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BIOL 3210
Nov 6, 2023

Microbial community composition & diversity via amplicon sequencing (e.g., 16S & 18S rRNA gene regions, ITS regions).

Method:

Typical amplicon sequencing methodology to analyze microbial community composition and diversity selects specific regions of ribosomal RNA genes, 16S, 18S, and ITS (Internal Transcribed Spacer), for taxonomic and phylogenetic analysis of microorganisms. These regions are most commonly used since they are highly conserved across a wide range of species and contain many variable regions that are used to distinguish between different taxa. In the protocol, the microbial cells of the desired samples are broken, and the specific genetic material (e.g., 16S, 18S, or ITS) is isolated through either organic (phenol–chloroform), nonorganic (salting out and proteinase K treatment), and adsorption techniques (silica–gel membrane) (Gupta 2019). The isolated gene then undergoes four rounds of Polymerase Chain Reaction (PCR) amplification. The first round consists of amplifying the desired gene needing to be sequenced. In the second round, DNA adapters ligate to the ends of the DNA fragments. These adapters contain sequences necessary for sequencing, such as primer binding sites (Gunter 2022). In the third step, an Emulsion Polymerase Chain Reaction (ePCR) is conducted in which water droplets containing the DNA fragments, primers, and PCR reagents disperse within an oil phase, creating a water-in-oil emulsion (Iacumin 2020). Consequently, each water droplet acts as a small, isolated reactor. The ePCR rounds take place in individual wells of a microfluid chip simultaneously. After amplification, a detergent is added to break the emulsion, separating the oil and water phases (Iacumin 2020). Lastly, the newly amplified DNA is subjected to high-throughput sequencing technologies like Illumina or Ion Torrent. This sequencer reads the genetic code of each amplified gene. As specific nucleotides are being expressed in each well, there is usually either a camera or a pH meter that detects the specific nucleotide incorporated, generating a massive dataset of sequences. The PCR rounds repeat for a minimum of 30 times depending on the specific kind of sample allowing for exponential DNA amplification.

Information Obtained:

Once the dataset of sequences is generated, the data undergoes quality control to remove low-quality reads and sequencing errors. Following this, the sequences are grouped into Operational Taxonomic Units (OTUs) based on their similarity (Lie 2014). OTUs are the most used units when analyzing gene sequence datasets and are typically defined at an exact sequence

similarity threshold (e.g., 97% similarity for bacteria) (He 2015). This essentially clusters similar sequences together to represent different microbial taxa. Then each OTU is compared to reference databases such as GenBank and is assigned their specific taxonomic classification. This identity allows researchers to determine the types of microorganisms and the abundance of them present in the sample. Lastly, diversity metrics like Alpha and Beta Diversity may be calculated to gain more information.

Example:

Elevated blood glucose levels in individuals with diabetes frequently lead to the accumulation of fatty deposits within their blood vessels. This process can result in the narrowing of blood vessels, impairing blood circulation. Consequently, reduced blood flow hinders the healing process and elevates the susceptibility to infections, as cells may die due to insufficient blood supply. One infection that may occur in the bone is osteomyelitis. The diagnosis of this relies on bone bed biopsies but, due to the fastidious nature of bacteria, more rapid techniques are required (Gramberg 2023). This study investigated the high concordance of Illumina 16S rRNA gene amplicon sequencing as an alternative to the standard methodology of conducting bone biopsies (Gramberg 2023). The protocol was able to detect various bacterial strains that were not initially detected in the biopsies. Therefore, amplicon gene sequencing can be effectively conducted in sequencing low levels of microbial biomass samples of bone and ulcer bed biopsy samples in people with diabetic foot osteomyelitis.

Advantages:

Two advantages of this technique are its high throughput and usage in environmental monitoring. This methodology can rapidly process and parallel sequence millions of DNA fragments efficiently. This technique allows for the identification of specific marker genes or regions that can be used to distinguish different taxonomic groups. Also, these biomarkers can then serve as indicators of environmental health or specific conditions.

Disadvantages:

One disadvantage is that it is most efficient when working with short reads. Short reads may not capture long-range structural information within the genome, causing limitations to the capabilities of amplicon sequencing. Another disadvantage is that the multiple rounds of PCR can amass errors through point mutations or stochastic variations. These errors can later affect the accuracy of the amplified DNA sequences.

Metagenomics for microbial or viral communities (NOT 16S and 18S rRNA sequencing)

Method:

One highly effective methodology for delving into the metagenomics of microbial and viral communities is known as shotgun metagenomic sequencing. Unlike 16S sequencing, which only targets 16S rRNA genes, shotgun metagenomic sequencing sequences all given genomic DNA from a sample. The library preparation workflow for this method mirrors that of conventional whole-genome sequencing, encompassing random fragmentation and adapter ligation. This approach enables microbiologists to evaluate bacterial diversity and ascertain the abundance of microbes across diverse environments. The process begins with sample collection. Subsequently, DNA is extracted from the collected samples, with its concentration measured and purity assessed via techniques like UV-Visible spectroscopy (Perez-Cobas 2020). Once ensuring that the DNA is of high quality, the extracted DNA is fragmented into smaller pieces, typically ranging from 300 to 600 base pairs (Perez-Cobas 2020). This can be achieved through physical shearing or enzymatic methods. Adapters are then added to the DNA fragments for subsequent sequencing. Depending on the sequencing platform, size selection is performed to retain DNA fragments within the desired size range. This step contributes to achieving uniform sequencing coverage. A Polymerase Chain Reaction is then performed to amplify the DNA fragments with attached adapters, effectively priming the DNA for the sequencing process. Common platforms for shotgun metagenomics include Illumina, Oxford Nanopore, and PacBio (Perez-Cobas 2020). Lastly, a high-throughput sequencing is performed of the prepared libraries, generating a vast amount of short-read data (Illumina) or long-read data (Oxford Nanopore or PacBio) (Perez-Cobas 2020).

Information Obtained:

The quality of the sequencing data is assessed using bioinformatics tools. Any low-quality reads are either trimmed or filtered, thereby ensuring the removal of adapter sequences. For short-read data (Illumina), metagenomic assemblers like MEGAHIT, IDBA-UD, or MetaSPAdes are used to construct scaffolds or longer sequences representing microbial or viral genomes (Quince 2017). To shed light on the taxonomic composition and functional potential of the community, sequences are often assigned to taxonomies and genes through bioinformatic tools like Kraken, Kaiju, MG-RAST, or Prokka (Quince 2017). The metagenomic data is then compared to existing databases or reference genomes to identify unique features, trends, and ecological relationships.

At times, metagenomic binning is performed to group scaffolds into 'bins' representing individual genomes (Quince 2017). This helps in characterizing species and strains within the community. Lastly, phylogenetic trees may be constructed based on marker genes identified in the metagenomic data to understand the evolutionary relationships within the community.

Example:

Diarrhea in newborn piglets presents a significant and pressing concern within the agricultural industry. It is believed that this disease is associated with changes in the intestinal microbiome. To tackle this issue, the current study undertook a comprehensive exploration of the microbiome in newborn piglets suffering from diarrhea in order to identify relevant markers of the disease, using metagenomics. The study tried to identify differences between suckling piglets with diarrhea and a group of healthy ones and used the MGISEQ shotgun sequencing system, specifically, to do so (Gryaznova 2023). They concluded that, at the bacterial and fungal levels, the fecal microbiota of piglets afflicted by diarrhea exhibited no substantial differences when compared to the microbiota of their healthy counterparts (Gryaznova 2023). They only observed a higher rate of bacterial alpha diversity in the group of diarrheic piglets. This heightened diversity could be attributed to factors like dysbacteriosis (microbial imbalance) or inflammation. This suggests that while the overall composition of the microbiota may not differ significantly, the diversity of bacterial species may play a role in the manifestation of diarrhea in these piglets.

Advantages:

One advantage of metagenomics is that it provides a means to study unculturable microorganisms that are otherwise difficult or impossible to analyze. Also, unlike capillary sequencing or PCR-based approaches, this procedure allows researchers to sequence thousands of organisms in parallel. With the ability to combine many samples in a single sequencing run, metagenomic sequencing can detect very low abundance members of the microbial community that may be missed or are too expensive to identify using other methods.

Disadvantages:

A disadvantage to using metagenomics is that it generates a vast amount of data, which can be computationally intensive and challenging to manage. Analysis of large datasets requires substantial computational resources and expertise. Additionally, metagenomics provides a snapshot of the community's genetic content but does not reveal spatial or ecological information about the organisms in their natural habitat.

Microbial community transcriptomics (whole-transcriptome shotgun sequencing; RNA-seq).

Method:

Whole-transcriptome shotgun sequencing, often abbreviated as RNA-seq, is a next-generation sequencing (NGS) technique used to analyze the entire transcriptome of an organism or a sample. The transcriptome represents the complete set of RNA molecules, including messenger RNA (mRNA) and various non-coding RNAs, expressed in a cell, tissue, or organism at a specific moment in time. RNA-seq allows researchers to investigate gene expression, alternative splicing, post-transcriptional modifications, and other aspects of RNA biology. In other words, RNA-seq is a technique used to determine the quantity and exact sequence of RNA in a sample. The first step of an RNA-seq workflow is RNA extraction which is most commonly done by using guanidinium thiocyanate-phenol-chloroform extraction (Peirson and Butler 2007). This filter paper-based lysis and elution method features high throughput capacity. Once the RNA sample is obtained, it must be converted into complimentary DNA (cDNA) fragments (a cDNA library) to be later sequenced (Mackenzie 2018). This is done by reverse transcription, the synthesis of DNA from an RNA template. This process is driven by RNA-dependent DNA polymerases, also known as reverse transcriptases. The cDNA is then fragmented, and adapters are added to each end of the fragments. These adapters contain sequences necessary for sequencing, such as primer binding sites (Gunter 2022). Following processes of amplification by Polymerase Chain Reaction (PCR) and size selection, clean-up, and quality checking, the cDNA library is then analyzed by NGS, producing short sequences that correspond to all or part of the fragment from which it was derived (Mackenzie 2018). After sequencing, the raw data is processed to remove low-quality reads and adapter sequences.

Information Obtained:

The acquired clean reads are then mapped to a reference genome (if available) or de novo assembled into transcripts. The number of sequencing reads that map to each gene or transcript is quantified. This information is used to estimate gene expression levels. The expression data can be presented as counts, transcripts per million (TPM), or fragments per kilobase of transcript per million mapped reads (FPKM) (Mackenzie 2018). Read alignment is an essential step in RNA-seq downstream analysis. RNA-seq data typically lack information about the order and origin of the reads, including the specific part, homolog, or strand of the genome from which they originate

(Deshpande 2023). Computational alignment of the reads to an annotated reference transcriptome can establish where on the genome the reads originated (Deshpande 2023). Alignment of the reads to a reference sequence also reveals how many reads overlap each position on the reference sequence, which is known as the coverage. This can be done by using bioinformatic tools like GenomeScope, Smudgeplot, and Merqury (Deshpande 2023).

Example:

Critical limb ischemia (CLI) is a very serious form of peripheral artery disease (PAD), a condition where the blood vessels in the legs are blocked, leading to severe health problems. CLI can cause a lot of suffering and even death. Unfortunately, Black people tend to get CLI more often than others. To understand why and find the best treatments for Black patients, a study looked at the genes in the gastrocnemius muscles of healthy White and Black volunteers and those with CLI (Terwilliger 2021). They used whole transcriptome shotgun sequencing and looked for changes in specific pathways related to how our bodies work. They found that when comparing Black and White patients with CLI to their healthy counterparts of the same race, they had similar problems with their genes and how their muscles use oxygen (Terwilliger 2021). This means that the factors affecting amputation outcomes were the same for both races.

Advantages

RNA-seq can tell us which genes are turned on in a cell, what their level of transcription is, and at what times they are activated or shut off. This allows scientists to understand the biology of a cell more deeply and assess changes that may indicate disease (Mackenzie 2018). Also, unlike hybridization-based approaches, which may require species-specific probes, RNA-seq can detect transcripts from organisms with previously undetermined genomic sequences, making it fundamentally superior for the detection of novel transcripts (Zhao 2014).

Disadvantages:

Alignment of RNA-seq reads to a complementary reference sequence can help determine which transcripts are expressed and the degree to which they are expressed, but the alignment approach is ill-equipped to discover transcripts that are missing from the reference sequence. Furthermore, even the human reference transcriptome remains incomplete. Additionally, novel transcripts can be discovered by performing de novo assembly of RNA-seq reads to generate an entire transcriptome without alignment to a reference sequence; however, this can be challenging and requires large amounts of computational time and resources.

Microbial community proteomics

Method:

Microbial community proteomics, also termed metaproteomics, is an emerging field within the area of microbiology, which delves into the comprehensive protein profile of a complex environmental microbial community at a given time (Wang 2016). This approach allows researchers to gain insights into the functional activities, metabolic processes, and interactions within microbial communities. To initiate a metaproteomics study, researchers first acquire environmental samples and subsequently extract proteins. Proteins are extracted by breaking down cell walls and membranes to release intracellular proteins. This extraction typically involves cell lysis through methods like sonication, followed by filtration to remove larger debris, and centrifugation to separate cellular remnants from the supernatant (Martinez, 2023). Once a protein collection is obtained, the next step is to separate these proteins based on their physicochemical properties, like size, charge, and hydrophobicity. Two approaches are mainly used for the separation of proteins: gel-based and chromatography-based. For the gel-based approach, a two-dimensional gel electrophoresis (2D-PAGE) technique is used to separate proteins on the basis of two parameters: isoelectric point and molecular weight (Martinez 2023). In the first separation step, called isoelectric focusing (IEF), proteins are separated on the basis of their isoelectric point (pI), which is the pH at which the protein has no net charge (Tamang 2023). The second separation step is sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), where proteins are separated based on their molecular weight. In the chromatography-based approach, an ion-exchange chromatography or liquid chromatography is done. Lastly, to analyze the proteins, a mass spectrometry is performed in which the proteins are identified and quantified based on their mass-to-charge ratio (m/z).

Information Obtained:

There are many approaches to further analyzing the data obtained from proteomics. Firstly, the mass spectrometry data can be compared to reference databases of known proteins to determine the identity of the proteins present in the sample. Common database searching algorithms like SEQUEST or Mascot are used (Aslam 2017). A differential in-gel electrophoresis can also be done. It is a method used to detect differences in protein expression patterns between two samples. This involves labeling proteins from two different samples with different fluorescent dyes and

mixing them together before separating them by electrophoresis on a two-dimensional gel (Tamang 2023).

Example:

Analyzing the regenerative capacity of the neonatal heart has been an ongoing characteristic that researchers have been studying. It is of interest because this research can help in treating myocardial infarctions; heart attacks that occur when one or more areas of the heart muscle does not get enough oxygen. This happens when blood flow to the heart muscle is blocked. All existing clinical treatments aim to restore blood supply to the infarcted area, but they cannot save the necrotic myocardium in the infarction zone (Liu 2023). Therefore, promoting endogenous proliferation of cardiomyocytes is being investigated. A group of researchers created a mouse model of heart regeneration and performed a myocardial infarction surgery on one-day-old mice and then analyzed the transcriptome and proteome at six days post the myocardial infarction (Liu 2023). Once the transcriptomic and proteomic results were analyzed, they disseminated that gene expression at the mRNA and proteins levels only had statistical significance at 16 genes in which 15 were upregulated and 1 was down regulated (Liu 2023). There was no situation in which gene expression was opposite at the two levels, thus displaying the promising effects of endogenous myocardial regeneration therapy for myocardial infarctions.

Advantages:

One advantage of using proteomics is that is can be widely used in medical research to identify biomarkers associated with diseases. These biomarkers can then aid in early diagnosis, disease monitoring, and the development of targeted therapies. Additionally, proteomics allows the study of post-translational modifications such as phosphorylation, glycosylation, and acetylation. Understanding post-transcriptional modifications is crucial for deciphering protein regulation and function.

Disadvantages:

In the gel-based approach of proteomic analysis, two-dimensional gel electrophoresis has limitations that it cannot detect proteins with low molecular weight or those that are too large. Also, proteomics data alone may not always provide enough biological context. Integrating proteomics with other omics data (genomics, transcriptomics, metabolomics) is often necessary for a comprehensive understanding, as seen in the example study discussed above.

Microbial community metabolomics

Method:

Microbial community metabolomics is the studying of the complete set of metabolites within a microorganism and monitoring the global outcome of interactions between its development processes and the environment (Tang 2011). This approach provides insights into the metabolic activities, interactions, and functional processes within microbial ecosystems. Conducting metabolomics begins with sample collection with consequent extraction of metabolites via quenching of cells. Although perchloric acid and liquid nitrogen can be used, methanol is the golden standard for this extraction procedure. In the methanol method, microbial culture is added into a methanol solution (60% v/v) or kept at low temperature of about −40 °C (Sellick 2011). The solution is then mixed by gently inverting the solution and placed in liquid nitrogen. This stops cell metabolism. When needed, the samples are thawed, and centrifugation is performed to separate cell biomass from the culture medium. Then the quenching solution is aspirated, the cell pellet is resuspended in methanol, and the new solution is centrifuged again. The supernatant is then removed, and the samples are dried in a centrifugal evaporator, ending metabolite extraction (Sellick 2011). Metabolites are then separated and purified through techniques such as liquid chromatography mass spectrometry (LC-MS) and gas chromatography mass spectrometry (GC-MS). These methods separate and quantify metabolites based on their physicochemical properties, especially their mass-to-charge ratio (m/z). Often times, to acquire information about the structure and abundance of metabolites in a non-destructive manner, nuclear magnetic resonance (NMR) spectrometry is also used (Sellick 2011).

Information Obtained:

After the instrumental analyses, the data (mass-spectra, NMR spectra, or chromatograms) is corrected for instrument drift and excess noise is removed. Statistical and bioinformatics analyses are performed to identify and quantify metabolites, detect patterns, and explore relationships between metabolites and experimental conditions (Hu 2022). The retention data and peak areas of the data are also compared to reference databases to identify the metabolites extracted. There are many metabolome databases such as Metlin, Human Metabolome Database (HMDB), KNApSack Database, and MassBank that can be used (Hu 2022). After aligning the information with these reliable databases and with multivariate statistical analysis, the obtained raw data can be converted to more meaningful conclusions, such as biomarkers. Multivariate

statistical analysis methods include principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) (Hu 2022). They can help us to visualize the metabolic data, trend, and cluster.

Example:

Depression is a common mental disorder characterized by persistent sadness and a lack of interest or pleasure in previously rewarding or enjoyable activities. Previous research has shown evidence of changes in the metabolites present in the blood of people suffering from depression, however, the exact pathways through which these metabolites interact remain unknown. A study sought out the use of metabolomics and reference databases to compare blood retrieved from people suffering from depression with the blood of patients not suffering from depression (Yang 2023). In this study, the MENDA reference database was used. Out of a total of 854 differential metabolite entries of peripheral blood of patients with depression, 215 entries had significantly enriched pathways when compared to the non-altered blood (Yang 2023). Specifically, they discovered four pathways through which the metabolites interacted: amino acid metabolism, nucleotide metabolism, energy metabolism, and others. In summary, this integrated analysis uncovered groups of related pathways associated with depression. This work adds to our understanding of the molecular mechanisms at play in depression, which is an important step in improving how we diagnose and treat this mental health condition.

Advantages:

When examining the specific steps of metabolomics, the quenching of cells using methanol is advantageous due to its low-temperature strategy which is beneficial in arresting cellular enzymatic activity in less than a second (Tang 2011). Also, when looking at metabolomics largely, metabolomics allows researchers to acquire a holistic snapshot of the metabolic state of an organism/system at a specific point in time, offering insights into its functional status (Tang 2011).

Disadvantages:

One disadvantage is that the metabolome, the set of all the metabolites present in cells, is sensitive to various genetic and environmental stimuli. Therefore, the execution of a metabolomic study requires the consideration of several factors so that confounders can be limited, and information recovery is optimized. Another disadvantage is the required sample drying. The repeated centrifugation, aspiration, and drying of the samples during metabolomic extraction can allow for human error and sample loss.

Stable Isotope Probing for tracking microbial metabolism through biomarker analysis

Method:

Stable isotope probing (SIP) is a powerful technique used to monitor and analyze microbial metabolism in complex environments. SIP allows researchers to establish direct connections between the metabolic capabilities of microorganisms and their phylogenetic and metagenomic characteristics. This is achieved by tracing isotopically labeled substances to phylogenetically and functionally informative biomarkers (Uhlik, 2012). SIP is based on the use of stable isotopes, such as carbon-13 (13C) or nitrogen-15 (15N), which are non-radioactive and safe to work with. The fundamental principle is that microbial cells can assimilate these stable isotopes into their biomass during metabolic processes (Uhlik, 2012). By providing labeled substrates containing the stable isotope, researchers can selectively trace the metabolic activities of certain microbial groups or taxa. To begin, a substrate of interest (e.g., a carbon source or amino acid) is labeled with a stable isotope, typically 13C or 15N. This labeled substrate is then added to the environmental sample. The environmental sample, harboring a diverse microbial community, is incubated, and subjected to controlled conditions, including factors such as temperature and oxygen levels, to facilitate the microbial metabolism of the labeled substrate (Uhlik, 2012). Microbial cells that consume the labeled substrate will assimilate the stable isotope into their biomass during growth. This incorporation distinguishes them from “non-consumers”. After incubation, the microbial community is separated by density gradient centrifugation. This step separates the "heavy" fractions (microbes with labeled biomass) from the "light" fractions (microbes with unlabeled biomass) (Uhlik 2012). Then, DNA, RNA, and proteins can be extracted. This can be done by using an acidic solution, which consists mainly of guanidinium thiocyanate (GuSCN), sodium acetate, phenol, and chloroform, followed by centrifugation (Ali 2017).

Information Obtained:

Once the biomarkers (DNA and RNA) and proteins are extracted, the quality of the raw data needs to be verified to ensure it is reliable and free from contaminants. Data is trimmed or filtered as needed to remove low-quality and noisy information. The biomarkers can undergo molecular analysis, such as DNA sequencing and RNA sequencing, to identify the microbial taxa or functional genes associated with the labeled biomass. The metagenomic data is then compared to existing databases or reference genomes to identify unique features, trends, and ecological relationships. At times, metagenomic binning is performed to group scaffolds into 'bins'

representing individual genomes (Quince 2017). The proteins can undergo mass spectrometry and that data can be further compared to reference databases of known proteins to determine the identity of the proteins present in the sample. Common database searching algorithms like SEQUEST or Mascot are used (Aslam 2017).

Example:

Growth rate, how quickly organisms grow and reproduce, is a key feature of biology. However, there are only a few measurements of microbial growth rates in soil, despite its crucial importance to terrestrial ecosystems and global environmental change. A study disseminated that by measuring the uptake of isotopically labeled water, we can quantify microbial growth, even at exceedingly slow rates (Caro 2023). So, researchers measured the microbial growth rates in three distinct soil environments (conifer forest, grassland, and alpine tundra) using hydrogen-stable isotope probing of lipids with 2H-enriched water (Caro 2023). Between the three soil environments, growth in the alpine tundra was the slowest. Differences observed between specific compounds in all three soils indicate that different taxonomic groups within a given soil may exhibit growth rates between 0.1629 and 0.0017 d−1, corresponding to generation times between 4.3 and 402.1 d (Caro 2023). This vast range indicates highly variable growth rates across different constituents of the soil microbiome. Therefore, the majority of soil microorganisms in the study appear to be growing at extremely slow rates when compared to the maximum potential growth rates of many bacteria grown in culture (Caro 2023).

Advantages:

One notable advantage of SIP is its independence from culture-based methods, making it well-suited for investigating complex and uncultivated microbial communities. Additionally, SIP has the unique capability to meticulously trace the propagation of stable isotopes across intricate food webs, encompassing interactions among plants, herbivores, and predators. This functionality enriches our understanding of the intricacies of ecosystem dynamics.

Disadvantages:

One disadvantage is that the interpretation of SIP data may lead to false positives or negatives, particularly when dealing with low-abundance taxa or when labeled substrates are not fully assimilated. Also, because this methodology heavily relies on the stable isotopes used for labelling, choosing appropriate labeled substrates and ensuring their purity is crucial.

Reverse-transcriptase quantitative PCR for monitoring microbial gene expression

Method:

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) stands out as the most sensitive method for measuring mRNA levels, especially when it comes to studying gene expression. It's composed of two main parts: reverse transcription (RT) and quantitative PCR (qPCR). In RT-qPCR, the first step involves changing RNA into complementary DNA (cDNA), which makes it compatible with DNA-based PCR. This conversion is done with the help of an enzyme called reverse transcriptase. It takes a primer, typically a short DNA piece or a mix of oligo-dT and random primers, attaches it to the RNA template, and then crafts a matching DNA strand from the RNA template. Once we have the cDNA, we use quantitative PCR to gauge the amount of cDNA made. This part offers precise measurements of the initial RNA levels. It works by using specific DNA primers and special fluorescent probes that zero in on the target gene or RNA. These fluorescent probes can be TaqMan probes or SYBR Green, and they produce fluorescent signals during the amplification process, which helps with quantification. The qPCR machine goes through various temperature changes to amplify the cDNA while simultaneously tracking the fluorescence. The results of RT-qPCR are expressed in cycle thresholds. A cycle threshold (Ct) is essentially the number of cycles required for the fluorescent signal to go past a certain level (in other words, rise above the background). In this context, lower Ct values mean there's more of the target nucleic acid in the sample, and vice versa.

Information Obtained:

The Ct values serve as a basis for calculating the relative expression levels of the RNA we are interested in. When the Ct values are lower, it means there was more of the RNA to start with. To ensure accuracy, we get rid of any data that might be of low quality or contain errors, like signals affected by background noise or contamination. After that, we normalize the Ct values to correct for any differences in the quality and quantity of the samples. To figure out the relative expression levels of the target genes, we compare them to a reference sample. Typically, we use a common method known as the comparative Ct method. It involves using a reference sample and an internal control to determine how much of the target nucleic acid is in a sample. In studies where we compare things, we can use statistical analysis methods like t-tests, ANOVA, or non-parametric tests to figure out if the differences between the experimental groups are meaningful.

Example:

The COVID-19 pandemic has highlighted the importance of finding reference genes to understand how host genes impact our overall gene expression. MicroRNAs (miRNAs) have been implicated in the mechanisms related to immune dysregulation, making them potential candidates for use as biomarkers or therapeutic agents in clinical settings. Therefore, it's crucial to delve deeper into the changes in miRNA expression in the context of COVID-19. In this study, researchers examined the expression of 798 miRNAs in the peripheral blood of 24 critically ill patients, with half of them being COVID-19-positive and the other half COVID-19-negative (Formosa 2023). They compiled a list of potential stable reference genes, which included ten miRNAs (Formosa 2023). Then, they subjected the top six candidates to reverse transcription quantitative polymerase chain reaction, allowing them to use established computational algorithms like geNorm, NormFinder, BestKeeper, and RefFinder (Formosa 2023). The results consistently pointed to two specific miRNAs, miR-186-5p and miR-148b-3p, as stable across all the algorithms (Formosa 2023). This research aids in identifying specific biomarkers that can provide valuable insights into the altered gene expression caused by COVID-19.

Advantages:

RT-qPCR offers a significant advantage in terms of its remarkable sensitivity. It excels at detecting exceedingly low levels of RNA, a characteristic that makes it an ideal choice for accurately quantifying gene expression, even when working with samples that have limited RNA content. This heightened sensitivity allows researchers to delve into gene expression with precision, even in cases where the starting RNA material is quite scarce. Another key benefit lies in the specificity of RT-qPCR. This technique relies on specific primers and probes designed to exclusively target the genes of interest. This precision is vital as it significantly reduces the likelihood of non-specific amplification, where genetic material unrelated to the study's objectives can be mistakenly amplified.

Disadvantages:

Given the array of computational algorithms available for research, the selection of suitable reference genes for normalization becomes a critical task. It's important to recognize that the stability of these reference genes may fluctuate under different experimental conditions. Also, RT-qPCR is contingent upon the existence of specific primers or probes tailored for the genes of interest. This requirement limits its applicability to genes and sequences that are already known and characterized.

Fluorescence in situ hybridization (FISH) microscopy

Method:

Fluorescence in situ hybridization (FISH) microscopy is a laboratory technique designed for the detection and precise localization of specific DNA sequences on a chromosome (Dutra 2023). The method involves affixing the complete set of chromosomes from an organism onto a glass slide and subsequently exposing them to a "probe." This probe is a minute segment of purified DNA, distinguished by the attachment of a fluorescent dye (Bhat and Wani 2017). The fluorescently labeled probe actively seeks out and binds to its complementary sequence within the chromosomal set. Utilizing a fluorescence microscope, the precise site on the chromosome or sub-chromosome where the fluorescent probe has affixed can be visualized (Bhat and Wani 2017). The process commences with the creation of specific DNA or RNA probes via recombinant DNA techniques, where they are integrated into plasmids. These probes are meticulously designed to be a perfect match for the target sequence of interest and are evidently marked with fluorescent dyes. Subsequently, the biological sample of interest is collected, fixed, and spread onto a microscope slide to create a monolayer. This sample can encompass cells, tissues, or chromosomes. The labeled probes are then gently applied to the sample. In instances where the target sequence is present in the sample, the probes will readily hybridize (bind), forming a secure bond with their complementary sequences (Bhat and Wani 2017). Unbound probes are thoroughly rinsed away to diminish background noise, and the sample is counterstained with a DNA marker to facilitate the visualization of cell nuclei (Bhat and Wani 2017). Ultimately, microscopic imaging is accomplished using fluorescence microscopes, which effectively capture the fluorescent signals emitted by the affixed probes. Each probe's unique fluorescent color imparts the ability to pinpoint the exact location of the target sequence within the cell or tissue.

Information Obtained:

Upon obtaining high-quality images of the FISH-stained samples, several crucial adjustments are carried out, including background subtraction, noise reduction, and fine-tuning of image contrast and brightness (Adiga and Chaudhuri 2000). The subsequent step involves segmentation, where areas of particular interest, such as cell nuclei, are extracted from the background. Within the segmented regions, the FISH probe signals, represented by fluorescent spots, are accurately detected using a myriad of algorithms. Next, quantification of the FISH signals is carried out. This quantification may encompass aspects like signal intensity, signal-to-background ratios, or the number of signals per cell (Adiga and Chaudhuri 2000). Lastly, the

obtained quantitative data from FISH microscopy can be subjected to comprehensive statistical analysis to calculate measures like central tendency and variation, along with various statistical tests designed to assess distinctions or correlations between samples or experimental conditions.

Example:

The total enumeration of bacteria in probiotics is critical for the industry to ensure products are consistently and effectively able to confer a benefit to the host. However, when researchers count bacteria in probiotic products, the usual method can be a bit difficult. Enumeration of probiotic bacteria has routinely been achieved via counting the total number of colony-forming units (CFUs) grown on an agar plate with selective media (Pasulka 2021). Unfortunately, this method tends to favor the speedy bacteria that grow well in the lab, and it often gives a lower count than the actual number of bacteria. That is why a group of researchers decided to test the use of FISH microscopy on probiotics (Pasulka 2021). The researchers made three new FISH probes to see and count three types of *Bacillus* bacteria (*Bacillus amyloliquefaciens*, *Bacillus pumilus*, and *Bacillus licheniformis*) in probiotic products (Pasulka 2021). Interestingly, when they looked at the bacteria under a microscope, the count was the same as or even higher than what the product label claimed. This was true for various bacteria in different products. Therefore, FISH microscopy is a good way for researchers to count bacteria in probiotic products and see how they grow after you use them.

Advantages:

FISH microscopy offers some key benefits in research. Firstly, it gives researchers insights into the spatial details of biological samples, helping them study where nucleic acid sequences are located inside cells or tissues. This is crucial for understanding how cells are organized and how they work. Secondly, FISH has a range of applications, including its use in cytogenetics to pinpoint the positions of particular genes or chromosomal issues. This makes it valuable for identifying genetic disorders and analyzing chromosomal changes.

Disadvantages:

Much like RT-qPCR, FISH relies on special probes that match the target sequence. This means it can only detect sequences we already know about, not new ones. There's also a chance of false results because background noise or probes sticking to the wrong places can make it seem like there's something there when there isn't. On the other hand, if the signal is very weak or completely missing, it can mistakenly show that something isn't there when it actually is.

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Abstracts

Method #1 - Concordance between culture, Molecular culture and Illumina 16S rRNA gene amplicon sequencing of bone and ulcer bed biopsies in people with diabetic foot osteomyelitis.

Abstract

Background In clinical practice the diagnosis of diabetic foot osteomyelitis (DFO) relies on cultures of bone or ulcer bed (UB) biopsies, of which bone biopsy is reference standard. The slow growth or fastidious nature of some bacteria, hamper expeditious detection and identification. Rapid molecular techniques may solve both issues, but their additional value for everyday practice is unknown.

We investigated the concordance between conventional culture, the molecular techniques Molecular Culture (MC), and illumina 16S rRNA gene amplicon (16S) sequencing in people with DFO.

Methods In the BeBoP trial, bone and UB biopsies were obtained from people with DFO who visited Amsterdam UMC. These biopsies were analysed using 1) conventional culture, 2)MC, a rapid broad range PCR analysing the 16S-23S ribosomal-interspace-region, and 3) 16S sequencing, and evaluated concordance among these techniques.

Results We analysed 20 samples (11 bone and 9 UB) of 18 people. A total of 84 infectious agents were identified, 45 (54%) by all techniques, an additional 22 (26.5%, overall 80.5%) by both MC and 16S, and the remaining 16 species by culture and MC or 16S, or by a single method only. MC and 16S identified anaerobes not detected by culturing in 5 samples, and the presence of bacteria in 7 of 8 culture-negative (6 bone, 2 UB) samples.

Method #2 - Characteristics of the Fecal Microbiome of Piglets with Diarrhea Identified Using Shotgun Metagenomics Sequencing

Abstract: Diarrhea in piglets is one of the most common diseases leading to high mortality and, as a result, to economic losses. Shotgun metagenomic sequencing was performed on the DNBSEQ-G50, MGI system to study the role of the fecal microbiome in the development of diarrhea in newborn piglets. Analysis of the study data showed that the composition of the fecal microbiome at the level of bacteria and fungi did not differ in piglets with diarrhea from the healthy group. Bacteria belonging to the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, and *Fusobacteria* were the most abundant. However, a higher level of bacterial alpha diversity was observed in the group of piglets with diarrhea, which may be due to dysbacteriosis and inflammation. The study of the virome showed the difference between the two types of phages: *Bacteroides* B40-8 prevailed in diseased piglets, while *Escherichia* virus BP4 was found in greater numbers in healthy piglets. The results of our study suggest that the association between the fecal microbiome and susceptibility to diarrhea in suckling piglets may have been previously overestimated.

Method #3 - Racial differences in the limb skeletal muscle transcriptional programs of patients with critical limb ischemia

Abstract Go to:

Critical limb ischemia (CLI) is the most severe manifestation of peripheral artery disease (PAD) and is characterized by high rates of morbidity and mortality. As with most severe cardiovascular disease manifestations, Black individuals disproportionately present with CLI. Accordingly, there remains a clear need to better understand the reasons for this discrepancy and to facilitate personalized therapeutic options specific for this population. Gastrocnemius muscle was obtained from White and Black healthy adult volunteers and patients with CLI for whole transcriptome shotgun sequencing (WTSS) and enrichment analysis was performed to identify alterations in specific Reactome pathways. When compared to their race-matched healthy controls, both White and Black patients with CLI demonstrated similar reductions in nuclear and mitochondrial encoded genes and mitochondrial oxygen consumption across multiple substrates, indicating a common bioenergetic paradigm associated with amputation outcomes regardless of race. Direct comparisons between tissues of White and Black patients with CLI revealed hemostasis, extracellular matrix organization, platelet regulation, and vascular wall interactions to be uniquely altered in limb muscles of Black individuals. Among traditional vascular growth factor signaling targets, WTSS revealed only *Tie1* to be significantly altered from White levels in Black limb muscle tissues. Quantitative reverse transcription polymerase chain reaction validation of select identified targets verified WTSS directional changes and supports reductions in *MMP9* and increases in *NUDT4P1* and *GRIK2* as unique to limb muscles of Black patients with CLI. This represents a critical first step in better understanding the transcriptional program similarities and differences between Black and White patients in the setting of amputations related to CLI and provides a promising start for therapeutic development in this population.

Method #4 - Integrated transcriptomic and proteomic analysis reveals potential targets for heart regeneration

Abstract

Research on the regenerative capacity of the neonatal heart could open new avenues for the treatment of myocardial infarction (MI). However, the mechanism of cardiac regeneration remains unclear. In the present study, we constructed a mouse model of heart regeneration and then performed transcriptomic and proteomic analyses on them. Gene Ontology (GO) enrichment analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, and Gene Set Enrichment Analysis (GSEA) of differentially expressed genes (DEGs) were conducted. Western blot (WB) and qPCR analyses were used to validate the hub genes expression. As a result, gene expression at the mRNA level and protein level is not the same. We identified 3186 DEGs and 42 differentially expressed proteins (DEPs). Through functional analysis of DEGs and DEPs, we speculate that biological processes such as ubiquitination, cell cycle, and oxygen metabolism are involved in heart regeneration. Integrated transcriptomic and proteomic analysis identified 19 hub genes and Ankrd1, Gpx3, and Trim72 were screened out as potential regulators of cardiac regeneration through further expression verification. In conclusion, we combined transcriptomic and proteomic analyses to characterize the molecular features during heart regeneration in neonatal mice. Finally, Ankrd1, Gpx3, and Trim72 were identified as potential targets for heart regeneration therapy.

Method #5 – Integrated pathway and network analyses of metabolomic alterations in peripheral blood of patients with depression.

Abstract

Depression is a serious mental illness, but the molecular mechanisms of depression remain unclear. Previous research has reported metabolomic changes in the blood of patients with depression, while integrated analysis based on these altered metabolites was still lacking. The objective of this study was to integrate metabolomic changes to reveal the underlying molecular alternations of depression. We retrieved altered metabolites in the blood of patients with depression from the MENDA database. Pathway analysis was conducted to explore enriched pathways based on candidate metabolites. Pathway crosstalk analysis was performed to explore potential correlations of these enriched pathways, based on their shared candidate metabolites. Moreover, potential interactions of candidate metabolites with other biomolecules such as proteins were assessed by network analysis. A total of 854 differential metabolite entries were retrieved in peripheral blood of patients with depression, including 555 unique candidate metabolites. Pathway analysis identified 215 significantly enriched pathways, then pathway crosstalk analysis revealed that these pathways were clustered into four modules, including amino acid metabolism, nucleotide metabolism, energy metabolism and others. Additionally, eight molecular networks were identified in the molecular network analysis. The main functions of these networks involved amino acid metabolism, molecular transport, inflammatory responses and others. Based on integrated analysis, our study revealed pathway-based modules and molecular networks associated with depression. These results will contribute to the underlying knowledge of the molecular mechanisms in depression.

Method #6 – Hydrogen stable isotope probing of lipids demonstrates slow rates of microbial growth in soil

Abstract

The rate at which microorganisms grow and reproduce is fundamental to our understanding of microbial physiology and ecology. While soil microbiologists routinely quantify soil microbial biomass levels and the growth rates of individual taxa in culture, there is a limited understanding of how quickly microbes actually grow in soil. For this work, we posed the simple question: what are the growth rates of soil microorganisms? In this study, we measure these rates in three distinct soil environments using hydrogen-stable isotope probing of lipids with ^{2}H-enriched water. This technique provides a taxa-agnostic quantification of in situ microbial growth from the degree of ^{2}H enrichment of intact polar lipid compounds ascribed to bacteria and fungi. We find that growth rates in soil are quite slow and correspond to average generation times of 14 to 45 d but are also highly variable at the compound-specific level (4 to 402 d), suggesting differential growth rates among community subsets. We observe that low-biomass microbial communities exhibit more rapid growth rates than high-biomass communities, highlighting that biomass quantity alone does not predict microbial productivity in soil. Furthermore, within a given soil, the rates at which specific lipids are being synthesized do not relate to their quantity, suggesting a general decoupling of microbial abundance and growth in soil microbiomes. More generally, we demonstrate the utility of lipid-stable isotope probing for measuring microbial growth rates in soil and highlight the importance of measuring growth rates to complement more standard analyses of soil microbial communities.

Method #7 - Validation of reference gene stability for miRNA quantification by reverse transcription quantitative PCR in the peripheral blood of patients with COVID-19 critical illness

Abstract

The COVID-19 pandemic has created an urgency to study the host gene response that leads to variable clinical presentations of the disease, particularly the critical illness response. miRNAs have been implicated in the mechanism of host immune dysregulation and thus hold potential as biomarkers and/or therapeutic agents with clinical application. Hence, further analyses of their altered expression in COVID-19 is warranted. An important basis for this is identifying appropriate reference genes for high quality expression analysis studies. In the current report, NanoString technology was used to study the expression of 798 miRNAs in the peripheral blood of 24 critically ill patients, 12 had COVID-19 and 12 were COVID-19 negative. A list of potentially stable candidate reference genes was generated that included ten miRNAs. The top six were analyzed using reverse transcription quantitative polymerase chain reaction (RT-qPCR) in a total of 41 patients so as to apply standard computational algorithms for validating reference genes, namely geNorm, NormFinder, BestKeeper and RefFinder. There was general agreement among all four algorithms in the ranking of four stable miRNAs: miR-186-5p, miR-148b-3p, miR-194-5p and miR-448. A detailed analysis of their output rankings led to the conclusion that miR-186-5p and miR-148b-3p are appropriate reference genes for miRNA expression studies using PaxGene tubes in the peripheral blood of patients critically ill with COVID-19 disease.

Method #8 - Visualization of probiotics via epifluorescence microscopy and fluorescence in situ hybridization (FISH)

Abstract

Aerobic plate counts, the standard for bacterial enumeration in the probiotic industry, can be biased towards fast-growing organisms that replicate on synthetic media and can significantly underestimate total bacterial abundance. Culture-independent approaches such as fluorescence in situ hybridization (FISH) hold promise as a means to rapidly and accurately enumerate bacteria in probiotic products. In addition, FISH has the potential to more accurately represent bacterial growth dynamics in the environment in which products are applied without imposing additional growth constraints that are required for enumeration via plate counts. In this study, we designed and optimized three new FISH probes to visualize and quantify Bacillus amyloliquefaciens, Bacillus pumilus, and Bacillus licheniformis within probiotic products. Microscopy-based estimates were consistent or higher than label claims for Pediococcus acidilactici, Pediococcus pentosaceus, Lactobacillus plantarum, Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus licheniformis and Bacillus pumilus in both a direct fed microbial (DFM) product as well as a crop microbial biostimulant (CMB) product. Quantification with FISH after a germination experiment revealed the potential for this approach to be used after application of the product.