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1 Abstract

High throughput sequencing is a technology that allows for the generation of millions of reads of genomic data regarding a study of interest. Subsequent analysis of this data can yield information ranging from transcription profiles, microbial diversity, or even cellular abundance in culture. Despite the compositional nature of sequencing data, many tools inappropriately treat the underlying features in sequencing data as counts and thus model them as such. This paper proposes a log-ratio transformation method that allows for feature comparisons between samples in a compositional context. The log-ratio transformation will be implemented as an extension to a general compositional data analysis tool known as ALDEx2 or ANOVA Like Differential Expression. It will be demonstrated using both simulated and real data that the (insert name here) log-ratio transformation provides results that are more robust and in line with expectations relative to ALDEx2.

2 Background

RNA-Seq is one fo the many tools that uses high throughput sequencing technology in order to generate information regarding the transcriptome of an organism. Traditionally, samples are comprised of a set of features whose identity depends on the experimental design. For example, features can considered genes in the case of RNA-seq and differential expression or operational taxonomic units (OTUs) when the objective is identifying microbial diversity. Count based data for RNA-Seq must fit the assumption that it is independent and identically distributed for statistical tests [2, 7]. However, in the context of RNA-Seq, counts per feature are not independent in the sense that they are dependent on the sequencing capacity of the machine. The probability of detecting rare transcripts in RNA-Seq is lower in the presence of highly expressed transcripts [8]. Absolute feature counts returned from a sequencing machine are uninformative due to the violation of the assumption of independence. Thus, RNA-Seq data is inherently compositional and must be treated as such. Traditional tools do not address the compositional nature of RNA-seq data [5].

Formally, a composition is defined as a vector whose components are proportions. These proportions sum to an arbitrary constrained constant c. This constant c is arbitrary in the sense that it can represent 1 if the components are proportions, 100 if the components are percentages or other constants such as 10^9 for parts-per-billion. Absolute values of components in a composition are uninformative. The only information provided in compositional data is the relative magnitudes of the components ratios between all pairs of components [1]. As compositional data carries relative information between components, analyzing the data as log-ratios is suggested by Aitchison [1]. The scale-invariant Centered-Log-Ratio (CLR) transformation proposed by Aitchison is defined as:

$$\begin{aligned} y &= \{\frac{x_i}{g(x)}\}_{i=1,\ldots,D} \\ \text{where } y &= \text{A composition transformed by CLR} \\ x &= \text{A component of the non-transformed composition (x)} \\ D &= \text{The number of components of x} \\ g(x) &= \text{Geometric mean of D components of x} \end{aligned} \tag{1}$$

For a given sample, ALDEx2 considers the sample to be a composition, and its features to be the components [5]. For each feature in a sample, ALDEx2 converts its count into a set of probabilities through Monte Carlo sampling from the Dirichlet distribution. Monte Carlo methods are defined as repeated random sampling from a probability distribution. Sampling features as Monte-Carlo instances from a Dirichlet distribution allows for sampling variance to be modeled [4]. Furthermore, the sum of each instance per sample is equivalent to one, making the features proportions. ALDEx2 transforms each proportional instance into log-ratios with Equation 1. This compositional approach is appropriate for any data derived from a high throughput sequencing machine.

This transformation normalizes the features of each sample to the geometric mean of each sample, and allows for comparisons of expression between and within RNA-Seq samples [3]. The CLR-transformed data accounts for differences in sequencing depth between samples [4, 6]. Much like how fluorescence in a qPCR reaction provides an internal standard to determine the quantity of amplified DNA, the geometric mean provides an internal standard to determine the abundance of a feature as a ratio to all other features in the sample. Taking the CLR of each feature ensures a one-to-one correspondance between features in the composition. This allows for feature changes to be observed relative to the geometric mean [5].

(Describe the effect plot here or just reference effect plot paper?)

ALDEx2 generates effect plots which are visual representations of a feature's difference to dispersion ratio relative to other features.

3 Problem

The assumption being made during the CLR transformation is that most features are either invariant or randomly varying. If there is systematic variation between groups simply due to presence or absence of features, then this assumption is broken. For example, when comparing microbial diversity between sampling sites, organisms present in one subsite may be absent from another. In the case of transcriptomics, organisms that reside in one condition may have a different expression profile than those that reside in a second condition. These differences are represented by either zeroes or low count features between samples. It will be demonstrated that exaggerated differences can be achieved with seemingly little variation within simulated data. Additionally, zeroes are problematic as they cannot be represented in logarithmic space. Therefore, prior to the log ratio transformation, zero count features are discarded if present in exclusive or conditions, or adjusted with an uninformative prior. Ideally, the goal is to identify a non-deterministic geometric mean that best represents each sample so features can be accurately compared.

In its current implementation, ALDEx2 computes a per-sample geometric mean for a samples' set of features and declares this as the baseline for feature comparisons. However as demonstrated by Figure 1b, systematic variation between groups can influence the baseline such that the comparison between features is biased. In this case the majority of features cluster below the location 0. These figures represent a RNA-Seq experiment simulated such that 119 samples from one condition contain genes that are completely absent in the second condition. The assumption that most features are invariant is broken as the histogram of the mean expression per sample is not centered about the location 0 as depicted in Figure 1b. This shift of mean expression is problematic as it may lead to exaggerated differences and in turn, false positives. If there are a large proportion of features in a sample that do not follow the central tendency of the data, the geometric mean can be unreliable as a baseline. Therefore, identifying the features with the most consistent variance across all samples and generating the geometric mean from this subset will account for this systematic variation.

4 Methods

The geometric mean is the nth root of the product of a set of n numbers, and it represents the central tendency of the set of numbers. ALDEx2 computes the geometric mean on a per-sample basis using the entire set of features that constitute that sample. This method has been demonstrated to be effective on datasets in which there is no systematic variation across the entire dataset. The proposed (insert name here) log-ratio transformation subsets the features such that only the sets of features with the most typical variance are considered in the computation of the geometric mean. For each sample, the centered-log ratio transformation is applied to its set of features. The per-feature variance is computed across the set of log-ratio transformed data, and the resulting features whose variance falls into the inter-quartile range is declared as the invariant set. These are the features that are used to compute the (robust?) geometric mean.

$$\begin{aligned} y &= \{\frac{x_i}{g'(x)}\}_{i=1,\ldots,D} \\ \text{where } y &= \text{A composition transformed by ILR} \\ x &= \text{A component of the non-transformed composition (x)} \\ D &= \text{The number of components of x} \\ g'(x) &= \text{Geometric mean of IQR components of x} \end{aligned} \tag{2}$$

Figure S1a depicts how taking the (robust?) log-ratio transformation on the invariant set of variates estimates the central tendency of the data better. A set of 135 random variates is drawn with a population mean of 20 and a standard deviation of 2. An additional 15 variables with a value of 0.5 are added to simulate a 10% sparsity across the 150 variables. The geometric mean computed across the entire set of the 150 variables does NOT encapsulate the centrality of the data. However, taking only the subset of features whose variance falls within the IQR of the variance allows for a geometric mean that is more representative of the sample as demonstrated in Figure S1b.

4.1 Simulated Data

A series of RNA-Seq simulation data was generated for benchmarking purposes. Assemblies from *Saccharomyces cerevisiae* uid 128 and a complete reference genome of *S. cerevisiae* were drawn from NCBI. Using R package `polyester`, an RNA-Seq experiment with 2 groups of 10 replicates was generated, with a 20x sequencing coverage across the simulation experiment. Using bowtie2 these simulated reads were aligned to the *S. cerevisiae* reference genome. Labeling each group as A and B is arbitrary and hence samples s_1, s_2, ..., s_10 belong to condition A, and samples s_11, s_12, ..., s_20 belong to condition B. There are a total of 6349 features in these simulated data. On average, samples in the unmodified base dataset have a simulated read depth of approximately 870000 reads.

For the basic simulated dataset, all features in condition A were simulated to have no fold differences, where features 1-10, 11-20, 21-30, 31-40 in condition B were simulated with a fold difference of 2, 3, 4, 5 respectively. These 40 features serve as an internal control of true positives for each dataset as their fold changes are explicit and should always be displayed as differentially expressed. This dataset will be referred to as the base or control dataset. Figure 1a is an effect plot demonstrating how the 40 internal control features are considered statistically significant and differentially expressed. An additional dataset with simulated variation was generated by taking the base dataset, and removing 120 features from samples in one condition. Approximately 2% of the features in condition A are simulated to have zero counts in the dataset *reads_2*. It is apparent from Figure 1b that the simulated variation within this dataset breaks the assumption that most features are invariant.

An additional 98 datasets derived from the base dataset are generated in order to benchmark how well the IQR (robust?) log-ratio transformation supports the assumption that most features are invariant and unchanging. As the original dataset has approximately 6000 nonzero features, 60 features are incrementally removed from the samples of condition A in each simulated dataset for a resultant set of datasets with sparsity ranging from 0% to 98% sparse in condition A.

4.2 Selex Data

(FIX THE EXPLANATION FOR THIS SELEX DATA, WHAT ARE THE VARIANTS?)

Selective growth experiments (selexes) are experiments in which permutations of oligonucleotides are screened for some phenotype. This real dataset analyzed represents a selex experiment in which a library of LAGLIDAG homing endonuclease sequence variants is screened for relative abundance through growth on non-permissive media [**selex**]. Four residues in the LAGLIDADG homing endonuclease gene were modified, two randomly and two with a fixed mutation yielding a library of 1600 possible permutations. These can thus be considered features of samples (technical replicates) residing in both a permissive growth condition and a non-permissive growth condition. ALDEx2 can be applied to this dataset as the process of sequencing also generates compositional data. In this dataset, differential abundance is measured in place of differential expression. Gene variants should exhibit differential abundance if they allow their host bacterium to escape selection from a bacteriostatic medium. By using a bacteriostatic medium, change in abundance per variant is confined to the positive dimension. Variants escaping selection should not become less abundant relative to one another, only more abundant. The geometric mean G(x) represents the mean abundance per technical replicate, and features of each replicate are classified as more or less abundant than the mean of the replicate. In essence, using a geometric mean that encapsulates the true mean of the sample is advantageous as it provides a more accurate estimate of relative abundance per sample.

5 Results

For the sake of brevity, this section will focus on the results obtained from the (robust?) log-ratio transformed analysis of simulated dataset reads_2. This is a dataset with approximately 2% sparsity, or 120 features removed from condition A relative to condition B. However conclusions derived from these results are generalizable to all simulated datasets, and a summary of results can be located in the discussion.

5.1 Dataset: reads (control)

Given a dataset with no systematic variation, IQR (robust?) log-ratio transformation will provide a result that is consistent with the centred log-ratio transformation. Using the set of invariant features which can be considered a trimmed estimator over the set of all features yields a geometric mean that is for all intents and purposes equivalent to the one generated by the set of all features. (Figure was deleted)

5.2 Dataset: reads_2

Figure 2 illustrates how the Inter Quantile Log Ratio transformation accounts for the systematic variation introduced through the removal of features in condition A of reads_2. As indicated in the mean expression histograms for both Figures 1a and 2, the IQR-Adjustment translates the majority of the features such that they are centred along the location x=0. The effect of zero or low count features are mitigated by centering against features that are relatively invariant across each sample. This correction prevents the exaggeration of features that are simply differential due to systematic variation and provides a more reproducible baseline for feature comparisons. The set of 40 internal control features are determined as differentially expressed along with the set of 120 zero-count features in 2.

5.3 Dataset: reads_2_min

A dataset based off the control dataset with 2% of its features simulated as minimal counts. Instead of removing 120 features from condition A of the data, their values are substituted with 1. Zero-removal adjustment of this dataset is not sufficient as these features are not explicitly zero across samples s_1, s_2, ..., s_10. Therefore, the dataset is not affected by zero-removal adjustment. Figure 3a demonstrates how the zero removal adjustment has no effect on this dataset, and is equivalent to the original ALDEx2 adjustment in Figure 3b.

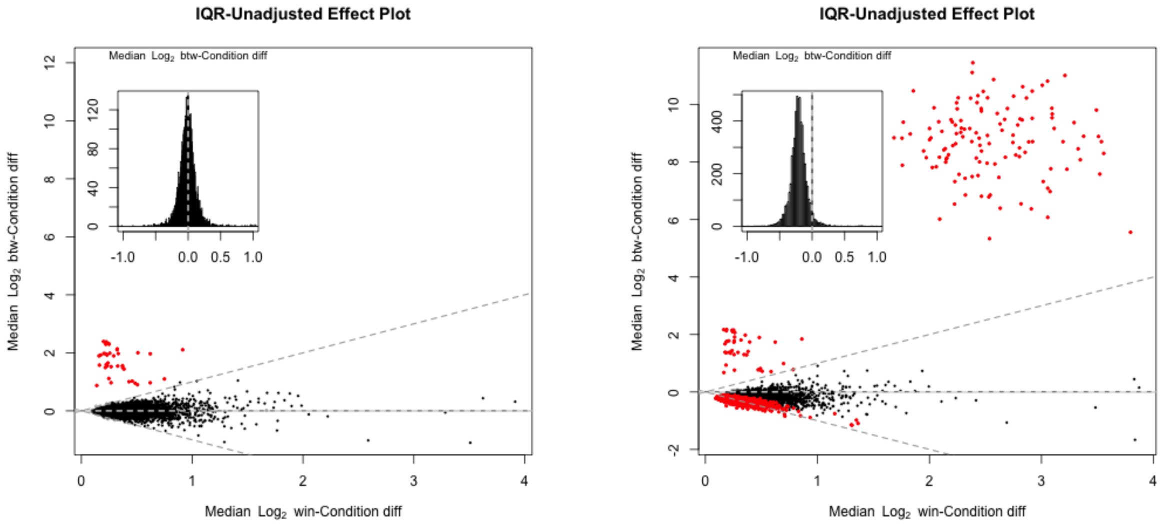
5.4 Dataset: selex

The (robust?) log-ratio transformation also behaves as expected in a real dataset. The discrepancies between Figures 4a and 4b can be explained by the inclusion of low count features between the selective and nonselective conditions. In the selective condition, there are some variants that result in a nonfunctional enzyme and therefore no growth. The directionality of growth is fixed as the growth media is bacteriostatic. Therefore variants (or features in this instance) should become more abundant, and not less abundant. Thus features with low counts should not be located as below the location of y=0 because this incorrectly implies that they have a negative directionality. By using the geometric mean computed from IQR invariant set of features, the emphasis of the low count features is mitigated and the feature comparisons are more biologically relevant.

6 Discussion

Quantifying true biological differences between experiments is difficult since there is no objective standard of difference that is extendable to various experimental designs. In transcriptomics and diversity, feature abundance is a continuous variable, making it difficult to set a threshold that defines differential abundance or microbial difference. Existing tools for transcriptomics seek to identify statistically significant features across experimental conditions based on parametric assumptions (reference here). However, significance only gives information about whether or not a phenomenon is simply due to random chance. P-values provide little to no information regarding the magnitude of difference between conditions. Additionally, the power of a statistical test is proportional on the sample size of the test. Therefore, it is theoretically possible for a test to identify statistically significant features even in the absence of true differences given a sufficient number of samples. For this reason, ALDEx2 reports the effect sizes of a feature to prevent confounding with large sample sizes, as effect sizes are independent of sample size. Through effect plots generated by ALDEx2, statistical and biological significance of features can be visualized. Statistical significance of a feature is tested with a parametric Welch's t-test, and biological significance is visualized through a features' location on the ALDEx2 effect plot. In Figure 1a, a set of 40 positive control features are identified as differentially expressed on the effect plot. Features 1-10, 11-20, 21-30, 31-40 are simulated to have fold changes between conditions of 2, 3, 4 and 5 respectfully.

7 Figures



(a) *Based on reads*

(b) *Based on reads_2*

Figure 1: 1a: depicts the ALDEx2 effect plot on the original *reads* dataset. 1b: demonstrates how systematic variation by introducing 2% sparsity can affect the effect plot. The majority of features are shifted below the location y=0. This is an artifact that reflects how dissimilar the samples are, and must be accounted for in order to make accurate comparisons.

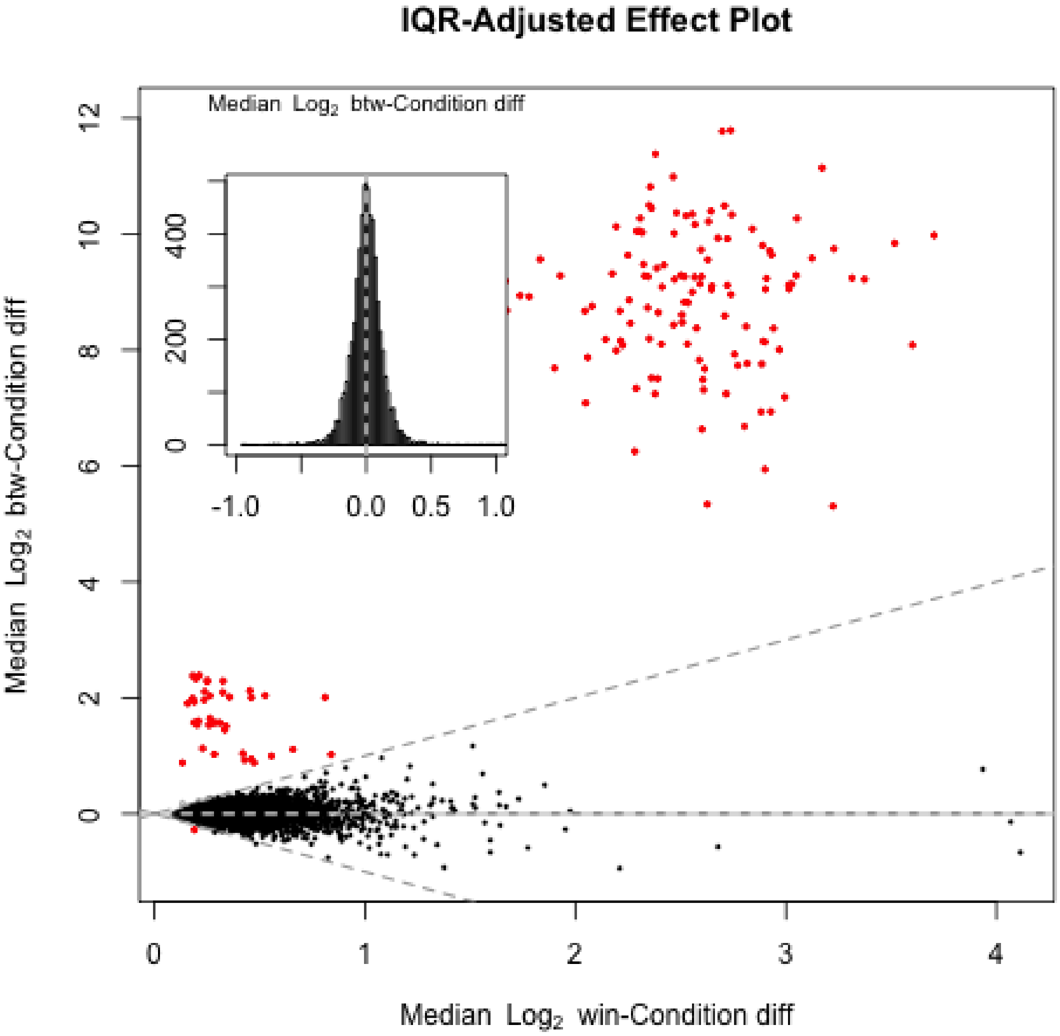
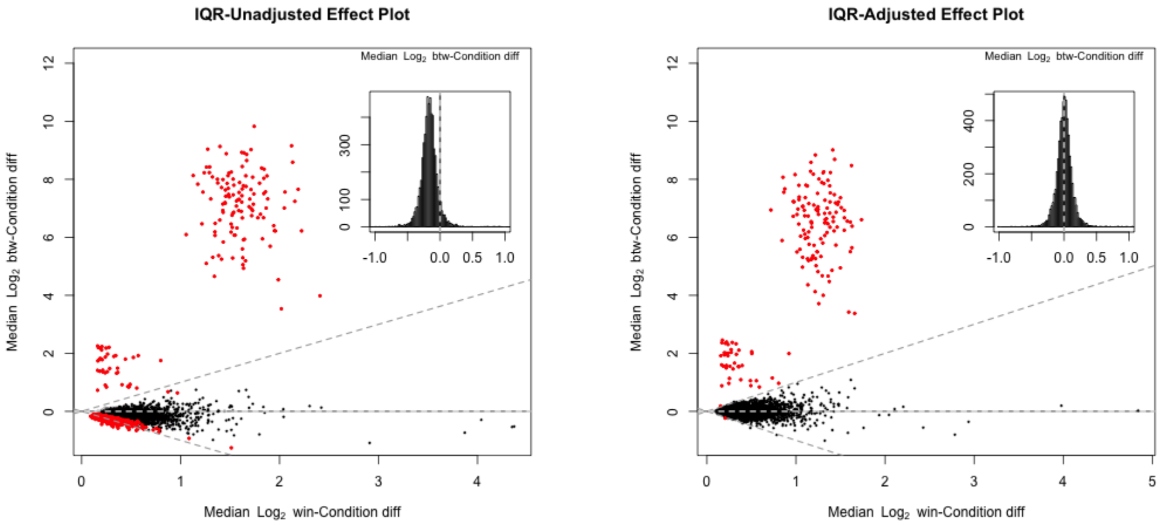


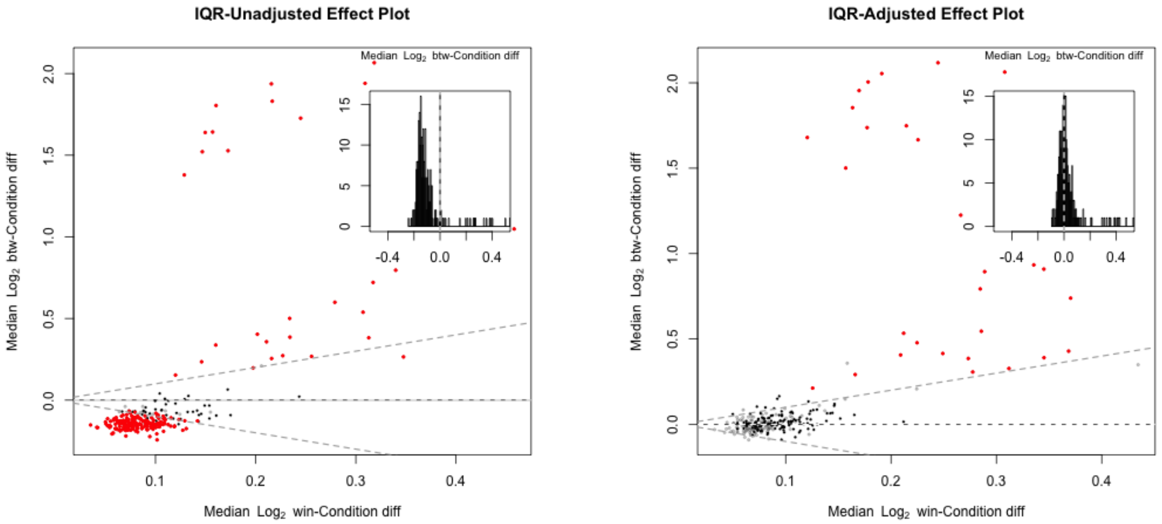
Figure 2: *Based on reads_2.* Effect plot and mean expression per sample histogram of a dataset using the invariant set features to compute the geometric mean. The Inter-Quantile Log Ratio transformation corrects the problem observed in 1b



(a) *Based on reads_2_min*

(b) *Based on reads_2_min*

Figure 3: A figure depicting how zero-removal adjustment is a special case of IQR transformation. 3a depicts the zero-removal adjustment on a dataset where 2% sparsity is simulated, however the sparsity is in the form of low count features as opposed to zero count features. As these features are not explicitly zero across samples, this dataset is not corrected by zero-removal adjustment. 3b demonstrates how adjusting features to the Inter-Quantile Log Ratio geometric mean accounts for this sparsity.



(a) *Based on selex*

(b) *Based on selex*

Figure 4: A SELEX dataset where condition one contains a various amount of low count but not zero count features per sample. The low count features should be unchanging but due to the high range between feature counts across samples, these features are negatively exaggerated as indicated by 4b

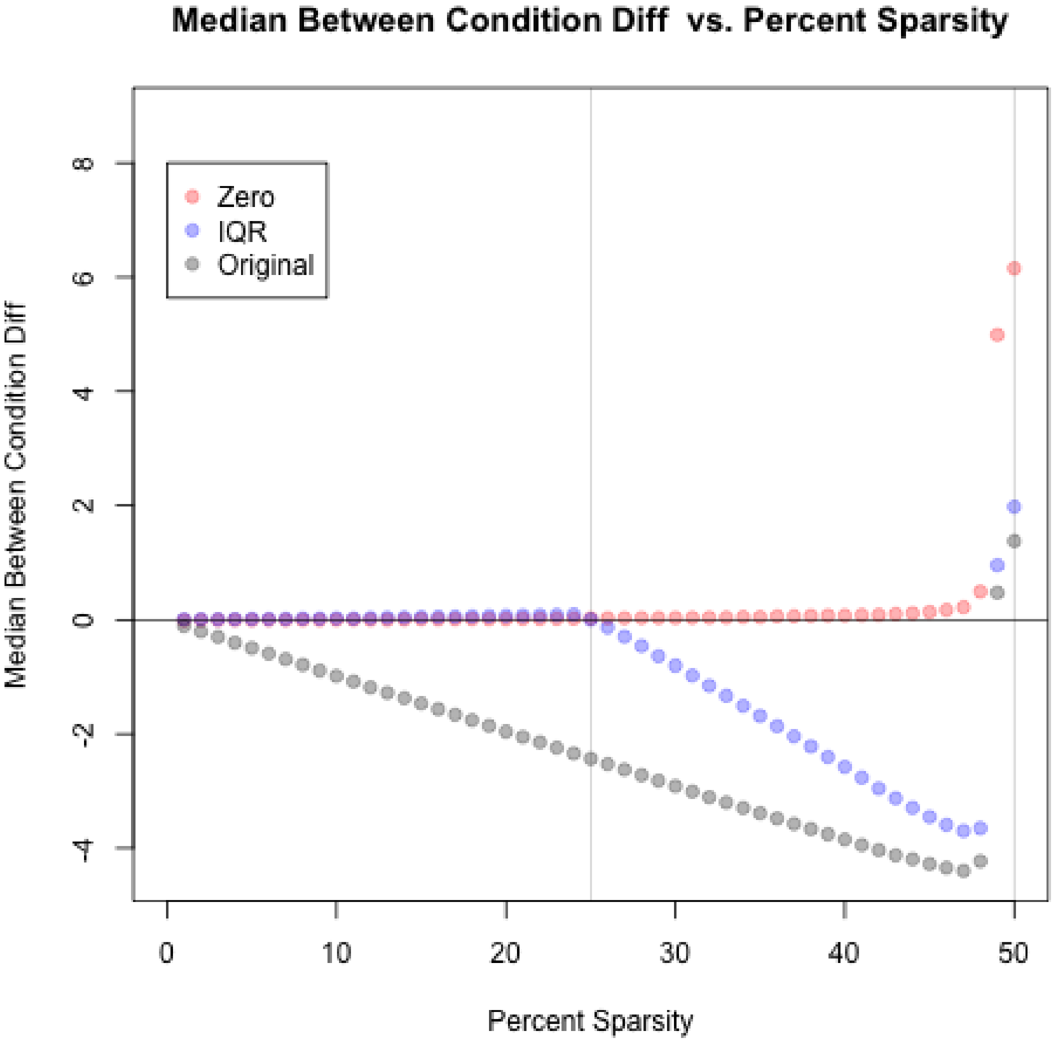
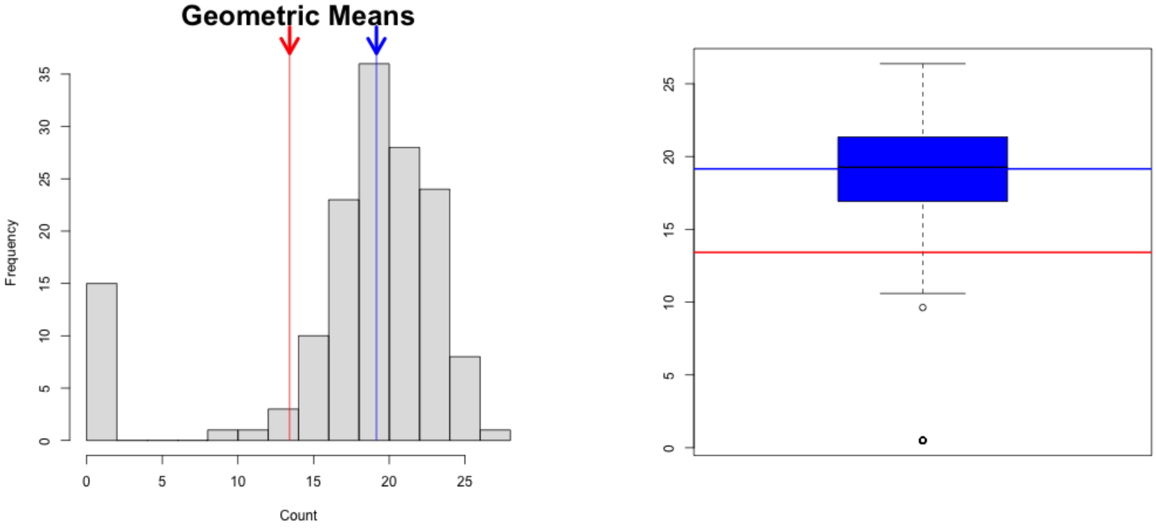


Figure 5: A figure depicting the behaviour of the transformations for datasets with varying sparsity. Each point represents the median between condition difference for a given transformation given a dataset with a specified sparsity. Points closer to the location y=0 are favourable as it implies that the transformations support the assumption that the majority of features are invariant and unchanging. The original ALDEx2 transformation fails as soon as sparsity is introduced. Inter Quantile Log Ratio transformation is effective on datasets with up to 25% sparsity from zeroes or extreme count features. Zero-removal transformation is effective on datasets with up to 50% sparsity exclusively from zeroes. Zero removal is an extended special case of IQR centering as it only affects datasets whose sparsity is due to zeroes.

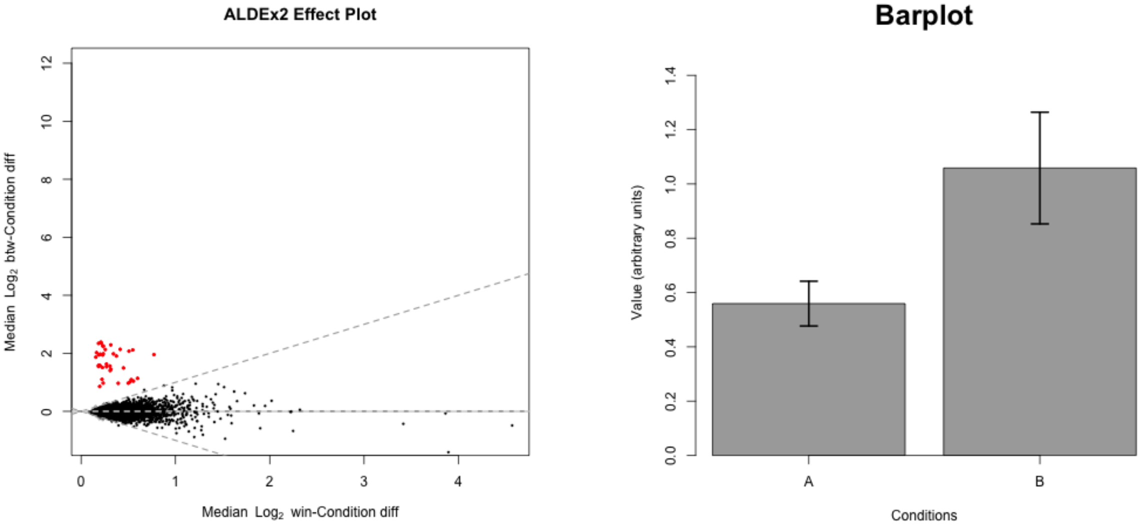
8 Supplementary Information



(a)

(b)

Figure S1: S1a: A histogram of 135 random variates with a population mean of 20 and a standard deviation of 2. 15 additional values of 0.5 were added to simulate a 10% sparsity across the 150 variables. Red represents the geometric mean of the entire set of features. Blue represents the geometric mean of the variates that fall within the IQR of the dataset. S1b: Blue is the geometric mean of the IQR features, Red is the geometric mean of the entire set of features.



(a) *Based on reads*

(b)

Figure S2: S2a: An ALDEx2 effect plot. Each individual point can be considered a feature whose location on the plot explains its effect size. The X-axis refers to dispersion, or the error bars in a barplot. The Y-Axis refers to difference, or the absolute difference between conditions A and B on the bar chart. Points that fall above the line of y=x have an effect size >1, where points that fall below the line of y=-x have an effect size of <-1. Red features are ones denoted as statistically significant by a Welch's T-test.

References

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