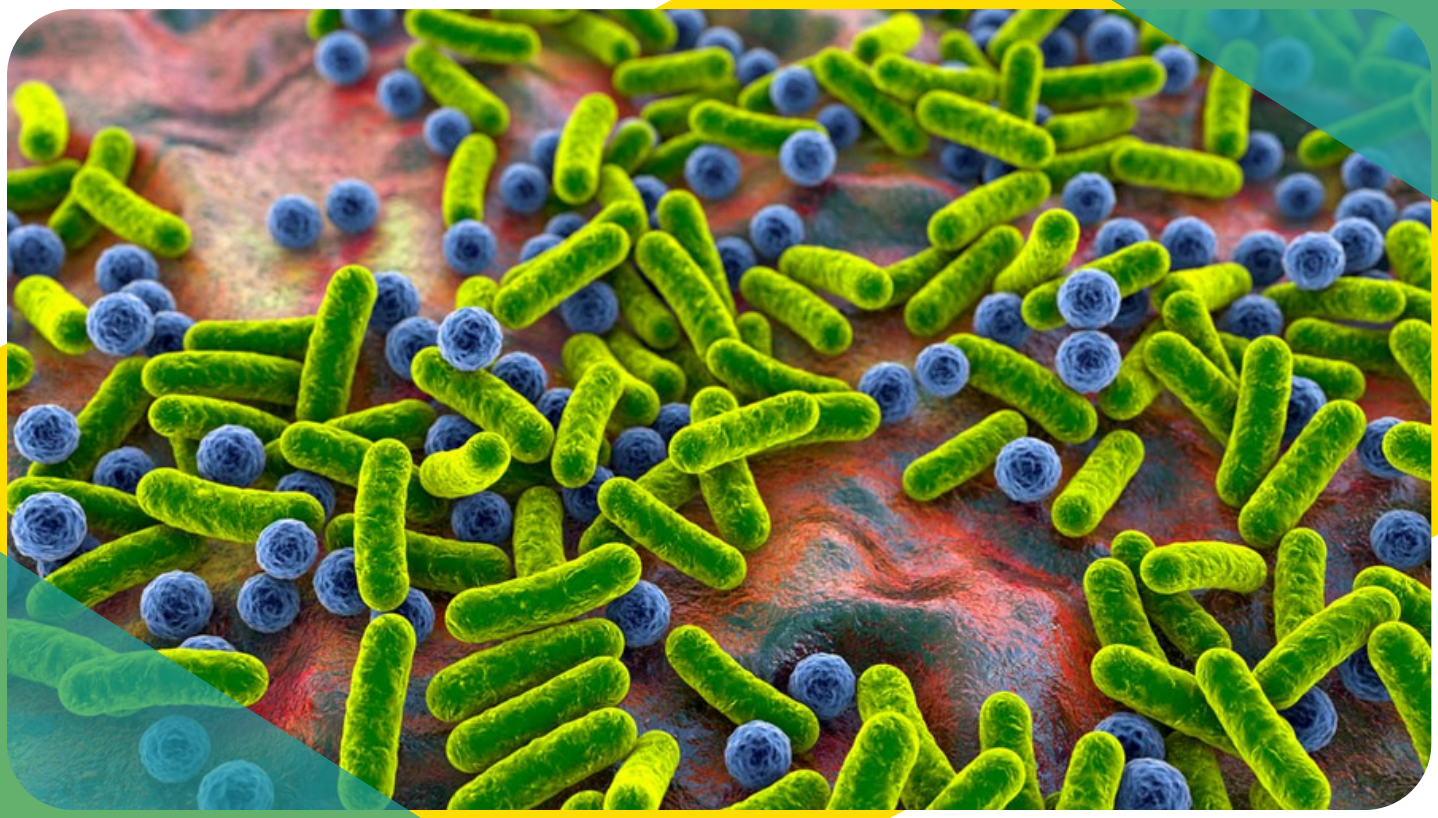
BioTechniques
The International Journal of Life Science Methods



Investigating the gut microbiome

ZYMO RESEARCH

eBook: Investigating the gut microbiome

Contents

Introduction

Living inside us are trillions of microorganisms collectively known as the microbiome. Various organs have distinct microbiomes; however, it is the gut microbiome that has garnered the most attention. The gut microbiome plays a crucial role in human health and disease, from controlling immune function to influencing behavior through the gut–brain axis.

The field of microbiomics has expanded rapidly in the past decade, and while these studies have yielded fascinating insights into the relationship between human health and the microbiome, results are often difficult to reproduce and datasets from different studies are not comparable. Poor reproducibility in microbiome studies can arise for a number of reasons but is often due to a lack of proper controls and consistent comparison to microbiome reference materials.

Fortunately, over the past several years, this reproducibility crisis has gained the attention of key opinion leaders and the adoption of defined microbial standards is growing, leading to an increased ability to standardize protocols, techniques and workflows. However, as our understanding of the conditions required for true reproducibility develops, the need for a common point of reference for the human gut microbiome has become apparent.

In this eBook, in partnership with Zymo Research, we explore the gut microbiome, including important insights and techniques used, and highlight the importance of the use of standards and controls when carrying out microbiome research.

Annie Coulson
Digital Editor, *BioTechniques*
a.coulson@future-science-group.com

Could gut bacteria help us understand the causes of multiple sclerosis?

Research shows changes in the gut bacteria of multiple sclerosis patients compared with healthy controls. There are also microbiota differences between multiple sclerosis activity states.

DAN KYTKA-SHARPE, CONTRIBUTING EDITOR, *BIOTECHNIQUES*

Researchers led by Oluf Borbye Pedersen at the University of Copenhagen (Denmark) have identified that people with multiple sclerosis (MS) have different gut bacteria compared to healthy people. The study also found that MS patients have differing intestinal microbiomes depending on their disease activity.

MS is a chronic autoimmune disease of the central nervous system that results in physical and cognitive impairments. It is most frequently diagnosed in young adults in their 20s and 30s, but the underlying causes are not well understood. MS patients may have relapsing-remitting multiple sclerosis, characterized by periods without disease activity followed by a relapse that may cause further impairments.

The current case-controlled study compared the gut microbiomes of 148 Danish multiple sclerosis patients with the same number of healthy control subjects. Each participant provided blood and fecal samples when the study began and two years later. The scientists genetically analyzed the composition of the collected gut bacteria to assess their effect. They also measured the levels of known inflammatory markers in the blood plasma samples.

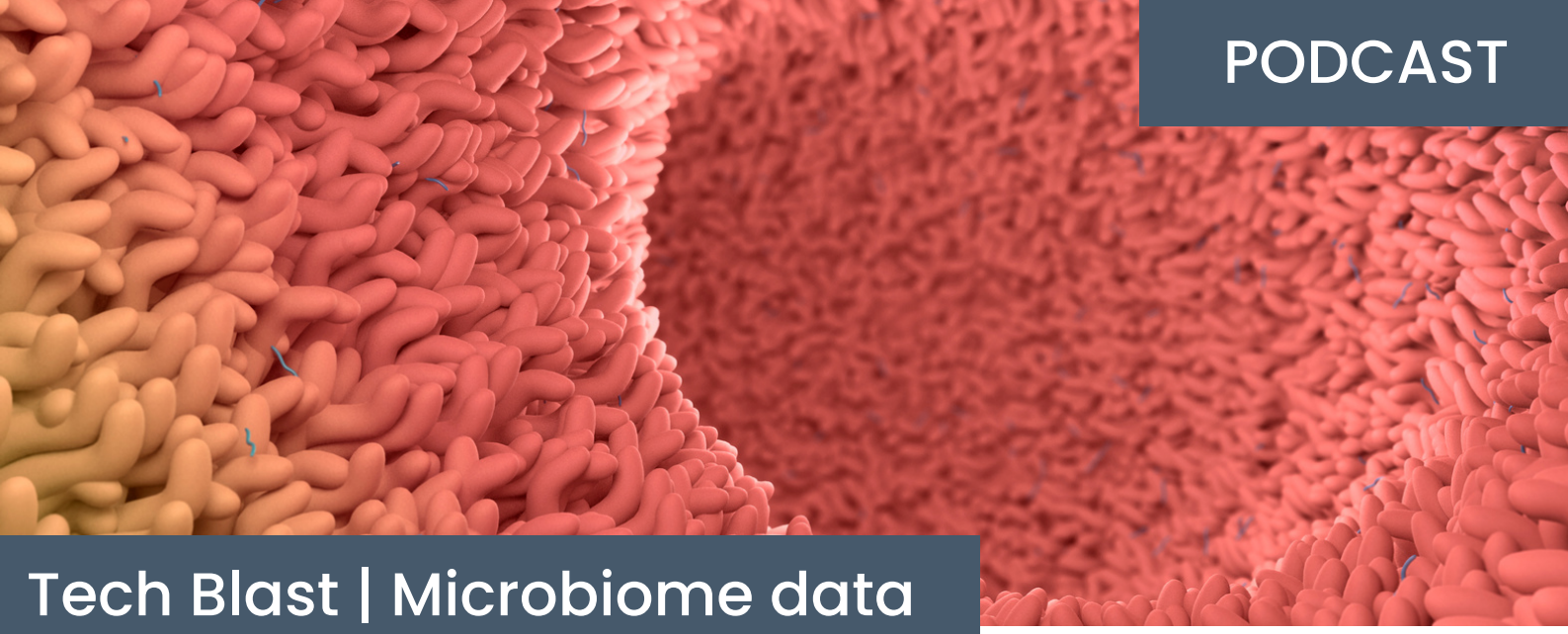
The team identified 61 gut bacteria species that were differentially abundant when comparing all the MS patients with the healthy controls. This included 31 species that were enriched in patients with the disease. Researchers also identified clusters of inflammation markers that were positively associated with a group of disease-associated bacteria.

Finn Sellebjerg, a clinical professor and study co-author, explained that "undergoing treatment for multiple sclerosis seems to be linked to a change in the composition of bacteria compared with patients who are not undergoing treatment." He added that some of the gut bacteria changes can be identifiably linked to the occurrence of inflammatory reactions in the body.

The researchers were most interested in the finding that there are two species of 'good' gut bacteria that are found more frequently in MS patients without active disease. These health-promoting bacteria produce fatty acids that the body cannot synthesize itself and anti-inflammatory metabolites, including urolithin.
Pedersen, the senior author, believes that this finding, if independently confirmed, offers a route to treatment trials. This could include an "anti-inflammatory, green diet and a cocktail of next-generation probiotics" to regulate immune competence. He cautioned that "unfortunately, there is still some way to go before we can provide specific advice on a health-enhancing lifestyle or bacteria supplement."

Sellebjerg suggested that this research provides "a handful more pieces in the 10,000-piece jigsaw puzzle of multiple sclerosis, but there are still large gaps." He added that, "the great difference is that the pieces we have found are starting to reveal systems that we can manipulate without the side effects some medicines can have."

Source: Thirion F, Sellebjerg F, Fan Y *et al.* The gut microbiota in multiple sclerosis varies with disease activity. *Genome Med.* 15(1), 1 (2023).



PODCAST

Tech Blast | Microbiome data

In this Tech Blast episode, we discuss generating, standardizing and sharing microbiome data with Raul Cano (left), Chief Scientific Officer at The BioCollective (CO, USA). Raul provides a brief overview of the reproducibility crisis and explores how the use of standards and databases can address the challenges it poses.

Get Raul's insight into the field and discover the steps that can be taken to make microbiome research more accessible and reproducible.

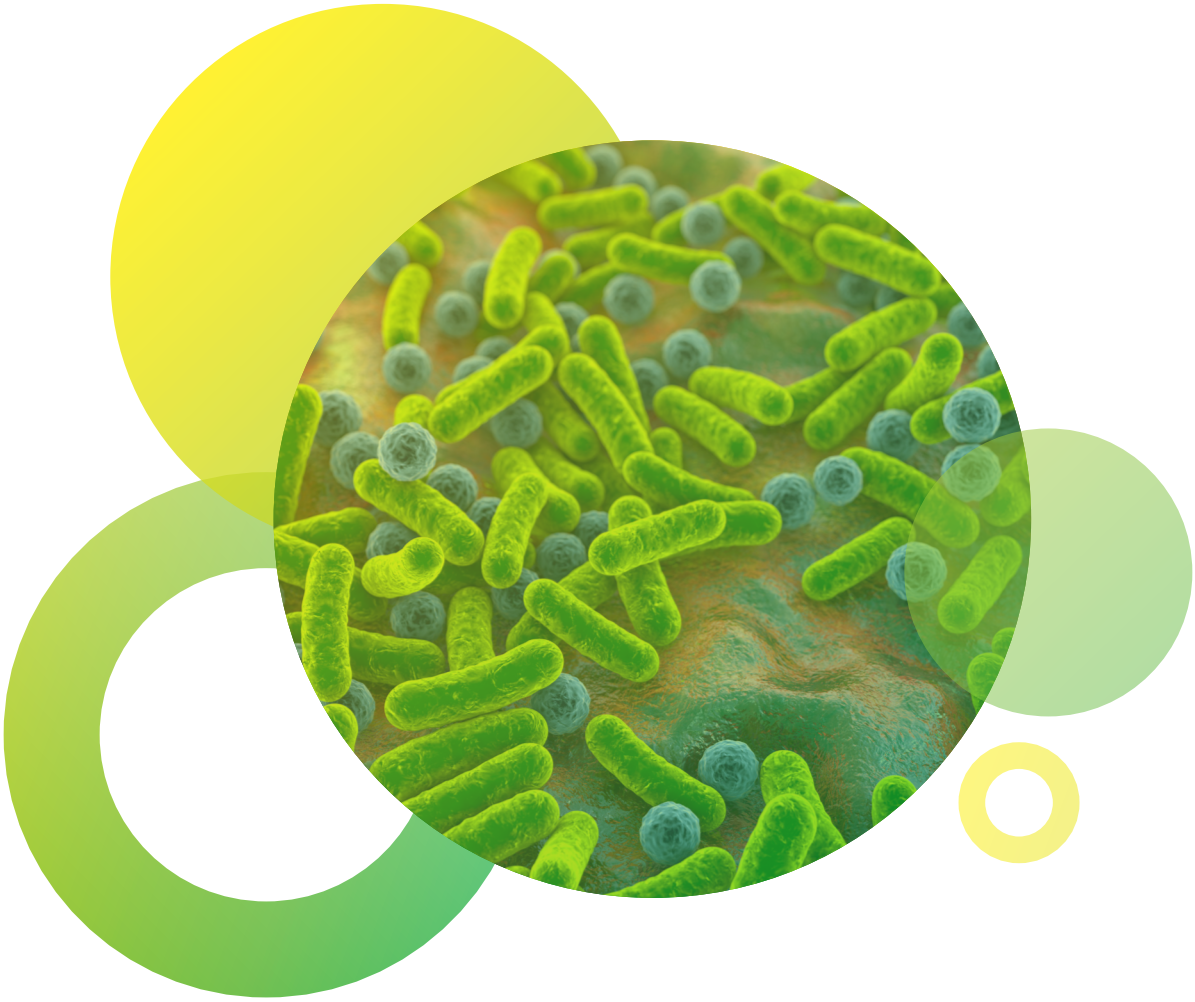
Contents:

Follow the QR code or click the link to access the episode:

ZYMO RESEARCH

The Beauty of Science is to Make Things Simple

How to Choose a Microbiome Standard



Controls and Standards in Microbiome Research

The advancement of NGS based technologies has led to a rapid growth in the field of microbiome research and deciphering microbial community composition, function, and interactions. Many studies conclude that technical variability in microbiome processing methods leads to significant variations in results[1-3]. Most of the discrepancies in reporting are explained by differences among the methods for nucleic acid extraction, NGS library preparation, bioinformatic data processing, and the choice of reference databases. Despite the complexity and variation introduced by varying protocols and methods for each step of the microbiomics workflow, data is being generated at an unprecedented pace. In many cases, a lack of proper controls or comparison to microbiome reference materials means that important and high-impact conclusions cannot be reproduced or reliably compared to similar data sets.

Commonly used and accepted controls or reference reagents are often called 'standards' because their inclusion and consideration allow for comparisons of methods, equipment, and protocols. Microbiome standards are imperative for microbial community profiling and analysis. Whereas the microbial compositions of experimental samples are variable and often unknown, microbiome standards provide a common, accurate, and consistent measurement as a basis for comparison. By providing a common control to measure and evaluate performance, microbiome standards indicate biases allowing users to verify and optimize methods, enable inter-lab comparisons, and ensure reproducibility.

How to Select the Appropriate Microbiome Controls

The principle of a microbiome standard is simple: use a well characterized, quantified, and known microbial input to perform experimental procedures and evaluate consistency of the output. Standards can then be run as a parallel quality control to experimental samples to evaluate the consistency of the method. The resulting profile provides a basis to calibrate and when needed, begin troubleshooting. Several different types of NGS Microbiome controls are available, each detecting different and sometimes overlapping parts of the complex microbiome processing workflow. This article is meant to aid in selecting the appropriate reference reagents and controls for your microbiome experiments.

Mock Communities, True Diversity Reference, and Spike-in Controls

Several categories of microbiome reference reagents are available including microbial mock communities, true diversity reference material, and spike-in controls. Each category has overlapping characteristics, such as the use as positive controls, and each detects different biases throughout the microbiome analysis workflow.

Table 1 – Microbiome Standards and Controls Suggested Use

Standards	Suggested Applications
Mock Community Standards (Cellular)	
ZymoBIOMICS™ Microbial Community Standard	• General optimization and benchmarking • Positive control for microbial lysis
ZymoBIOMICS™ Gut Microbiome Standard	• General optimization and benchmarking for gut microbiome workflows • Assess cross-kingdom, strain-level resolution, and pathogen detection
ZymoBIOMICS™ Microbial Community Standard II (Log Distribution)	• Assessing detection limit of whole workflows beginning with DNA extractions
Mock Community Standards (DNA)	
ZymoBIOMICS™ Microbial Community DNA Standard	• Optimization and positive control for library preparation and bioinformatics
ZymoBIOMICS™ HMW DNA Standard	• Optimization and positive control for long-read sequencing library preparation and bioinformatics
ZymoBIOMICS™ Microbial Community DNA Standard II (Log Distribution)	• Assessing detection limits of library preparation and bioinformatics
True Diversity Reference	
ZymoBIOMICS™ Fecal Reference with TruMatrix™ Technology	• Assessing taxonomic assignment and bioinformatic processing parameters • Enable inter-lab and inter-study data comparisons
Spike-In Controls	
ZymoBIOMICS™ Spike-in Control I (High Microbial Load)	• *In situ* extraction control and absolute quantification for high biomass samples
ZymoBIOMICS™ Spike-in Control II (Low Microbial Load)	• *In situ* extraction control and absolute quantification for low biomass samples

The categories of microbiome standards and suggested applications are listed in Table 1.

Mock communities are accurately quantified and well-defined artificial microbial communities that act as ground truths of known composition and abundance. On the other hand, a true diversity reference is created from a specified natural source, such as human stool, stabilized and homogenized to be a common and consistent control material containing a true-to-to life microbial profile and diversity. Finally, while mock communities and true diversity references are meant to be used in parallel to experimental samples, spike-in controls are added directly to experimental samples and processed within each sample. The defined abundance of the spike-ins' unique species allows for absolute cell number quantification and quality control for each individual sample.

Cellular Mock Community Standards

Mock communities generated from whole cells are the most commonly used microbiome standard because they function as positive controls for the entire workflow. But perhaps more importantly, cellular mock communities such as the ZymoBIOMICS™ Microbial Community Standard are used to optimize and compare microbial lysis methods[4-5] because they contain equal abundances of species with a wide range of cell wall recalcitrance and cell size. By comparing the resulting profile to the theoretical profile, the ability of the lysis method can be assessed. For example, if the Gram-negative bacteria in the mock community profile are observed to be in excess while the Gram-positive bacteria are deficient compared to the theoretical abundance, the lysis method may struggle to break open thicker cell walls.

Additionally, site-specific microbial standards are another type of mock communities with their own uses. For example, the ZymoBIOMICS™ Gut Microbiome Standard contains 21 microbial strains from 3 kingdoms to allow for the evaluation of methods analyzing the gut microbiome and to act as a general positive control[6-7].

Finally, log-distributed mock community standards, such as the ZymoBIOMICS™ Microbial Community Standard II (Log Distribution), contain species at different abundances ranging from 10^2 – 10^8 cells per prep. This logarithmic

Table 2 – ZymoBIOMICS™ Standards, References, and Controls

Application	Mock Community (Cellular)			Mock Community (DNA)			True Diversity Reference	Spike-In Controls	
	ZymoBIOMICS™ Microbial Community Standard	ZymoBIOMICS™ Microbial Community Standard II (Log Distribution)	ZymoBIOMICS™ Gut Microbiome Standard	ZymoBIOMICS™ Microbial Community DNA Standard	ZymoBIOMICS™ Microbial Community DNA Standard II (Log Distribution)	ZymoBIOMICS™ HMW DNA Standard	ZymoBIOMICS™ Genuine Fecal Standard with TruMatrix™ Technology	ZymoBIOMICS™ Spike-in Control I (High Microbial Load)	ZymoBIOMICS™ Spike-in Control II (Low Microbial Load)
	D6300	D6310	D6331	D6305	D6311	D6322	D6323	D6320	D6321
General Microbiome Samples	✓			✓					
Fecal Samples	✓		✓	✓			✓	✓	
Assessing Detection Limit		✓			✓				
Long Read Sequencing	✓		✓			✓			
High Diversity							✓		
Internal Spike-ins								✓	✓
Targeted (16S, ITS) Sequencing	✓	✓	✓	✓	✓	✓	✓	✓	✓
Metagenomic (Shotgun) Sequencing	✓	✓	✓	✓	✓	✓	✓	✓	✓

distribution of species enables users to evaluate the detection limits of their microbiome analysis workflow[8].

DNA Mock Community Standards

Mock community standards made with purified microbial genomic DNA are more often used to detect biases and as optimization tools because they are utilized as input for library preparation rather than at the beginning of the workflow. DNA mock community standards such as the ZymoBIOMICS™ Microbial Community DNA Standard can be utilized to control biases associated with library prep and bioinformatics[9-10]. The optimization can be focused on library prep by first aligning NGS reads generated from the standard only to the genomes within the standard. After library prep has been optimized, the bioinformatics pipeline can be evaluated by aligning NGS reads against an entire reference database.

Similar to the cellular version, log distributed DNA standards, such as the ZymoBIOMICS™ Microbial Community DNA Standard II (Log Distribution), are used to assess detection limits but for library prep and bioinformatics pipelines.

Furthermore, an emerging technology for metagenomic analysis and genome assembly is long-read sequencing, often referred to as 3rd gen sequencing. Critical to long-read sequencing library prep and bioinformatics is high molecular weight DNA. The ZymoBIOMICS™ HMW DNA Standard is the only commercially available high molecular weight mock community, and has been used to evaluate sequencing chemistries and bioinformatic tools for long read sequencing[11-12].

True Diversity Reference

A true diversity reference is control material from a specified natural source that contains a complete, unchanging microbiome. In contrast to mock communities which have a quantified, known, and defined composition, the microbial composition of a true diversity reference is naturally derived. The ZymoBIOMICS™ Fecal Reference with TruMatrix™ Technology* is the first commercially available true diversity reference stabilized for long-term and lot-to-lot consistency. This reference features the high microbial diversity of a real fecal sample as well as a wide range of abundance.

Run-to-run and user-to-user consistency can be assessed on the same sample for each experiment. Reference materials can also be used to test system suitability by challenging experimental methods with actual source material. Bioinformatic analysis and taxonomy assignment are challenged with the added complexity of an unchanging true diversity sample. Since the microbial composition is static, the abundance and composition are stable and therefore allow users to assess method and analysis consistency.

Spike-in Controls

Unlike mock communities and true diversity references, spike-in controls offer different functions when added directly to experimental samples. The ZymoBIOMICS™ Spike-in Controls are composed of very unique species, alien to the human microbiome as well as many others. This enables them to be spiked into samples without interfering with the native microbiome. The defined composition of these species enables the quantification of the absolute cell number within the unknown sample, when analyzed with NGS-based microbiome methods. Furthermore, an emerging use of these spike-in controls is as *in situ* quality controls, meaning that it can be used as a positive control for every sample rather than a positive control for a whole run. This is very useful for NGS-based pathogen diagnosis.

Two spike-in controls are available for different sample types. The ZymoBIOMICS™ Spike-in Control I (High Microbial Load) is meant for high biomass samples such as stool. The ZymoBIOMICS™ Spike-in Control II (Low Microbial Load) is meant for low microbial biomass samples such as sputum and bronchoalveolar lavage (BAL) fluid.

Choosing a Microbiome Standard

The past several years has seen an explosion in the demand for microbiome standards, controls, and references that provide different and specific utilities. The scientists at Zymo Research share a passion for creating and providing the world with tools to improve microbiome data accuracy and reproducibility. As a result, the ZymoBIOMICS™ line of standards, references, and controls provides a range of utility for various microbiome applications. Additional information about the standards and applications can be found in Table 2.

*TruMatrix™ is a trademark of The BioCollective.

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BioTechniques

REPRODUCIBILITY: THE SEARCH FOR MICROBIOME STANDARDS

The reproducibility crisis is resulting in a lot of discussion in various scientific fields. This feature explores the latest technologies, methods and projects relevant to the biological laboratory and aiming to solve this problem.

There is a growing concern about results that cannot be reproduced. The scientific community is suffering from a reproducibility crisis. The combination of more complex research, a heightened pressure to publish and advanced statistics has resulted in enormous concern about the challenges of irreproducible research and scientific transparency among almost all involved in the scientific process.

Recently American Type Culture Collection (ATCC; VA, USA), a global biological materials resource and standards organization, conducted a reproducibility survey with the purpose of providing insight into scientists' perceptions about reproducibility problems.

The results highlighted the impact this problem is having on the scientific community. Of the 415 scientists questioned about reproducibility, 41% are losing faith in the scientific process and 52% said it risks the integrity of their research. Some of the key reasons cited included the pressure to publish or achieve quickly rather than more value being assigned to quality. More alarmingly, 85% of respondents under the age of 30 years said that reproducibility is an urgent problem.

It is important to note that, with rare exceptions, there is no evidence to suggest that irreproducibility is caused by scientific misconduct. When conducting research, there are numerous opportunities for experimental biases to alter the results of a study. Recognizing these factors is an essential step towards controlling them. This article will explore the causes of irreproducible studies and efforts to improve, with particular focus on the variability in data obtained in studies of the microbiome and what can be done to normalize our approach to science to ensure that these important measurements are reproducible.

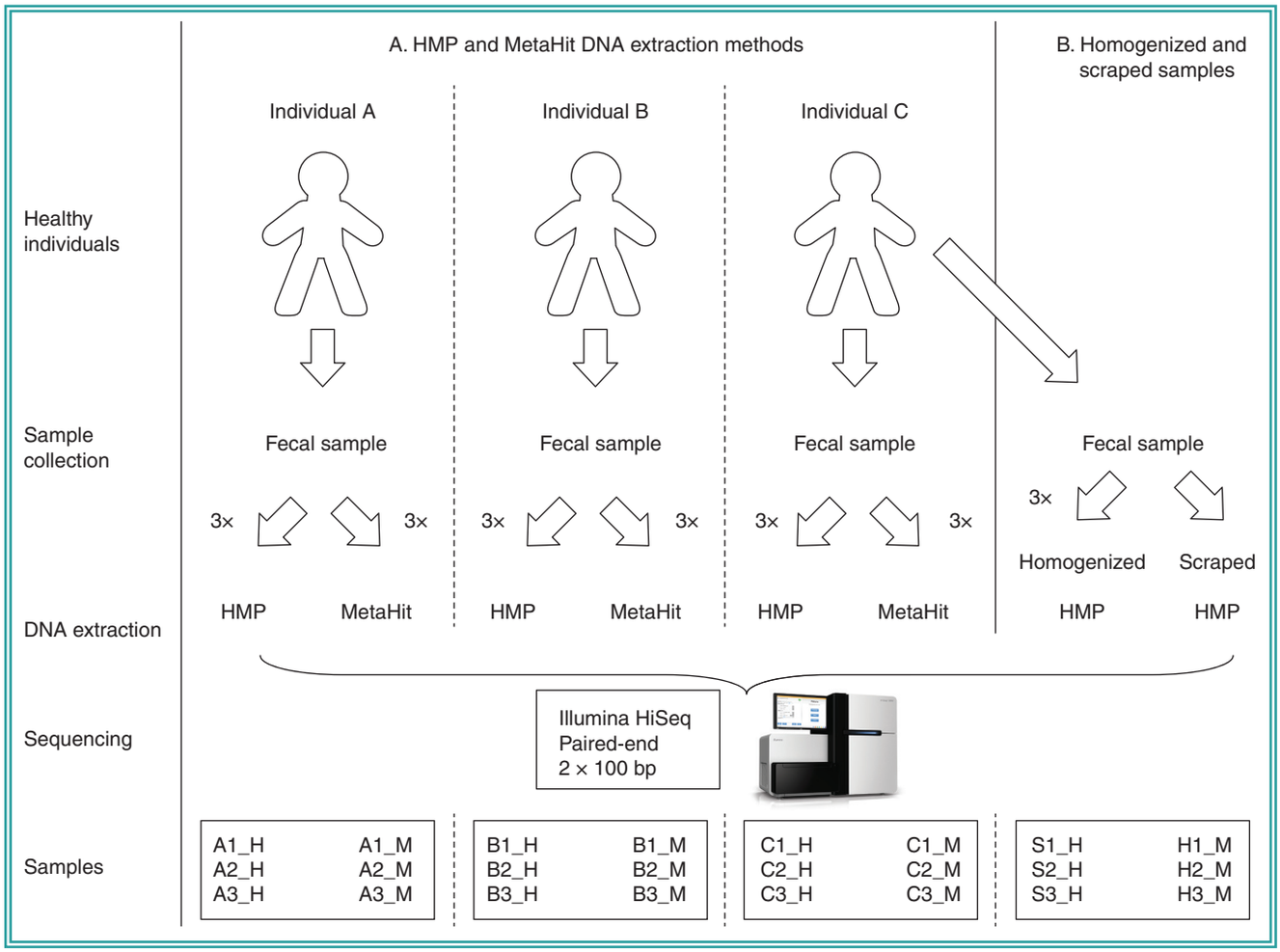
Should we be surprised experimental findings are difficult to reproduce? Are we all doing enough?

GETTING TO THE GUT OF REPRODUCIBILITY

Microbiomics is one field that is particularly impacted by poor-quality data in relation to reproducibility across labs. This is a result of both complicated measurements and substantial bias that can be introduced at each step of the workflow.

Despite the plethora of research that has yielded interesting insights into the relationship between microbiota, human health and the environment, there have been questions raised regarding the variability of data obtained by different labs. The application of next-generation sequencing (NGS) for mapping of bacterial phylogeny and function has opened new doors to this field of research. Due to this rapid expansion and the demand for high-performance protocols at every level of sample processing, specially designed controls and reagents are needed from sample collection, DNA and RNA extraction, to NGS library preparation.

Ryan Kemp, Director of Nucleic Acid Solutions at Zymo Research (CA, USA) commented on the importance of scientists using and adhering to standards when working with the microbiome: "NGS-based microbiome analysis has great potential to improve fields such as medicine, agriculture and environmental safety, by quickly generating many times more data than traditional methods. But, in order to make decisions that can potentially affect public health and safety, it is critical that methodology is validated to be accurate and reproducible using known ground truths, namely microbiome standards. Thousands of microbiome publications are effectively incomparable due to the wide range of workflows deployed and the associated workflow biases. With the emergence of microbiome-driven diagnostics, health monitoring services and interventions, the field is transitioning from basic discovery to application and the success of the field is dependent on accurate, reproducible measurements."



Schematic representation of the study design. (A) Comparison of the HMP and MetaHIT DNA extraction methods. (B) Comparison of homogenized and scraped samples. Reproduced from [1].

There are numerous opportunities for experimental biases to profoundly alter the results of a study. In addition to these factors, research groups will often alter experimental processes depending on their specific study. Therefore, recognition and documentation of these factors can help towards controlling the variability between labs.

One example of interest that highlighted the severity of this issue focused on the DNA extraction methods used by two major collaborative efforts: The European MetaHIT and the American Human Microbiome Project (HMP) [1]. Interestingly, depending on the method, significant differences in the distribution of bacterial taxa were observed. For example, DNA from bacteria within the Bacteroidetes phylum was most enriched by the HMP protocol.

In another study, Costea *et al.* tested 21 representative DNA extraction protocols on the same fecal samples. Variations resulting from different extraction methods were then compared, with differences attributed to library preparation and sample storage. The researchers demonstrated that DNA extraction had the largest effect on the outcome of metagenomic analysis [2].

Studies that highlight these technical variations accelerate the push for standardized methods – in this case, a DNA extraction method for human fecal samples.

Kemp explained: "The lack of congruency between methods and the chronic absence of workflow controls and standards has led to protocols that are not optimized for microbiome measurements that demand new levels of rigor. Measurement accuracy and reproducibility is a serious concern for the future health of the microbiome field and, unfortunately, many are unaware of the challenges."

Kemp continued to describe what is being done to tackle this worrying issue: "Fortunately, the US National Institute of Standards and Technology (MD, USA) has been hosting workshops on standards for microbiome measurements annually to draw awareness to the problems within the field."

In an effort to improve microbiome measurement accuracy and reproducibility across the field, companies such as Zymo Research are driving initiatives to encourage the use of and increase accessibility of well-defined microbial standards.

"We are urging researchers to question and validate their methodology using mock-microbial community standards. Currently, Zymo Research is providing thousands of free microbiome standards and controls to researchers through the Microbiomics Standards & Controls Initiative. Using microbiome standards as a ground truth, Zymo Research has built an entire workflow of highly accurate

tools and kits for collection, extraction and library preparation for measuring a microbiome," explained Kemp.

While the goal is simple – to achieve accurate, reproducible measurements in this exciting field – the road to success might be longer than we think. It will take a combined effort between the publications, the publishers, the funders, reviewers and the institutions. "With this effort, the field will coalesce on basic standards of best practice to ensure quality measurements, such as the use of microbiome community standards as routinely as one would perform positive and negative controls," Kemp continued.

"Transparent and open sharing of quality data and materials is a cornerstone of reproducible science. Different scientific fields have different sharing cultures," commented Joanne Kamens, Executive Director of Addgene (MA, USA).

"We're always happy to see a field that is growing engage in dialog about best practices towards openness, sharing and reproducibility. We'd be eager to highlight a microbiome research collection of plasmids, should members of that community step up to help us create and curate it."

SETTING THE STANDARD

So what can we do to begin to rectify and solve some of these reproducibility issues?

Cell lines were also a subject of the ATCC survey. The problems with cell line misidentification have been known for a long time, contributing to the growing concerns about irreproducible experiments and false conclusions. The current call for action is focused on improving the verification of cell lines.

Mark Capriani, Senior Director of Marketing at ATCC, commented: "It's starting with credible materials that allows you to do incredible things. If you don't know what's in your materials, you could squander a lot of time and a lot of money trying on science that may have been compromised for a variety of reasons. Without authenticated materials, there's a variety of challenges to ensure the science is reproducible."

Interestingly, the ATCC survey also revealed an insight into the proportion of researchers who might place too much trust in their peers when borrowing cell lines: nine out of ten scientists reported borrowing cell lines from their colleagues and only 29% of them will reauthenticate before use.

Capriani also highlighted the importance of reauthenticating borrowed cell lines: "In our experience, most of the scientists who borrowed cell lines didn't seem to blame the irreproducibility of their research on technique or the protocols. There is a common misconception that you can trust cell lines from scientific colleagues without needing to reauthenticate, but it is actually distinctly possible that it is contributing to the issue."

The key to tackling the crisis lies in making authentication easy for researchers. Again, it is important to note that it is both publishers and researchers alike who need to start seeing standards as an asset rather than a chore.

STEPS IN THE RIGHT DIRECTION

As mentioned previously, the pressure to publish, fueled by the academic incentive system, encourages rapid submission of research findings to the detriment of careful replication. Limited platforms for publishing negative data is also a problem. This is where both funding agencies and publishers can make a positive impact.

Figure 2. Profeza: a product that provides a set of workflow software tools to help create more easily reproducible protocols [4].

Efforts to tackle the reproducibility problem are not restricted to researchers; funding agencies are also playing their part. The NIH (MD, USA) is developing a training module on enhancing reproducibility and transparency of research findings, with a particular focus on good experimental design [3].

Improving data availability and protocol sharing are just two steps that can be taken by publishers to help tackle the reproducibility crisis, the latter being a recent step that *BioTechniques* is taking to ensure that all of the information needed to replicate a method is presented in every article.

BioTechniques has recently partnered with Profeza [4], a product that provides a set of workflow software tools to help create more easily reproducible protocols. It makes the improvement of research outputs a continuous process rather than a one-time event, allowing other researchers to follow the protocol step-by-step and provide feedback on certain aspects that either worked well or could do with improvement. The authors of the protocol are then able to constantly edit their protocol, based on their own research or feedback from others. We encourage all of our authors to submit a protocol alongside their manuscript that can then be published on Profeza [5].

Additionally, we are actively working together with members of our editorial board to create a reproducibility advisory group, highlighting the most important issues in reproducibility and what can be done from a publisher's perspective to develop clear guidelines for authors and to emphasize the importance of depositing and sharing reagents, and encouraging cell-line authentication prior to publication.

Despite the abovementioned steps, there is still a lot more work to be done and improving the reproducibility of our research outputs will remain a priority for *BioTechniques*.

Written by Joseph Martin

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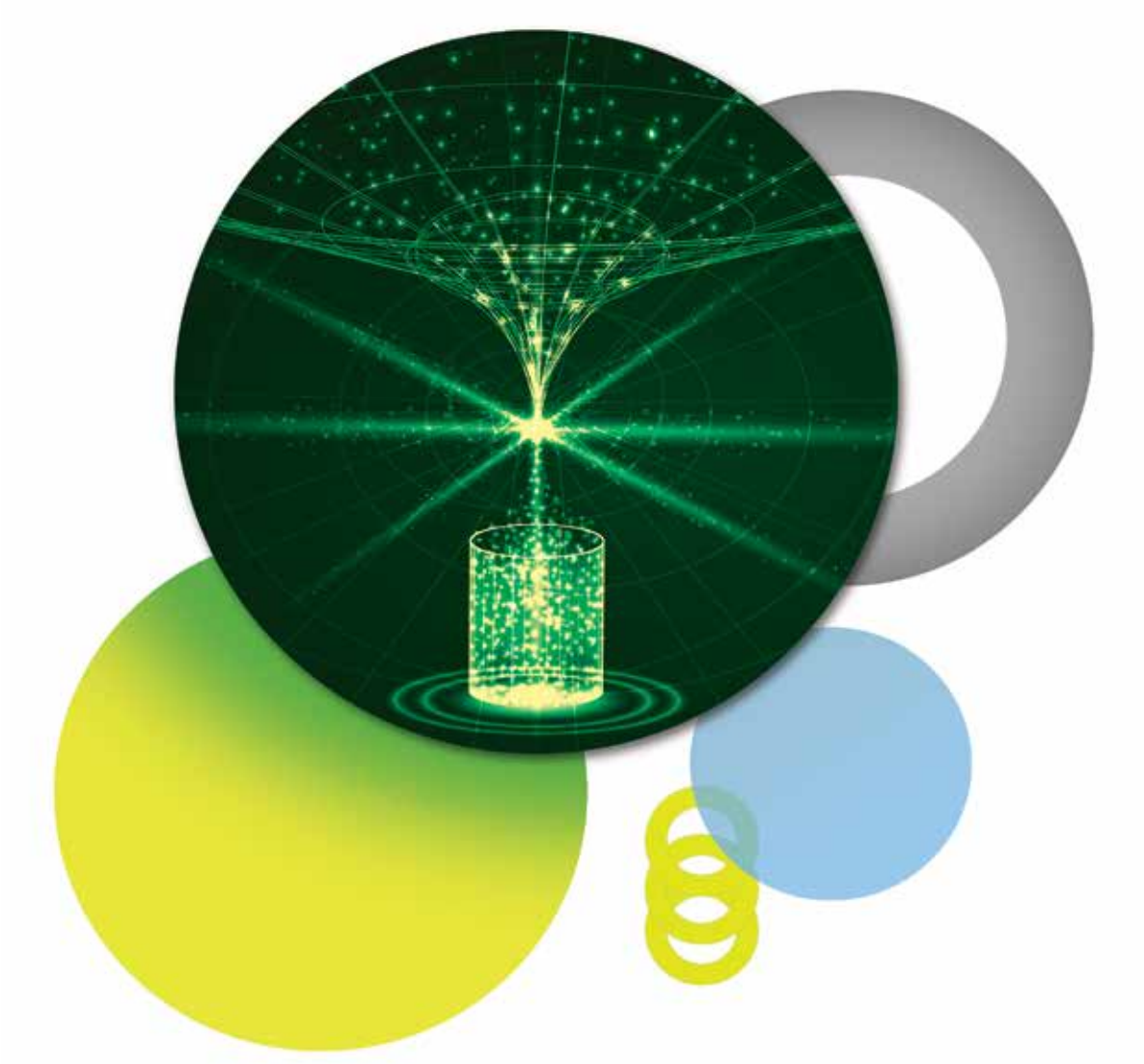
Development of Human Fecal Microbiome Reference Material and Database



Introduction

The human fecal/gut microbiome is a complex and diverse microbial community that plays an important role in human health. In recent years, there have been innumerable studies that link the gut microbiota to a wide range of conditions such as Alzheimer's Disease, asthma, cancers, and Gestational Diabetes Mellitus (GDM), as well as neurological disorders such as autism, depression, and Parkinson's Disease[1-5]. Microbiome profiling featuring Next Generation Sequencing (NGS) is the essential technology that is underlying these recent advances. Whereas traditional microbiology focuses on one organism at a time, NGS has become a routine analysis for profiling large microbial communities.

However, one persistent problem with the microbial profiling workflow is that seemingly small methodological differences may have considerable impact on the results obtained. This workflow includes sample collection, transportation, storage, DNA/RNA extraction, NGS library preparation, sequencing, and bioinformatic analysis. Due to the lack of strict quality control measures and well-established microbiome standards, researchers have found poor data reproducibility between microbiome datasets generated by different labs. To address these challenges, Zymo Research has released several microbiome standards featuring mock microbial communities with pre-defined compositions. One of which is the ZymoBIOMICS Gut Microbiome Standard (D6331), which contains 21 microbial strains to mimic a gut microbiome. The mock community has an accurate pre-defined composition which makes it ideal for assessing the accuracy and bias of a gut microbiome profiling workflow.

Unfortunately, due to the limited strains that can be included in a mock community, a microbiome standard cannot mimic all aspects of a real fecal sample. An important and undeniable feature of a fecal sample is its high microbial diversity, which represents a significant challenge in both wet-lab and dry-lab parts of a microbiome profiling workflow. To address this limitation, Zymo Research has partnered with the BioCollective to introduce the first whole stool microbiome reference material – The ZymoBIOMICS™ Fecal Reference with TruMatrix™ Technology (D6323). 200,000 aliquots have been created that are derived from a huge homogeneous mixture of human feces. Each aliquot contains enough material for 10 uses, allowing for a total of 2 million identical analyses from one sample source. This ensures all sequencing labs have access to the same homogenous human fecal sample.

Characterization

There are several key differences between mock communities such as the ZymoBIOMICS Gut Microbiome Standard (D6331) and microbiome reference materials. Microbiome mock communities are composed of select quantified microbial species, whereas reference materials are native source material, such as feces or soil. The ZymoBIOMICS Fecal Reference with TruMatrix Technology (D6323) consists of real feces with natural diversity. Because it is natural material, and not cultured and quantified in a lab, its true microbial composition is unknown. Thorough sequencing and characterization are required to resolve this complex microbial community and, eventually, with enough depth and consensus, approach a ground truth.

In order to accurately characterize the reference material, an unbiased mechanical lysis process (5 minutes of bead beating with FastPrep-24) was utilized to ensure complete lysis of all microbial cells. DNA and RNA were then extracted and purified, and DNA library preparation was performed using KAPA HyperPlus kit, which we found introduced the least bias among shotgun library prep kits, according to our previous studies. RNA library preparation was performed using Zymo-Seq RiboFree Total RNA Library Kit, and to avoid bias ribosomal RNA depletion was avoided. The DNA and RNA libraries were then sequenced deeply using Illumina sequencing with greater than 30 million reads each. The microbial composition of these two datasets were determined using an in-house bioinformatic pipeline as shown in Figure 1.

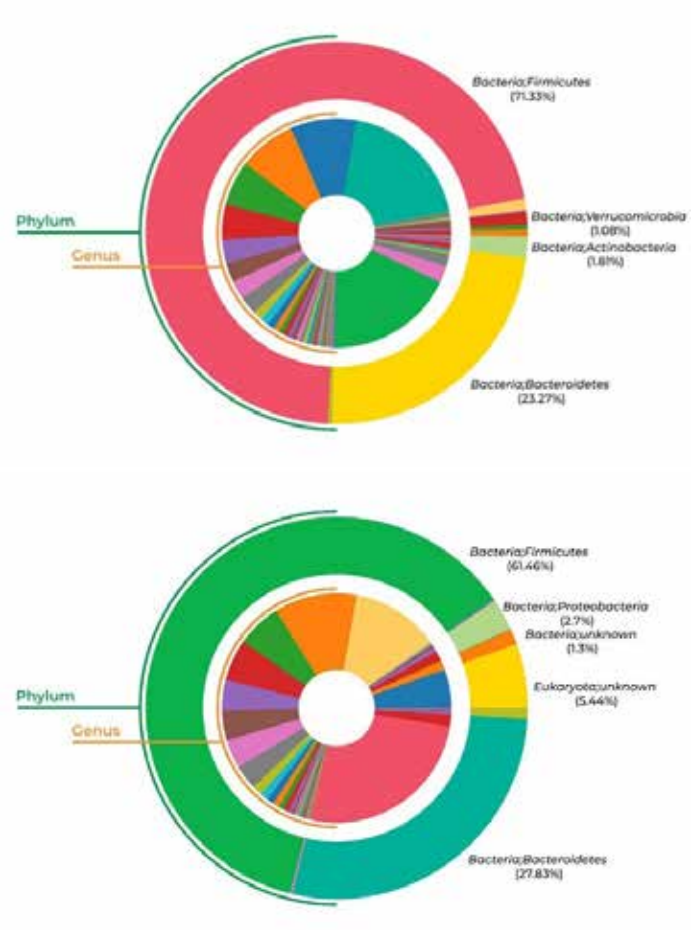


Figure 1 Phylum and genus level taxonomy profiles determined by metagenomic sequencing (top) and metatranscriptomic sequencing (bottom).

Online Portal for Data Sharing

Due to the complexity of this reference material, no single characterization can represent the "ground-truth" composition of the product. The effective profiling of this product requires a joint effort from the whole microbiome research community. Different extraction/purification and library prep methods, sequencing platforms, and bioinformatic tools are required for more exhaustive characterization. Data sharing is necessary and critical to the success of building such a joint effort. To facilitate this, Zymo Research has built an online portal specifically for this fecal reference, to enable sequencing data submission, metadata recording, record searching, and data download. Zymo Research's internal characterization and detailed method description are all in the portal and available to all who sign up. Overtime, as more researchers deposit their characterization data into the portal, this will be an opportunity to approach ground truth and a very valuable public resource for all microbiome researchers. Learn more and access the database at https://www.fecalreferencedb.com/.

Characterization with PacBio HiFi Sequencing

Zymo Research and PacBio have united over a shared goal to advance the field of metagenomics. The collaborative efforts, aim to sequence complex biological samples (e.g. soils and feces), and produce complete genomes for all taxa present in the sample. Zymo Research and PacBio have already achieved over 200 circularized genomes from this fecal reference standard that were assembled using PacBio HiFi sequencing, and additional data is being processed for even greater insight. As both accurate profiling and high molecular weight DNA were considerations, the ZymoBIOMICS DNA Miniprep Kit (D4300) was used for extraction. Lysis was performed with a Vortex Genie II, using 40 minutes of uninterrupted bead beating. The resulting DNA samples have a size of 8-15kb, which can be fed directly into PacBio SMRTbell® library preparation, without additional processing or shearing considerations. Two SMRT cells of PacBio HiFi data were used for the original characterization and additional data generation is still ongoing. The current data is now available in Zymo Research's online public portal.

Assessing Reproducibility and Consistency

The fecal reference material captures the true diversity of a fecal sample, making it ideal for assessing the reproducibility and consistency of a fecal microbiome workflow. It validates microbiome workflows in strictly regulated settings, such as CLIA-CAP and GLP facilities. For example, two studies are provided that assess data reproducibility in a single lab and across multiple labs.

In the first study, DNA extraction was performed from 100 µl of the fecal reference in a single lab. Bead beating was performed on a Vortex Genie II with a horizontal tube adaptor at the maximum speed for 40 minutes and sequenced by 16S rRNA gene sequencing targeting the V3-V4 hypervariable region. This process was repeated six times to collect the data from 6 separate

runs. The microbial composition is shown at the Phylum level and the results show consistent relative abundances across the 6 runs (Figure 2).

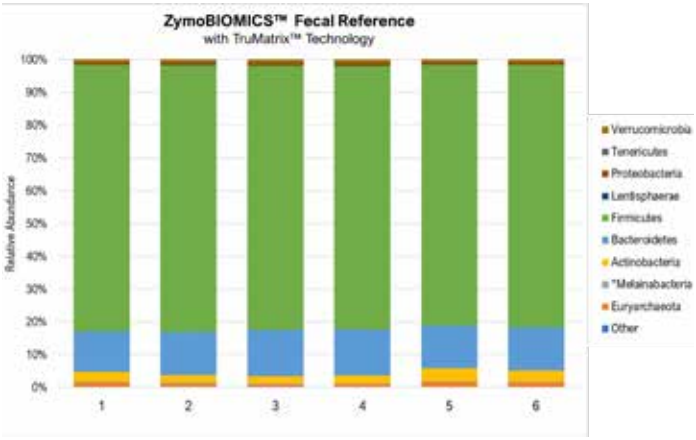


Figure 2: Stability and consistency of taxonomy profiles at the phylum-level of the ZymoBIOMICS™ Fecal Reference with TruMatrix™ Technology across different runs of 16S rRNA gene sequencing

In the second study, the fecal reference was used to assess the consistency of microbiome profiling across labs. Four different labs used the same workflow for microbiome profiling except for a small variation in the microbial lysis process: a different mechanical lysis device was used by each lab. The profile generated from Lab Q appears to have more deviation compared to others. It has a lower abundance of Firmicutes, which are Gram-positive and therefore, generally tougher to lyse compared to Bacteroidetes and similar easy-to-lyse Gram-negative bacteria. This is a common indication that the microbial lysis is incomplete, resulting in the overestimation of the abundance of easy-to-lyse microbes, and underestimation of difficult-to-lyse microbes.

The ZymoBIOMICS Fecal Reference with Trumatrix technology is a true diversity human stool reference material in sufficient quantity for all microbiome researchers to validate and assess consistency of sample processing and compare to other researchers results on the same sample material. The Fecal Reference database prepared specifically for this microbiome reference material provides an easy way to access data of other researchers and share your characterization.

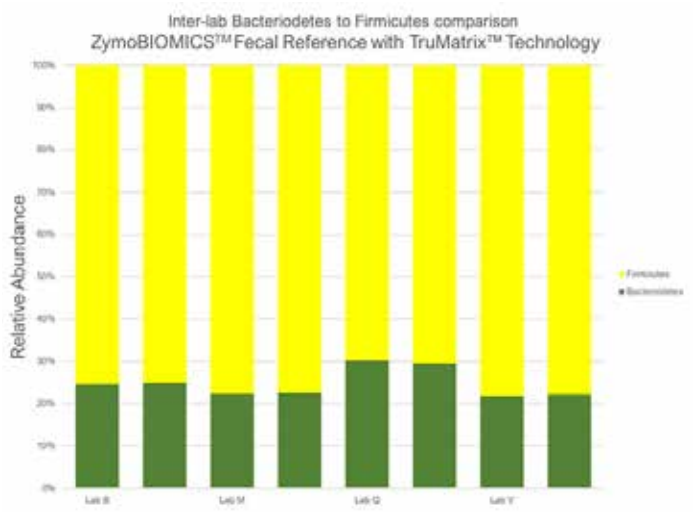


Figure 3: Inter-lab data comparison utilizing different bead beater devices. Phylum-level taxonomic profiles of the ZymoBIOMICS™ Fecal Reference Material with TruMatrix™ Technology were generate with metagenomic sequencing

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Reports

BioTechniques

Prediction of high fecal propionate-to-butyrate ratios using 16S rRNA-based detection of bacterial groups with liquid array diagnostics

Pranvera Hiseni*[,1,2], Lars Snipen[2], Robert C Wilson[3], Kari Furu[1], Finn Terje Hegge[1] & Knut Rudi[2,3]

*[1]Genetic Analysis AS, Kabelgata 8, Oslo, 0580, Norway; [2]Department of Chemistry, Biotechnology & Food Sciences, Norwegian University of Life Sciences, PO Box 5003, Aas, 1432, Norway; [3]Department of Biotechnology, Inland Norway University of Applied Sciences, PO Box 400 Vestad, Elverum, 2418, Norway; *Author for correspondence: Tel.: +47 4864 4835; ph@genetic-analysis.com*

BioTechniques 74: 9–21 (January 2023) 10.2144/btn-2022-0045

First draft submitted: 4 April 2022; Accepted for publication: 5 December 2022; Published online: 5 January 2023

ABSTRACT

Butyrate and propionate represent two of three main short-chain fatty acids produced by the intestinal microbiota. In healthy populations, their levels are reportedly equimolar, whereas a deviation in their ratio has been observed in various diseased cohorts. Monitoring such a ratio represents a valuable metric; however, it remains a challenge to adopt short-chain fatty acid detection techniques in clinical settings because of the volatile nature of these acids. Here we aimed to estimate short-chain fatty acid information indirectly through a novel, simple quantitative PCR-compatible assay (liquid array diagnostics) targeting a limited number of microbiome 16S markers. Utilizing 15 liquid array diagnostics probes to target microbiome markers selected by a model that combines partial least squares and linear discriminant analysis, the classes (normal vs high propionate-to-butyrate ratio) separated at a threshold of 2.6 with a prediction accuracy of 96%.

METHOD SUMMARY

We present a quantitative PCR-compatible test based on the liquid array diagnostics method to be used as a tool for detecting/classifying fecal samples with an atypically high propionate-to-butyrate ratio. The liquid array diagnostics-based test presented here targets the 16S rRNA gene of a limited number of bacterial markers to infer their presence and abundance in fecal samples. The classification of samples (normal vs high propionate-to-butyrate ratio) is performed utilizing an algorithm combining partial least squares and linear discriminant analysis.

KEYWORDS:

butyrate ● gut microbiome ● LAD ● propionate ● qPCR ● SCFA

The human gut microbiome affects the health of the host through a variety of mechanisms, including the fermentation of nondigestible carbohydrates that escape small intestinal digestion and absorption [1]. The end products of this fermentation, short-chain fatty acids (SCFAs), serve a variety of functions, including acting as the main energy source for colonocytes [2], enhancing the intestinal epithelial barrier [3], regulating mucus production [4], modulating inflammatory responses [5], inducing apoptosis in colon cancer cells [6], regulating blood pressure [7], mediating gut–brain cross-talk [8], regulating glucose homeostasis [9] and lipid metabolism and adjusting satiety levels [10].

In healthy adult populations, it is estimated that the three major SCFAs (acetic, propionic and butyric acid) accumulate in a 3:1:1 molar ratio [11–13]. A deviation in such proportions, with a significant decrease in butyrate levels, has been observed in people consuming a diet high in protein and low in carbohydrates [14]. Butyrate production is solely dependent on the intake of nondigestible fiber, whereas the major propionate producers, such as Bacteroidetes, metabolize peptides as well, thus leaving propionate levels unaltered [15]. Lower butyrate levels have also been linked to a slower fecal transition time, and both are associated with a higher colonic pH, which in turn promotes the production of propionate [16]. A low pH environment protects against the overgrowth of pathogens [17]; thus, in this context, an increase in the propionate-to-butyrate (P:B) ratio may indicate a vulnerable gastrointestinal state.

A deviant ratio in favor of propionate was proposed to act as a diagnostic marker for irritable bowel syndrome (IBS) [18]. Increased levels of this acid (but not butyrate) were also reported in overweight and obese people [11], individuals with an increased risk of type 2 diabetes [19], patients with Alzheimer's disease [20] and those with nonalcoholic fatty liver disease [21]. Additionally, a reduced butyrate (but not propionate) concentration was observed in people with a high risk of stroke [22]. Although the evidence linking disproportionately low levels of butyrate and/or high levels of propionate with various diseases is expanding, routine diagnostic measurement for SCFA content remains challenging, mainly due to the high volatility of SCFAs and the complex sample clean-up procedure [23,24].

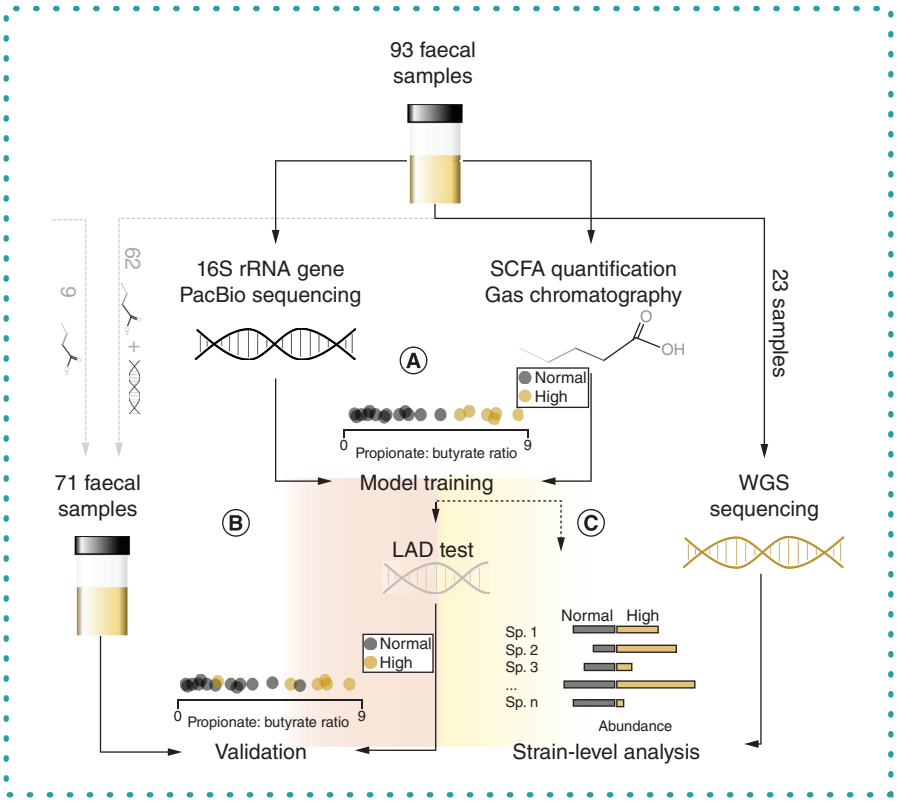

Figure 1. Building and validating a propionate-to-butyrate ratio prediction model. (A) Identification of taxonomic biomarkers for propionate-to-butyrate ratio. In this step, 93 fecal samples were analyzed for both their taxonomic composition (16S rRNA gene sequencing with PacBio single-molecule real-time technology) and SCFA content (GC). A model combining partial least squares and linear discriminant analysis was built, selecting a limited number of operational taxonomic units to act as predictors of normal versus high propionate-to-butyrate ratio. **(B)** Validation of the prediction model using an LAD-based test. In total, 71 fecal samples, nine of which were not PacBio-sequenced, were tested with a set of LAD probes designed to target operational taxonomic units selected by the combined partial least squares and linear discriminant analysis model in the previous step. **(C)** Functional and strain resolution associations with propionate-to-butyrate ratios.
LAD: Liquid array diagnostics; SCFA: Short-chain fatty acid; SP: Species; WGS: Whole-genome shotgun.

Here we aimed to infer SCFA levels by targeting a limited number of key bacteria using a novel quantitative PCR (qPCR)-compatible method, liquid array diagnostics (LAD) [25], circumventing the need to utilize GC-based methods. A LAD test targets variable regions within the 16S rRNA gene and allows the detection of up to 25 bacterial markers in a single tube. We focused on the P:B ratio, a single variable with the potential of providing an indication of functional dysbiosis in clinical samples. The analytical strategy followed in this study is outlined in Figure 1.

Materials & methods

Fecal samples & gDNA extraction

In total, 115 anonymized adult fecal samples, biobanked at Genetic Analysis AS, Oslo, Norway (research biobank no. 4071), were used for this study. Samples were collected and anonymized in accordance with the ruling by the Norwegian Regional Committee for Medical and Health Research Ethics (reference no. 2010/3209).

All fecal samples were stored at -40°C prior to gDNA extraction or GC sample prep. The gDNA of all fecal samples was extracted using a mag midi kit (LGC Biosearch Technologies, Hoddesdon, UK) following the steps suggested by the manufacturer. Genomic extracts were further analyzed with LAD, PacBio single-molecule real-time technology (PacBio, CA, USA) [26], or whole-genome shotgun sequencing (Illumina, CA, USA).

Measurement of SCFA content with GC

The SCFA content of 115 fecal samples was measured with GC (TRACE™ 1310 with autosampler; Thermo Fisher Scientific, MA, USA). Fecal samples were diluted in water (1:10) to a total volume of 1500 µl and then homogenized for 2 × 40 s at 1800 rpm using a Fastprep®-96 (MP Biomedicals, CA, USA). After a gentle spin, 300 µl of supernatant was transferred to a new tube, to which 300 µl of internal standard was added. The internal standard consisted of 0.4% formic acid and 2 mM 2-methylvaleric acid. The samples were centrifuged at 13,000 rpm for 10 min. Subsequently, 300 µl of supernatant was transferred to spin columns (0.2-µm filters) and centrifuged at

10,000 rpm for 5 min. The solution that passed the membrane was transferred to GC vials for SCFA measurement. An internal standard (1 mM 2-methylvaleric acid) was used as a reference for sample-to-sample normalization of results. A total of nine samples did not pass quality control by failing to produce a measurement on acetic acid. Given that this acid is the most volatile, its depletion was taken as an indication that the samples were compromised; therefore, they were excluded from further processing. In addition, a sample erroneously handled during laboratory work was removed. The P:B ratio of the 105 remaining samples was computed and used for further data analyses.

PacBio sequencing of 16S rRNA gene

Ninety-six samples randomly selected from 115 with SCFA content determined by GC were sent for PacBio Sequencing (Full-Length 16S Amplification SMRTbell® Library Preparation and Sequencing) at the Norwegian Sequencing Center (Oslo, Norway) (www.sequencing.uio.no). The first round of amplification was performed using the in-house 16S primers (GA-map® forward primer 5′-TCCTACGGGAGGCAGCAG-3′, GA-map® reverse primer 5′-CGGTTACCTTGTTACGACTT-3′, both protected by patent US20110104692 A1) tailed with universal sequences, as recommended in the PacBio protocol.

On average, there were approximately 28,480 sequencing reads per sample with an average length of 1175 nucleotides. The reads sharing at least 0.97 sequence identity were clustered into operational taxonomic units (OTUs) using the open-source metagenomics tool VSEARCH [27]. Excluding singletons, clustering resulted in 598 total OTUs, and the average number of OTUs per sample was 184. The OTU read counts were centered log ratio-transformed [28] (after the addition of one pseudo-read count) prior to further processing. One of the samples was unfortunately mislabeled when sent for sequencing, and two of the 96 samples did not meet the GC criteria (no measured acetate) and were thus excluded from the downstream analysis.

Identification of bacterial targets through combined partial least squares & linear discriminant analysis modeling

Centered log ratio-transformed OTU read counts from 93 samples were used as input for a combined partial least squares and linear discriminant analysis algorithm, with the aim of selecting variables (OTUs) to act as markers for classifying samples with normal versus high P:B ratios [29]. The aim was to correctly identify and classify the samples with the highest ratios, as they represent the deviation from the norm. The border between the two types of samples (normal vs high ratio) was allowed to go as low as possible without losing model prediction accuracy. The highest accuracy was reached at a P:B border of 2.5, with 37 OTUs acting as predictors, spanning 15 dimensions (leave-one-out cross-validated sensitivity = 90%, specificity = 99%, positive prediction rate = 90% and negative prediction rate = 99%). These OTUs were subsequently considered targets for LAD assay development.

Probe design for LAD

Eight-mer sequences containing a C at their 3′ ends – shared between only 16S *in silico* amplicons of target organisms – were computed using the in-house TNTProbeTool [30]. These sequences were considered the 3′ end segments of potential LAD labeling probes (LPs). Probes had to have a minimum melting temperature (*T*m) of 70°C (computed by the nearest neighbor method) hybridizing to the target group and a maximum *T*m of 30°C hybridizing to a nontarget group. The final LP sequences did not contain a C at their 3′ ends. In this way, the presence of the corresponding bacterial target would ensure that they became extended with a quencher-labeled ddCTP.

A reverse complementary reporter probe (RP) was designed for each of the LPs. The RPs were designed with a fluorophore tag on their 5′ ends, ensuring proximity to the quencher in duplexes harboring a 3′-labeled, RP-complementary LP. Duplexes containing the same fluorophore were designed with varying lengths to produce distinct temperature-dependent signals on the same qPCR channel of detection. The quenching effect of a longer and more stable duplex is observed as a dissociation curve with a higher *T*m. The DNA duplex *T*ms were calculated using the web-based OligoAnalyzer Tool™ 3.1 (Integrated DNA Technologies, Inc., IA, USA) based on the nearest neighbor method.

Basic Local Alignment Search Tool searches (nucleotide collection [nt/nr] database with Blastn) with each OTU sequence as query were performed to infer OTU taxonomy. Initially, 21 probes were designed, covering all 37 OTUs. However, six of the probes, targeting 11 OTUs (*Coprococcus eutactus*, *Alistipes indistinctus*, *Bacteroides eggerthii*, [*Clostridium*] *spiroforme*, *Ruthenibacterium lactatiformans*, [*Clostridium*] *glycyrrhizinilyticum*), failed to produce a signal; therefore, they were excluded from the assay.

Because of sequence similarity between F_3_1 and R_12_1 LPs, which were designed to detect *Dorea longicatena* and *Fusicatenibacter saccharivorans*, respectively, it was impossible to keep them in a single test tube, as this would risk producing double signals when only one target was present. Therefore the test was split into two tubes and the number of probes was divided proportionally between them (eight probes in group 1, seven probes in group 2). We used ROX_12_1 as an RP for both LPs. A list of final probes and their *T*ms and target species are presented in Table 1.

Generation of templates for LAD labeling reaction

Genomic DNA from 71 available samples was PCR-amplified. The SCFA content of these samples had been measured in previous steps; however, nine of the samples were not PacBio-sequenced. Each PCR reaction, with a total volume of 25 µl, consisted of 5 µl bacterial lysate (catalog no. 01-02-00500; Solis BioDyne, Tartu, Estonia), 3.75 U HOT FIREPol® DNA Polymerase (catalog no. 01-02-00500; Solis BioDyne), 1× B1 buffer (catalog no. 01-02-00500; Solis BioDyne), 2.5 mM $MgCl_2$ (catalog no. 01-02-00500; Solis BioDyne), 0.2 mM dNTPs

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Table 1. Liquid array diagnostics test probes.

LP name	LP sequence, 5′–3′	RP name	RP sequence, 5′–3′[†]	*Tm* (°C)	Target species	Group
F_2_8	GCTACACACGTGCTACAATGGCGCATA	FAM_2_8	tTATGCGCCATTG	43.7	*Escherichia coli*	1
F_3_1	CGGGACTGCATTTGGAACTGCTGAG	ROX_12_1	tAGCCAGACAGTTTCCAATGCAGTCCCA	52.9	*Dorea longicatena*	1
H_6_13	GGTGGATGCTGGATGTGGGGAC	HEX_6_13	ttGTCCCCACATCC	45.5	*Bifidobacterium adolescentis*	2
R_9_2	CCGGGACTGCTTTGGAAACTATGCAG	ROX_9_2	ttCTGCATACTTTCCAAAGC	43	*Coprococcus comes*	2
R_10_3	GGAGCGTAGAAGGCATTGCAAGC	ROX_10_3	ttGCTTGCAATGCCTTC	52.8	*Blautia* sp. Marseille-P3313	2
R_12_1	TGGGACTGCATTGGAAACTGTCTGGCT	ROX_12_1	tAGCCAGACAGTTTCCAATGCAGTCCCA	69.3	*Fusicatenibacter saccharivorans*	2
C_14_5	CCCGTCACTCCATGAGAGTTGGAGATAC	CY5_14_5	ttGTATCTCCAACTCTC	45.5	Uncultured bacterium clone AP07S.190	1
C_15_4	CCGTACTGGCTCTGGAAACTGTTCAG	CY5_15_4	ttCTGAACAGTTTCCAGAGC	55.4	*Holdemanella biformis*	1
C_17_1	GGCCACACACGTACTACAATGGTGGTTAA	CY5_17_1	tTTAACCACCATTGTAGTACGTGTGTGG	64.6	*Flavonifractor plautii*, *Flintibacter* sp. KGMB00164	1
C.5_18_8	TGGAAGCCGGGAGTACCTGAAG	CY5.5_18_8	ttCTTCAGGTACcCCC	35.9	*Barnesiella* sp. strain mt172, *Barnesiella* sp. strain mt155	1
C.5_19_2	CGCGAGGGGGAGCAAAACTGGAAAA	CY5.5_19_2	tTTTTCCAGTTTTGC	44.8	Uncultured bacterium isolate DGGE gel band RB1-25	1
C.5_20_2	GCGGACTACTGGGCACCAA	CY5.5_20_2	tTTGGTGCCCAGTAGTC	55.2	*Faecalibacterium prausnitzii*; uncultured bacteria clones 2-002-f10, A3-213 and TS3_a01c08	2
C.5_21_1	GGAAGCGACTGGGCAACCAGAAG	CY5.5_21_1	ttCTTCTGGTTGCCCAGTCGCTTC	64.9	Uncultured organism clone ELU0116-T290-S-NI_000152	1
Fecali291	TTGCTCCACCTCGCGGTCTTGCTTCTCTTTGTTTAA	Fecali291 CY5	TTAAACAAAGAGAAGCAAGACCGCGAGGTGGAGCAA	72.2°C	*Faecalibacterium prausnitzii*; [*Eubacterium*] *siraeum* V10Sc8a; Ruminococcaceae bacterium strain MT139; uncultured bacteria clones PB1_aai26e05, C3-2 16S, A3-213, TS3_a01c08, SJTU_A2_03_71 and A5_016	2
Rum1167	CACTCTAGCCTGACAGTT	Rum1167 CY5	AACTGTCAGGCTAG	47.1°C	[*Ruminococcus*] gnavus; uncultured bacteria clones SJTU_G_10_25, Cadhufec15ml and CFT114H1	2

[†] Lowercase t nucleotides represent 5′-end T-tails. These were introduced with the purpose of securing physical distance between the fluorophore and Gs (within either the RP sequence itself or the G's in the 3′ end of the complementary LP sequence). Gs are known to have an intrinsic quenching property.
LP: Labeling probe; RP: Reporter probe; *Tm*: Melting temperature.

(catalog no. 18427088; Thermo Fisher Scientific) and 0.2 μM in-house primers (GA-map® forward primer 5′-TCCTACGGGAGGCAGCAG-3′, GA-map® reverse primer 5′-CGGTTACCTTGTTACGACTT-3′, both protected by patent US20110104692 A1). The amplification was carried out using an Applied Biosystems Veriti™ Thermal Cycler (Thermo Fisher Scientific) with an initiation period of 15 min at 95°C followed by 30 cycles of 30-s denaturation at 95°C, 30-s annealing at 55°C and 80-s elongation at 72°C, ending with a final step of elongation at 72°C for 7 min. PCR products were then treated with 2.7 U Exonuclease I (catalog no. M0293L; New England Biolabs, MA, USA) and 7.36 U recombinant shrimp alkaline phosphatase (catalog no. M0371L; New England Biolabs) and set for incubation at 37°C for 10 min, followed by 15 min at 80°C to inactivate the enzymes.

Single nucleotide extension of LPs & melting curve analysis with LAD

A total of 10 μl of PCR products treated with Exonuclease I–shrimp alkaline phosphatase (14.5–25.6 ng/μl) were used as templates for LP labeling. The labeling reaction also comprised LPs at a final concentration of 0.1 μM (biomers.net GmbH, Ulm, Germany), 1× buffer C (catalog no. 01-06-00500; Solis BioDyne), 1 mM $MgCl_2$ (catalog no. 01-06-00500; Solis BioDyne), 7.5 U HOT TERMIPol® DNA Polymerase (catalog no. 01-06-00500; Solis BioDyne) and 0.96 μM ddCTP-DYQ660 (catalog no. NU-850–660Q; Jena Bioscience, Jena, Germany). The reaction was performed in a PCR instrument with an initiation step at 95°C for 12 min followed by 40 cycles of denaturation (96°C for 20 s) and annealing/elongation (68°C for 40 s).

Following labeling, a mixture of RPs and $MgCl_2$ was added to the reactions to achieve final concentrations of 0.01 μM and 5 mM, respectively. Reagent S, available from Inland Norway University of Applied Sciences (Hamar, Norway), was also added to a final concentration of 1%. The melting curve analysis (31–85°C) was performed using a CFX96 qPCR instrument (Bio-Rad Laboratories, Inc., CA, USA).

The extraction of peaks and determination of positive signals were performed as described by Hiseni *et al.* [25] with a slight modification. Prior to extracting the signals, the fluorescence measurements within each channel were centered with the purpose of minimizing the range of measurements across wells at any given temperature. Next, the baseline within each channel was corrected (flattened) by subtracting the centered values of each sample from the average no template control centered values. As an ultimate step, for group 1 samples only, a further correction of FAM and CY5 baselines was performed by subtracting the values from one another (FAM = FAM - CY5 and CY5 = CY5 - FAM).

Bioinformatics evaluation of probe specificity

OTU sequences (PacBio) were used as subjects to check for sequences complementary to 3′ C-labeled probes. A search for the occurrence of probes, allowing one mismatch anywhere along the sequence was performed (excluding the probe 3′-C). The intention of this step was to prove that probes precisely targeted the intended bacteria. OTU sequences containing sites complementary to probe sequences were considered to act as 'labeling templates'. The read counts of all such sequences were considered *in silico* signals, which were then used to compute the correlation with real LAD signals.

Whole-genome shotgun sequencing

A total of 24 samples were sent for whole-genome shotgun sequencing at the Norwegian Sequencing Center. Libraries were prepared using a Nextera™ DNA Flex Library Preparation Kit (Illumina) following the protocol recommended by the manufacturer. Samples had different SCFA levels that spanned well the P:B values. One of the samples failed the GC quality check (no measured acetate) and was excluded from further analysis.

Processing of whole-genome shotgun sequencing results

DIAMOND software [31] was used to search for genes related to propionate and butyrate. Raw whole-genome shotgun sequencing reads were used as an input. For propionate, we searched for the genes *mmdA*, *lcdA* and *pduP* (markers for the succinate, acrylate and propanediol pathways, respectively [32]). For butyrate, the process involved searching for *but* and *buk* genes. For each read, only the hit with the highest bit score per pathway was kept (e-value $\leq$1e-05). For each sample, the reads that got a hit with one of the genes were counted and then grouped and summed according to the SCFAs to which they were related. After normalizing for the query sequence size and sequencing depth, the total number of hits related to propionate and butyrate was compared with the relative abundance of these acids. Taxonomic assignment of the sequencing reads was performed with a combination of Kraken2 [33], KrakenUniq [34] and Bracken [35] using HumGut_975 as a custom database, as described by Hiseni et al. [36].

Results & discussion

Identification of taxonomic biomarkers for P:B ratio

We examined the microbiome composition (PacBio sequencing of 16S rRNA gene) and SCFA content of 93 adult fecal samples. The aim was to identify potential associations between different members of the microbiome and levels of propionate and butyrate, and use this information to build a simple, predictive test based on LAD technology.

We computed the correlation between centered log ratio-transformed OTU read counts and relative abundance of propionate and butyrate. Only OTUs with $>$0.2 or $<$-0.2 correlations ($p < 0.05$) were considered. A total of 65 OTUs correlated with propionate levels (39

positively, 26 negatively), and 62 correlated with butyrate levels (28 positively, 34 negatively). Of these, 11 correlated with both butyrate and propionate, albeit in opposite directions. A simplified network of SCFA/OTU relationships is presented in Supplementary Figure 1.

We performed a Basic Local Alignment Search Tool search for highly similar sequences (nucleotide collection [nt/nr] database) using OTU sequences as queries. Among the OTUs positively correlated with butyrate, we found some that shared high sequence identity (>98.5%) with typical butyrate producers, including *Faecalibacterium prausnitzii* [37,38] (correlation = 0.21; $p < 0.05$), *Agathobaculum butyriciproducens* [39] (correlation = 0.23; $p < 0.05$) and *Coprococcus catus* [40] (correlation = 0.21; $p < 0.05$). However, we also found a positive relationship between butyrate and the read counts of sequences sharing high identity (>99%) with *Lactobacillus acidophilus* (correlation = 0.22; $p < 0.05$), *Fusicatenibacter saccharivorans* (correlation = 0.33; $p < 0.005$) and *Blautia wexlerae* (correlation = 0.26; $p < 0.05$) – species not known to produce this acid [41–43]. Furthermore, *Dysosmobacter welbionis* (correlation = -0.25; $p < 0.05$) and *Flavonifractor plautii* (correlation = -0.3; $p < 0.005$) – both butyrate producers [44,45] – exhibited a negative correlation with the relative abundance of butyrate. Similarly, propionate levels did not exclusively correlate with well-described propionate producers.

In light of this complex outcome, we decided to build a model based on a binary classification system (i.e., classifying samples as having a high or normal acid level). Aiming for a simple method, we chose to detect and classify samples based on a single variable that inferred information about both acid concentrations: the P:B ratio. Classification of samples based on this ratio makes biological sense, as the molar ratio between propionate and butyrate in healthy adults is nearly 1.0 [11–13]. Given the role of butyrate in maintaining human health [2,6,46–48], our goal was to detect samples with depleted butyrate levels, inferred by a deviant P:B ratio in favor of propionate (i.e., P:B ratio >>1.0).

We computed the P:B ratio from GC data for all samples. We then built a model combining partial least squares and linear discriminant analysis (PLS + LDA) using OTU read counts as predictors and aimed to find the ratio that best separated the two groups (normal vs high ratio) while selecting a reasonably small number of OTUs to act as markers. These marker OTUs did not exclusively represent propionate and butyrate producers. GC measurements for each sample are presented in Supplementary Table 1, and a list of all OTUs correlated with propionate and/or butyrate is presented in Supplementary Table 2.

Building a predictive LAD-based test

We designed 21 LAD probes to cover the 37 OTUs selected by the combined partial least squares and linear discriminant analysis model, with the intention of converting the dry lab results to a routine molecular diagnostic tool for classification. Six of the probes failed to produce a signal, so they were removed from the assay. The remaining LAD probes were used to analyze 71 random samples, nine of which were not PacBio-sequenced. The performance of the LAD probes is presented in Supplementary Figure 2.

When signals from the 15 LAD probes were used as an input, the best separation, yielding the highest model prediction accuracy (leave-one-out cross-validated), was observed at 2.6 (Figure 2A). This value corresponded well to the value derived by applying a formula designed to find outliers in positively skewed data like ours (i.e., median value + 3 × median absolute deviation → 0.92 + 3 × 0.54 = 2.54) [49]. A detailed distribution of P:B ratios among the samples tested is presented in Figure 2B.

To ensure that a high P:B ratio (≥2.6) implied increased levels of propionate relative to butyrate (and not acetate), we computed the average levels of these acids within the different groups. Indeed, the average butyrate concentration for the normal ratio group was 20%, whereas the average butyrate concentration for the high ratio group was 7.2%. Samples with a normal P:B ratio had, on average, a propionate level of 16.6%, whereas samples with a high P:B ratio had a level of 29.8% (Figure 2C). These results support our theory that a disturbed ratio between propionate and butyrate elucidates information about the levels of both acids.

Validation of the prediction model using LAD

Given the limited number of samples, we validated the LAD test by performing leave-one-out cross-validation; that is, classification of each sample was performed by taking the rest of the samples into account, excluding from the training set the one to be classified. Of nine samples with a P:B ratio ≥2.6, the algorithm correctly classified seven and missed two; however, of 62 samples with a P:B ratio <2.6, 61 were classified correctly (Figure 3). The positive predictive value showed that for any sample classified as having a high ratio, the chance of that sample indeed having a ratio >2.6 was 87.5%. The negative predictive value was 97%. All nine samples that were not PacBio-sequenced and therefore not included in the initial model for selecting OTU markers were correctly classified (all normal ratio).

We acknowledge that the number of tested samples not forming the basis for marker selection by the PLS + LDA algorithm is low (i.e., nine of 71). Therefore, testing of more independent samples will be crucial in the next phase of LAD characterization as well as further development and implementation. However, we here present a solid proof of concept to serve as a foundation for future work.

We do not possess clinical details regarding the individuals whose samples were tested, and that may present another limitation of this study. It would be of particular interest to learn whether these people suffer from health conditions for which high propionate or low butyrate has been reported. Nevertheless, we screened the metadata of 130 samples used by Zeng *et al.*, who reported that significantly increased propionate levels were associated with a high risk of stroke [22]. We found that, on average, people with a low risk of stroke had a P:B ratio <2.6, whereas significantly higher P:B ratios were observed in people with a medium and high risk of stroke (average P:B ratios of 2.04, 3.22 and 2.84 for low, medium and high risk, respectively) ($p < 0.05$).

A P:B ratio threshold of approximately 2.6 was determined using two different approaches (PLS + LDA algorithm and outlier formula). It represents a limit separating normal samples from biological outliers in terms of both SCFAs and microbiome composition. It is

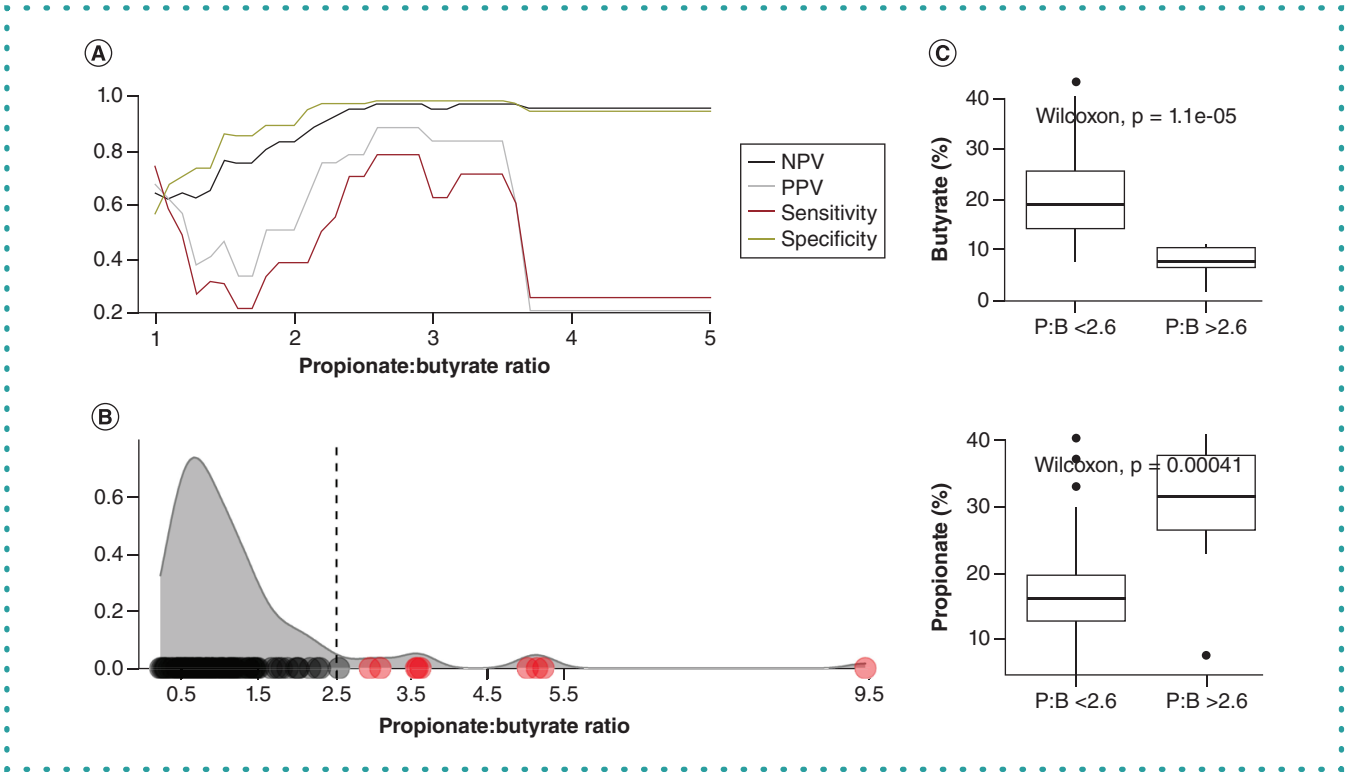

Figure 2. Propionate-to-butyrate ratio model selection. (A) Diagnostic testing accuracy of various P:B ratio thresholds using liquid array diagnostics signals as predictors. Different thresholds were tested to determine the border between high and normal ratio samples for further combined partial least squares and linear discriminant analysis classification. The dashed vertical line depicts the 2.6 ratio, the lowest ratio to yield the highest sensitivity, specificity, NPV and PPV. **(B)** Ratio density among 71 tested samples. Most of the samples (three quartiles) had a ratio <1.5, whereas the median ratio was 0.9. The dashed vertical line at 2.54 separates the outliers from the data (median + 3 × median absolute deviation). Dots along the x-axis show measured ratios for each sample, colored based on classes they belong to according to the combined partial least squares and linear discriminant analysis model (black = normal P:B ratio, red = high P:B ratio). **(C)** Box plots showing difference in distribution of butyrate (upper panel) and propionate (lower panel) levels across two different groups of samples (normal = P:B ratio <2.6, high = P:B ratio >2.6).
NPV: Negative predictive value; P:B: Propionate-to-butyrate; PPV: Positive predictive value.

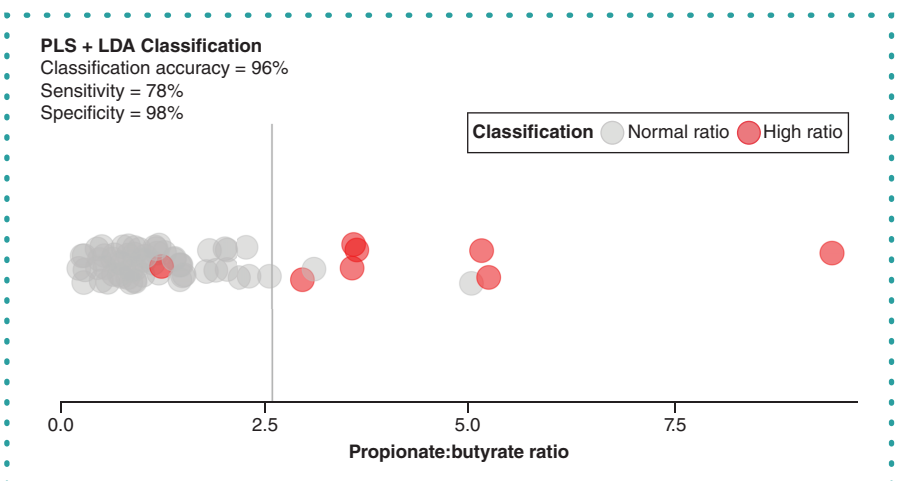

Figure 3. Combined partial least squares and linear discriminant analysis model prediction accuracy with liquid array diagnostics probe signals used as an input. The vertical line placed at 2.6 marks the border between normal and high propionate-to-butyrate ratio. The positioning of each dot shows the real sample ratio (x-axis), whereas the dot color indicates the classification by the model (gray = normal, red = high). Most (seven of nine) samples were correctly classified as having a high ratio (red dots on the right side of the 2.6 border). Only one normal ratio sample was misclassified as a high ratio sample (red dot on the left).
LDA: Linear discriminant analysis; PLS: Partial least squares.

tempting to speculate that this threshold may very well reflect an important biological threshold with a direct implication for the etiology of complex diseases.

Functional & strain resolution associations with P:B ratio

We chose to further analyze 23 randomly selected samples of various P:B ratios (17 normal, six high) by performing whole-genome shotgun sequencing in an attempt to further explore the biological differences between the two classes (i.e., normal and high ratio). On average, samples with a normal P:B ratio displayed 205 species, whereas samples with a high ratio harbored ten fewer species, suggesting a lower diversity in the latter. However, this difference did not exhibit an acceptable significance level ($p > 0.1$).

Looking deeper into the composition, we found that high ratio samples were significantly richer in *Escherichia coli*, *Phocaeicola dorei* (a known propionate producer, formerly named *Bacteroides dorei* [50]), *Enterocloster* sp001517625 (named *Clostridium bouchedurhonense* at National Center for Biotechnology Information), *Blautia_A* sp900066165 and *Anaerotruncus colihominis* (butyrate producer [51]). There was also a tendency for lower richness of *F. prausnitzii_C* (butyrate producer [37,38]) and *Eisenbergiella sp900066775* and higher richness of *Akkermansia muciniphila* (propionate producer [52]) ($p < 0.1$) (Figure 4).

Among these strains, our test is designed to detect both *E. coli* and *F. prausnitzii.* These two species have commonly been found to act as markers in a wide range of diseases [53–55]. Next, we used the sequencing reads to search for genes related to propionate and butyrate production using DIAMOND software [31], and no linear relationship was found (Figure 5).

This finding corroborates those retrieved from PacBio sequencing, where the majority of OTUs correlated to either propionate or butyrate were not known to be producers of such acids. Furthermore, the abundance of bacteria known to produce specific SCFAs was not always in a positive correlation with the fecal levels of such short-chain fatty acids. This was the case with *D. welbionis* and *F. plautii* – both butyrate producers – where relative abundance was found to be negatively correlated with butyrate levels. The latter species was instead found in a positive correlation with propionate levels, and is a target in our assay.

The seemingly complicated relationship between bacterial species and butyrate and propionate levels suggests that levels of SCFAs in fecal samples cannot be inferred by quantifying known acid producers alone, presumably because of the complex cross-feeding mechanisms involved [56]. For example, we believe that the inclusion of *Bifidobacterium adolescentis* (lactate and acetate producer) as a target of our test, is tightly related to cross-feeding between this bacterium and well-described butyrate producers (i.e., the production of butyrate is enhanced by *B. adolescentis* activity) [57,58].

Clinical utility

Currently, it appears that the most relevant clinical application of the P:B ratio would be related to neurodegenerative diseases such as Alzheimer's [20] and Parkinson's [59]. A contributing cause of neurodegenerative disease in elderly individuals is their reduced ability to metabolize propionate as a result of decreased methylmalonyl-CoA mutase activity [60]. This leads to potential accumulation of toxic methylmalonic acid, which has been associated with decreased cognitive function in older adults [61]. By contrast, it has been shown that butyrate, a histone deacetylase inhibitor, can act as a therapeutic agent by reducing levels of abnormally deposited brain amyloid-β [62,63]. Flagging samples with a high P:B ratio in a timely manner would assist clinicians to offer the necessary dietary advice to the elderly.

Other diseases and disorders can also potentially be linked to high P:B levels. An association with a significant propionate increase or butyrate decrease has been reported for the ailments listed in Table 2. The most pronounced association was reported between a high P:B ratio and IBS [18]. In addition to being a biomarker, there could also be a causality between the P:B ratio and IBS severity. Thus, this ratio could potentially have utility in treatment of these patients through, for example, dietary advice.

To the best of our knowledge, detecting samples with high P:B ratios can only be achieved by directly quantifying SCFAs and computing the ratios afterward. Measuring the level of SCFAs in fecal samples is usually accomplished by employing GC, LC, capillary electrophoresis or NMR [68]. However, given the complex sample clean-up and preparation procedure combined with high volatility of these acids, SCFA measurement using today's technology remains a challenging task [23,24,69]. This is why knowledge in the field continues to be derived from fragmented, small-scale studies that are insufficiently standardized across laboratories.

The lack of robust methods for use in clinical settings creates a gap between the state-of-the-art knowledge in the field and its practical utility and application. A simple molecular diagnostic method like the LAD test presented here allows inexpensive, high-throughput screening of fecal samples, bridging this gap. The major benefits of LAD in a clinical setting are related to simplicity and cost as well as the ability to detect the microorganisms underlying the P:B ratio, which in turn can be used in therapeutics.

Our approach offers a solution for at least two problems. First, it focuses on the ratio between propionate and butyrate, ignoring their absolute values, which are known to fluctuate based on the time of day a sample is collected and processed [70]. Second, it circumvents the need to measure SCFA levels, utilizing a robust molecular diagnostic system instead.

We offer an indirect way of detecting both propionate and butyrate levels by identifying biological outliers, that is samples with highest propionate and/or lowest butyrate ratios. The tool we present here is not aimed at replacing other conventional 16S rRNA gene or SCFA analyses; nor does it have the capacity to do so, as it is strictly focused on inferring a narrow segment of microbial functionality. Rather, it represents an applicable solution that integrates both types of methodologies into a single measurement of high clinical utility.

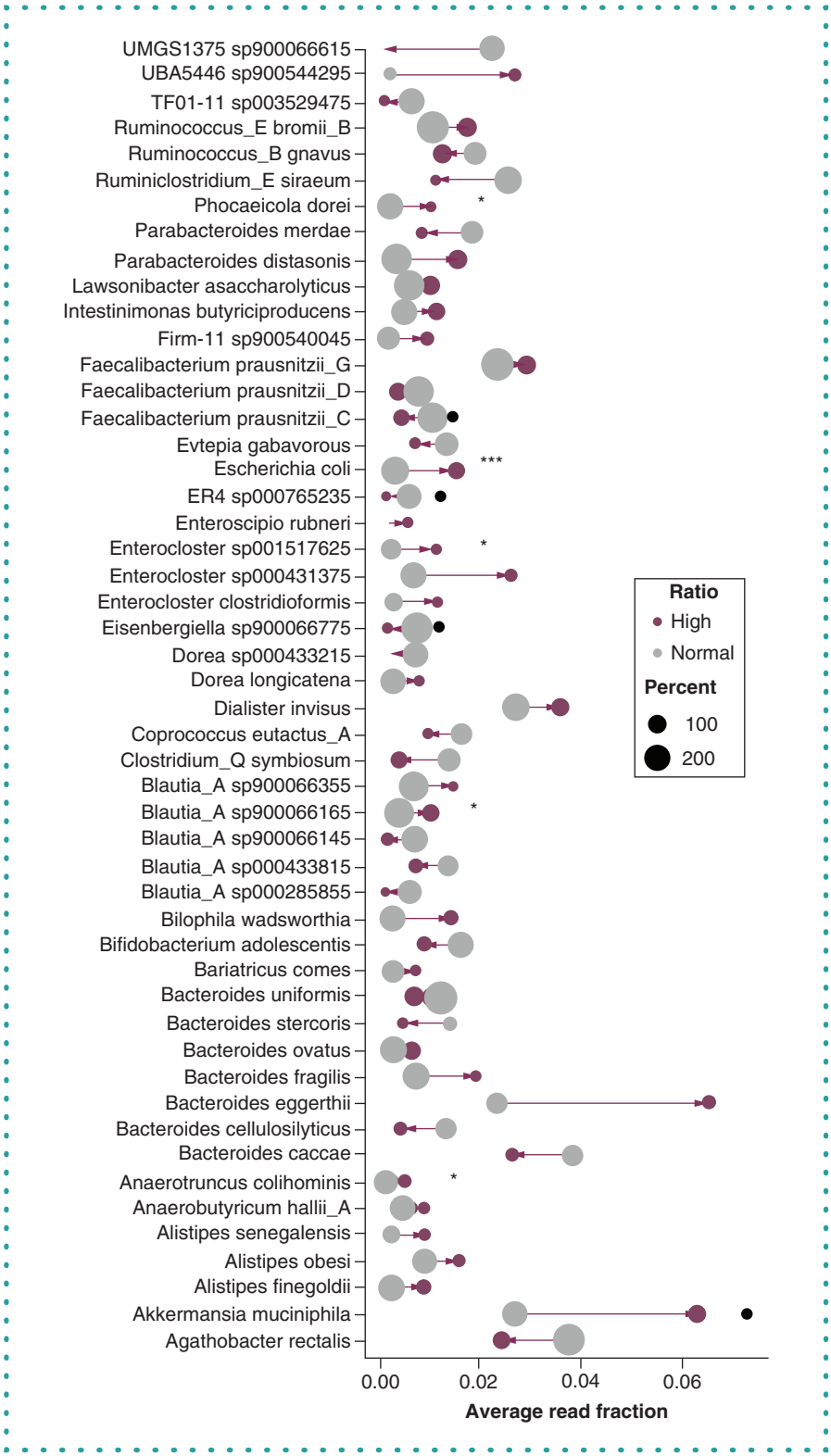

Figure 4. Top 50 species with the greatest difference in average abundance between groups (samples with normal vs high propionate-to-butyrate ratio). Gray circles indicate the average abundance of normal ratio samples, and burgundy represents the abundance of samples with a high ratio. The circle size shows the percentage of samples within the group where the bacterium was found. Arrows point toward samples with a high ratio. Dot and star symbols indicate significant differences as determined by Wilcoxon test.
*$p < 0.05$; ***$p < 0.005$; •$p < 0.1$.

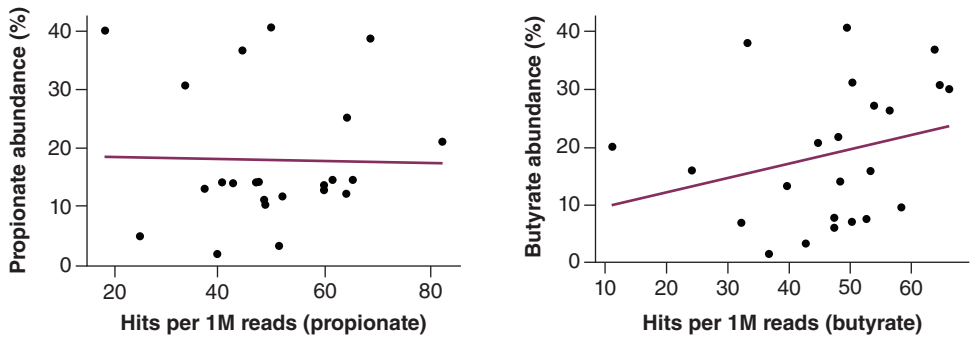

Figure 5. Relationship between relative abundance of propionate and butyrate and corresponding number of reads with a hit (highest bit score e-value ≤1e-05) with respective marker genes. The number of hits was normalized after considering the sequence length of queries and sequencing depth. M: Million.

Table 2. Studies associating diseases with an increase/decrease in fecal short-chain fatty acid levels (high propionate-to-butyrate ratio).

Health disorder	Individuals tested	Significant change compared with controls	Ref.
Obesity	30 lean 35 overweight 33 obese	↑Total SCFAs ↑Propionate	[11]
Type 2 diabetes	952 from LifeLines DEEP cohort	↑Propionate	[19]
NAFLD	27 healthy 32 NAFLD	↑Acetate ↑Propionate	[21]
IBS	25 healthy 25 IBS	↓Butyrate	[18]
Stroke	51 low risk of stroke 54 medium risk of stroke 36 high risk of stroke	↓Butyrate	[22]
ASD	20 healthy 30 ASD	↓Acetate ↓Butyrate ↑Valerate	[64]
CKD	61 healthy 128 CKD	↓Butyrate	[65]
Rett syndrome	29 healthy 50 Rett syndrome	↑Total SCFAs ↑Propionate ↑Isovalerate ↑Isobutyrate	[66]
IBS	26 healthy 26 IBS	↑Total SCFAs ↑Acetate ↑Propionate	[67]
PD	34 healthy 34 PD	↓Total SCFAs ↓Butyrate	[59]

ASD: Autism spectrum disorder; CKD: Chronic kidney disease; IBS: Irritable bowel syndrome; NAFLD: Nonalcoholic fatty liver disease; PD: Parkinson's disease; SCFAs: Short-chain fatty acids.

Conclusion

Here we present a novel qPCR-compatible, single-tube multiplex test that predicts samples with increased ratios of propionate relative to butyrate. Circumventing the need to directly measure the SCFA content in fecal samples, a robust and simple test like LAD will enable high-throughput analysis and regular monitoring of functional dysbiosis in the gut.

Executive summary

- Healthy adult fecal propionate and butyrate levels are expected to be equimolar.
- An increased propionate-to-butyrate ratio has been linked to several health disorders.
- Measurement of levels of short-chain fatty acids is challenging because of their highly volatile nature, presenting a major bottleneck for high-throughput studies.
- The challenges related to short-chain fatty acid measurements create a gap between knowledge acquired in the field and its clinical utility.
- This article presents a method for predicting and classifying samples with significantly elevated propionate-to-butyrate ratios by directly targeting predictor bacteria, circumventing the need to measure short-chain fatty acid levels.
- The method is based on a liquid array diagnostics assay, a quantitative PCR-compatible test capable of detecting multiple targets in a single-tube multiplex reaction.
- The test predicting samples with high propionate-to-butyrate ratios showed 78% sensitivity and 98% specificity (leave-one-out cross-validated).
- The assay presented here has the potential to be utilized in high-throughput studies, validating reported findings in the literature in addition to serving as a robust screening tool for routine diagnostics.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2022-0045

Author contributions

The study was conceived by K Rudi. P Hiseni planned and conducted the lab work. All authors discussed and aided in interpreting the results. P Hiseni wrote the manuscript with equal input from all authors.

Financial & competing interests disclosure

This work was funded by the Norwegian Research Council (research and development grant no. 283783). At the time of writing the manuscript, P Hiseni, K Furu and FT Hegge were employed by Genetic Analysis AS, a licensee of the liquid array diagnostics method. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The collection and handling of fecal samples were performed in accordance with the Norwegian Regional Committee for Medical and Health Research Ethics (reference no. 2010/3209).

Data sharing statement

PacBio and Illumina whole-genome shotgun sequencing data are deposited at Sequence Read Archive (PRJNA820539).

Open access

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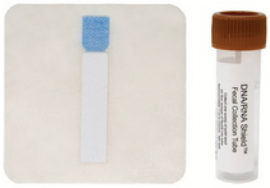

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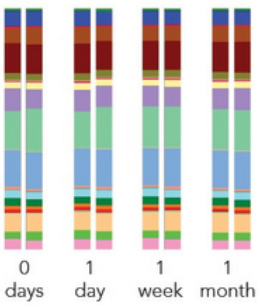

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Sample Collection Products	Cat. No.
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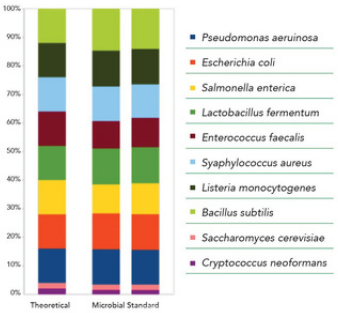

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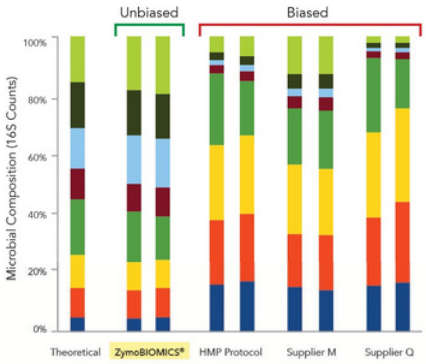

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DNA/RNA Extraction Products	Cat. No.
ZymoBIOMICS™ DNA Miniprep Kit	D4300
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Future MICROBIOLOGY

Vitamin C improves gut *Bifidobacteria* in humans

Sabine Hazan*[,1,2], Sonya Dave[2], Andreas J Papoutsis[1], Nirupama Deshpande[1], Mark C Howell Jr[1] & Leisha MA Martin[3]

[1]ProgenaBIome, LLC, Ventura, CA 93003, USA

[2]Mcrobiome Research Foundation, Ventura, CA 93003, USA,

[3]Texas A&M University – Corpus Christi, Department of Life Sciences, Corpus Christi, TX 78412, USA

*Author for correspondence: DrHazan@ProgenaBiome.com

Aims: Numerous beneficial effects of vitamin C (ascorbic acid) supplementation have been reported in the literature. However, data on its effects toward the gut microbiome are limited. We assessed the effect of vitamin C supplementation on the abundance of beneficial bacterial species in the gut microbiome. **Materials and methods:** Stool samples were analyzed for relative abundance of gut microbiome bacteria using next-generation sequencing-based profiling and metagenomic shotgun analysis. **Results:** Supplementation with vitamin C increased the abundance of bacteria of the genus *Bifidobacterium* ($p = 0.0001$) and affected various species. **Conclusion:** The beneficial effects of vitamin C supplementation may be attributed to modulation of the gut microbiome and the consequent health benefits thereof.

Plain language summary: Vitamin C, also known as ascorbic acid, is used as a supplement for fighting infectious disorders. Many disorders, including COVID-19 and cancer, harmfully disrupt the levels of bacteria that naturally reside in the gut, which may contribute to symptoms. The aim of the study was to understand whether high-dose vitamin C could improve the types of bacteria in the human gut. To do this we characterized the gut bacteria before and after 23 individuals took vitamin C, as prescribed by their respective physicians. We observed that vitamin C increased levels of a gut bacterium called *Bifidobacterium* which has positive health benefits, including fighting infection. This study suggests the possibility that vitamin C could be successful for improving infection outcomes, possibly even COVID-19, partially because it improves the gut bacteria present.

Tweetable abstract: Patients receiving ascorbic acid supplementation had increased abundance of *Bifidobacterium* in their gut microbiome, which may help to explain some of the apparent health benefits and antiviral properties of vitamin C.

First draft submitted: 10 September 2022; Accepted for publication: 3 November 2022; Published online: 8 December 2022

Keywords: ascorbic acid • bacterial diversity • *Bifidobacterium* • gut microbiome • Lachnospiraceae • vitamin C

Eminent chemist and two-time Nobel laureate Linus Pauling's controversial scientific conjecture about the health benefits of vitamin C (ascorbic acid) has been the subject of much debate [1,2]. Pauling's book [1] provoked resentment among several professionals because it was written for the lay public and it presented information that was not widely accepted by the medical establishment [3]. Since then, at least 100 studies have sought to determine the potential role of vitamin C in reducing the incidence, severity or duration of the common cold [3]. Two meta-analyses, published more than 40 years after Pauling's book, reported that vitamin C supplementation only reduced the duration of colds in the general population by an average of 8% [2] and that extra doses of vitamin C given at the onset of cold symptoms could reduce duration and relieve symptoms [4]. The meta-analysis used a cutoff of <0.2 g of vitamin C per day, a dosage much lower than the 2–18 g per day recommended by Pauling [2]. Although a few studies have shown statistically significant reductions in incidence [5–7], a consistent decrease in duration or symptom reduction can be observed in many more studies [5,6,8–21], verifying some of Pauling's claims.

Many studies have also failed to demonstrate beneficial effects, and it may be possible that the public's perception of beneficial effects of vitamin C may be responsible for some of the favorable findings [22].

Low vitamin C concentrations have been reported in cognitively impaired patients, such as those with Alzheimer's disease and dementia [23], and in advanced cancer [24] and severe SARS-CoV-2 infection [25]. Between 0.8 and26% of adults in high-income countries appear to be vitamin C deficient, as defined by levels <11 μmol/l [26]. A US survey found that about 13% of the population was deficient, with the overall occurrence of age-adjusted vitamin C deficiency being closer to 7% and higher among lower socioeconomic classes [27]. However, it has also been suggested that because depletion of tissue stores can happen rapidly, short-term or intermittent vitamin C deficiency prevalence in the population could be much higher [28,29].

An increasing body of evidence has shown that the gut microbiome is a key regulator of immunity and host defense mechanisms. Disturbance of homeostasis involving interactions between the gut microbiome and the immune system can adversely influence resistance to viral infections, increase disease risk and alter neurocognitive function [30–32]. Although previous studies have recognized that vitamin supplementation can alter the gut microbiome, no vitamins are presently classified as prebiotics (agents that promote the growth of beneficial microorganisms in the gut) by the International Scientific Association for Probiotics and Prebiotics [33,34]. Vitamin supplementation in patients with Crohn's disease resulted in an altered gut microbiome composition when patients were administered riboflavin [35] or vitamin D [36]. Several additional studies on vitamin D and the gut microbiome have been performed [37] linking the mucosal immune system and the microbiome in inflammatory bowel disease [38], identifying host–microbe interactions and mutations in the vitamin D receptor as risk factors for inflammatory bowel disease [39] and suggesting that the vitamin D receptor can affect the gut microbiome [40]. Therefore substantial interest in the clinical significance of this finding should prompt further studies on vitamin-mediated modulation of the microbiome for the treatment and prevention of dysbiotic microbiota-related diseases.

Although the most common SARS-CoV-2 infection symptoms are respiratory, SARS-CoV-2 infections also target the gastrointestinal tract; investigations have shown that SARS-CoV-2 infection causes changes to the gut microbiota, including an overall decline in microbial diversity, enrichment of opportunistic pathogens and depletion of beneficial commensal microorganisms [41–45]. It has been found that there is an association between SARS-CoV-2 infection severity and the abundance of certain bacteria, with symptomatic SARS-CoV-2-infected patients having significantly less bacterial diversity and lower relative abundances of *Bifidobacterium* and *Faecalibacterium,* while having increased *Bacteroides* [41,42,45]. This particular dysbiosis pattern may be amenable to pre- or post-infection intervention through probiotic supplementation or fecal microbiota transplantation [41,43].

It has been hypothesized that the use of vitamin C could reduce SARS-CoV-2 infection via its beneficial immunomodulating properties, which include neutralization of the inflammatory response, reduction of oxidative stress and stimulation of antiviral cytokines [46–48]. During viral infections vitamin C has been shown to increase production of α/β interferons and downregulate the production of proinflammatory cytokines such as TNF and IL1-α/β [49,50]. However, more evidence-based clinical data are needed to support these findings.

A recent study by Pham and colleagues compared the effects of colon-targeted vitamins C, B2 and D on the human gut microbiome and reported that vitamin C produced the most distinct effect on the microbiome, increasing microbial alpha-diversity and short-chain fatty acids [51]. However, one meta-analysis found that vitamin C therapy did not reduce major health-related poor outcomes in SARS-CoV-2-infected patients [52]. However this meta-analysis did not consider the gut microbiome, specifically baseline *Bifidobacterium* levels. Also, it has been noted that larger prospective randomized trials are needed to evaluate the effect of isolated vitamin C administration [46–48,52]. It is possible that vitamin C might help in a certain population of SARS-CoV-2-infected patients via modulation of the SARS-CoV-2-induced dysbiotic gut microbiome.

A large variety of factors act to shape and potentially disrupt individuals' microbiomes, such as genetics, aging, diet, infections and medications [53]. We hypothesized that vitamin C administration could modulate the gut microbiome, which is a known regulator of immunity [53–55]. It is possible that such microbiome changes could contribute to protection from viral illnesses associated with microbiome changes, including SARS-CoV-2 infection [41,56,57], and this is worthy of exploration.

Materials & methods

Study design & participants

Twenty-three participants (11 males and 12 females) with varying pre-existing medical conditions who were taking oral vitamin C supplementation daily, prescribed by their primary care or naturopath doctor, were enrolled in our

study to understand how this vitamin consumption was affecting their microbiomes. No subjects were declined for participation, and subjects were recruited from 1 November 2019 until 31 January 2022. The dose of vitamin C ranged from 3 to 25 g/day and was administered either orally (daily) or intravenously (weekly). The duration of vitamin C supplementation varied among the subjects, ranging from 5 days to 1.5 years. There was no other change in the medication regimens of subjects during the period of vitamin C administration.

This study was approved by the 'Ethical and Independent' (E&I) review board (https://eandireview.com/).

Stool sample collection & processing

The procedure for collection and processing of fecal samples was published previously [41]. Stool samples from subjects were collected prior to baseline and 24 h after the last dose of vitamin C was given. Specifically, for baseline, the samples were collected 1 week to 7 months prior to vitamin C administration, which is valid in light of our analysis and data processing methods; specifically, our microbiome data were processed to remove the component of bacteria due to daily dietary and other fluctuations. We have internally validated these methods and demonstrated consistency in subjects' microbiome readings over extended time periods, in the absence of major interventions or disease.

DNA/RNA Shield™ fecal collection tubes (Zymo Research, Cat # R1101, CA, USA) were used to collect 1 ml of fresh stool sample. DNA was then extracted from samples using the QIAmp® PowerFecal® Pro DNA extraction kit (Qiagen, Cat#51804, MD, USA). The isolated DNA was then quantified and normalized for downstream library fabrication using shotgun methodology. The prepared libraries were then pooled and sequenced using NextSeq 500/550 High Output v2.5 300 cycle kit (Illumina, Cat# 20024905, CA, USA) and run on the Illumina NextSeq 550 system as we previously reported [58]. Briefly, run setup parameters on the NextSeq Control Software (Illumina Local Run Manager) included paired-end sequencing set to 150 cycles with both Index 1 and 2 at 10 bp. Sequencing acceptance criteria were a Q-score (AQ30) $\geq$75%, cluster density between 120 and 240 K/mm^2, and clusters passing filter (PF%) $\geq$80%. Following successful next-generation sequencing quality control, sequences were mapped utilizing the minimap2 sequencing alignment tool in One Codex's (CA, USA) bioinformatics analysis pipeline (open source, available at http://github.com/onecodex). A detailed description of the bioinformatics methods is available at http://docs.onecodex.com.

Data analysis

The DNA sequences of microbial strains were analyzed using metagenomic sequencing analysis and then compared for bacterial species present before and after vitamin C supplementation at all taxonomic levels. The data were uploaded to One Codex and analyzed against the One Codex database, which contains more than 115,000 complete microbial reference genomes. During processing, reads were first screened against the human genome, then mapped to the microbial reference database using a k-mer-based classification. Individual sequences (next-generation sequencing read or contig) were compared against the One Codex database by exact alignment using k-mers, where k = 31. Based on the relative frequency, unique k-mers were filtered to control for sequencing or reference genome artifacts. The relative abundance for the microbial taxa was then assessed, based on the depth and coverage for the available reference genomes in the database, as we previously reported [41]. Bacterial diversity was assessed using the Shannon (richness of bacterial composition) and Simpson alpha-diversity (evenness of bacterial composition) indices, calculated at the genus level [59].

The bioinformatic pipeline employed for bacterial identification (One Codex) matches all overlapping k-mers in a given read to the most specific organism and taxonomic level possible. Because not all k-mers are unique to an individual species or strain, each k-mer is classified to the lowest common ancestor within a taxonomic/phylogenetic tree. Finally, aggregated individual k-mers are matched across a given read and the most specific, consistent taxonomic ID is assigned to the read (i.e., the highest weighted root-to-leaf path of k-mer matches across the taxonomic tree). This bioinformatic approach utilizing the lowest common ancestor is our employed operational taxonomic unit, with more stringent criteria to increase taxonomic accuracy.

One Codex calculates relative abundance for all bacteria in each sample, many of which are at practically zero abundance. We collected data on 730 families, 2734 genera and 16,527 species. To focus our analysis, statistical analysis was performed on the families, genera and species studied by Otten *et al.* [60] in their similar, albeit preliminary, study that pioneered the question of vitamin C's effect on the microbiome.

GraphPad Prism v. 9.4 (GraphPad Software, CA, USA) was used for statistical analysis and graphical image generation; p-values were calculated using Wilcoxon matched-pairs signed rank test, and $p < 0.05$ was considered

Table 1. Demographics, clinical characteristics and dosage of vitamin C supplementation in subjects.

Age (years)	Gender	Race	Past medical history	Ascorbic acid dose*	BMI (kg/m^2)
6	M	Lebanese	ASD; allergic to penicillin; tonsils and adenoids removed	12,000 mg/day p.o. for 10 days	18.2
80	F	Caucasian	Bipolar disorder; allergy to sulfa; elevated cholesterol; arthritis; osteopenia; cyst near pancreas	10,000 mg/day p.o. for 5 days	24.1
38	M	Caucasian	Lyme, mast cell and cardiovascular disease; CIDP; appendectomy; sepsis; seizures; kyphoplasty; GERD; cyclic vomiting syndrome; hypertension	10,000 mg/ day p.o. for 5 days	24.4
55	F	Moroccan	Hashimoto's thyroiditis	10,000 mg/day p.o. for 5 days	NA
5	M	Lebanese	Healthy	12,000 mg/day p.o. for 10 days	21.7
34	F	Caucasian	Overweight	10,000 mg/day p.o. for 10 days	27.5
43	F	Caucasian	Healthy	5000 mg/week iv. + 3000 mg/day p.o. for 3 weeks	26.6
53	F	NA	Hypothyroid	10,000 mg/day p.o. for 10 days	NA
27	F	Caucasian/Hispanic	Healthy	5000 mg/day p.o. for 5 days	NA
53	F	Caucasian	Hypothyroid; IBD; PCOS	3000 mg/day p.o. for 1 year	32.0
54	F	Caucasian	Mold illness; cesarean section surgery	15,000 mg/week iv. + 3000 mg/day p.o. for 4 weeks	20.5
57	M	Caucasian	Anxiety; hernia repair; knee, shoulder and forearm surgeries	15,000 mg/week iv. + 3000 mg/day p.o. for 4 weeks	23.2
44	F	Caucasian/Asian	Past SARS-CoV-2 infection; cesarean section surgeries	20,000 mg/week iv. + 3000 mg/day p.o. for 3 weeks	22.3
54	M	Caucasian	Uses alcohol	3000 mg/week p.o. for 1.5 years	34.4
50	M	Caucasian	Anxiety; uses nicotine	10,000 mg/week iv. + 3000 mg/day p.o. for 3 weeks	27.1
54	M	Caucasian	Healthy	5000 mg/week p.o. for 2 weeks	29.0
46	M	Caucasian	Uses nicotine	20,000 mg/week iv. + 3000 mg/day p.o. for 3 weeks	21.8
55	M	Caucasian	Parkinson's disease	20,000 mg/week iv. + 3000 mg/day p.o. for 3 weeks	20.2
58	F	Caucasian	Parkinson's disease	20,000 mg/week iv. + 3000 mg/day p.o. for 3 weeks	20.0
63	M	Caucasian	Guillain–Barré syndrome; bacteremia	20,000 mg/week iv. + 3000 mg/day p.o. for 3 weeks	25.6
61	F	African–American	Low RBC, low WBC; hypothyroid; fibrocystic breasts	20,000 mg/week iv. + 3000 mg/day p.o. for 3 weeks	26.2
73	F	Caucasian	Diverticulitis and irregular heartbeats; hypothyroid	10,000 mg/week iv. + 3000 mg/day p.o. for 3 weeks	24.0
64	M	Caucasian	Colon polyps; hypertension; hypothyroid; high cholesterol; depression; long COVID	25,000 mg/week iv. + 3000 mg/day p.o. for 1 week	35.6

ASD: Autism spectrum disorder; CIDP: Chronic inflammatory demyelinating polyneuropathy; F: Female; GERD: Gastroesophageal reflux disease; IBD: Inflammatory bowel disease; iv.: Intravenous; M: Male; NA: Not available; PCOS: Polycystic ovary syndrome; p.o.: *Per orem*; RBC: Red blood cells; WBC: White blood cells.

statistically significant. All analyses and comparisons were performed for each subject individually, both prior to and after vitamin C supplementation. Mean and standard error of mean (SEM) are used when describing patient characteristics, and median and interquartile range are used when describing changes in relative abundance or diversity of bacteria. When calculating p-values, multiple comparisons were incorporated, using false discovery rate. Fold changes were calculated for each subject, and then the median was found for all subjects for a given bacterium. Individual points with zero initial relative abundance of bacteria were excluded. Throughout the study, relative abundances are presented on a scale of 0–1.

Results

This observational study enrolled 23 participants aged 5–80 years with varying medical conditions who received outpatient care at sites in California or via telehealth. Demographic clinical characteristics of subjects (n = 23) are presented in Table 1. The mean (SEM) age of participants was 49 ± 3.7 years and the mean ± SEM BMI was 25.2 ± 1.1 kg/m^2 (Table 2).

Table 2. Summary of patient demographics and clinical characteristics.

Parameter	Value
Age (years): mean ± SEM; range	49 ± 3.7; 5–80
Male	11 (47.8%)
Female	12 (52.2%)
Relatively healthy†	7 (30.4%)
BMI (kg/m^2): mean +/- SEM; range	25.2 ± 1.1; 18.2–35.6
Race:	
Caucasian	16 (69.5%)
Other	6 (26.1%)
Not specified	1 (4.3%)

†Includes healthy individuals, or those with history of only one or more of the following: hypothyroidism, past surgeries and/or nicotine/alcohol use. Otherwise healthy overweight/obese individuals are not counted as relatively healthy.
SEM: Standard error of the mean.

Changes in relative abundances of bacteria, grouped by rank (family, genus, species) before and a vitamin C administration are listed in Table 3. At the family level, we found that vitamin C supplementation significantly increases the Lachnospiraceae and Bifidobacteriaceae families (Figure 1A). Vitamin C supplementation also appears to alter the degree of abundance of the genus *Bifidobacterium,* as well as the species *Collinsella aerofaciens* and *Barnesiella intestinihominis* (Figure 1B & C). Specific to the *Bifidobacterium* genus, vitamin C supplementation increased the species *Bifidobacterium adolescentis* (Figure 1D).

Changes in relative abundance for all bacteria analyzed, grouped by rank and calculated as median of fold changes for individual subjects, are shown in Figure 2A. Relative abundance of genera of bacteria for each subject before and after and vitamin C administration is shown in Figure 2B. Figure 2B provides an overview, focused on the most common bacterial genera. Figure 2A & Table 3 focus on bacteria of interest, which are similar to the bacteria analyzed by Otten *et al.* [60].

Of note, a few subjects (Figure 2B) appeared to have a sizably different response to vitamin C administration. For instance, subject 19's *Bifidobacterium* relative abundance drastically decreased, while other subjects' abundance all increased. Regardless, the change in *Bifidobacterium* abundance remained highly significant. Likewise, the *Bacteroides* abundance typically decreased after vitamin C administration; however, subjects 4 and, again, 19 showed opposite results.

Three bacterial types were chosen for closer analysis: genera *Bifidobacterium* and *Bacteroides*, and family Lachnospiraceae. A significant increase in abundance of the genus *Bifidobacterium* ($p = 0.0001$) and family Lachnospiraceae ($p = 0.0301$) and a strong trend of decrease in the bacterial genus *Bacteroides* ($p = 0.0501$) were seen after supplementation of vitamin C (Figure 3A–C). Vitamin C supplementation appears to increase the abundance of *Bifidobacterium* approximately threefold higher compared with baseline (Figure 3A).

There was no difference in the Shannon diversity index ($p = 0.7069$) or Simpson index ($p = 0.5839$) pre and post vitamin C supplementation (Figure 4A & B).

Discussion

With the advent of high-throughput sequencing technology and bioinformatics, it is now feasible to explore the function of the gut microbiome at a more detailed level. This observational study explored the effect of the micronutrient vitamin C on the composition and diversity of the gut microbiome. Our results indicate that vitamin C increases the abundance of gut bacteria of the genera *Bifidobacterium*. A study by Otten *et al.* investigated vitamin C supplementation at a dose of 1 g per day for 2 weeks. They observed a more than fourfold increase in the mean relative abundance of *Bifidobacterium*, supporting our findings [60]. Differences between our study cohort and the cohort in that study included the mean age of participants, and participants being moderately active and healthy in Otten *et al*. versus participants with pre-existing medical conditions in this study [60].

Our observational study shows that vitamin C has microbiome-modulating properties, presenting a new potential mechanism for its therapeutic value. The use of probiotics has been shown to result in a statistically significant reduction in the incidence of upper respiratory tract infections ($p = 0.002$) [61]. In another study investigating probiotic treatment, $CD8^+$ cytotoxic T cells and T suppressor cells were enhanced with probiotic treatment during the first 14 days of supplementation [62]. This microbiome mechanism may explain its potential role in improving the common cold and other respiratory viral illnesses.

Table 3. Changes in relative abundances of bacteria, grouped by rank (family, genus, species), before and after vitamin C administration.

Rank		Before vitamin C		After vitamin C		p-value
		Median	IQR	Median	IQR	
Family	Bacteroidaceae	0.1508	(0.0669, 0.2165)	0.1001	(0.0754, 0.1306)	0.1982
	Bifidobacteriaceae	0.0044	(0.0010, 0.0342)	0.0468	(0.0328, 0.0704)	**0.0001**
	Lachnospiraceae	0.1557	(0.1088, 0.2091)	0.1983	(0.1456, 0.2336)	**0.0301**
	Oscillospiraceae	0.0051	(0.0023, 0.0101)	0.0029	(0.0014, 0.0049)	0.2722
	Ruminococcaceae	0.1262	(0.0492, 0.1946)	0.1578	(0.1019, 0.2239)	0.7314
Genus	*Bacteroides*	0.1508	(0.0669, 0.2165)	0.1001	(0.0754, 0.1307)	0.0501
	Bifidobacterium	0.0044	(0.0010, 0.0342)	0.0468	(0.0328, 0.0704)	**0.0001**
	Blautia	0.0247	(0.0113, 0.0332)	0.0301	(0.0179, 0.0542)	0.2726
	Dialister	0.0000	(0.0000, 0.0000)	0.0000	(0.0000, 0.0023)	0.2500
	Enterococcus	0.0000	(0.0000, 0.0001)	0.0000	(0.0000, 0.0000)	0.7695
	Roseburia	0.0251	(0.0113, 0.0406)	0.0138	(0.0083, 0.0365)	0.6650
	Veillonella	0.0000	(0.0000, 0.0002)	0.0000	(0.0000, 0.0002)	0.9219
Species	*Akkermansia muciniphila*	0.0000	(0.0000, 0.0000)	0.0000	(0.0000, 0.0014)	0.8125
	Bacteroides fragilis	0.0003	(0.0000, 0.0024)	0.0003	(0.0000, 0.0012)	0.4954
	Bacteroides vulgatus	0.0350	(0.0023, 0.0679)	0.0370	(0.0094, 0.0677)	0.8288
	Barnesiella intestinihominis	0.0000	(0.0000, 0.0000)	0.0000	(0.0000, 0.0007)	**0.0313**
	Bifidobacterium adolescentis	0.0000	(0.0000, 0.0016)	0.0000	(0.0000, 0.0265)	**0.0029**
	Bifidobacterium animalis	0.0000	(0.0000, 0.0000)	0.0000	(0.0000, 0.0154)	0.0580
	Bifidobacterium bifidum	0.0000	(0.0000, 0.0000)	0.0000	(0.0000, 0.0116)	0.0781
	Bifidobacterium breve	0.0000	(0.0000, 0.0000)	0.0000	(0.0000, 0.0000)	0.3125
	Bifidobacterium dentium	0.0000	(0.0000, 0.0000)	0.0000	(0.0000, 0.0000)	0.7500
	Bifidobacterium longum	0.0022	(0.0000, 0.0160)	0.0068	(0.0000, 0.0235)	0.0507
	Bifidobacterium pseudocatenulatum	0.0000	(0.0000, 0.0004)	0.0000	(0.0000, 0.0007)	0.3828
	Christensenella minuta	0.0000	(0.0000, 0.0000)	0.0000	(0.0000, 0.0000)	0.0625
	Collinsella aerofaciens	0.0000	(0.0000, 0.0063)	0.0000	(0.0000, 0.0266)	**0.0322**
	Faecalibacterium prausnitzii	0.0630	(0.0149, 0.0717)	0.0573	(0.0164, 0.0789)	0.1601
	Gemmiger formicilis	0.0012	(0.0000, 0.0099)	0.0020	(0.0000, 0.0111)	0.4657
	Holdemanella biformis	0.0000	(0.0000, 0.0000)	0.0000	(0.0000, 0.0000)	0.8750
	Intestinibacter bartlettii	0.0000	(0.0000, 0.0005)	0.0001	(0.0000, 0.0020)	0.0785
	Prevotella copri	0.0000	(0.0000, 0.0000)	0.0000	(0.0000, 0.0000)	0.2500
	Streptococcus thermophilus	0.0000	(0.0000, 0.0006)	0.0003	(0.0000, 0.0022)	0.3778

Median and IQR of relative abundance (depicted on a scale 0–1) are shown, with bold p-values and bacterial names indicative of significant change.
IQR: Interquartile range.

Members of the genus *Bifidobacterium* are considered beneficial bacteria and are an indicator of a healthy gut [54]. *Bifidobacterium* are among the first microbes to colonize the human gastrointestinal tract [54,63] and are used as probiotics due to their health-promoting properties [64]. *Bifidobacterium* plays a role in several beneficial functions such as increased ATP production, modulation of the immune system, mucosal barrier integrity and production of short-chain fatty acids [54,55,64–66].

The administration of probiotics containing *Lactobacillus gasseri* PA 16/8, *Bifidobacterium longum* SP 07/3, *Bifidobacterium bifidum* MF 20/5 (5 × 107 CFU/tablet) for at least 3 months has been shown to reduce the severity of cold symptoms and shorten the duration by almost 2 days [62]. Several studies in mice have revealed that *Bifidobacterium* can protect mice from influenza infection by increasing anti-influenza IgG [67], balancing Th1/Th2 responses against infection and decreasing IL-6 production in the lungs, leading to increased survival rates [68]. *Bifidobacterium* also mediates anti-influenza effects through the production of the metabolites valine and coenzyme A [69]. A human trial demonstrated that oral administration of *B. longum* BB536 resulted in lower influenza infection rates in the elderly [70]. Neutrophil, phagocytic cell and NK cell activity remained higher through the end of the study in the BB536 group compared with the control group. Recently, studies have reported a decrease in abundance of *Bifidobacterium* associated with SARS-CoV-2 infection and severity [41,56,57]. The promising results

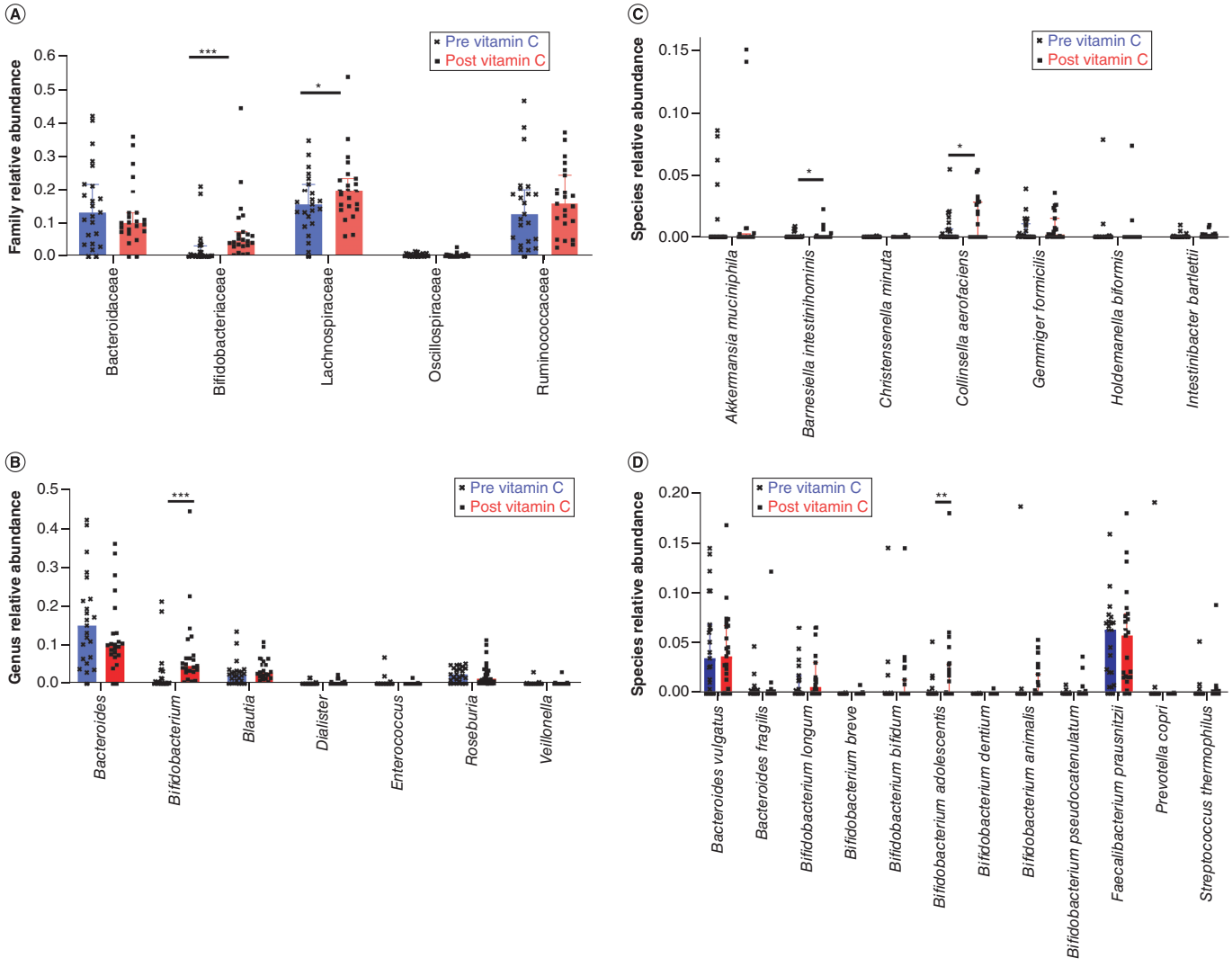

Figure 1. Relative abundances (expressed as a fraction) of bacteria, grouped by rank. (A) Family. **(B)** Genus. **(C & D)** Species. Bars indicate median + interquartile range. Symbols indicate individual points: x = prior to vitamin C, square = after vitamin C.
*p < 0.05; **p<0.01; ***p < 0.001.

of recent preclinical and clinical trials investigating dietary supplementation of probiotics, including *Bifidobacteria*, for treating SARS-CoV-2 infection have provided hope that these bacteria can be an important means to fight SARS-CoV-2 infection; however, further work is needed [43,71–73]. Moreover, this study would suggest vitamin C could be used for the process of restoring microbe levels, such as *Bifidobacterium*, a process we call Refloralization™.

Work by Shu *et al.* [74] found that vitamin C concentration can increase the growth of *B. bifidum* BB01 and BB02 *in vitro*; however, it is also affected by many factors, including oxygen levels and pH. However, obligate anaerobes are especially susceptible to oxidative stress, and the presence of oxygen can severely compromise the growth of *Bifidobacterium* [75–79]. The redox potential of the gut is linked to the ratio of aerobic or facultative anaerobic and anaerobic species [77]. As an antioxidant, vitamin C would be expected to exert a direct effect on the redox balance in the gut and may modulate the microbiome via this mechanism. It is important to note that electrons from ascorbate also have the capacity to reduce metals, some of which may be present in the stool (e.g., iron and copper), which may lead to the production of superoxide and hydrogen peroxide and the subsequent generation of reactive oxygen species when high concentrations are achieved [80,81]. More work is needed to determine the concentrations of ascorbate in the gut that are achieved by supplementation, the role of the route of administration (oral vs intravenous) and how this influences the redox potential, ascorbate's antioxidant versus pro-oxidant behavior and, ultimately, the gut microbiome and metabolomics.

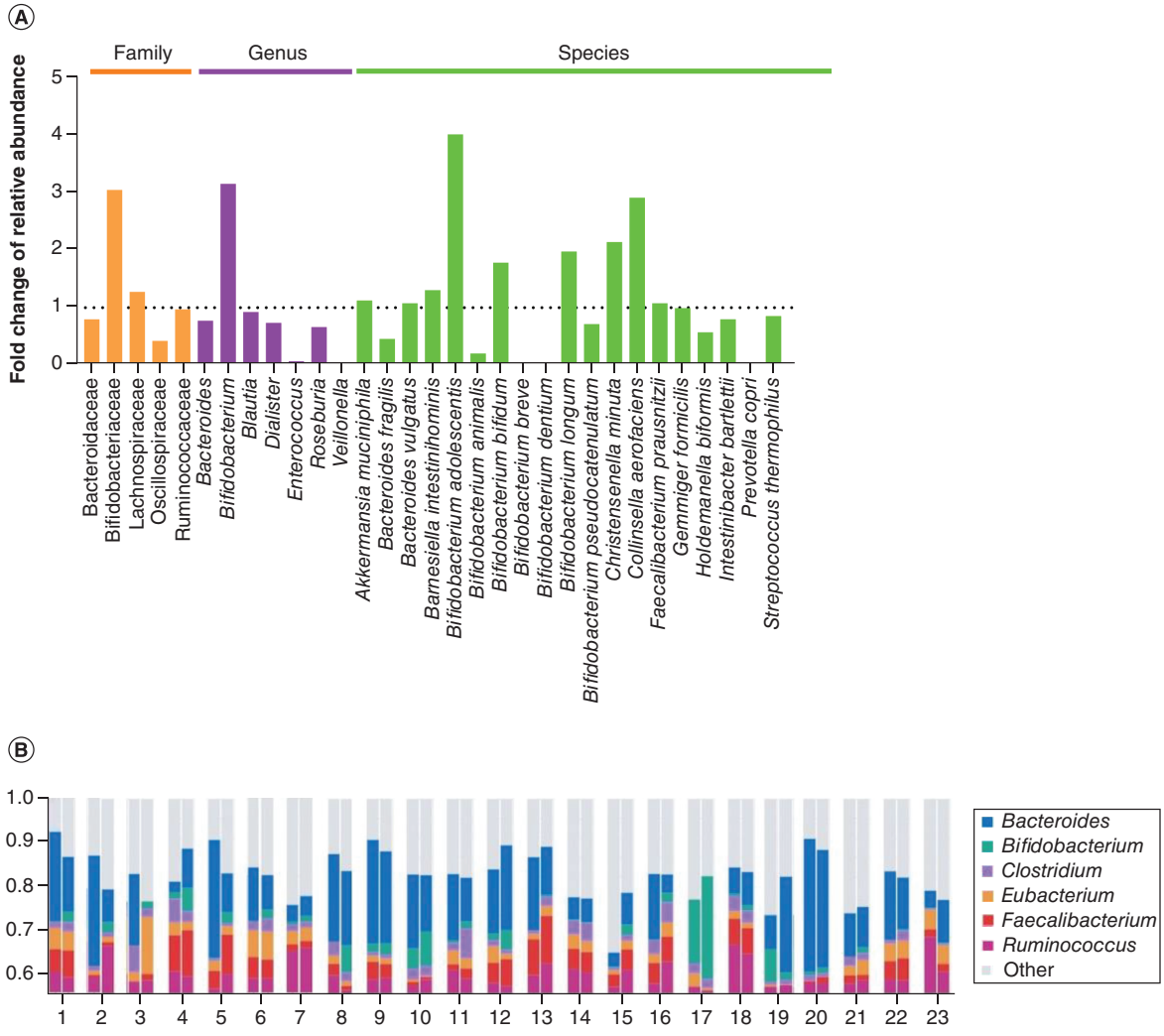

Figure 2. Changes in relative abundance of bacteria in response to Vitamin C adminstration. (A) Fold change of relative abundance for all bacteria analyzed, calculated as median of fold changes for individual subjects. **(B)** Relative abundance of genera of bacteria for each subject (left) before and (right) after vitamin C administration.

Also consistent with the study by Otten *et al.*, we observed an increase in the relative abundance of the family Lachnospiraceae. Lachnospiraceae are the predominant bacteria which likely produce short-chain fatty acids in the gut of healthy subjects [82]. Increased populations of Lachnospiraceae may be associated with markers of good health, such as improved epigenetic states, increased fatty acid metabolism and decreased inflammatory markers [82]. Nonetheless, when one examines Lachnospiraceae in various human diseases and disease models, the results prove more complex: either high or low levels are associated with the presence of various disorders [82]. Whether the increase in Lachnospiraceae due to vitamin C is beneficial or harmful would require further studies, and may depend on the individual's diet, microbiome, health history and other parameters.

Gut microbiome diversity and mammalian microbial co-metabolism are closely interconnected with overall human health and wellbeing. Reduced levels of microbial diversity are linked to several acute and chronic diseases, including SARS-CoV-2 infection [41,42,83]. The study by Pham *et al.* that investigated colon-targeted vitamins found that vitamin C significantly increased microbial alpha-diversity and composition [51]. Although we observed no changes in the overall diversity of the gut flora post vitamin C supplementation, specific supplementation parameters may make it possible to achieve this effect.

Given that this was an observational study and vitamin C was being administered for clinical as well as research purposes, there was a sizable variation in not only the length, route of administration and dose of vitamin C, but also the ages, weights and medical histories of the patients. As such, these parameters are worth discussing. One

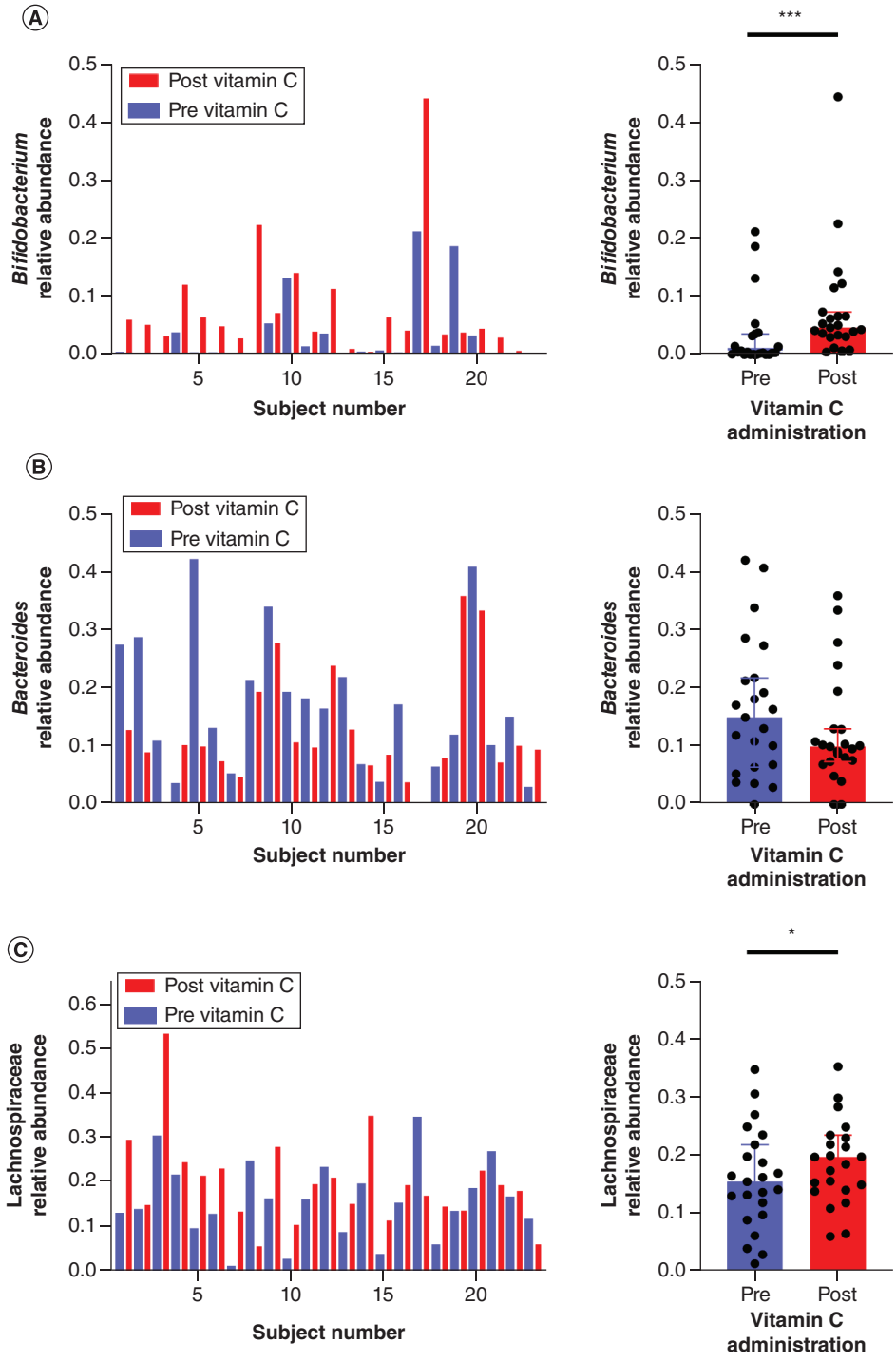

Figure 3. Relative abundance of bacteria for organisms potentially affected by vitamin C. Relative abundance before and after vitamin C administration shown for individual subjects for **(A)** *Bifidobacterium* (p = 0.0001), **(B)** *Bacteroides* (p = 0.0501) and **(C)** Lachnospiraceae (p = 0.0301). Graphs on right panel plot median + interquartile range.
*p < 0.05; ***p < 0.001.

systematic review and meta-analysis of six randomized controlled clinical trials involving a total of 572 SARS-CoV-2-infected patients reported dosing of vitamin C ranging from 50 mg/kg/day to 24 g/day, with routes of administration including both intravenous (four studies) and oral (two studies) [52]. This meta-analysis showed that administration of vitamin C did not have any effect on major health outcomes (positive or negative) in

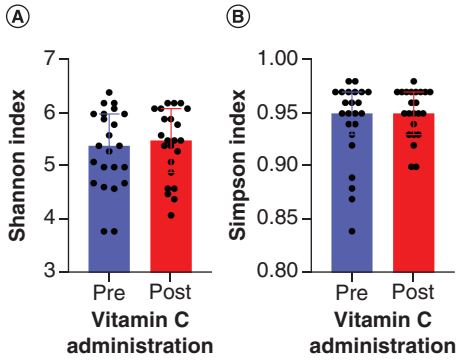

Figure 4. Alpha diversity at the genus level. As measured by **(A)** Shannon diversity index (p = 0.7069) and **(B)** Simpson diversity index (p = 0.5839) for each subject before and after vitamin C administration.

SARS-CoV-2-infected patients, compared with either placebo or standard therapy, irrespective of its dosage, route of administration and disease severity. A recent phase I clinical trial found that intravenous vitamin C, administered in a dose of 1.5 g/kg three times weekly, appears to be safe and essentially free of adverse side effects [84].

Our study observed both intravenous and orally administered dosages, which likely have different pharmacokinetic profiles. Recent pharmacological modeling revealed that orally administered vitamin C, even at very large and frequent dosing, will increase plasma concentrations only modestly, from 0.07 to a maximum of 0.22 mM, while intravenously infused doses were predicted to result in peak plasma vitamin C levels over 60-times higher and urine concentrations 140-fold higher than oral doses, via pharmacokinetic modeling [85]. The actual bioavailability of the molecule is controlled by numerous factors, including absorption by the intestine and other tissues, kidney absorption and excretion, and other patient-specific factors [86–90]. Studies of supplemental iron showed that oral iron affects the diversity of gut bacteria and the production of gut metabolites differently compared with intravenous iron [91,92]. In addition to the differences in concentration delivered via intravenous versus oral administration, oral administration delivers vitamin C directly to the gut microbiome, as opposed to via the bloodstream; thus its effect on the gut microbes could relate to kinetics and concentrations completely different from those affecting plasma levels. More work is needed to determine routes of administration and dosage schedules that achieve optimum, persistent vitamin C concentrations and a consistent physiological effect in the gut. Despite the potential for variation in results due to methods of administration, we observed clear statistical differences in the relative abundances of the genus *Bifidobacteria*.

Although the sample size available was small, we were able to observe statistically significant alterations in two bacteria present in the stool samples. Despite having a range of participants who varied in terms of age, gender, ethnicity, route of administration and pre-existing medical conditions, these trends were clearly identifiable. A larger-scale study would allow us to explore these correlations in more detail. Our use of metagenomic sequencing methods (as opposed to 16s ribosome sequencing) is a less commonly used approach that may allow for a more detailed characterization of the microbiome, including possible physiologically important components [93]. Future studies should seek to identify differences based on BMI, gender, diet and particular medical conditions. Specific factors that may affect the microbiome results are vitamin C supplementation dose and duration, blood and baseline vitamin C levels and the contribution of diet to overall vitamin C intake. Future studies should also look at disease-specific effects, such as the effects on prevention or reduction of respiratory viral illness.

Conclusion

Vitamin C as a therapeutic agent should be explored specifically for its potential to reverse or ameliorate disorders linked to microbiome dysbiosis, especially *Bifidobacterium* deficiencies. It may be able to restore the gut microbiome (i.e. carry out Refloralization) after *Bifidobacterium* depletion due to various conditions or acute illness, including respiratory viral illnesses such as SARS-CoV-2 infection.

In conclusion, we postulate that the microbiome, specifically *Bifidobacterium*, is a mediator of the numerous reported beneficial effects of supplementation and mega-dose administration of vitamin C and that vitamin C supplementation can be used to restore *Bifidobacterium*. Longer-term and larger studies are still needed to understand the effects of vitamin C on the microbiome. This study also points out the need for the measurement of baseline *Bifidobacterium* levels in placebo-controlled trials, to compare similar populations.

Summary points

- Low vitamin C concentrations have been reported in cognitively impaired patients, such as those with Alzheimer's disease and dementia, and in patients with advanced cancer and severe SARS-CoV-2 infection.
- We hypothesized that vitamin C administration could modulate the gut microbiome contributing to protection from severe outcomes associated with viral illness, including SARS-CoV-2 infection.
- Supplementation with vitamin C increased the relative abundance of bacteria of the genus *Bifidobacterium*. Families Lachnospiraceae and Bifidobacteriaceae also significantly increased, and various species changed.
- Our observational study shows that vitamin C has microbiome-modulating properties, presenting a new potential mechanism for its therapeutic value.
- Moreover, the data demonstrate that vitamin C has a potential for creating Refloralization™ (restoration of the human gut microbiome) after *Bifidobacterium* depletion.

Author contributions

S Hazan: conceptualization, data curation, investigation, methodology, project administration, resources, software, formal analysis, writing (original draft), writing (review and editing). S Dave: formal analysis, investigation, software, writing (original draft), writing (review and editing). L Martin: investigation, writing (review and editing). M Howell: writing (review and editing). N Deshpande: writing (original draft), writing (review and editing). A Papoutsis: writing (review and editing), formal analysis

Acknowledgments

The authors would like to thank K Hendricks for writing and editorial support and helpful discussion.

Financial & competing interests disclosure

S Hazan declares that she has pecuniary interest in Topelia Pty Ltd in Australia and Topelia Pty Ltd in USA. She is the founder and owner of Microbiome Research Foundation, ProgenaBiome and Ventura Clinical Trials. S Dave declares she has corporate affiliation to McKesson Specialty Health/Ontada and North End Advisory, LLC. A Papoutsis declares he has corporate affiliation to ProgenaBiome, LLC. N Deshpande and M Howell declare they have corporate affiliation to North End Advisory, LLC. L Martin declares corporate affiliation with LEI NanoTech, LLC and MNT SmartSolutions, LLC. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Medical writing support was provided by Dr. Sonya Dave of North End Advisory, LLC and was funded by the corresponding author and her institutions, ProgenaBiome, LLC and Microbiome Research Foundation. Additional medical writing support was provided by Dr. Nirupama Deshpande and Dr. Mark Howell, also of North End Advisory, LLC and were funded by ProgenaBiome, LLC.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval (Ethical and Independent IRB: study number 20110) and have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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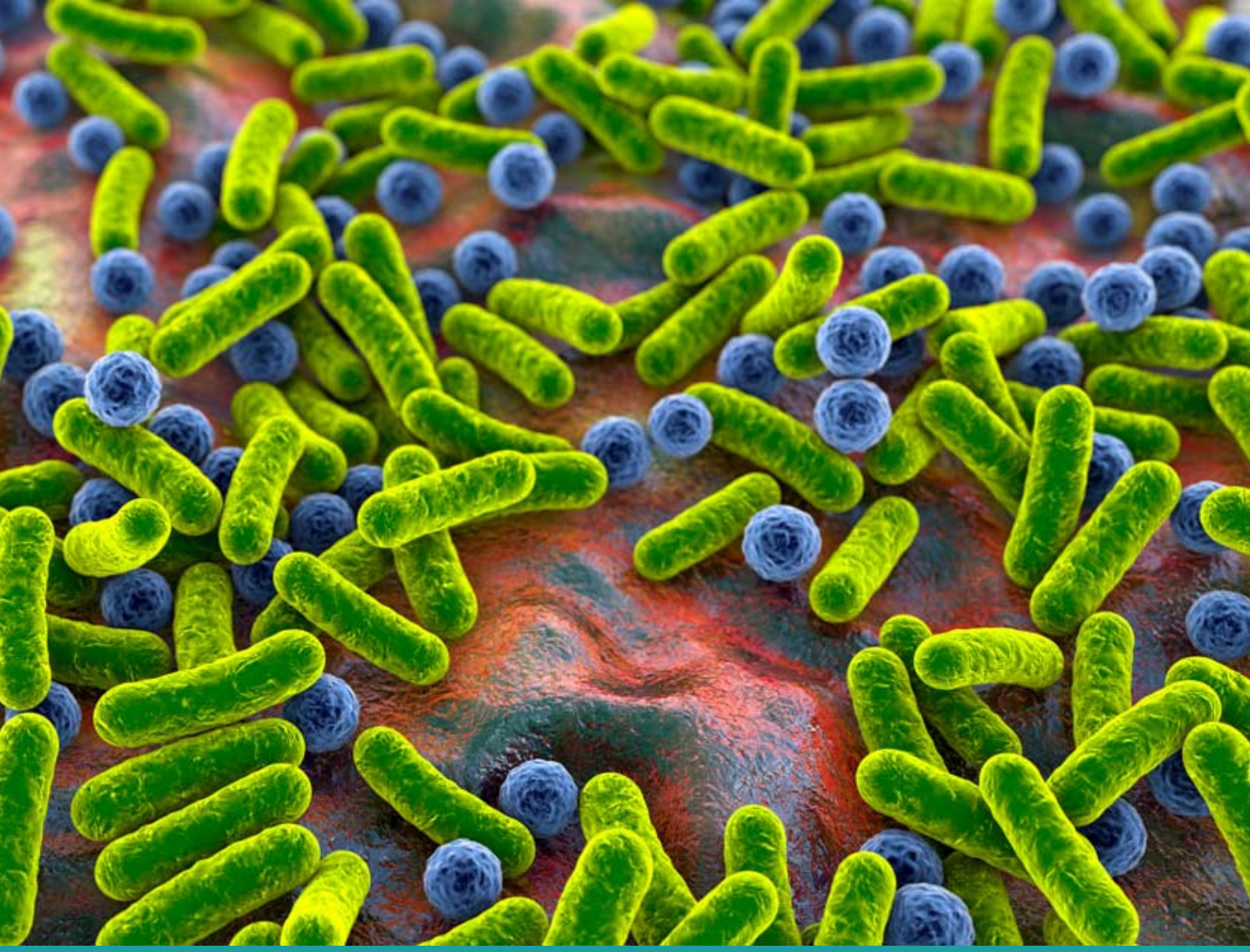

Contact us

Editorial Department

Senior Editor
Tristan Free
tfree@biotechniques.com

Business Development and Support

Commercial Director
Evelina Rubio Hakansson
e.rubiohakansson@future-science-group.com

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