mean}_{\text{case}}(\log \mathbf{W}_{dn}) - \text{mean}_{\text{control}}(\log \mathbf{W}_{dn})$$

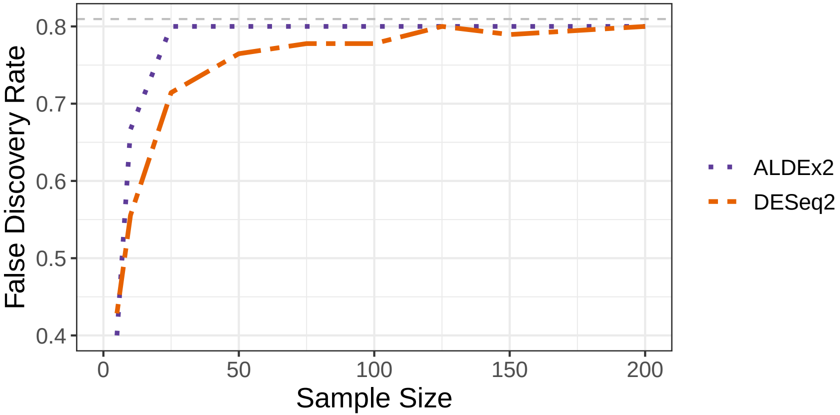
Implied Assumptions about Scale, cont.

Since $\log \mathbf{W}_{dn} = \log \mathbf{W}^{\parallel}_{dn} + \log W^{\perp}_{n}$, the CLR normalization implies:

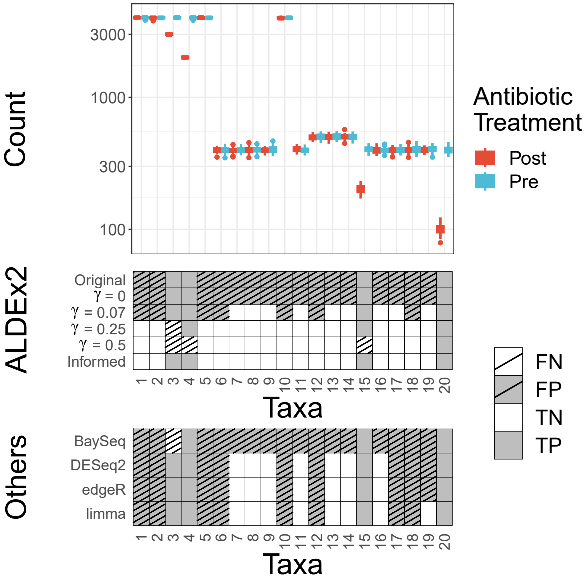
$$\begin{aligned}\log W_{dn} &= \log \mathbf{W}^{\parallel}_{dn} - \text{mean}(\log \mathbf{W}^{\parallel}_{\cdot n}) \\ \log W^{\perp}_{n} &= -\text{mean}(\log \mathbf{W}^{\parallel}_{\cdot n}).\end{aligned}$$

What happens when this is wrong?

Unacknowledged bias!



Adding Uncertainty in Scale can Help.



Section 2

Scale Reliant Inference

Scale Reliant Inference: The Basics

- **The CoDA perspective:** Research questions that depend on $W^{\perp}$ (scale) cannot be answered rigorously.
- **The Normalization perspective:** Research questions that depend on $W^{\perp}$ (scale) can be answered after normalization.
- Who is right?

Scale Reliant Inference: The Basics

- **The CoDA perspective:** Research questions that depend on $W^{\perp}$ (scale) cannot be answered rigorously.
- **The Normalization perspective:** Research questions that depend on $W^{\perp}$ (scale) can be answered after normalization.
- Who is right?
- **The CoDA perspective:** Rigourous, but scientifically limiting.
- **The Normalization perspective:** Practical, but unacknowledged bias.

Scale Reliant Inference: The Basics

- For LFCs, θ depends on $W^{\perp}$:

$$\begin{aligned}
\theta_d &= \text{mean}_{\text{case}}(\log \mathbf{W}_{dn}) - \text{mean}_{\text{control}}(\log \mathbf{W}_{dn}) \\
&= \dots \\
&= \underbrace{\text{mean}_{\text{case}}(\log \mathbf{W}^{\parallel}_{dn}) - \text{mean}_{\text{control}}(\log \mathbf{W}^{\parallel}_{dn})}_{\theta^{\parallel}} \\
&\quad + \underbrace{\text{mean}_{\text{case}}(\log W^{\perp}_{n}) - \text{mean}_{\text{control}}(\log W^{\perp}_{n})}_{\theta^{\perp}}
\end{aligned}$$

Scale Reliant Inference: Theory Intro

Recall for LFCs:

$$\begin{aligned}\theta_d &= \text{mean}_{\text{case}}(\log \mathbf{W}_{dn}) - \text{mean}_{\text{control}}(\log \mathbf{W}_{dn}) \\ &= \theta^{\parallel} + \theta^{\perp}\end{aligned}$$

- What can we say about θ from $\theta^{\parallel}$ alone?

Scale Reliant Inference: Theory Intro

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- What can we say about θ from $\theta^{\parallel}$ alone?
- Statistical perspective: θ is not identifiable without $\theta^{\perp}$.
- Practical issues: unbiased estimators, calibrated confidence sets, and type-I error control **NOT** possible!
- See Nixon et al. (2023) for details.

$\theta^{\perp}$: The Missing Piece

$$\theta^{\perp} = \text{mean}_{\text{case}}(\log W_n^{\perp}) - \text{mean}_{\text{control}}(\log W_n^{\perp})$$

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- The change in scales between conditions matters for estimating LFCs.
- The scale only needs to be known up to a constant (see Nixon et. al (2023)).

$\theta^{\perp}$: The Missing Piece

$$\theta^{\perp} = \text{mean}_{\text{case}}(\log W_n^{\perp}) - \text{mean}_{\text{control}}(\log W_n^{\perp})$$

- The change in scales between conditions matters for estimating LFCs.
- The scale only needs to be known up to a constant (see Nixon et. al (2023)).
- Each normalization implies a value of $\theta^{\perp}$ (e.g., CLR):

$$\theta^{\perp}_{\text{CLR}} = \text{mean}_{\text{case}}(-\log \text{GM}(\mathbf{W}^{\parallel}_{\cdot n})) - \text{mean}_{\text{control}}(-\log \text{GM}(\mathbf{W}^{\parallel}_{\cdot n}))$$

Scale Simulation Random Variables

Goal: Estimate $\theta = f(\mathbf{W}^{\parallel}, W^{\perp})$.

1. Draw samples of $\mathbf{W}^{\parallel}$ from a measurement model (can depend on Y).
2. Draw samples of $W^{\perp}$ from a scale model (can depend on $\mathbf{W}^{\parallel}$).
3. Estimate samples of $\theta = f(\mathbf{W}^{\parallel}, W^{\perp})$.

Comparison to ALDEx2

The ALDEx2 Model

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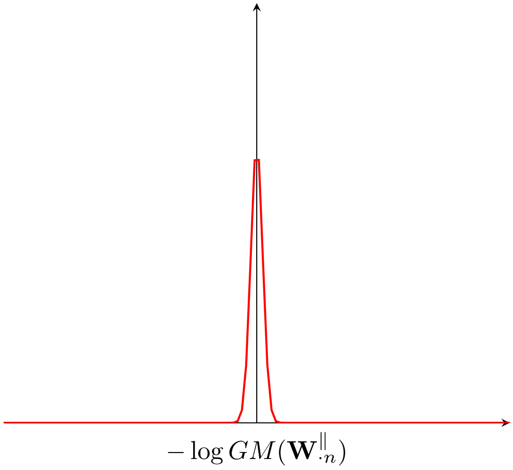
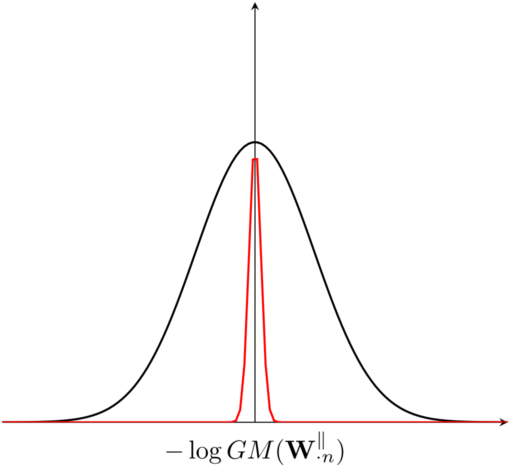
Step 3: Calculate LFCs and Test if Different from Zero.

The Original Scale Model



Extending the Original Scale Model



ALDEx2 as an SSRV

Step 1: Model Sampling Uncertainty

$$\mathbf{Y}_{\cdot n} \sim \text{Multinomial}(\mathbf{W}^{\parallel}_{\cdot n})$$
$$\mathbf{W}^{\parallel}_{\cdot n} \sim \text{Dirichlet}(\alpha)$$

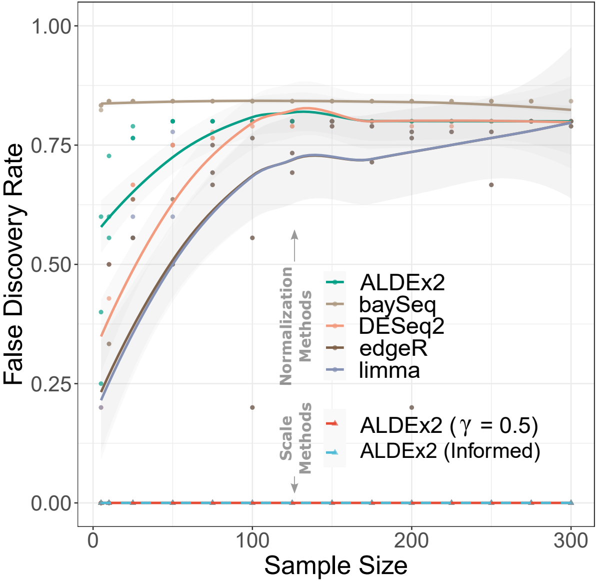
Step 2: Draw Samples from a Scale Model

$$\log W^{\perp}_{n} = -\text{mean}(\log \mathbf{W}^{\parallel}_{\cdot n}) + \epsilon,\ \epsilon \sim N(0, \gamma^2)$$
$$\log \mathbf{W}_{\cdot n} = \log \mathbf{W}^{\parallel}_{\cdot n} + \log W^{\perp}_{n}$$

Step 3: Calculate LFCs and Test if Different from Zero.

$$\theta_d = \text{mean}_{\text{case}}(\log \mathbf{W}_{dn}) - \text{mean}_{\text{control}}(\log \mathbf{W}_{dn})$$

Benefits of Moving Past Normalizations to Scale



Section 3

Updated ALDEx2 Model

ALDEx2 as an SSRV

Step 1: Model Sampling Uncertainty

$$\mathbf{Y}_{\cdot n} \sim \text{Multinomial}(\mathbf{W}^{\parallel}_{\cdot n})$$
$$\mathbf{W}^{\parallel}_{\cdot n} \sim \text{Dirichlet}(\alpha)$$

Step 2: Draw Samples from a Scale Model

$$\log W^{\perp}_{n} \sim Q$$
$$\log \mathbf{W}_{\cdot n} = \log \mathbf{W}^{\parallel}_{\cdot n} + \log W^{\perp}_{n}$$

Step 3: Calculate LFCs and Test if Different from Zero.

$$\theta_d = \text{mean}_{\text{case}}(\log \mathbf{W}_{dn}) - \text{mean}_{\text{control}}(\log \mathbf{W}_{dn})$$

Intro to Scale Models

There are no restrictions on what scale models can be, although there are some helpful options:

1. Based on normalizations. (Stochastic normalizations)
2. Based on biological knowledge.
3. Based on outside measurements.

Scale Models based on Biological Knowledge

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- A past study showed that a certain disease (e.g., Crohn's disease) leads to lower microbial load in the gut.

$$
\begin{aligned}
\log W^{\perp}_{\text{Healthy}} &\sim N(1, \gamma^2) \\
\log W^{\perp}_{\text{Crohn's}} &\sim N(0.7, \gamma^2)
\end{aligned}
$$

Scale Models based on Outside Measurements

How can outside measurements be used to quantify scale?

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1. These measurements can be used *if* they relate to your scale of interest.
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$$\log W_n^{\perp} \sim N(\log \mu_{FC,n}, \sigma_{FC,n}^2)$$

Section 4

Changes to the ALDEx2 Interface

Including scale

The new ALDEx2 model removes normalizations in lieu of scale models.

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Major updates:

1. A new argument `gamma` which makes it easy to incorporate scale uncertainty (`aldex` and `aldex.clr` functions).
   - `gamma` can either be a single numeric or a matrix.
     1. Single numeric: controls the noise on the default scale model.
     2. Matrix: A $N \times S$ matrix of samples of $W^{\perp}$.
2. A new function `aldex.senAnalysis` to see how analysis results change as a function of scale uncertainty.

Option 1: Default Scale Model

The default scale model is based on errors in the CLR normalization.

$$\log \hat{W}_n^{\perp(s)} = -\text{mean}\left(\log \hat{W}_{\cdot n}^{\parallel(s)}\right) + \Lambda^{\perp} x_n$$

$$\Lambda^{\perp} \sim \; N(0, \gamma^2).$$

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1. When $\gamma = 0$, behavior matches the original ALDEx2 model.
2. For any value of $\gamma > 0$, it models potential error in the CLR assumption (false positives will decrease compared to the CLR normalization.)
3. It has a concrete interpretation to contextualize scale assumptions.

Interpreting the Default Scale Model

$$
\begin{aligned}
\theta^{\perp}_{\text{Default Scale}} &= \text{mean}_{\text{case}}(-\text{GM}(\mathbf{W}^{\parallel}_{\cdot n})) - \text{mean}_{\text{control}}(-\text{GM}(\mathbf{W}^{\parallel}_{\cdot n})) + \epsilon \\
&= \theta^{\perp}_{\text{CLR}} + \epsilon \\
\epsilon &\sim N(0, \gamma^2)
\end{aligned}
$$

Interpreting the Default Scale Model

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The default scale model implies that:

- With 95% certainty, the value of $\theta^{\perp}$ is within $\pm 2\gamma$ of the value of $\theta^{\perp}_{\text{CLR}}$.

Interpreting the Default Scale Model

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The default scale model implies that:

- With 95% certainty, the value of $\theta^{\perp}$ is within $\pm 2\gamma$ of the value of $\theta^{\perp}_{\text{CLR}}$.
- With 95% certainty, the true difference in scales falls within the the range $2^{\theta^{\perp}_{\text{CLR}} \pm 2\gamma}$.

Example: Interpreting the Default Scale Model

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Example: Interpreting the Default Scale Model

With 95% certainty, the true difference in scales falls within the the range $2^{\theta^{\perp}_{\text{CLR}} \pm 2\gamma}$.

- Suppose that we are performing differential abundance in a case/control study where $\theta^{\perp}_{\text{CLR}} = 0.04$.
- Suppose we set $\gamma = 0.5$.

Example: Interpreting the Default Scale Model

With 95% certainty, the true difference in scales falls within the the range $2^{\theta^{\perp}_{\text{CLR}} \pm 2\gamma}$.

- Suppose that we are performing differential abundance in a case/control study where $\theta^{\perp}_{\text{CLR}} = 0.04$.
- Suppose we set $\gamma = 0.5$.
- Then, this implies that, with 95% certainty, we believe that the scale of the case condition is within a factor of $[2^{0.04-2\times 0.5}, 2^{0.04+2\times 0.5}] = [0.51, 2.05]$ of the control condition.

Using the Default Scale Model

```r
## Adding noise via the default scale model
mod.defaultScale <- aldex(Y, conds, gamma = 0.5)
```

Option 2: More Complex Scale Models

Alternatively, can pass a matrix of scale samples to `gamma` so long as:

1. The dimension is $N \times S$.
2. They are samples of $W^{\perp}$ not $\log W^{\perp}$.

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1. The dimension is $N \times S$.
2. They are samples of $W^{\perp}$ not $\log W^{\perp}$.

Reasons to do this:

1. **Biological beliefs:** Scale is guided by the biological system or the researcher's prior beliefs.
2. **Outside Measurements:** These can be used in building a scale model *if* they are informative on the scale of interest (e.g., qPCR, flow cytometry).

Sensitivity Analyses

- Instead of picking γ, why not test over a range instead?

Sensitivity Analyses

Step 1: Model Sampling Uncertainty

$$\mathbf{Y}_{\cdot n} \sim \text{Multinomial}(\mathbf{W}^{\parallel}_{\cdot n})$$
$$\mathbf{W}^{\parallel}_{\cdot n} \sim \text{Dirichlet}(\alpha)$$

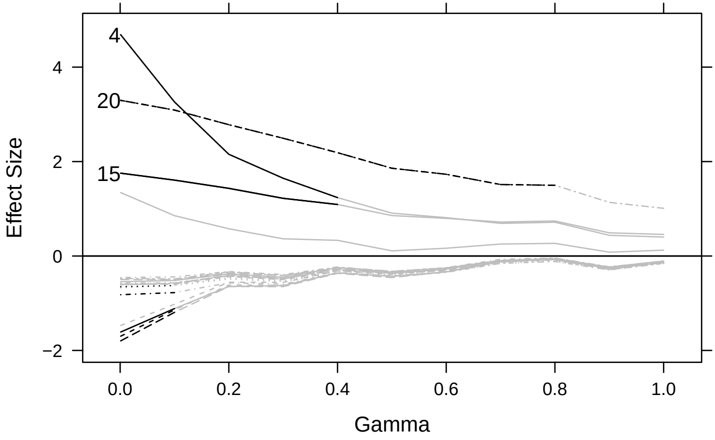
Step 2: Draw Samples from a Scale Model For a given γ:

$$\log W_n^{\perp,\gamma} = -\mathrm{mean}\left(\log \hat{W}_{\cdot n}^{\parallel(s)}\right) + \Lambda^{\perp} x_n$$
$$\Lambda^{\perp} \sim \ N(0,\gamma^2)$$
$$\log \mathbf{W}^{\gamma}{}_{\cdot n} = \log \mathbf{W}^{\parallel}_{\cdot n} + \log W_n^{\perp,\gamma}$$

Step 3: Calculate LFCs and Test if Different from Zero.

Step 4: Repeat for all desired values of γ.

Example: Sensitivity Analyses



Section 5

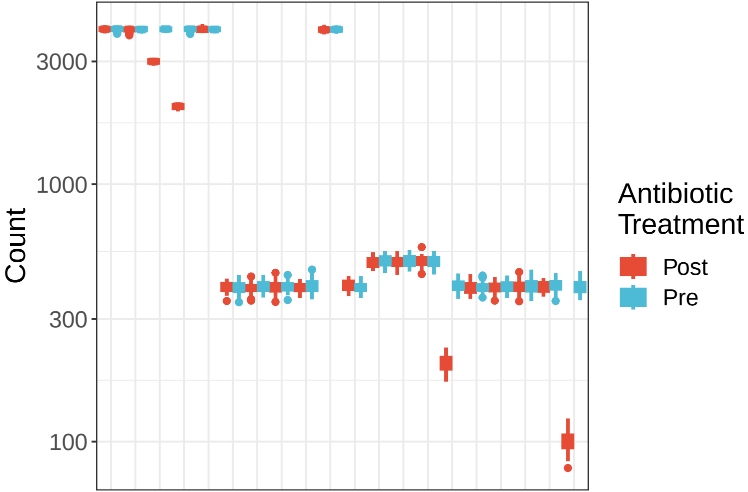
Data Examples

Simulation Study

Consider a simple study of the microbiome pre/post antibiotic administration.

- **Research question:** Which taxa change in absolute abundance after taking an antibiotic?
- 100 study participants, 50 in each condition (pre/post antibiotics).
- 20 taxa total with 4 taxa truly changing (decreasing)

Data



Adding Scale is Easy

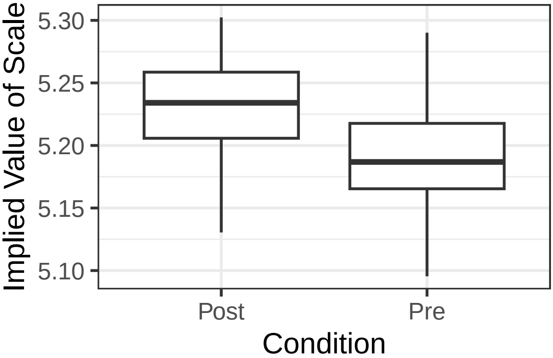
```r
## Adding noise via the default scale model
mod.ss.high <- aldex(Y, conds, gamma = 0.5)
```

Investigating Assumptions about Scale

```r
## Looking at the implied scale
clr <- aldex.clr(Y, conds, gamma = 1e-3)
clr@scaleSamps[1:6, 1:4]
```

```
##          [,1]     [,2]     [,3]     [,4]
## [1,] 5.174279 5.124890 5.199780 5.175163
## [2,] 5.175705 5.144470 5.184953 5.167715
## [3,] 5.178751 5.171188 5.130795 5.100749
## [4,] 5.158594 5.195139 5.164371 5.145696
## [5,] 5.120674 5.175533 5.189581 5.171154
## [6,] 5.208741 5.273464 5.207085 5.162631
```

Investigating Assumptions about Scale, cont.



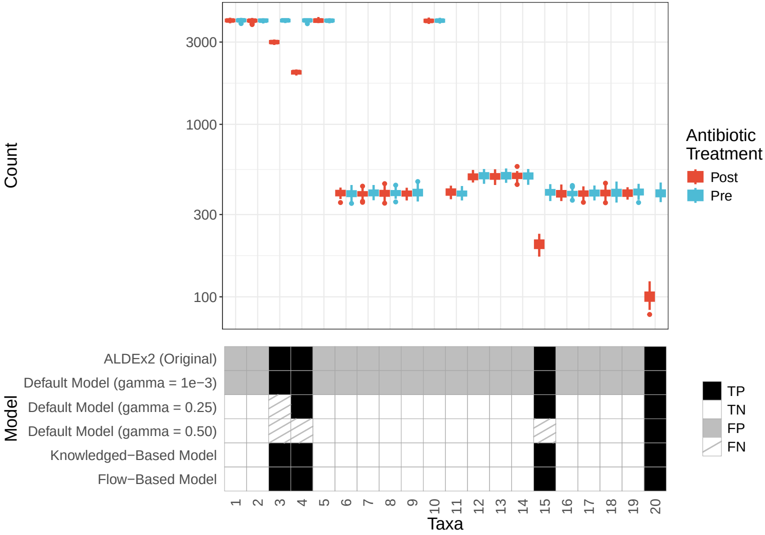
Scale Model based on Biology

```
## Creating an informed model using biological reasoning
scales <- c(rep(1, 50), rep(0.9, 50))
scale_samps <- aldex.makeScaleMatrix(
  gamma = .15,
  mu = scales,
  conditions = conds,
  log = FALSE
)

mod.know <- aldex(Y, conds, gamma = scale_samps)
```

Scale Model based on Outside Measurements

```r
scale_samps <- matrix(NA,
  nrow = nrow(flow_data_collapse),
  ncol = 128
)

for (i in 1:nrow(scale_samps)) {
  scale_samps[i, ] <- rnorm(
    n = 128,
    mean = flow_data_collapse$mean[i],
    sd = flow_data_collapse$stdev[i]
  )
}
mod.flow <- aldex(Y, conds, gamma = scale_samps)
```

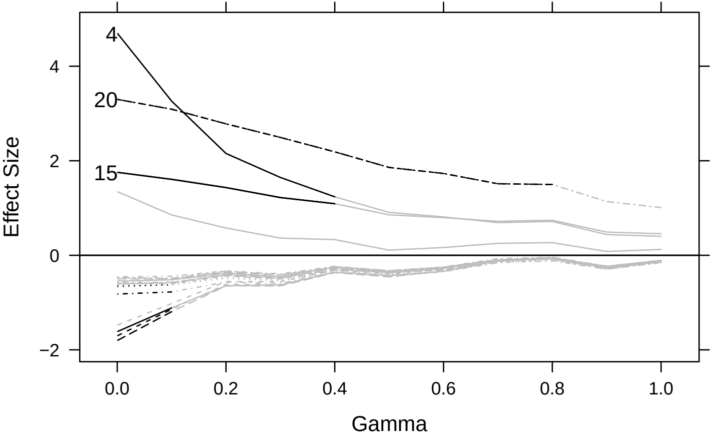
Plotting Results



Sensitivity Analyses

```
## First, specifying different values for the noise
in the scale
gamma_to_test <- c(1e-3, seq(0.1, 1, by = .1))

## Run the CLR function
clr <- aldex.clr(Y, conds)

## Run sensitivity analysis function
sen_res <- aldex.senAnalysis(clr,
  gamma = gamma_to_test
)
plotGamma(sen_res,
  thresh = .1,
  blackWhite = TRUE, taxa_to_label = 3
)
```

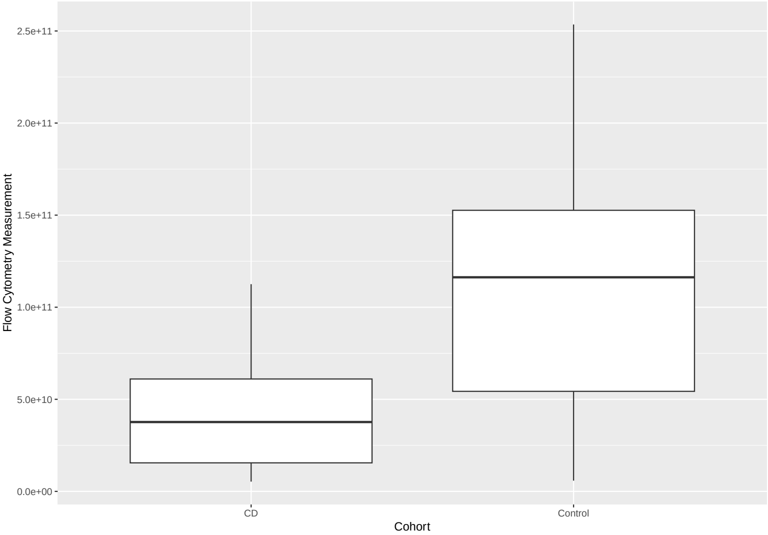
Sensitivity Analyses, cont.



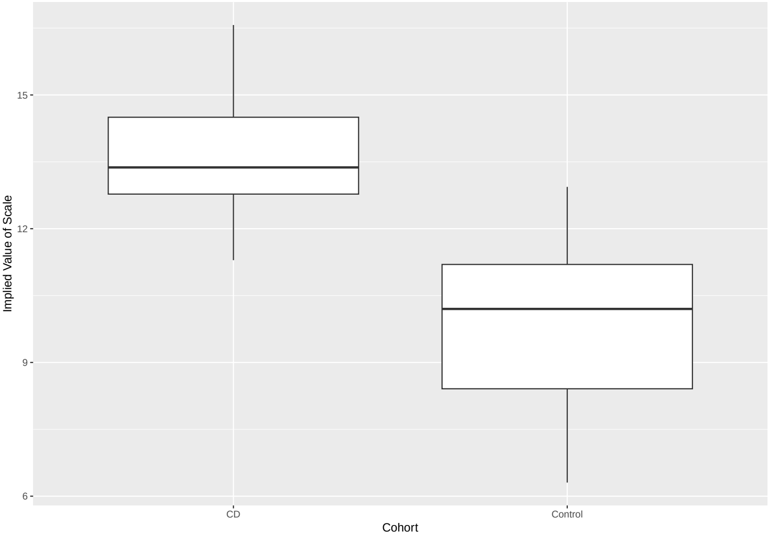
Real Example: Vandputte

1. Comparison study of 29 Crohn's disease patients and 66 healthy controls.
2. For each patient, they sequenced the fecal sample and obtained flow cytometry measurements.
3. Proposed an approach that supplemented sequence count data with flow cytometry measurements.

Difference in Scale Implied by Flow Cytometry



Difference in Scale Implied by CLR



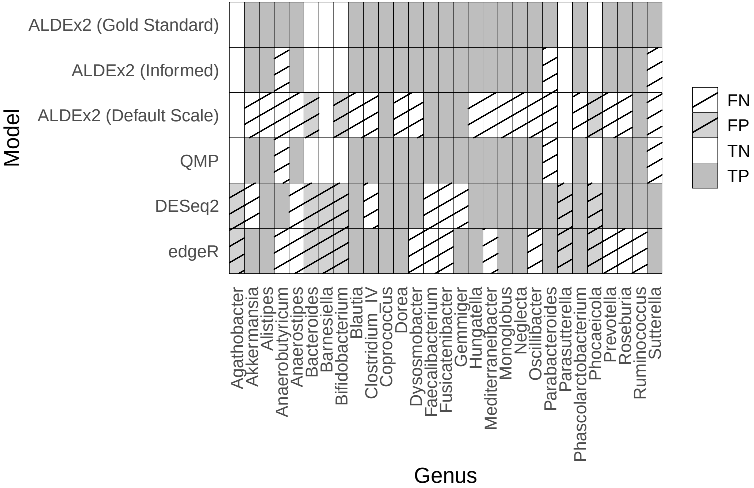
Creating a Gold Standard Model

```r
scale_mean <- log2(sample_data(phylo)$CellCount)
scale_var <- rep(0.7, 95)

scale_samples <- matrix(NA, nrow = 95, ncol = 1000)
for (i in 1:95) {
  scale_samples[i, ] <- 2^rnorm(
    1000,
    scale_mean[i],
    scale_var[i]
  )
}
```

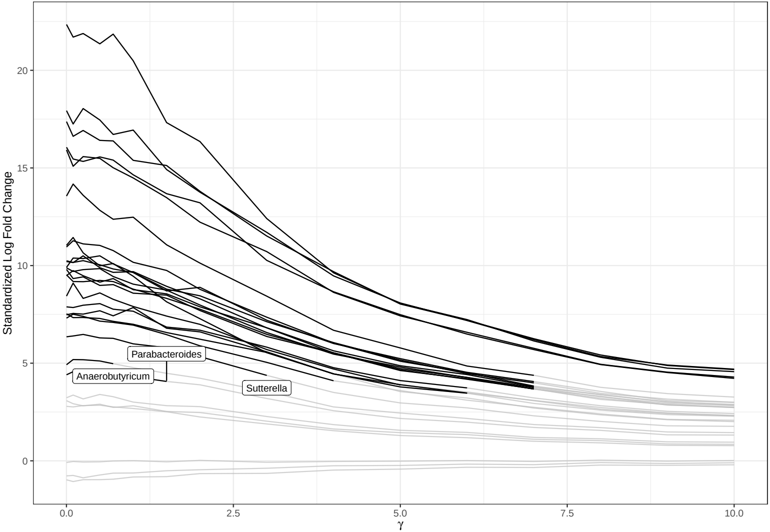
Creating an Informed Model

```
scale.cd <- 2^matrix(rnorm(1000 * 29,
  mean = log2(.7), sd = .125
), nrow = 29)
scale.control <- 2^matrix(rnorm(1000 * 66,
  mean = log2(1), sd = .125
), nrow = 66)

scale.informed <- rbind(scale.cd, scale.control)
aldex_informed <- aldex(Y, X,
  mc.samples = 1000,
  gamma = scale.informed
)
```

Comparing to Other Methods



Sensitivity Analyses



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- Gloor, Nixon, and Silverman. (2023) “Scale is Not What You Think; Explicit Scale Simulation in ALDEx2.” *BioRXiv Preprint 2023.10.21.563431*.
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