Challenges and limitations for improving feed efficiency from metagenomics data

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Summary

Feed efficiency is one of the traits that is gaining more attention in dairy cattle breeding programs. High phenotyping cost and the necessity for adequate infrastructure reduce the throughput of this breeding goal. Apart from the genetic predisposition of the animal to efficiently utilize feed, ruminal and gut microbiota play a fundamental role in feed digestion and by-products available to the animal. Advances in high throughput techniques allow investigating the microbiota composition and its potential implications on feed efficiency in cattle. Simultaneously, the host genotype has an effect on the microbiota composition, building a host-microbiota binomio responsible for feed efficiency.

In this paper, we determined that microbiota is more correlated to feed efficiency than to residual feed intake. A core microbiota was detected using different statistical methods, composed by some microorganisms from *Prevotella*, *Lachnospira*, *Coprococcus*, *Shuttleworthia*, *Ruminococcus*, *Methanobrevibacter* and *CF231* genera, plus some unspecified genera from the *Paraprevotellaceae*, *RF16*, *BS11* and *Christensenellaceae* families. This core microbiota affecting feed efficiency explained 50% more variance than that explained by the genotypes, increased the goodness of fit of the model, and the correlation between observed and estimated feed efficiency phenotypes by 5%.

Some of the challenges and limitations we faced in the metagenomic studies are presented. Lastly, some strategies to improve feed efficiency through perturbation of the microbiota composition are envisioned, including biotechnology strategies.

Introduction

Feed efficiency is one of the traits that has gained attention in recent years. There is increased interest in reducing feed cost in all livestock species, while improving sustainability with a lower use of land and human edible food. The economic importance of feed efficiency in dairy cattle has been estimated to be between 5-18 % in a global selection index (Gonzalez-Recio et al., 2014b; Bell et al., 2014). Feed efficiency, measured as residual feed intake (RFI), has already been incorporated as a direct trait in Australia, and other countries are planning to include it soon (Pryce et al., 2015). This trait can be defined as the additional dry matter intake for an animal given her milk yield, in energy equivalent, lactation stage, body weight and change in body weight. The lower the RFI the more efficiency in the dry matter utilization. Residual feed intake is associated to mobilization of body reserves during lactation, and is highly correlated to energy balance. However, a different feed efficiency (FE) value is usually used by nutritionists, calculated as the ratio between output (milk yield equivalent energy) and input (dry matter intake or DMI). Although this ratio trait poses some

difficulties from a breeding perspective, as more emphasis is usually placed on the trait with larger variance (milk yield or DMI). Including the microbiome composition as a proxy could tackle this limitations.

Recording feed efficiency traits is expensive to measure in the commercial population. High investment in terms of infrastructure and labour render this trait unfeasible to be recorded in the whole population. Although a reference population can be created to implement genomic selection for feed efficiency, proxy traits that help with the genetic prediction of the selected traits could improve accuracies of genomic prediction. Rumen microbiome composition is proposed as a proxy to improve feed digestion related traits such as feed efficiency or methane emissions (Ross et al., 2013; Wallace et al., 2015). However, the microbiome reflects more than a feed efficiency. It can be considered as a complementary trait, because the microbiome is actually a holobiont organism that has its own complex machinery that is responsible for feed digestion and that interacts with the animal for an enhanced or impaired feed efficiency. One of these interactions occurs at the level of the host genetic background, which modulates the microbiome composition (Roehe et al., 2016; Bonder et al., 2016; Gonzalez-Recio et al., 2017). Breeding programs could perturb the rumen microbiota towards some target composition, if the characteristics of the beneficial microbiome are known. Advances in sequence technology allow discovering taxonomy of ruminal microbes at an affordable and decreasing cost. Potential microbial gene functions can also be obtained through whole sequenced metagenome. However, strategies to perturbate the microbiome for enhanced feed efficiency are not clear, and they need to demonstrate a clear benefit for a throughput implementation. The costs of obtaining the microbiome and metagenome compositions are still large, which entails a competitive disadvantage for developing throughput strategies. However, the cost of sequencing is decreasing at an incredible rate, and genomic selection can also add value to the study of the rumen and gut microbiomes.

This paper evaluates the potential, challenges and limitations of metagenomics in the multiomics era, as a complementary alternative to improve feed efficiency in dairy cattle.

Material and methods

Data from 70 cows from *BLANCA from the Pyrenees* experimental farm (Lerida, Spain) with comprehensive feed efficiency, genotypic and metagenomic information were used. Ruminal samples were collected from each cow using a stomach tube. Microbial DNA extraction was performed using the commercial Power Soil DNA Isolation kit (Qiagen Inc) following the manufacturer's instructions. The extracted DNA was subjected to paired-end Illumina sequencing of the V3 and V4 hypervariable regions of the 16S rRNA gene. Sequence data were processed using MOTHUR version 1.38.1.1 (Schloss et al., 2009; Kozich et al., 2013), and analyzed with the microbiome package in R. Genotypes from animals under study were also obtained from the Illumina 9K chip (Illumina, Inc, San Diego, CA, USA), and were imputed to the Illumina Bovine50K beadchip using the Eurogenomics reference population.

Microbiome correlations with feed efficiency traits

RFI and FE were phenotypically correlated in our sample (ρ=-0.37), but they are traits defining different biological processes. RFI, calculated as observed minus expected DMI based on milk yield and bodyweight, is mainly correlated with DMI (ρ=0.79) and uncorrelated to milk yield (ρ=0.09) (Figure 1a and 1b), whereas FE is more correlated to milk

yield (ρ=0.86) but uncorrelated to DMI (ρ=0.08) (Figure 1c and 1d). Microbiome composition is more correlated with FE than with RFI (Figure2), as this holobiont is responsible for feed digestion; products and subproducts thereof are further absorbed by the host animal along the rumen and gut. On the other hand, the biological processes that generate variation in RFI are mainly controlled by the host animal's ability to mobilize body reserves during lactation. Some correlation might still exist if the composition of the microbiome is partially controlled by the host animal.

There are no previous studies, to the best of our knowledge, showing to which trait the microbiome is more correlated. RFI is thus far the preferred trait to include in the merit index, because it presents lower correlation to milk production level, which allows incorporating it in the selection indices without double counting the importance of milk yield, which would increase the genetic gain of both traits (Gonzalez-Recio et al., 2014b). The lower genetic correlation with the main traits related to profit offers easier interpretation of index weights for the farmers.

Based on these premises, feed efficiency studies involving the microbiome should probably consider FE as the target trait, rather than RFI. The more favourable microbiota composition could then be selected for using genetic selection on the host. Genetic correlations between the target microbiota and other traits, including RFI, should be estimated to apply proper selection weights. Microbiota composition can be recorded in a larger number of cows in the population than FE, and at a lower cost, which would favour genomic selection strategies.

Microbes associated to feed efficiency

We studied a core group of Operational Taxonomic Units (OUT) that were associated to feed efficiency using linear regression, random forest and zero-inflated regression models. Here, milk and DMI variables were standardized to calculate FE. Significant Spearman correlations with FE were found for *Coprococcus* (0.34), *Shuttleworthia* (0.35), unclassified *Bacteroidetes spp* (-0.34), *BS11 Spp* (-0.32), *F16 Spp* (-0.35) and *Paraprevotellaceae Spp* (-0.34) (Figure 2). Random Forest analyses showed variable importance >20% for 19 OTUs at explaining FE, including *Lachnospira*, *Coprococcus*, *Prevotella*, *BS11 Spp*, *RF16 Spp*, *Paraprevotellaceae Spp* and other unspecified genera belonging mainly to Firmicutes, Proteobacteria and Bacteroidetes phyla (Figure 3). Bayesian regression of OTU relative abundance (RA) on FE assuming zero inflated Gaussian distribution on the microbial RA showed that highest density (90%) of posterior probability distribution for FE as covariate did not contain zero for the genera of *Prevotella*, *Lachnospira*, *Shuttleworthia*, *CF231*, *Sharpea*, *Coprococcus*, *Ruminococcus*, *Ruminococcaceae Spp*, *RF16 Spp*, *Christensenellaceae spp* and *Succinivibrionaceae spp* among others (Table 1).

The variance of FE explained by this microbial core community was studied using different methods:

1) A Fixed model with environmental effects of lactation number and lactation stage
2) A GBLUP model adding the genomic breeding value (GEBV) to 1).
3) Relative abundance of the microbes in this core microbiome was included in 2) as covariates applying a double exponential prior distribution.
4) A nonmetric multidimensional scaling kernel matrix accounting for microbiome similarities was included in 2), using a reproducing kernel Hilbert space approach.

Including the microbiome information improved the goodness of fit of model 1 and 2, and the residual variance decreased by 28% with respect to model 1 (Table 2). Genomic

heritability for FE was estimated between 0.17 and 0.24, whereas microbial community explained a larger variance than the genotype. The inclusion of microbiome information increased the correlation between the predicted and observed phenotype by 5% (Table 3).

Nonetheless, microbial communities interact with each other, and determining a global favourable composition is substantially more challenging. Studying the functional variation of those communities may help to disentangle what genera are more favourable. Whole metagenome sequencing could improve these results.

Challenges of metagenomics studies that need to be tackled

In the last decades, the advances in sequencing technology have facilitated the study of microbes in their natural habitats, instead of under lab conditions and specific cultures. These advances have allowed a more in depth knowledge of microbial processes in the ruminant gut. Nonetheless, metagenomic research applied to feed efficiency in cattle is still in its infancy, and many questions need to be tackled to achieve a widespread implementation in breeding programs. In this section we discuss some of the challenges that can be faced with the available technology; and some of them might require a large economical cost to be overcome.

Microbiote sites

Most of the studies in cattle have sampled microbiota from the rumen to describe its composition and function. Most of the fiber degradation (typically around 30-35% of the nutrients consumed by dairy cattle) is done in the rumen, with most of the energy derived from this digestion (along with that from soluble carbohydrates) absorbed in the rumen, whereas protein (both dietary and microbial) is absorbed in the intestine. (Ross et al., 2012) showed differences between faecal and rumen microbiotas. Other studies have shown differences between the microbiota at different intestine sites. It is not clear which microbiota site explains a larger proportion of the variability in FE or RFI. It is likely that feed efficiency is best explained by different microbiota at several gut sites, and a proper balance among them must be achieved.

Futures studies need to determine the relative importance of the microbiota at different gut sites to explain the variability of feed efficiency, and placing proper weights on a potential microbial efficiency index.

Longitudinal effect

Most of the microbiota composition colonizes the rumen and overall gut during the early stage of the animal (Malmuthuge et al., 2017; Yáñez-Ruiz et al., 2010). Opposite to the host genotype, the holobiont metagenome varies throughout the life of the animal, depending on age, diet, lactation state, hygiene conditions and even pen mates. Although these modifications occur, the core microbiome is usually maintained along the animal lifetime (Saraswati and Sitaraman, 2015; Odamaki et al., 2016). The taxonomical characterization may change, but the general and even specific functionality of the microbiome could remain with different microbial taxa. Whether this longitudinal variability within the host animal across time is relevant to the overall feed efficiency or whether relevant changes in the Spearman correlation of hosts occurs need to be yet determined.

Bio-informatics

The conclusions that we can draw from microbiome research rely on computational tools that

provide accurate characteristics from large data sets of DNA sequences from the community under investigation. The microbiota composition will then be used as an intermediate phenotype for animal breeding. Several authors have reviewed the specifications of different bioinformatics tools to analyze 16S rRNA gene sequences eg (Lozupone and Knight, 2005; Oulas et al., 2015; Nilakanta et al., 2014). Among these tools, MOTHUR (Schloss et al., 2009) and QIIME (Caporaso et al., 2010) are currently two of the most used suits of tools to analyze metagenomic information from rRNA amplicons. However, comparisons of these tools on real data sets are scarce. For instance, (Lindgreen et al., 2016) performed a benchmark study previously in order to investigate the performance of several tools in terms of taxonomy and function on synthetic whole sequence metagenomes. (Plummer and Twin, 2015) evaluated QIIME and MOTHUR in faecal samples collected from preterm infants, showing slight differences in terms of the effective number of genera, richness and relative abundance detected. Pérez et al. (submitted) evaluated the same two softwares in rumen samples, showing important differences in the relative abundance of less frequent microbes, which may have a large impact at comparing differences between microbiota samples from different animals or treatments (Figure 4).

Evaluating the performance of different software at recovering the true microbial composition can be used with mock samples that represent as closely as possible the composition of the environments to be analyzed.

Microbial complexity

Most studies evaluating the host genetic effect on the microbiome have considered the relative abundance of individual genera independently. This is a simplistic approach that misses the complexity of the microbiome, in terms of functionality and interacting relationships among microorganisms. Further, it does not consider the decrease in the RA of certain genera when others increase and *vice versa*, or that bacteria may have more than a single copy of DNA and thus some RA of specific genera may be overestimated. This simultaneous relationship should be considered, and microbial composition needs be considered as a whole. Further, different microbial composition may perform similarly in terms of efficiency, as different sort of microbes may have similar functions.

There are traditional microbiology tools that allow comparing microbial richness and diversity within and between samples, such as the alpha and beta diversity, rarefaction curves, Shannon distance, Bray-Curtis distance. Future research needs to evaluate whether these parameters suit the needs to evaluate the differences between the feed efficiency of microbiotas. Otherwise, ad-hoc measurements or strategies need to be developed.

Host genetic effect

As said previously, the host genotype affects the composition of the microbiota. This regulation can happen at different levels. For instance, the size and shape of the gut, the internal conformation of the rumen and intestine walls, feed transit speed, appetite, taste transduction or physiological processes are importantly regulated by the genes that the host carries. They can simultaneously selectively favour certain microbes.

The microbiome can also explain some of the traditional GxE effect, and opportunities arise for in-depth interaction studies that were not possible to face before the metagenomics era. The interactive picture seems to be complex, and multiple interactions can be thought of, such as interactions between the microbiome and the host genotype or between the microbiome and diet (or environment). The more complete the phenotypic information the more accurately the models can separate all sources of information, and can increase the

accuracy of genetic and genomic prediction for feed efficiency. It is probably too ambitious trying to disentangle the whole situation during the next few years, but some research might help to understand these complex relationships from a biological point of view. Whether this will translate in some in-farm application to improve feed efficiency needs yet to be proved.

Statistical models

Microbiome studies pose some challenges in animal breeding. Microbial relative abundance is the output from some bioinformatics analyses and is obtained with some error. These data are then considered as an intermediate phenotype, which may have implications for the traditional methods.

Microbial RA is seldom normally distributed, and transformation thereof is necessary. Some OTUs are not observed in all individuals, increasing the density on zero for the distribution of the intermediate phenotype, which requires the implementation of zero inflated models and their extensions to incorporate genetic effects should be straightforward.

Another challenge comes from the existing interaction between the host genetics and the microbial composition. Models that simultaneously account for GxE and ExE must be developed to analyze the effect of the microbiota on complex traits.

Genetic opportunities to shape the rumen microbial system

Breeding

Dairy breeding programs have selected for more efficient cows along the last decades. However, they have also impaired fertility and energy balance as collateral consequences. The efforts in the last years at breeding for a recovered fertility had a negative impact on RFI and maintenance requirements as shown in (Pryce et al., 2014). Phenotypic and genetic variability still exist that allow for precision breeding. A favourable microbial composition in the rumen and gut of cattle can be included as an additional selection trait related to increase benefits from higher milk yield at the same feed cost, or lower feed costs at constant milk yield levels. This can be done following the traditional steps in the breeding programs: 1) define what is the favourable microbial composite, 2) calculate the economic importance of a more favourable microbiome, 3) estimate covariance components with traits in the breeding goal and in the index, and 4) apply proper selection weight. The most challenging aspect is number 1), and efforts should be placed in determining what microbes or microbiota types are preferred to increase feed efficiency. A microbiota composite index with different characteristics along the gut seems sensible at this point. Metagenome and microbial RNA amplicons can be sequenced at an affordable cost that is expected to decrease in the next years. Evaluating the genetic potential to host a favourable microbiome in the whole population is possible by phenotyping a large enough population and applying genomic selection. Although rumen and (mainly) faecal microbiota can be sampled at a relatively low cost, phenotyping cost and its margin must be evaluated (Gonzalez-Recio et al., 2014a).

Biotechnology (CRISPR)

One of the most promising techniques for animal breeding in the last years is genome editing. It is unclear how genome edited animals will be regulated in the future. Currently, the FDA is drafting the rules that will regulate the use and application of genome editing technologies. It will open the potential to edit genomic regions in the host genome that favour more efficient microbial compositions. This poses the limitation that some genes that explain a large enough amount of genetic variability must be discovered.

Biotechnology also offers the possibility of editing the genome of microorganisms relevant for feed efficiency. Recently, Delgado and González-Recio (2017) explored the possibility of editing methanogenic archaeas with CRISPR/Cas9 to study the potential and limitations of reducing methanogenesis in the rumen. Genome editing technology can be applied in vitro and could assist on modifying metagenomic pathways involved in feed efficiency. The metabolic routes that increase or decrease the efficiency of feed digestion in cattle can be determined. This would also allow determining what potential consequences are expected at altering the microbiota in a given direction. This technology can complement the results shown here and help at understanding what and why some given microbiota composition are preferred over others, as well as avoiding to perturb microbiota in an undesirable direction.

However, this technology also shows some limitations. Several studies have already reported that some individuals are resistant to changes using CRISPR (Champer et al., 2017), and it is thought to be dependent on the genetic line.

Conclusions

Metagenomic research is in its infancy, but it shows promising strategies to improve feed efficiency in dairy cattle. This study presents novel result correlating microbiota composition to feed efficiency. The main challenges that need to be addressed in the near future are discussed; and some guidelines on incorporating microbiota information in the breeding programs are proposed. The main questions that need to be addressed are 1) to determine the composition of the most favourable microbiota to improve feed efficiency, which might depend on the forage-concentrate ratio; 2) to standardize the protocols and bioinformatics for analysing microbiota; and 3) to weight the information within a global merit index.

Table 1. Posterior mean of FE ratio effect for different microbial genera using Bayesian regression.

	posterior mean	**HPD95[1]**	**HPD90[1]**
g__Ruminococcus	-1.09	N.S.	*
f__Succinivibrionaceae_unclassified	1.31	*	*
g__Coprococcus	0.73	*	*
o__GMD14H09_unclassified	-2.01	N.S.	*
p__Firmicutes_unclassified	-0.53	N.S.	*
f__Christensenellaceae_unclassified	-0.21	*	*
p__SR1_unclassified	-0.27	N.S.	*
f__RF16_unclassified	-0.30	*	*
g__Lachnospira	0.38	*	*
g__Sharpea	0.29	N.S.	*
f__Ruminococcaceae_unclassified	-1.00	*	*
g__CF231	-0.31	*	*
g__Shuttleworthia	1.20	*	*
p__Bacteroidetes_unclassified	-2.80	*	*
g__Prevotella	5.78	*	*

[1] An '*' states that the highest probability density at 90 or 95% did not contain zero, N.S. otherwise.

Table 2. Variance components and DIC for the different models tested.

	DIC	Residual Variance	Genomic Variance	Genomic heritability
Fixed Model	152.2	0.46	NA	NA
GBLUP	146.3	0.386	0.11	0.22
Microbiome	147.7	0.42.6	NA	NA
GBLUP + Regression on microbes	146.2	0.396	0.08	0.17
GBLUP + microbialKernel	144	0.368	0.07	0.16

Table 3. Correlation between feed efficiency phenotype (y) and the genomic breeding value (GEBV), Microbiome effect, and the sum of all effects in the model (y_hat).

	Cor(y,GEBV)	Cor(y, Microbiome)	Cor(y, y_hat)
Fixed Model	-	-	0.54
GBLUP	0.81	-	0.77
Microbiome (RKHS)	-	0.52	0.67
GBLUP + Regression on microbes	0.80	0.40	0.76
GBLUP + microbialKernel	0.79	0.49	0.79

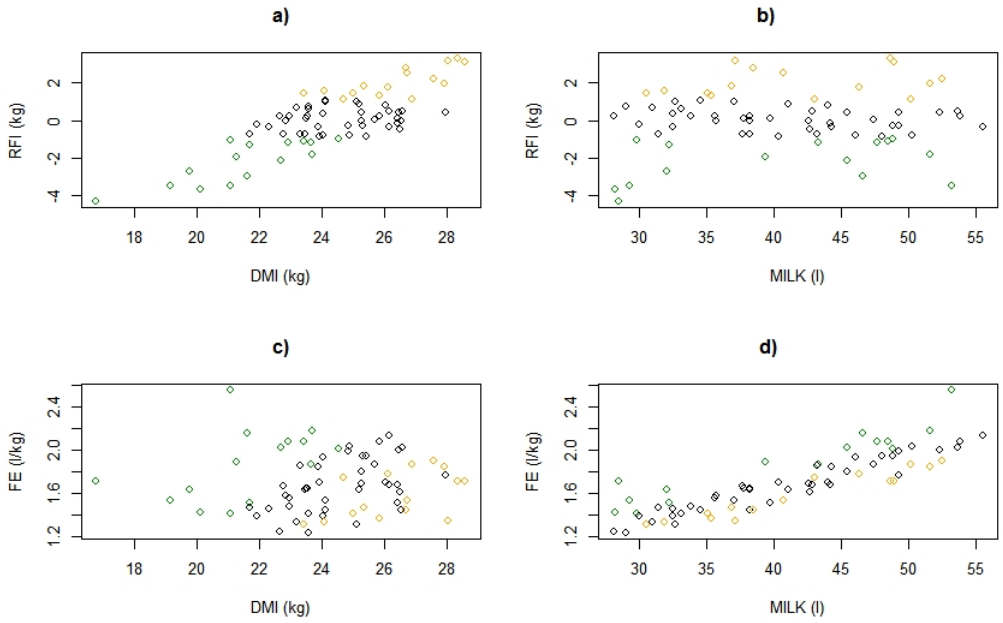

Figure 1. Scatter plots for a) RFI and DMI, b) RFI and milk, c) FE and DMI and d) FE and milk yield. Cows with the lowest RFI (more efficient) are plotted on green dots, whereas less efficient cows are plotted on orange dots.

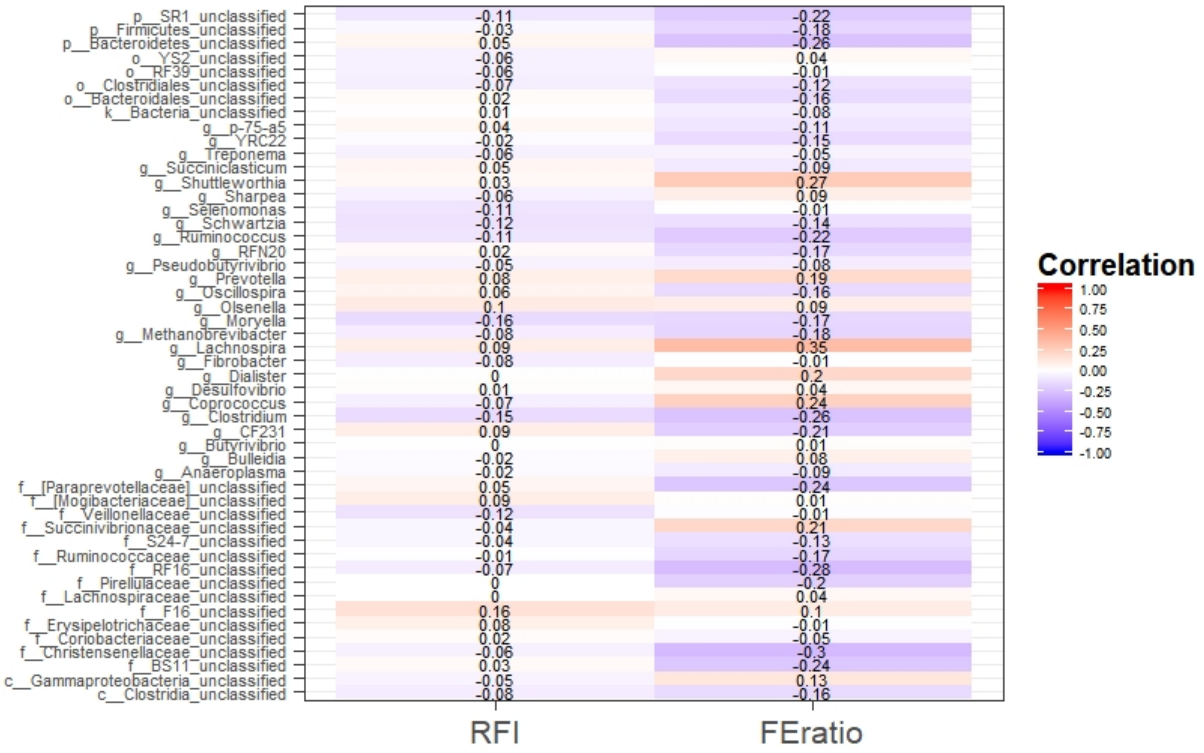

Figure 2. Correlations between core microbial genera and RFI or FE.

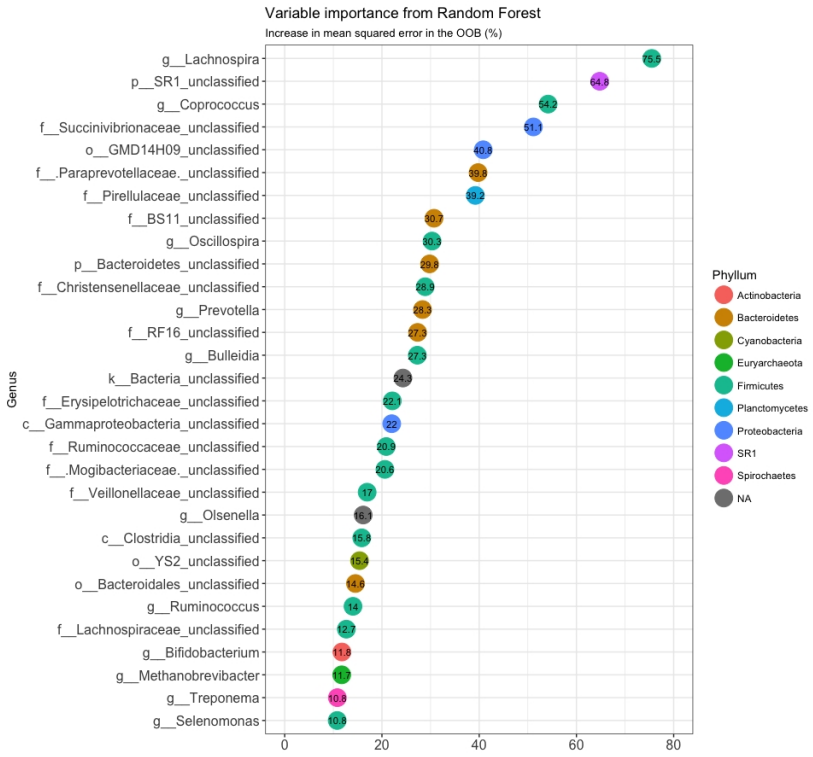

Figure 3. Variable importance from Random Forest regression trees on FE.

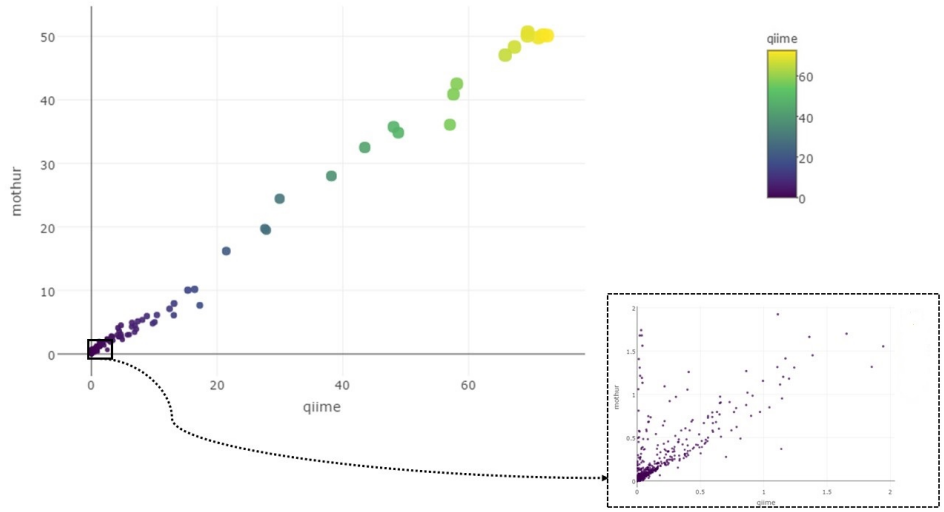

Figure 4. Differences between Mothur and Qiime (Adapted from Pérez et al., submitted).

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