SIMULATING NUTRIENT PREFERENCES TO INFORM CO-CULTURE DESIGN FOR PROBIOTIC MANUFACTURING

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Bachelor of Science in Biomedical Engineering

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On my honor as a University student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments.

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ABSTRACT

The gut microbiome plays an important role in human health, prompting interest in using gut microbes in therapeutics. Next generation probiotics, also sometimes referred to as live biotherapeutics, are probiotics that act in a pharmaceutical capacity by shifting the gut microbiome to address specific needs. To bring these probiotics to market, a method for improving the growth of gut microbes in co-culture is needed to increase scalability and decrease costs during the manufacturing process. This study aims to use optimization techniques such as flux variability analysis (FVA) and parsimonious flux balance analysis (pFBA) to simulate nutrient preferences using genome scale metabolic network models from gut microbes. We developed a computational pipeline involving an iterative process of pFBA that can be applied to various probiotic strains to develop genome scale metabolic network models (GENREs) and nutrient preferences. These nutrient preferences were validated by using experimental data to determine the correlation between the nutrient preference rankings from the pFBA analysis and experimental results. Nutrient preferences were simulated for a multispecies probiotic to look at the results in context. There was a 20% reduction in the number of consumption-coupled nutrients per gut microbial species using the iterative pFBA method over FVA. The iterative pFBA method resulted in significantly more production-coupled nutrients overall. Determining the nutrient preferences for any gut microbe will allow the nutrient preferences of different microbes to be compared to determine if there will be competition for nutrients or if there will be a cooperative process of producing and consuming different nutrients. These results will help find combinations of species that are best suited for co-culturing. This will serve to lower costs in manufacturing by reducing nutrients required per batch and improve scalability by utilizing a more robust combination of strains to help make live biotherapeutic strategies more accessible.

AUTHOR SUMMARY

Next generation probiotics, also sometimes referred to as live biotherapeutics, are probiotics that act in a pharmaceutical capacity to address specific needs. In the medical field, probiotics are being used as a treatment for gastrointestinal conditions such as IBS, but also, diseases such as cancer, heart disease, and diabetes. To bring these next-generation probiotics to market, key obstacles to adoption include high cost and poor scalability. We created a pipeline to generate a set of nutrient preferences that can be examined to understand potential interactions between gut microbial species. These preferences can be used to determine how combinations of species may grow efficiently together. This process will allow for the development of co-cultures in larger communities of microbes. Probiotic products that use a larger number of different species, such as those used to combat malnutrition, can be developed more efficiently through this process. The use of co-cultures in manufacturing will serve to lower costs by reducing nutrients required per batch and improve scalability by utilizing a more robust system of community growth. This more efficient process of manufacturing with gut microbial strains will help to make next generation probiotics more feasible for large scale manufacturing.

INTRODUCTION

The gut microbiome plays a tremendous role in human health, and the amount of genomic data on gut microbial strains continues to increase (1). Methodologies to study these gut microbial communities play a large role in furthering our understanding of the functional capacity of the gut microbiome and the development of targeted therapies.

One strategy for harnessing the genomic data of gut microbial strains is through use of metabolic network models. Genome-scale metabolic network reconstructions are used to analyze the metabolism of organisms using annotated genomes to reconstruct metabolic networks. These metabolic networks, combined with metabolomic data, provide insight on essential metabolic pathways and key metabolites (2).

These insights can be used to inform the development of therapies that incorporate live bacteria. These therapies include the transfer of live gut microbes to the gut using next-generation probiotics. Next-generation probiotics are probiotics that act in a pharmaceutical capacity to address specific needs, using bacteria grown specifically for that function. To become feasible for large-scale administration, new strategies must be employed to increase manufacturing yield of these human gut bacteria for probiotic use (3).

Currently, gut microbes are manufactured individually, in a process called monoculture, and then mixed together in a process that is complex, costly, and not scalable. This project presents methods to better understand how bacterial species interact, allowing for the potential for species to be grown together in co-culture. Interactions between different bacterial species in co-culture can improve biomass yield, subsequently lowering the cost of the production. These bacterial communities are additionally more robust in response to environmental variations and less susceptible to invasions by pathogens than monocultures, resulting in increased scalability (4).

Although GENRES are important tools for modeling systems level metabolic interactions in bacterial species, the annotated genomes that are used to build GENRES are often incomplete. Therefore, these draft reconstructions often require additional experimental metabolomics data and manual curation in order to provide a more accurate representation of organism specific metabolism (5). The process of manual curation can take up a significant amount of time, therefore, refining models through manual curation for performing metabolic analysis using hundreds of bacterial species would not be a good use of time. A recent study, by Moutinho et al., proposed a method called PROTEAN (Probabilistic Reconstruction Of constituent Anabolic Networks) which used GENREs that were not well curated to provide biological insights and generate nutrient preferences. The study highlights the potential of using automatically generated GENREs without extensive curation to predict metabolic interactions in different bacterial species (6). Another study by Plata et al., also used automatically generated GENREs for 300 phylogenetically diverse bacterial species to assess how phenotypic traits have evolved over time (7).

In this study, we used optimization techniques such as FVA and pFBA to simulate nutrient preferences using automatically reconstructed GENREs. We introduced a method using pFBA in an iterative process to generate a set of nutrient preferences that can be examined to understand

potential interactions between gut microbial species. Using the predicted nutrient preferences, we are able to make suggestions for co-culturing conditions and provide a better understanding of the metabolic interactions between various gut microbes.

METHODS

Generating Genome-scale Metabolic Network Reconstructions

GENRES were created for gut microbial strains released from a study by Tramontano et al., reporting a set of 96 phylogenetically diverse gut microbial strains and another study by Poyet et al., reporting a comprehensive collection of multi-omics data and 3832 genome sequences corresponding to gut bacterial isolates, which are found in the BROAD Institute OpenBiome Microbiome Library (8,9). Annotated genomes of each microbial species are retrieved from the PATRIC database by finding the unique genome ID associated with each strain (10). Genome IDs for the strains reported in the Tramontano et al. study were found by manually searching through PATRIC. Nine strains from the study were not included in the analysis because annotated genomes for those strains were not found in PATRIC. Annotated genomes from the Poyet et al. study were retrieved from PATRIC by using their whole genome shotgun (WGS) identification numbers and modifying them so that PATRIC genome IDs for these strains could be automatically retrieved using the PATRIC command line interface. Mackinac, a software package that integrates features from ModelSEED, COBRApy, and PATRIC, was used to automatically generate draft genome reconstructions (11). All of the metabolic reactions and exchange reactions corresponding to annotated genes were compiled. Missing reactions were then gapfilled in complete media, using the ModelSEED servers, to allow the model to consume any nutrient available through a transport reaction. The resulting reactions and metabolic byproducts are then compiled into a stoichiometric matrix which is represented using a GENRE. A summary of this process is outlined in Figure 1.

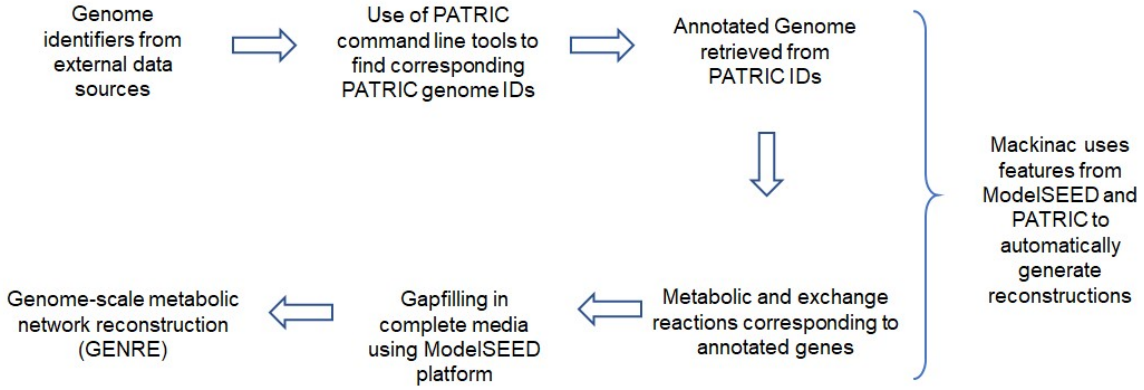

Figure 1: Genome-scale metabolic network reconstruction pipeline. Genome identifiers (WGS IDs from the Poyet et al. study) for each gut microbe strain are used to retrieve the corresponding PATRIC genome IDs using the PATRIC command line tools. The PATRIC genome IDs are then used to retrieve the annotated genomes. Metabolic and exchange reactions corresponding to the annotated genes are compiled using features provided by the software package mackinac to create draft reconstructions. Missing reactions are then gapfilled in complete media using features from the ModelSEED servers. The resulting reactions and metabolites are then constructed into a GENRE.

Flux Variability Analysis (FVA)

Flux variability analysis (FVA) is used to find the minimum and maximum flux for reactions in the network while maintaining some state of the network, such as optimizing growth rate (12). Flux variability analysis was performed using the *flux_variability_analysis* function from the package COBRApy (13). The parameter for the fraction of optimum was set at 0.5. Only the exchange reactions were included in this analysis.

Parsimonious Flux Balance Analysis (pFBA)

We first used parsimonious flux balance analysis (pFBA), a variant of FBA, to determine the flux through each reaction at the maximal growth rate (14). pFBA is based on the assumption that under exponential growth, there is a selection for the fastest-growing strains and most efficient strains, or strains that use the lowest combined flux (14). pFBA was performed using the *pfba* function from the package COBRApy (13).

Iterative pFBA Method

The steps of the method can be seen in Figure 2. In our method, we included only the reactions corresponding to constituents of the medium. After performing pFBA an initial time, this method then modifies a copy of the model of each microbial species and excludes one metabolite at a time from the medium. The first metabolite excluded is the metabolite that had the highest flux in the initial pFBA results. These results were obtained using cobrapy. pFBA is run again to determine the impact of each metabolite on growth. If the new growth rate drops below 50% of the original growth rate when a metabolite is removed, the organism is considered auxotrophic for that metabolite and the metabolite is left in the media for future steps. If the new growth rate is above this threshold, the metabolite with the next highest flux will be excluded. This process will repeat until no more metabolites can be dropped from the model without decreasing growth beyond the arbitrary threshold used to establish auxotrophic metabolites. This will result in a list of fluxes for the metabolites that together result in a growth level of above 50% of the original growth rate.

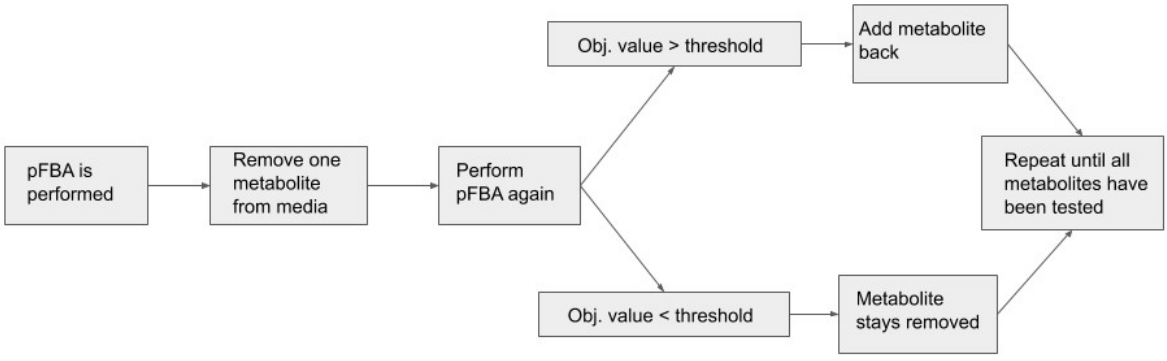

Figure 2: Iterative pFBA Method. pFBA was applied in an iterative process following the deletion of one metabolite at a time from the medium. The threshold for growth was set at 50% of the original growth rate.

Experimental Comparison of Nutrient Preferences

To compare simulated nutrient preferences to experimentally determined preferences, Biolog data from Plata et al. was used. In Plata et al., the authors maintain a collection of 40 different microbial species that are picked to represent a phylogenetically diverse group (7). The authors collected data on each species' ability to metabolize 62 different carbon sources, using Biolog GENIII Phenotype microarrays. Biolog GENIII Phenotype microarrays help to describe the usage of nutrient sources using a tetrazolium dye to assess metabolic activity. The Biolog data provides experimental growth profiles for 62 nutrients across all 40 strains.

The 40 strains in the Plata et al. paper have been previously identified in PATRIC by Medlock and Papin (15). The genomes were relayed to Mackinac by their PATRIC identification numbers to generate draft reconstructions using modelSEED and COBRApy. Using the FVA and iterative pFBA methods as described above, nutrient preferences were determined for these 40 strains.

To compare experimental and simulated results, the intersection between the metabolites represented in the Biolog data and the metabolites from simulated nutrient preferences was found for each species. These nutrients were then ranked from least to most preferred, with Biolog nutrients with the highest metabolic activity and simulated nutrients most highly consumption-coupled as most preferred. These rankings were then compared using Spearman correlation. The Spearman correlation coefficient for each species was then averaged together for a representative correlation metric between simulated and experimental nutrient preferences.

RESULTS

Predicted Nutrient Preferences Using FVA and Iterative pFBA Method

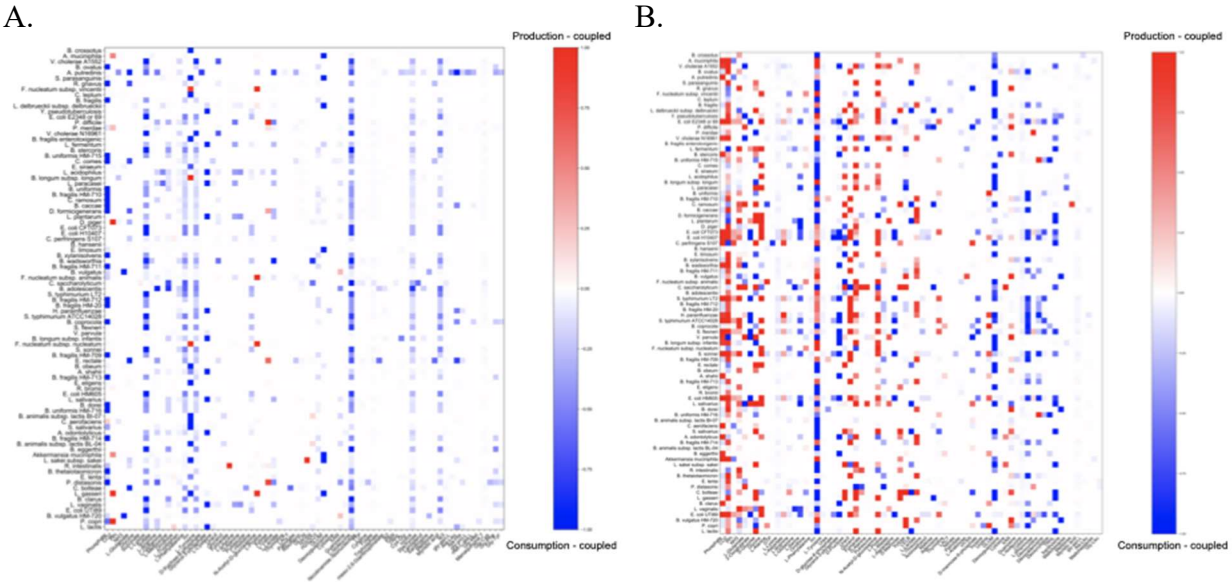

Figure 3: Simulated Nutrient Preferences. The red represents production-coupled nutrients. The blue represents production-coupled nutrients. A. The nutrient preferences were generated for 87 gut microbial strains using FVA with a threshold of 0.5 resulting including 81 nutrients. B. The nutrient preferences were generated through an iterative process using parsimonious FBA to determine the impact of each metabolite on growth for 87 gut microbial strains including 69 nutrients.

Nutrient preferences were simulated using FVA and the iterative pFBA method, described in the Methods section and shown in Figure 2, for 87 of the 96 strains referenced in the paper by Tramontano et al. (8). We were unable to find a genome ID for the other nine strains on PATRIC. The preferences as a result of FVA can be seen in Figure 3A, while the preferences using the iterative pFBA method can be seen in Figure 3B. The nutrients with fluxes less than 5% of the maximal flux were removed before displaying. The results of using FVA for 87 gut microbial strains included 81 nutrients overall, whereas the results of using the iterative pFBA method included 69 nutrients overall. There was a 20% reduction in the number of consumption-coupled nutrients per gut microbial species using the iterative pFBA method over FVA. The iterative pFBA method resulted in significantly more production-coupled nutrients as can be seen in Figure 3B. However, FVA remains more consistent than the iterative method with a lower variance for the number of nutrients for each species. The iterative method contained an outlier that had 47 nutrients for a single species. In addition to comparing the flux variability analysis, we compared some of the iterative pFBA results to what is known about microbial consumption and production. CO_2 is produced by the majority of the species included in the analysis as expected. Glycerol has long been known to be produced by microbes, and the results match this with 35 species producing glycerol (16). Overall, the iterative method was successful in generating nutrient preferences for the gut microbes to better understand species interactions.

Experimental Comparison of Simulated Nutrient Preferences

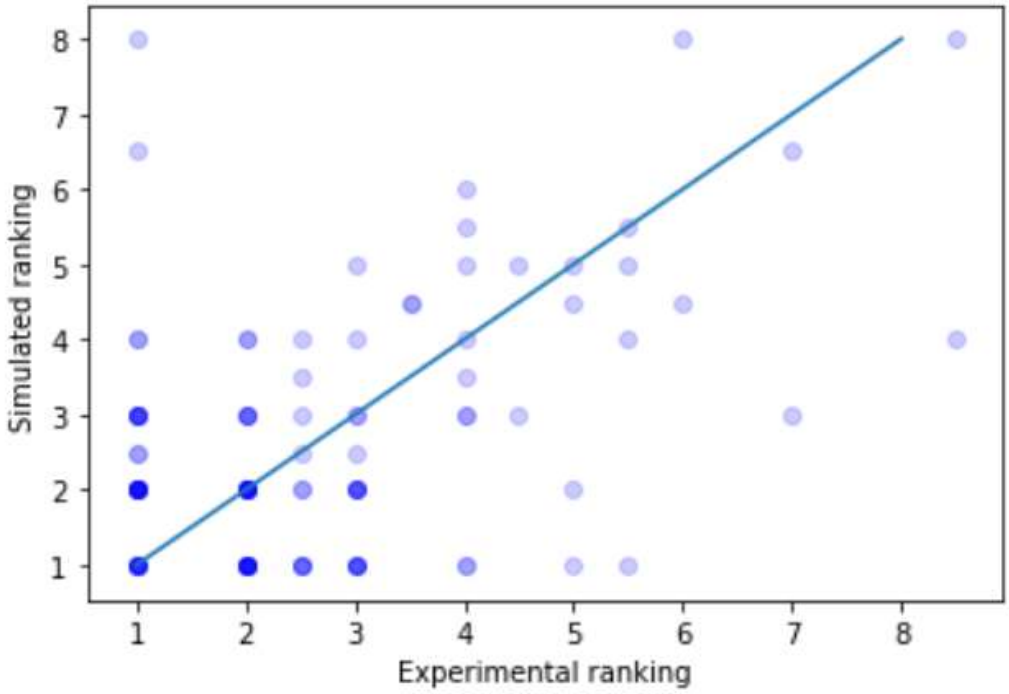

Figure 4: Correlation of experimental and pFBA nutrient preference rankings. The average Spearman correlation between rankings was -0.08, indicating little to no correlation between experimental and simulated nutrient preferences. Data is clustered at the bottom left due to the limited number of common nutrients and subsequent smaller list of rankings per species.

The number of metabolites in common ranged from 0-2 nutrients for FVA and 0-9 nutrients for pFBA, and thus there were not enough common nutrients to adequately compare nutrient preference rankings for FVA. The resulting ranking correlation for pFBA can be seen in Figure 4. The average Spearman correlation among all species for iterative pFBA was -0.08, demonstrating no strong correlation between experimental and simulated nutrient preferences. This can be attributed primarily to the limited number of common metabolites. Future analysis with specific nutrient preferences data for the 150 metabolites identified from pFBA would provide more substantial evidence on whether pFBA is a good predictor of nutrient preferences.

Application of Simulating Nutrient Preferences for Similac Strains

Nutrient preferences were simulated using the FVA method and iterative pFBA method for 3 strains present in Similac, a multispecies probiotic for treating necrotizing enterocolitis in preterm infants (17). The predicted nutrients using FVA and iterative pFBA are shown in Figure 5. The FVA method produced 23 total nutrients and the iterative pFBA produced 20 total nutrients, which illustrates the ability of using the iterative pFBA method for limiting total nutrients and reactions. In the resulting nutrient preferences using FVA, Xanthine was the only metabolite where there was an overlap in consumption-coupled growth and production-coupled growth between two species (Bifidobacterium lactis, Streptococcus thermophilus), suggesting potential metabolic cooperation. Octadecanoate and sulfate were associated with consumption-coupled growth for both Streptococcus thermophilus and Bifidobacterium lactis, suggesting potential competition between species. In the predicted nutrient preferences using pFBA, there were 3 metabolites (CO_2, NH_3, and N-acetyl-D-glucosamine) where there was an overlap between consumption-coupled growth and production-coupled growth between two of the species. Two metabolites (cellobiose, glutamate) were associated with consumption-coupled growth between two of the species. In total, there were more incidences of metabolic cooperation and competition interactions in the predicted nutrient preferences using iterative pFBA procedure than FVA. There were more consumption-coupled metabolites for S. thermophilus in the simulated nutrient preferences using FVA than using the iterative pFBA method. However, the consumption-coupled metabolites in the simulated nutrient preferences using FVA were not considered to be auxotrophic in the simulated nutrient preferences using pFBA. This suggests that the metabolites that produce the highest flux in Streptococcus thermophilus are primarily consumption-coupled, but they are not necessarily the metabolites that are the most essential for the growth of Streptococcus thermophilus.

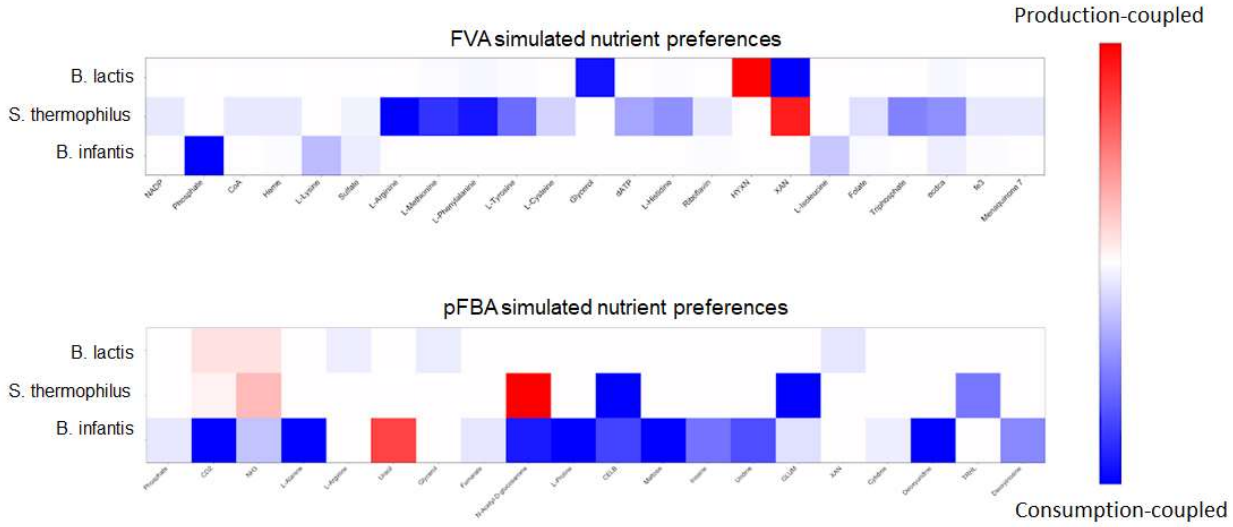

Figure 5: Simulated nutrient preferences for Similac strains. The top heatmap shows the simulated nutrient preferences using FVA. The bottom heatmap shows the simulated nutrient preferences using the iterative pFBA method. Metabolites shown in red are associated with production-coupled growth, metabolites shown in blue are associated with consumption-coupled growth, and metabolites in white have no association to growth.

Comparison of Predicted Nutrient Preferences to Current Media Conditions for Similac Strains

We also compared the predicted nutrient preferences generated by these optimization methods with current defined media conditions used to culture these three strains. The three strains are most commonly grown in milk mediums (18–20). Due to the prevalence of bifidobacterium species in the intestinal microbiota of breastfed infants, multiple studies have shown that culture mediums containing human milk oligosaccharides (HMOs) promote increased growth of bifidobacterium species (21,22). Since N-acetyl-D-glucosamine is one of the major constituents of HMOs, it could explain the potential synergistic behaviour between the production-coupled growth of Streptococcus thermophilus and the consumption-coupled growth of Bifidobacterium infantis with N-acetyl-D-glucosamine (22). Streptococcus thermophilus grown in milk mediums supplemented with yeast extract and peptone has also been shown to produce exopolysaccharides composed of N-acetyl-D-glucosamine (23).

A study by Letort and Julliard, proposed an optimal minimal chemically defined medium for Streptococcus thermophilus growth (24). The medium consisted of 20 components, which included one carbohydrate source, four buffer compounds, urea, two metal ions, six vitamins, and six amino acids. In addition, they also showed that most strains of Streptococcus thermophilus were able to grow in mediums without any branch-chained amino acids. One parallel between the predicted nutrient preferences using the iterative pFBA method and the medium proposed by Letort and Julliard, is the production of ammonia which is a byproduct of urea metabolism. The only amino acid present in the predicted nutrient preferences using the iterative pFBA method for Streptococcus thermophilus was glutamate, suggesting that amino acids may not be the most essential nutrient for the growth of Streptococcus thermophilus.

Another study by Oliveira et al. grew Streptococcus thermophilus and Bifidobacterium lactis in co-culture using milk mediums supplemented with inulin (18). They showed that growing Streptococcus thermophilus and Bifidobacterium lactis in co-culture increased biomass of both species relative to their respective monocultures demonstrating a strong case of mutualism between the two species rather than enhanced yield via competition avoidance. One of the potential causes of synergistic effects between the two species is the formation of free amino acids by lactic acid bacteria which has been shown to increase growth in lactobacilli and bifidobacteria strains (18). In addition, the increased viscosity due to the release of exopolysaccharides could provide additional protection to the two strains when grown in co-culture (18). In the simulated nutrient preferences using FVA and iterative pFBA, Streptococcus thermophilus and Bifidobacterium lactis had no incidences of potential competition for shared nutrients, suggesting potential synergistic effects when grown together. In addition, Streptococcus thermophilus and Bifidobacterium lactis have been administered in clinical studies as a synbiotic blend to improve gut function and sepsis in critically ill patients, and reduce antibiotic-associated diarrhea in infants (25,26).

DISCUSSION

We developed a computational pipeline involving an iterative process of pFBA that can be applied to various probiotic strains to develop genome scale metabolic network models (GENREs) and nutrient preferences. The iterative pFBA method is able to predict relative nutrient preferences for any microbe that has an available genome on PATRIC or the genome can be collected and added to PATRIC. This pipeline to determine the nutrient preferences for any gut microbe will allow the preferences of different microbes to be compared to predict if there will be competition for nutrients or if there will be a cooperative process of producing and consuming different nutrients. This knowledge will allow a determination to be made as to whether these different microbial species are likely to grow successfully together in co-culture. This will also provide the ideal media components for the co-culture for both or all species to achieve growth.

The iterative pFBA method was better suited for our goals than FVA. FVA has been previously used for classifying strains based on their differences, exploring alternate solutions, and showing the potential consumption patterns of different species (27–29). The increased number of production-coupled nutrients as a result of the iterative pFBA method suggests this method will be able to determine which microbial species may cooperate in co-culture by producing nutrients that are required for growth by other species. In addition, there was an average reduction in consumption-coupled nutrients per species with the iterative method. These predictions will allow for the finding of species with reduced rates of competition for nutrients and reduced costs due to less components needed in the media. However, FVA consistently returns a more similar number of nutrients required while the iterative pFBA process results in a greater range of nutrients depending on the species. This suggests that, while on average there is a reduction in the number of nutrients required with the iterative method, FVA may still be better suited for use on some species.

Additionally, the iterative pFBA methods effectively reduced the total number of predicted nutrient preferences for the three Similac strains. After comparing the simulated nutrient

preferences for the three Similac strains there was general agreement with the media components found in literature. Although further experimental validation needs to be done in addition to generating metabolomics data for these species, this comparative analysis highlights the potential application for using the iterative pFBA method for simulating nutrient preferences to improve the manufacturing of multispecies probiotics.

This work utilized genomes retrieved from PATRIC for 87 different gut microbial species. The pipeline relies on this access, thus any genomes missing from this service will limit the ability to apply this process to any species. While there are currently 331,644 bacterial genomes in PATRIC, this does not refer to the number of individual species that are represented. For instance, we were unable to find the genomes for five of the original 96 species referenced in Tramontano et al. in the PATRIC database. In addition, when genomes are available through PATRIC, there are still some concerns as to the quality of the data. PATRIC has five different metrics that mark different aspects of the quality, although not all genomes have a value for each metric. These five metrics are genome quality, coarse consistency, fine consistency, CheckM Completeness, and CheckM Contamination. The PATRIC genomes are analyzed for quality automatically when using the Binning Service or Genome Annotation. Their tools determine if the genome looks correct based on the functional roles present. The quality is determined by whether or not roles occur as predicted. PATRIC uses two other tools to determine the completeness and contamination using an updated version of the CheckM algorithm. CheckM uses a broader set of marker genes specific to the position of a genome within a reference genome tree and information about the collocation of these genes to estimate measures of completeness and contamination (30). PATRIC uses another quality tool, EvalCon to determine the consistency of the genome (31). Only one of the genomes retrieved for our analysis was marked as poor quality, while the other 86 were marked as good quality. The average scores for coarse consistency, fine consistency, and completeness were 99, 98, and 99, respectively. There were also scores for contamination for 28 of the 87 genomes that averaged to 2.775. While there is often concern over the quality of genomes, the genomes on PATRIC that we used were largely of good quality. Yet, when this is applied to different species, this will still be a characteristic that needs to be watched as an inaccurate genome would change the results for the reconstruction and subsequent analyses.

Finally, the number of contigs used in constructing the genome can be another problem. The number of contigs is often inversely related to quality (32). We aimed to pick the genomes for retrieval from PACTRIC that had a smaller number of contigs, but that was not always possible. Over the 87 genomes we used, there was an average of 67 contigs for each genome. While the number of contigs does not solely determine the quality of the genome, the lack of availability of genomes created with a small number of contigs limits the ability to use any strain most reliably.

The results are limited by the choice of threshold for both FVA and the iterative pFBA method. The fraction_of_optimum threshold for FVA and the threshold used to establish auxotrophic organisms for the iterative pFBA were both set at 0.5. These were set to equal values to compare the results. Beyond being equal, the thresholds were arbitrarily chosen. The nutrient preferences are sensitive to this value for both optimization techniques. More experimentation is needed to determine which threshold will achieve the most effective result for replicating the actual preferences of the gut microbes.

Differences in the predicted nutrient preferences for the three Similac strains versus the media conditions found in literature could be explained by our method of using complete media to gapfill missing reactions in the draft reconstructions. Gap filling on complete media allows for any metabolite involved in any reaction to contribute to growth regardless of whether those metabolites are actually correlated with the growth of the species. Gapfilling these strains using media conditions found in literature may provide a more accurate representation of the nutrient preferences and metabolic capabilities of these three strains grown in co-culture.

In the future, coupling these results to experimental metabolomics data could help provide a better representation of the nutrient preferences of these species. Additionally, adjustments to the threshold in the iterative pFBA analysis can also help to further refine the predicted nutrient preferences. The method of predicting nutrient preferences exploits metabolic capabilities of gut microbial species when they are cultured together, and can be used to rationally design co-culture combinations for manufacturing multi-species probiotics.

ACKNOWLEDGEMENTS

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