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Sequencing the Future of Probiotics: A Practical Handbook to Next Generation Testing for Safety and Quality

APEC Sub-Committee on Standards and Conformance

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Expert Committee on Application of Next-Generation-Sequencing in Probiotics Testing for Quality and Safety Assurance
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List of Abbreviations

16S	16S ribosomal RNA
ANI	Average Nucleotide Identity
APEC	Asia-Pacific Economic Cooperation
ASV	Amplicon Sequence Variant
BL medium	Blood-liver medium
bp	Base pair
BP	British Pharmacopoeia
CBER	Center for Biologics Evaluation and Research
CFSAN	Center for Food Safety and Applied Nutrition
CFU	Colony forming Unit
cgMLST	Core Genome Multilocus Sequence Typing
CLI	Command Line Interface
CRC	Colorectal Cancer
DBT	Department of Biotechnology
DNA	Deoxyribose Nucleic Acid
FDA	Food and Drug Administration
FFC	Food with Functional Claims
FGD	Focus Group Discussion
FOSHU	Food for Specified Health Uses
g	gram
GRAS	Generally Recognized as Safe
ICMR	Indian Council of Medical Research
IDF	International Dairy Federation
ISO	International Standards Organisation
LBP	Live Biotherapeutics
LC-MS	Liquid chromatography-mass spectrometry
LC-MS	Liquid Chromatography-Mass Spectrometry
MALDI -TOF	Matrix-Assisted Laser Desorption Ionization–Time-of-Flight Mass Spectrometry
MFDA	Ministry of Food and Drug Safety
MIC	Minimum inhibitory concentration
mL	millilitre
MRS	De Man Rogosa Sharpe
NADFC	National Agency of Drug and Food Control
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NHC	National Health Commissions of the People's Republic of China
NMDS	Non-Metric Multidimensional Scaling
ONT	Oxford Nanopore Technology

ORF	Open Reading Frame
PacBio	Pacific Biosciences
PCA	Principal Component Analysis
PCoA	Principal Coordinate Analysis
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel-Electrophoresis
PLS-DA	Partial-Least-Squares Discriminant Analysis
PRC	People's Republic of China
qPCR	Quantitative polymerase chain reaction
R&D	Research and Development
RNA	Ribonucleic Acid
SAMR	China State Administration for Market Regulation
SAMR	China State Administration for Market Regulation
SCSC	Sub Committee on Standards and Conformance
SFDA	China State Food and Drug Administration
SNP	Single Nucleotide Polymorphism
SOP	Standard Operating Procedure
spp.	Species
TGS	Third Generation Sequencing
UNEP	United Nations Environment Programme
UPGMA	Unweighted Pair-group Method with Arithmetic Means
US	United States
US FDA	United States Food and Drug Administration
USP	United States Pharmacopoeia
wgANI	Whole Genome Average Nucleotide Identity
wgMLST	Whole Genome Multilocus Sequence Typing
WGS	Whole Genome Sequencing
wgSNP	Whole Genome Single Nucleotide Polymorphism

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Executive Summary

The rapid growth of the global probiotics market, currently valued at approximately USD75 billion annually, is indicative of the increasing recognition of probiotics' health benefits. It is projected that by 2024, over 12 million tons of probiotic products will be consumed. The high demand for these products is driven by various factors, including increasing awareness of the benefits of probiotics for human health, as well as the diverse availability of probiotic products, ranging from supplements to yoghurt, ice cream, and infant formula. However, this vibrant market landscape is characterized by intense competition, motivating companies to distinguish themselves by offering products with diverse probiotic strains.

While probiotics are generally recognized as safe for consumption, there are risks associated with unknown or less-studied strains, genetic mutations, and contamination by harmful microbes. Therefore, it is essential to ensure that probiotic food products are safe to consume and meet quality requirements. To this end, food safety authorities perform lab analysis to verify the contents as stated on the labels.

Existing laboratory analysis of probiotic products, rooted in culture-dependent approach, often proves time-consuming, costly, inaccurate and inefficient, particularly for novel products with new probiotic strains. Additionally, the use of different standard methods such as ISO15214, ISO20128, and ISO27205 further compounds confusion and hampers compatibility with emerging products. In response to the mounting global demand and trade volume of probiotic products, the need for rapid, efficient, and standardized product testing has become increasingly pronounced.

Emerging as a transformative solution, Next-Generation Sequencing (NGS) technology promises to transcend the limitations of current culture-dependent methods. This innovative approach empowers comprehensive analysis of the entire microbial community in a single test, capable of identifying known and novel microorganisms, genetic mutations, and harmful microbial contaminants.

The "Sequencing the Future of Probiotics" handbook is the culmination of a collaborative effort involving experts in probiotics and next-generation sequencing (NGS) technologies from APEC and non-APEC economies. This comprehensive guide offers invaluable insights into the rapidly evolving landscape of probiotics, with a particular focus on NGS-based testing for safety and quality assurance.

The Handbook's Genesis

Funded by APEC (SCSC 04 2021A), this project brought together leading minds in probiotics and NGS applications. The goal was to identify best practices and recommend protocols for NGS-based probiotics testing in the laboratory. The handbook is the tangible outcome of this collective expertise and effort.

Navigating the Handbook

This comprehensive handbook is organized into four main sections, each offering a unique perspective on the world of probiotics and the revolutionary impact of next-generation sequencing (NGS) technology. These sections are thoughtfully designed to guide readers from the fundamentals to advanced applications:

- **Section A: Probiotics Landscape**
  In this initial section, readers will delve into the essence of probiotics, making it accessible for newcomers while offering valuable insights to experts in the field.

- **Section B: Probiotics Testing**
  The heart of this handbook lies within this section. The second section equips readers with an in-depth exploration of probiotic testing methodologies, empowering you to navigate challenges effectively.

- **Section C: NGS Application in Probiotics and Microbiome Research**
  The third section allow readers to explore the broader applications of NGS technology in probiotics and microbiome research.

- **Section D: Conclusion and Way Forward**
  In the concluding section, readers will find a synthesis of the key takeaways from this handbook, along with valuable recommendations for continued exploration and dialogue.

With this structured handbook, readers will embark on a journey that traverses the probiotic landscape, explores cutting-edge NGS applications, and propels the field toward new horizons. It's not just a handbook; it's your compass in the dynamic world of probiotics and NGS-based testing.

Recommendations for the Future

At the heart of this handbook are a set of crucial recommendations aimed at promoting and advancing the application of NGS-based probiotics testing. These recommendations, generated through online surveys, focus group discussions, roundtable discussions, and expert committee meetings, serve as a roadmap for the future of probiotics testing.

Conclusion

"Sequencing the Future of Probiotics" is not just a handbook; it's a testament to the collaborative spirit of experts from around the world. It provides a foundation for laboratories, researchers, and professionals to embrace NGS-based testing, ensuring the safety and quality of probiotic products. With these insights and recommendations, we embark on a journey to reshape the probiotics landscape, setting new standards for safety and quality assurance in this dynamic field.

Project Background

Malaysia, as Project Overseer through the Department of Standards Malaysia (JSM), and on behalf of the project co-sponsors Australia; People's Republic of China; Thailand; The United States; and Viet Nam is pleased to present this Handbook entitled *Sequencing the Future of Probiotics: A Practical Handbook to Next Generation Testing for Safety and Quality* as a project deliverable to help build NGS-testing capacity in APEC economies and therefore help to increase the market opportunity of novel local products from these economies to the global market.

This Handbook covers the practical considerations, guide, and recommendation of implementing NGS in probiotic products testing, summary of the focus group discussions participants and experts, as well as the comments and discussions from the capacity building workshop conducted in June 2023.

An expert committee was established in September 2022, consisting of experts from food microbiology, safety, NGS, probiotics and bioinformatics. This expert committee facilitates the discussion and capacity building of NGS implementation for probiotics testing in the APEC economies. A survey and two focus group discussions (FGDs) were carried out from November 2022- Jan 2023 by the Expert Committee Members of Application of NGS in Probiotics Testing for Quality and Safety Assurance, followed by a capacity building workshop in June 2023.

From the survey and focus group discussion, the expert committee selected key topics to be discussed at the workshop. The presentations and key discussions during the workshop are also incorporated in this handbook.

SECTION A: PROBIOTICS LANDSCAPE

Introduction to Probiotics

Probiotics, a term derived from the Greek words "pro" (for) and "bios" (life), encompass a fascinating array of live microorganisms that, when administered in adequate amounts, confer positive health benefits upon their host [1, 2]. These microscopic allies hold the potential to shape our well-being by establishing a harmonious balance within our intricate biological systems. Rooted in the concept that not all bacteria are detrimental, probiotics embody a paradigm shift in our understanding of the microbial world and its impact on human health.

Over time, researchers and experts have identified several strains of bacteria and yeast that exhibit probiotic properties. These strains, rigorously tested and shown to meet the stringent criteria set forth by the Joint FAO/WHO Expert Consultation [1, 2], have showcased their ability to enhance health outcomes in various ways. While the list of potential probiotics continues to evolve, some well-established genera of bacteria and yeast have emerged as key players in this arena.

As of now, several bacterial genera have garnered recognition as probiotics, demonstrating their potential to exert positive influences on the human body. These include [3–6]:

- *Lactobacillus*: A diverse genus comprising various species, Lactobacillus strains are known for their role in promoting gut health and aiding in digestion. They are commonly found in fermented foods like yogurt and kefir.

- *Bifidobacterium*: Another prolific genus, Bifidobacterium species are recognized for their contribution to maintaining gut health and supporting the immune system. They are often used in probiotic supplements and dairy products.

- *Lactococcus*: This genus encompasses bacteria that can aid in the fermentation of foods and also offer potential health benefits, particularly for gut health.

- Streptococcus: Certain strains of *Streptococcus* have been explored for their probiotic potential, particularly in the context of oral health.

The probiotic spectrum extends beyond bacteria to encompass certain yeast genera that have exhibited health-promoting attributes:

- *Saccharomyces*: Yeast strains belonging to the *Saccharomyces* genus, such as *Saccharomyces boulardii*, have gained recognition for their potential to support gut health and mitigate digestive disturbances.

- *Candida*: Some *Candida* species have shown promise in their probiotic potential, particularly in managing gut-related issues.

The landscape of probiotics is dynamic and subject to ongoing research and discovery. As the field advances, new strains from existing or novel genera may emerge as probiotic candidates, further enriching our understanding of the intricate relationship between these microorganisms and human health.

Probiotic health effects and functions, as evidenced by multiple clinical studies, are often highly specific to individual strains. Kumar et al. [7] and Reid et al. [8] have proposed that certain widespread mechanisms of probiotics, such as inhibiting pathogens and generating metabolites or enzymes, exhibit similarities across various taxonomic groups. Though the

impacts on immune systems, whether at the intestinal or extraintestinal level, tend to be distinct to each specific strain [9]. Presently, the market boasts numerous commercially available strains that have demonstrated well-established health effects (Table 1). This list is rapidly expanding, reflecting the ongoing growth in probiotic research and discovery.

Table 1. Commercially available probiotic strains and potential health benefits. [3–6, 10]

Bacterial Genera	Species	Commonly Available Strains	Potential Health Benefits
Lactobacillus	*L. acidophilus*	NCFM	Gut health, Immune support, Lactose digestion
		DDS-1	Digestive health, Immune enhancement
		LA-5	Digestive support, Vaginal health
	L. casei	DN-114001 (Immunitas)	Immune support, Digestive health
		Shirota (LcS)	Gut health, Immune enhancement
	L. plantarum	299v	Gut health, Immune modulation
		DSM 9843 (LP299v)	Digestive health, Immune support
	L. reuteri	DSM 17938 (L. reuteri Protectis)	Digestive comfort, Infant health
		ATCC PTA 6475	Gut health, Immune function
Bifidobacterium	*B. bifidum*	BB-02	Infant gut health
		BGN4	Gut health
	B. longum	BB536	Gut health, Immune support
		BORI	Digestive support, Immune modulation
	B. breve	M-16V	Gut health, Immune modulation
	B. lactis	BB-12	Gut health, Immune enhancement
		HN019	Digestive comfort, Immune support
Lactococcus	*L. lactis*	subsp. lactis	Dairy fermentation
Streptococcus	*S. thermophilus*		Dairy fermentation

Economies Regulatory Landscape towards Probiotics

Selected Asia-Pacific Economies

The regulatory guidelines for probiotic products in Asia and Australasia economies are not harmonized; each economy currently applies their own product regulatory processes summarized in Table 2.

Some regulatory processes are restrictive, whereas others are elaborative and have specific requirements companies need to demonstrate to get through the regulatory review process of marketing products: India, Japan, Thailand specifically prohibit probiotics for medical use. India's regulation requires information on nature and purpose for the intended use and specifies that probiotics can only be given to children between 2 to 5 years old under medical advice of a medical doctor, dietician, or nutritionist. Malaysia currently permits 2 general health claims. Singapore 3; whereas, Japan has approved 11 specific health claims, Chinese Taipei 13, People's Republic of China 24 (Table 2). All claims of probiotic products in Australia and New Zealand require pre-approval, while Republic of Korea emphasizes on false labelling and exaggerative advertisement.

Viet Nam demands proofs of effects for claims of finished products, and effects of respective ingredient not allow to be claimed as effects of final products. Table 2. summarizes the probiotic regulation in Asia and Australasia economies.

Table 2. Summary of probiotic regulation in Asia and Australasia economies [5].

Economy	Category	Live	Approved list	Dosage	Clinical trial	Permitted Health claims	Remarks	References
People's Republic of China	Health food	Yes	For adults: 11 yeasts spp. 38 bacterial spp. For infants & young children: 14 bacterial strains	10^6 CFU/mL	Novel food ingredient requires safety evaluation	24	For more details, please refer to 2.2.	[11–18]
India	Health Supplements; Nutraceuticals; Food for Special Dietary Use; Food for Special Medical Purpose; Prebiotic and Probiotic Food	Yes	31 bacterial spp.	≥10^8 CFU per day	New strain based on data collected in accordance with ICMR-DBT guidelines	Not for medical use. Provide sufficient information on nature and purpose for the intended use.	For children 2-5 yr only be given under medical advice by a recognized medical doctor or dietician or nutritionist.	[19–21]
Indonesia	Health supplements Processed food	Yes	91 bacterial strains & 3 yeast strains	Need to be stated on label	New registration requires detailed characterization, functionality (local double-blind randomized placebo-control phase 2/3 study) & safety data (Phase 1 trial on Indonesia population)	Maintaining gut health	Strain identification necessary. Not for baby &1 to 3-year-old Not for medical claim	[22, 23]

Japan	Food with health claims: Food for Specified Health Uses (FOSHU); Food with Function Claims (FFC)		Nil.		8 criteria: scientific & clinical evidence on effectiveness; safety; analytical determination	No medical claims. 11 health claims for FOSHU; >1,000 products for FFC		[24–26]
Korea	Health functional food	Yes	19 bacterial spp.	10^8-10^{10} CFU per day	Supported by human clinical studies based on the double-blind, randomized, placebo-controlled designs using human-originated probiotic strains. 9 safety characteristics	Shall not falsely label or exaggeratedly advertise the names, raw materials, manufacturing methods, nutrients, ingredients, usage methods or qualities of health functional foods & traceability of health functional foods	Identifying strain specificity use methods such as whole genome sequence	[27–32]
Malaysia	Pharmaceuticals Foods & beverages	Yes	32 bacterial strains	10^6 CFU/mL or CFU/g		Permits 2 general health claims. For additional health/other function claims may submit applications.		[33–35]

The Philippines	Food use Drug use	Yes	3 bacterial genera; 1 bacterial spp; 1 yeast spp.	In sufficient quantity	New strain requirement for food use 1. strain identification, 2. Functional in vitro/animal study, 3. Safety in vitro/animal/phase 1 human assessment. New strains requirement for drug use 1. Strain identification, 2. Functional in vitro/animal/human study, 3. Safety in vitro/animal/human assessment Double blind randomized placebo phase 2/3 human trials			[36–38]
Singapore	Food use Health supplement		Proven long history of safe use in food. Declare Probiotic species on label.	Food manufacturer or importer ensures the viable count in the product can bring about claimed effects	Dealers are responsible for safety and quality of the products. Do not contain prohibited ingredients, synthetic drugs, or toxic heavy metals	3 general health claims		[39–42]

					above legally permissible limits.			
Chinese Taipei	Ingredients or processing aid for food/ Functional food	Not stated	Not approval list for probiotic microorganisms to be used as food ingredients. *Information on Food Ingredients Inquiry Platform is used as reference only. The microorganisms listed on this platform to be used as food ingredients, are subjected to changes, and are not limited to probiotic microorganisms.	Not stated	Required no further test if already in use or reported in literature. New strain/culture must first be notified before marketing, and its safety & efficacy documented	13 health care effects specified for Health Food Registrations		[43–57]
Thailand	Food use	Yes	22 bacterial spp; 1 yeast spp	≥10^6 CFU/g	Other than the approved list, safety & properties evaluation i*n vitro* or *in vivo* and in human studies are required.	Specify this product is not for treat, heal, cure or prevent of disease.		[1, 2, 58–60]
Viet Nam	Supplemented food/ dietary supplement					Combined effects made only when there is scientific evidence of finished product, the effects of ingredients must not be claimed as effects of the product		[61, 62]

Australia & New Zealand	Food use	Yes	No positive list	≥10^8 CFU/g.	Must be safe and suitable for use in food	Pre-approval required		[63, 64]

Among the 13 economies in Asia and Australasia listed in Table 2, 10 provide a pre-approved list: The Philippines approved 3 bacteria genera and 2 microbial species, Republic of Korea 19 species, Thailand 24 species, Japan 28 strains, India 31 species, Malaysia 32 strains, and People's Republic of China 62 species or strains. Australia; New Zealand; Singapore, Chinese Taipei; and Viet Nam have no pre-approved probiotic list. New probiotics that are not included in the approved list, would require submitting documents of safety evaluation and effectiveness for the claims. Republic of Korea's regulation specifically states that evidence should be supported by human clinical studies based on the double-blind, randomized, placebo-controlled designs using human-originated probiotic strains, and 9 safety characteristics. Thailand also requires human studies. Japan listed 8 criteria for scientific & clinical studies on effectiveness, safety, and analytical determination.

In addition to strain identification, functional *in vitro* or animal studies, safety evaluations *in vitro*/animal models, and phase 1 human assessments, the Philippines also mandates the inclusion of a double-blind randomized placebo-controlled phase 2/3 human trial when considering the introduction of new probiotics for medical used.

Six of the economies stipulated minimal dosage of probiotics in the final products, and they vary from 10^6 CFU/mL or CFU/g for Malaysia, and Thailand, to 10^6 CFU/mL(g) for People's Republic of China, 10^8 CFU/mL(g) for Australia; India; Republic of Korea; and New Zealand.

People's Republic of China

Currently probiotics are widely used in the production of common food, infant formula food, special medical purposes, and health food. Some strains in clinical adjuvant treatment of diseases. Compared with other developed economies, the development of probiotics uses in People's Republic of China (PRC) started relatively late, but recent developments have been rapid. At present their approval process is a combination of list systems and a dynamic approval process in their efforts to control potential harmful microorganisms in food having probiotics ingredients. Details are described in the following section.

Common food, infant formula food and novel food

Common food, infant formula food and novel food are under the control of the National Health Commission (NHC). In 2010, the government published the "List of culture that can be used in food" [65], and the "List of culture that can be used in infant and young child food"[66] was published in 2011. If a company wants to use a new strain of probiotics in a corresponding food, they need to provide the necessary materials to the expert review committee, the new probiotic strain will be added to the list for use after approval. By the end of 2021, China has approved 38 strains of probiotics for commercial food production.

Due to the changes in taxonomy, the taxonomic status of the microorganism in China's list was updated in 2022, The updated list Including 6 species of Bifidobacterium, 8 species of *Lactobacillus*, 3 species of *Lacticaseiobacillus*, 2 species of *Limosilbacillus*, 1 species of *Lactiplantibacillus*, 1 species of *Ligiactobacillus*, 2 species of *Latilactobacillus*, 1 species of *Streptococcus*, 3 stains of *Lactococcus* lactis, 1 species of *Propionibacterium*, 1 species of *Acidipropionobacerium*, 1 species of *Leuconostoc*, 2 species of *Pediococcus*, 1 species of *Weizmannia*, 1 species of *Mammaliicoccus*, 2 species of *Staphylococcus* and 1 species of *Kluyveromyces* [67].

The list also states that strains traditionally used in food production and processing are allowed to continue to be used without declaration [67]. The corresponding economy standards clearly stipulates that the number of viable probiotics bacteria in the products should be higher than 10^6 CFU/mL(g) [68–75].

Health food

Before 2018, probiotics for health food were regulated by the PRC State Food and Drug Administration (SFDA) which later merged into the PRC State Administration for Market Regulation (SAMR) which currently administers probiotic use. The "List of probiotics that can be used in health food" and the "List of fungi that can be used in health food" were published in 2001 by the SFDA [76, 77]. In 2005, SFDA promulgated the "Measures on the Administration of Health Food Registration" [78] which was repealed in 2016. At the same time, "Administrative Measures on the Registration and Record Filing of Health Food" [79, 80] was promulgated and then revised in 2020.

In 2005, SFDA implemented the Declaration and Review of Probiotic Health Food (Trial) and SAMR issued the provisions draft for public comments based on the implementation in 2005 [81]. These provisions aim to strengthen the management of probiotic health food, standardize the declaration and review work, and ensure the safety, health function and quality control of probiotic health food. The provisions do not only provide a detailed definition of probiotic health food, but also outline the limit on the microorganism allowed in probiotic health food. It is clearly required in the provisions that the number of viable bacteria during the shelf life of the probiotic health food shall not be less than 10^6 CFU/mL(g) [75, 82].

Republic of Korea

In the Republic of Korea, probiotics are used in **general food** as lactic acid bacteria [28] and **health functional food** [29], regulated by the Ministry of Food and Drug Safety (MFDS) or previously known as Korea Food and Drug Administration (KFDA).

The food code currently does not specify any permitted lactic acid bacteria. However, if specific lactic acid bacteria are indicated on the label of the product, then the number of viable bacteria must be maintained **at least 10^8-10^{10} CFU/g** or **CFU/mL**, depending on the food products, throughout the shelf-life.

Republic of Korea's Health Functional Food Code is applied to the food manufactured with functional raw materials or functional ingredients for functional health, in which probiotics are used as the functional ingredients. In the case of two or more functional ingredients combined, the safety and health benefits should be verified and maintained. The Health Functional Food Code also specified the 19 permitted bacterial species that can be used as single strain probiotics or blend.

Table 3. Probiotic species permitted in Health Functional Food Code., [29].

Genus	Species
Lactobacillus	*L. acidophilus, L. casei, L. gasseri, L. fermentum, L. delbrueckii ssp. bulgaricus, L. helveticus, L. paracasei, L. plantarum, L. reuteri, L. rhamnosus, L. salivarius*
Lactococcus	*Lc. Lactis*
Enterococcus	*E. faecium, E. faecalis*
Streptococcus	*S. thermophilus*
Bifidobacterium	*B. bifidum, B. breve, B. longum, B. animalis ssp. Lactis*

The recommended daily intake amount ranges from 10^8 to 10^{10} CFU. Warning label should include [29]:

- Consult a health care practitioner prior to intake if you are on other medication or having any other disease(s),
- The individual who has an allergy may cause side-effect such as hypersensitivity reaction,
- Children's daily consumption to avoid over intake, and
- Consult a health care practitioner and stop consumption in case of adverse health events.

The United States

The Federal Food, Drug, and Cosmetic Act is the overarching legal framework that provides authority to the United States Food and Drug Administration (US FDA) to regulate a wide variety of products [83]. They carry out this capacity with a focus generally on intended use which will govern the respective regulatory and evidentiary burdens and pathways associated with the product categorization and eventual licensure.

Interestingly, a regulatory definition and use of the term "probiotic" (or prebiotic for that matter) **does not exist in the United States** (US) regulatory lexicon. However, as defined by prior expert consensus panels, probiotics can be defined as living microorganisms that when consumed in adequate amounts impart beneficial health effects on the host [84]. Using this definition carries certain evidential assumptions that do not align with the variety of categorizations that may exist for such products in the US. Hence, probiotics are not defined as a regulatory product category under the above act or the Public Health Service Act, since such products may be considered, for example, to be "foods" or food ingredients, "medical food," dietary supplement, "drug," or "biological product" under these acts, depending on the intended use of the product [85]. There are also considerations for animal foods that are beyond the scope of this section. In general terms, therefore, probiotics are commonly referred to as live microbial products in the US regulatory landscape.

Foods-based regulation

The vast majority of probiotic-type products in the US are handled as dietary supplements regulated through the Center for Food Safety and Applied Nutrition (CFSAN) at FDA. A dietary supplement is "a product (other than tobacco) intended to supplement the diet that bears or contains one or more…dietary ingredients" [86]. Herein, a dietary ingredient is considered a vitamin, a mineral, an herb or other botanical, an amino acid, a dietary substance for use by man to supplement the diet by increasing total dietary intake, or a concentrate, metabolite, constituent, extract, or combination of any of the above dietary ingredients. Probiotic formulations can fit into this definition and such products can be single strains or blends of multiple strains and require a premarket notification for a product that was not marketed in the US prior to 15 October 1994. After this point, the Dietary Supplement Health and Education Act involving Good Manufacturing Practices (GMPs) was introduced in an effort to exempt the dietary and herbal supplement industry from most FDA drug regulations without scientific requirement of their health and medical claims (see S.784 - 103rd Congress (1993-1994): Dietary Supplement Health and Education Act of 1994 | Congress.gov | Library of Congress). For new strains coming onto the market, a new dietary ingredient (NDI) notification is required.

The bases for NDI review under this category can be evaluated by five general criteria:

1. identity of the microorganisms with current updated taxonomy and systematics,
2. a documented history of use in conventional foods,
3. fermentation and processing detail,
4. assessment of potential pathogenicity features/repertoire including toxins, other virulence, antibiotic resistance genes, and associated mobile genetic architectures,
5. and toxicological and clinical data for establishing safe dosing for intended population.

It is important to note that some generalized health claims are acceptable for labeling and are not further evaluated or substantiated (unless requested by FDA) but specific structure-function claims that are associated typically with drugs and biologics are not permissible.

For new strains coming to market, it is expected that whole genome sequence (WGS) data will be used facilitating a more robust gold standard evaluation of the criteria mentioned above and comparative genomics to other well-characterized strains to assess risk. Safety will continue to be the primary driver that is established initially through proper identification and characterization including subchronic toxicological analysis for strains within species not traditionally associated with foods along with documenting manufacturing processes and expected product stability [87]. Strains derived from generally commensal species that contain known pathogenic members are heavily scrutinized and currently would not pass premarket review. An example of this would involve *Escherichia coli* with notable pathotypes yet they are also common commensals with known probiotic utility.

There are other uses of live microbials in the food spectrum that are handled through CFSAN as a food additive if they are intended as part of a food and if not already deemed "generally recognized as safe" (GRAS) as an ingredient (Generally Recognized as Safe (GRAS) | FDA). This elevated status is granted only if there is no recognized concern based on available scientific literature and generally accepted expert opinion. Examples may involve starters for cultured food or microbially fortified food products. GRAS can be self-determined by a manufacturer and does not require notification if the sponsor maintains adequate dossier supporting this self-determination – although FDA will review this premarket upon request of the sponsor. Lastly, there is a small and underrecognized classification of "medical foods" which is usually very narrowly interpreted and defined as needed for specific dietary management of disease [85]. This is usually conducted under medical supervision to address a distinct nutritional need/deficiency that otherwise cannot be obtained through the normal diet such as phenylketonuria. Regardless, in all the examples listed in this subsection, it is important to recognize that these products are intended for use in healthy populations and may have additional scrutiny for special populations such as use in infant formula or during pregnancy.

Live Biotherapeutic Products

Another emerging class of products involved is intended to be used as live biotherapeutic products (LBPs) that are handled through the Center for Biologics Evaluation and Research (CBER) at FDA. These are defined by CBER as containing live organisms used for prevention, treatment, or cure of disease in humans that is not a vaccine [88]. Safety of these products is a primary driver, but a higher bar has been established for the specific structure function of the products' claims of its ability to diagnose, cure, mitigate, treat, or prevent disease which meets the statutory definition of a drug [86]. Before being introduced into interstate commerce, such products meeting the definition above would need to be already approved or have an Investigational New Drug (IND) application. Strain characterization proceeds down a similar path with a need for genome sequencing to identify notable safety concerns for virulence and antibiotic resistance genetic features (along with their potential mobility). With the latter, the resistance profile to clinically relevant antibiotics is particularly important for treating any product-related infections.

Much of the clinical research necessary for products defined as drugs, like LBPs, has focused on commercially available probiotic formulations. For example, there are at least 787 such active studies that are planned or actively recruiting as of April 4th, 2023 predominately involving commercially available probiotics for > 700 conditions - with more than 1100 trials encompassing > 600 conditions already completed (www.clinicaltrials.gov). Noting, of course,

this is an underestimation since not all studies are required to be registered through this resource. However, the landscape is evolving given advancements in culturing and sequencing technology leveraged with economy investments in microbiome science that will undoubtedly produce next generation, precision probiotics/live biotherapeutics using human derived, novel, engineered strains and/or rationally defined functional consortia with potentially synergistic effects. Without available histories of or traditional use in food, moving beyond typical acute safety issues creates a challenge for designing long term safety assessments [89]. Much of the focus from CBER for LBPs has been on pre- and early-phase clinical investigations. Additionally, the safety thresholds coming from already-marketed products have been intended for use in healthy populations but will need to pivot towards functional utility in special populations and disease states for establishing safety and efficacy. Considering dietary supplements are intended for a generally healthy population, herein particular attention is paid with LBPs towards the vulnerability of the target population for which therapeutic concerns may involve important issues with gut barrier function and/or immunodeficiency [88].

It is important to note that, to date, there have been no approved microbial LBPs as described herein for rationally defined preparations. However relatedly, FDA CBER has issued enforcement discretion for fecal microbiota for transplantation (FMT) for treatment of Clostridioides difficile infection (CDI) that does not respond to standard therapies [90]. While this provides access to potentially efficacious therapy, such undefined microbial consortia have resulted in safety alerts from adverse events involving transmission of pathogenic and multidrug resistant organisms including, for example, enteropathogenic *Escherichia coli* and Shigatoxin-producing *Escherichia coli*. These events have resulted in additional protections involving donor and pathogen screening of stool [91] including subsequent additional protections for exposure to SARS-CoV-2 and monkeypox. Most recently in November 2022, FDA approved the first FMT product, REBYOTA, for prevention of recurrence of CDI in individuals 18 years of age and older, following antibiotic treatment for recurrent CDI.

Manufacturing and Quality

Products containing live microbials have unique considerations for manufacturing and certification that are unlike other dietary supplements or drugs. For example, products can vary in complexity, blending and matrices (with single or multiple strains), their associated scale-up and relative quantification, and their requirement to maintain viability at the expected dose for the life of the product. Moreover, their production (at scale) and the ability to distinguish closely related strains or potential contamination is exacerbated by limited and readily available standards and/or methodologies for such assessment. Given that quality and safety of probiotics resides with industry, it is important to verify and certify products through independent organizations to facilitate regulatory compliance in the US. There are many details in this certification process that are beyond the scope of this section and detailed elsewhere [92]. Regardless, the production process shall comply with relevant current GMPs. Many of these requirements for identify, purity, composition, and stability can be facilitated by non-governmental, non-profit, standard setting organizations. For example, the United States Pharmacopeia (USP) which serves an important mission in the regulatory-industry interaction and other similar organizations can use information from manufacturers to independently develop and provide the necessary standards for ingredients of finished products through the documented and referenceable monograph process forming a key connection and resource in this economy. The example of USP-industry interaction can produce validated analytical methods and acceptance criteria with related certification and "quality seals" that boost consumer confidence in label claims.

As an aside and mentioned previously, dietary supplement manufacturers are responsible for using GMPs in the US, but these do not specify type and level of contaminants but can use

validated analytical methods in official references such as AOAC and USP for developing the relevant testing program. For LBPs, CBER has focused particular attention on critical manufacturing processes to support INDs that include supportive chemistry, manufacturing, and controls (CMC) data. Notable here, is discretion that may allow for waivers of certain IND requirements related to CMCs for lawfully marketed, commercially available food and dietary supplement-related products to be shipped across state lines for clinical study. Nonetheless, products should include release specifications for identity, purity, and potency [88].

Public Health

Public health and regulatory authorities are well-aligned with complimentary missions to investigate when adverse events are reported and need additional analyses or signal to a larger public health concern involving outbreaks that implicate a respective product or strain. United States Centers for Disease Control and Prevention (CDC) will obtain and analyze clinical and related/relevant environmental samples as appropriate and in conjunction with FDA. These investigations generate bodies of evidence further used legally to result in seizure and/or injunction from interstate commerce by the FDA. Noting the previous product categories that available probiotic strains may fit into, much of the regulatory oversight is garnered with required premarket notifications and communication with the sponsor. Post-market surveillance generally is on voluntary bases for consumers and physicians reporting to MedWatch (MedWatch: The FDA Safety Information and Adverse Event Reporting Program | FDA) and/or CFSAN Adverse Event Reporting System (CFSAN Adverse Event Reporting System (CAERS) | FDA) specifically for food-related issues. Additionally, FDA provides publicly available information on recalls and safety alerts (Recalls, Market Withdrawals, & Safety Alerts | FDA). In a complementary fashion, CDC provides avenues for sharing information on urgent public health threats through different message types (alerts, advisories, updates, etc) in the Health Alert Network (Health Alert Network (HAN) | CDC). Additionally, CDC operates the Epidemic Information Exchange (Epi-X; CDC | Epidemic Information Exchange) that provides a powerful avenue to connect public health professionals to obtain and identify case information and scope economy-wide. In aggregate and specifically pertinent to FDA, these resources are designed to provide an important part of post-market surveillance that, in the case of dietary supplements at least, cover the primary oversight mechanisms along with the premarket notification process. If drug-based regulation is employed as with live biotherapeutics, post-market monitoring may occur upon agreement with the manufacturer for submitting safety updates.

SECTION B: PROBIOTICS TESTING

Current Laboratory Testing Methods for Probiotics Microbial Safety and Quality

Probiotics, as defined by the Joint Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) Expert Consultation, are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [88]. Meeting this definition necessitates that probiotics fulfill the following criteria:

- **Viability upon Administration**: Probiotic microorganisms must remain viable when administered.
- **Adequate Amount for Benefit**: They must be administered in sufficient quantities to exert beneficial effects.
- **Proven Health Benefit**: Scientific evidence must support their health benefits to the host.

In accordance with the FAO/WHO definition, laboratories conducting probiotic testing must employ appropriate methods. These methods not only ensure that the microbial strains claimed in the product are indeed probiotic strains but also confirm their viability at the time of testing. This testing approach is pivotal in ensuring the safety, quality, and efficacy of probiotic products, providing essential evidence for regulatory bodies to assess and approve these products for consumer consumption.

Probiotic products testing for microbial safety and quality encompasses three critical aspects:

- **Identification and Verification**: The accurate identification and verification of the strains present in the products.
- **Enumeration of Viable Cells**: Quantifying the number of live probiotic cells in the product.
- **Contamination Detection**: Ensuring there is no contamination of unintended strains that could pose hazards to consumers.

The safety and quality of probiotic products hinge on the precise identification and verification of the strains used. These aspects are especially crucial given the specificity of different strains in relation to health claims. The presence of specific strains directly influences the efficacy of a product for addressing particular health conditions. Moreover, different strains may carry varying safety profiles, including factors like virulence and antimicrobial resistance. Regulatory bodies require this information to ensure consumer safety and proper product labeling.

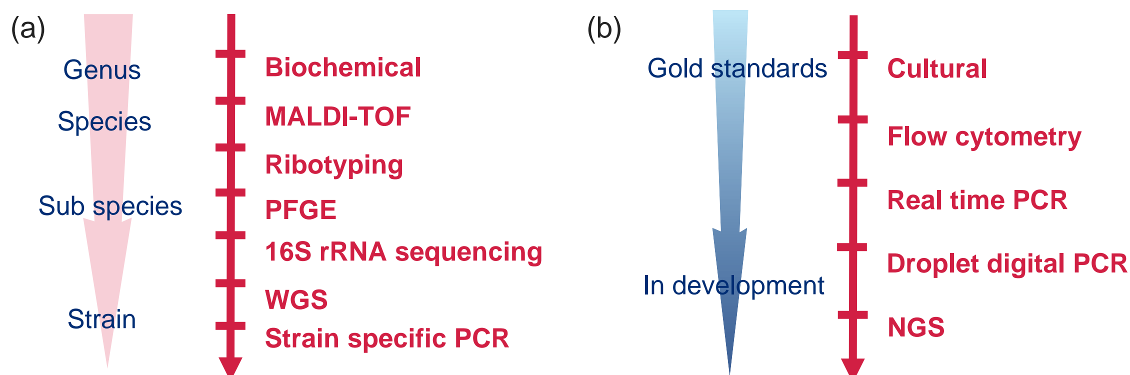
Current probiotics testing methods, primarily centered around genus- and species-level identification, may not suffice for comprehensive product assessment. Thus, achieving accurate strain-level identification is critical for both product development and compliance.

Identification involves precisely naming probiotics at various taxonomic levels, including genus, species, subspecies, and strain. Various technologies (4) are employed for this purpose, all dependent on isolating a pure culture in the laboratory before identification can occur. Therefore, successful identification relies on prior knowledge of the culturing conditions required for isolating the strains in the laboratory.

Table 4. Bacterial identification and verification methods in the laboratory.

Identification approach/ method	**Description**
Biochemical Analysis	It is commonly used for the identification of bacteria, particularly in clinical microbiology laboratories. This approach involves a series of tests that assess the metabolic and physiological characteristics of bacterial isolates. By analyzing the results of these tests, microbiologists can determine the identity of the bacterial species or strain. Some examples of commercially available kit or system are such as bioMérieux API® Microorganism Test Kits for identification of Gram positive and Gram negative bacteria and yeast.
Matrix-assisted Laser Desorption Ionization–Time-of-Flight Mass Spectrometry (MALDI-TOF)	It offers rapid and accurate identification at genus and species levels. It identifies bacteria based on their unique protein profiles, specifically the mass-to-charge ratio (m/z) of their ribosomal proteins.
Ribotyping	It is a molecular biology technique used for the characterization and differentiation of bacterial strains based on the patterns of their ribosomal RNA (rRNA) genes, specifically the 16S or 23S rRNA genes. This method is particularly useful for identifying and classifying bacteria at the species and sometimes subspecies levels.
Pulsed Field Gel-Electrophoresis (PFGE)	It is a powerful molecular biology technique used for the analysis and separation of large DNA molecules, particularly for genomic fingerprinting and strain typing of microorganisms, including bacteria. PFGE is a highly discriminatory method and is commonly employed in epidemiological investigations, outbreak tracing, and genetic studies of bacterial populations.
16S rRNA Sequencing	It is a molecular biology technique used for the identification and classification of bacteria and archaea based on the sequencing of a specific region of the 16S ribosomal RNA (rRNA) gene. This method is widely employed in microbiology and microbial ecology to study the diversity and taxonomy of bacterial communities.
Strain-specific PCR	It is a molecular biology technique used to detect and identify specific strains or subtypes of microorganisms, particularly bacteria. It is a highly specific and sensitive method that relies on the amplification of unique genetic markers or sequences that are characteristic of a particular strain or subtype. It is a preferred technique for strain-level identification, with standards

	like CEN/TS 15790 available. Strain-specific PCR is particularly valuable in situations where it is essential to distinguish between closely related strains or subtypes of microorganisms. This method is commonly used in clinical diagnostics, epidemiology, food safety, and environmental microbiology.
Whole Genome Sequencing (WGS)	It is a powerful and comprehensive DNA sequencing technique that involves the determination of the complete genetic information (genome) of an organism. This method sequences all the DNA in an organism's genome, including both coding and non-coding regions, providing detailed information about its genetic makeup. WGA provides high-resolution strain identification. ISO 23418 offers guidelines for this method.



Enumeration of viable cells is a fundamental aspect of probiotic testing to meet regulatory and efficacy requirements [1, 2]. While culturing methods have traditionally served as the gold standard for quantifying probiotic cells, various methods are now employed (Table 5).

Table 5 Microbial enumeration methods for probiotic products.

Enumeration approaches	**Description**
Culturing	Quantifies probiotic bacteria, with results expressed as colony-forming units (CFU). Numerous culturing methods are available. However, these methods often focus on lactic acid bacteria and may not encompass all probiotic strains. Yeast and fungal probiotics, increasingly relevant, are often inadequately covered by existing standards.
Flow Cytometry	It is a sophisticated analytical technique used to rapidly and accurately count and characterize particles, including cells and microorganisms, in a fluid sample. It utilizes the principles of hydrodynamics, optics, and electronics to measure various

	physical and chemical properties of individual particles as they pass through a flow cell.
Quantitative PCR (qPCR)	It is also known as real-time PCR, is a molecular biology technique used to measure and quantify the amount of specific DNA or RNA sequences in a biological sample. It is a powerful tool for accurately determining the quantity of a target nucleic acid sequence, which can be a gene, a viral RNA, a bacterial DNA, or any other specific genetic material of interest.
Droplet Digital PCR (ddPCR)	It is a highly sensitive and precise molecular biology technique used for quantifying and characterizing nucleic acids, such as DNA and RNA. It is a digital PCR technology that offers advantages over traditional quantitative PCR (qPCR) methods, particularly in situations where precise quantification of target nucleic acids is essential. ddPCR achieves this precision by partitioning a sample into thousands of tiny droplets, each of which can be independently analyzed.
Metagenomics	It aims to analyze and characterize the collective genetic content, or metagenome, of a microbial community within a specific environment. Metagenomics, while primarily used for characterizing microbial communities and their genetic potential, can also be employed to enumerate microbial cells in probiotic samples. This process involves quantifying the abundance of specific microbial species or strains within a probiotic product. Metagenomics-based enumeration is less biased and culture-independent, reducing the risk of underestimating microbial counts due to limitations of traditional culturing methods.

Contaminant detection/enumeration is crucial to identify unintended microorganisms, including foodborne pathogens. While guidance exists from organizations like the US Pharmacopoeia (USP) and the International Dairy Federation (IDF), sample preparation modifications may be necessary for probiotic testing. Unfortunately, limited guidance exists for these modifications, necessitating validation and verification.

To ensure the accurate detection and enumeration of contaminants in probiotic products, horizontal standards are essential (Table 5). The limited standard methods available for probiotic product testing indicate the complexity and weaknesses in the current culture-dependent approach.

In summary, standardization in probiotic identification and enumeration methods is crucial to reduce variation in results and guarantee the safety and quality of probiotic products. Efforts to develop and adopt these standards are essential for the continued growth and reliability of the probiotics industry.

Table 6 Overview of standard methods used in probiotics testing for strain identification, enumeration and contaminants detection.

Identification	Enumeration	Contaminants
ISO –PROBIOTICS NOT IN SCOPE! **ISO 23418:2022 Microbiology of the food chain —Whole genome sequencing for typing and genomic characterization of bacteria —General requirements and guidance** **Animal feeding stuffs** • **EN 17697 Animal feeding stuffs: Methods of analysis -PFGE typing of *Lactobacilli, Pediococci, Enterococci* and *Bacilli* in animal** feeds. • **CEN/TS 15790 PCR typing of probiotic strains of *Saccharomyces cerevisiae* (yeast)** **IDF** **Bulletin N°513/2021: Identification of probiotics at the strain level** **USP** **Several monographs covering *Bifidobacterium, Lactobacillus* and *Bacillus* (including *Weizmannia*) strains**	**GB** GB 4789.34-2016 National Food Safety Standard -Food Microbiological Examination -Examination of *Bifidobacterium.* **Animal feeding stuffs** • EN 15784 Animal feeding stuffs: Methods of sampling and analysis -Isolation and enumeration of presumptive *Bacillus* spp. • EN 15785 Animal feeding stuffs -Enumeration of *Pediococcus* spp. • EN 15786 Animal feeding stuffs -Enumeration of *Lactobacillus* spp. • EN 15787 Animal feeding stuffs -Enumeration of *Enterococcus (E. faecium)* spp. • EN 15788 Animal feeding stuffs -Enumeration of yeast probiotic strains **IDF** • ISO 29981/IDF 220 Milk products -Enumeration of presumptive *Bifidobacteria*-Colony count technique at 37 degrees • ISO 19344/IDF 232 Milk and milk products -starter cultures probiotics and fermented products - quantification of LAB by flow cytometry • ISO 20128/IDF 192 Milk products —Enumeration of presumptive *Lactobacillus acidophilus* on a selective medium —Colony-count technique at 37 degrees C **USP** Several monographs covering *Bifidobacterium, Lactobacillus* and *Bacillus* (including *Weizmannia*) strains	**ISO** ISO 6887 part 1, 3, 4, 5 Rules for the preparation of the initial suspension and decimal dilutions. **IDF** • ISO 13359/IDF 153 Butter, fermented milks and fresh cheese - Enumeration of contaminating microorganisms - Colony count technique at 30°C • ISO 27205/IDF 149 Fermented milk products - bacterial starter cultures - standard of identity **EN** EN ISO 6498:2012, Animal feeding stuffs- Guidelines for sample preparation **Horizontal standards need to be developed to provide guidance for the accurate detection and enumeration of contaminants in products containing products.

Limitations and Challenges in Probiotics Testing

Probiotics testing for identification, verification of effectiveness and safety need only to be performed for submission for regulatory approval. The cell count is the only parameter that requires routine testing. Conventional viable plate count is commonly applied for it is low cost, low in technical and equipment demand. It is also listed as the standard method for viable cell counting in many international standard protocols. It is also very useful for detection of contaminants which differ in growth conditions from the added probiotic. For example, a probiotic product consisting of lactic acid bacteria can be analyzed for yeast, mold and Gram-negative contaminants using selective media.

There remain limitations and challenges with the viable plate count technique. Because it is a viable count, it can take at least 1-2 days to obtain results, and even longer for the new generation of probiotics which are slow growing and require anaerobic growth conditions. Some testing laboratories may not have routine procedures for strict anaerobic growth. Since the viable count technique is a manual process, the accuracy is dependent on the operator and hence the element of human error needs to be considered. Some probiotic preparations contain strains that clump/ self-aggregate which can present challenges in quantifying the viable cells in the probiotic product. Hence inconsistent results will be obtained for the same product when analyzed by different laboratories. Further the viable count technique is largely only able to quantify at the genus level unless there is selective media available for the specific probiotic species. Even if such selective media were available, additional testing could be required for confirmation, hence extending the time required for analysis. Unless a specific selective medium is available for specific strains, the viable count technique cannot enumerate at the strain level of a probiotic. It is largely not possible to quantify individual probiotic strains in a product containing more than one species or strain of the same genus and without further testing it is not possible to validate claims of added probiotics. Currently the viable count technique cannot be used to assess the presence of genes for antibiotic resistance or virulence factors nor can it provide information about the functional aspects of the particular probiotic to support benefit claims.

In recent years, there has been a growing interest and market demand for multi-strain probiotic concoctions, including two to more than 30 probiotic strains. It is assumed that since probiotic effects are strain dependent, introduction of multiple strains could strengthen and complement the beneficial effects of the products. As it is not always possible to differentiate the different closely related probiotics in the mixture, the final total concentration of bacteria is considered the effective dosage. This is scientifically irrational, as different probiotic strains would die off in the preparation at different rates in different environmental conditions. This represents a regulatory loophole, for example a clinically effective probiotic strain of shorter shelf-life when mixed with a stable long shelf-life environmental strain could misrepresent as one with long shelf life. Moreover, metanalysis of clinical studies has revealed that closely related strains could nullify the clinically beneficial effects when administered in mixture [93]. It is probably due to steric hindrance for binding onto a receptor on mucosa-epithelia membrane leading to the phenomenon of competitive inhibition.

Quantitative PCR (qPCR) has been introduced in recent years to shorten the time for output of result, and to also allow better resolution on the quantification of different probiotic strains in a mixture by using specific primers. Next-generation sequencing (NGS) is a new technology used for DNA and RNA sequencing. NGS utilizes the advantages of unique sequencing chemistries, different sequencing matrices, and bioinformatics technology and allows a parallel sequencing of varying lengths of DNA or a whole genome within a relatively short

period of time. NGS has been offered as a solution for testing of probiotics because appropriate protocols could differentiate viable from dead cells, differentiate related strains, and enumerate concentrations with the use of appropriate primers, in one single test. It has been used to effectively analyze probiotic products for microbial content [94] and also for analysis of probiotics for safety consideration such as the presence of potentially undesirable genes [95]. Unfortunately, NGS has been deemed expensive for routine probiotic testing and also too difficult to implement in routine testing laboratories as it requires specialist technical support.

For NGS to be utilized for probiotic testing, it requires standardization of sample preparation, protocols and bioinformatic tool kits, since each step has its own challenges and limitations. The NGS technology includes a DNA extraction step, DNA fragmentation, library preparation, parallel sequencing, and bioinformatic analysis and interpretation. The success of the DNA extraction is affected by the protocols and kits used and for the best results would need to be tailored for the particular probiotics in the product. Alternatively, the protocol would need to be evaluated to effectively extract from very diverse probiotics including the spore forming microbes and the Gram-positive bacteria, since both require more stringent procedures for extracting the DNA from the cells. It is also important to flag that some DNA extraction methods can introduce inhibitors, which can negatively affect the subsequent enzymatic reactions. After extraction, a QC step can be added to remove contaminants and is recommended. The concentration and quality of the extracted DNA product will have a huge impact on the subsequent sequences generated. Samples of low or variable quality DNA can corrupt downstream processes such as library preparation and ultimately confound analysis. A sequencing run generally fails due to poor sample quality or inadequate primers. Occasionally, it can also fail due to a machine or human error. However, with appropriate protocols and workflows, the NGS technology has a lot to offer for analysis of probiotic products.

Next Generation Sequencing Technology for Probiotics Testing

Sequencing methods

Microbiome plays crucial roles in human life and various methods are employed to study the diversity of the microorganisms and their functions, depending on the objectives of the testing. Examples of these methods include full-length 16S sequencing and shotgun metagenome sequencing. For further investigation of a single microorganism, individual isolation, and whole genome sequencing (WGS) are typically performed. This approach provides detailed information about a single species of microorganism including its genetic information, virulence, antimicrobial profiles, and other important characteristics.

The application of NGS can be used in selection of probiotic strains, safety, quality assurance (Figure 2). Other applications of NGS in probiotics and microbiome research are discussed in section 9. NGS involves several steps, including sample collection, DNA isolation, sequencing, and data analysis (bioinformatics). The general workflow NGS is described below:

- Sample collection: Collection of samples from a source, whether it is a potential probiotic source or a product.
- DNA isolation: Isolation of genetic material from the sample using a suitable method to yield high-quality DNA for sequencing.
- Sequencing: Sequencing of samples using suitable high-throughput DNA-sequencing platform, e.g.: Illumina, Pacific BioSciences (PacBio), Oxford Nanopore Technologies (ONT), etc.
- Data analysis: Analysis of the sequenced data based on the objective of adopting a particular sequencing platform.

    - Taxonomic identification: The sequences are compared to a reference database of known microbial genomes to identify the taxonomical identity of the strains (16S ribosomal RNA (*16S rRNA*) & shotgun metagenome sequencing).
    - Functional annotation: The annotated sequences can be used to infer the functional potential of the microbial community, including the potential for probiotic activity (shotgun metagenome sequencing & WGS)
    - Safety assessment: The sequences can be analyzed for potential safety concerns including the presence of virulence, antibiotic resistance, or other potential harmful characteristics (shotgun metagenome sequencing and WGS).
    - Relative abundance: Sequencing can estimate the relative abundance of specific microbial strains and groups within a sample based on their DNA sequences, which can be compared to a reference database (shotgun metagenome sequencing).
    - Strain selection: Finally potential probiotic strains can be selected based on their taxonomic identity, functional potential, and safety profile [96] .

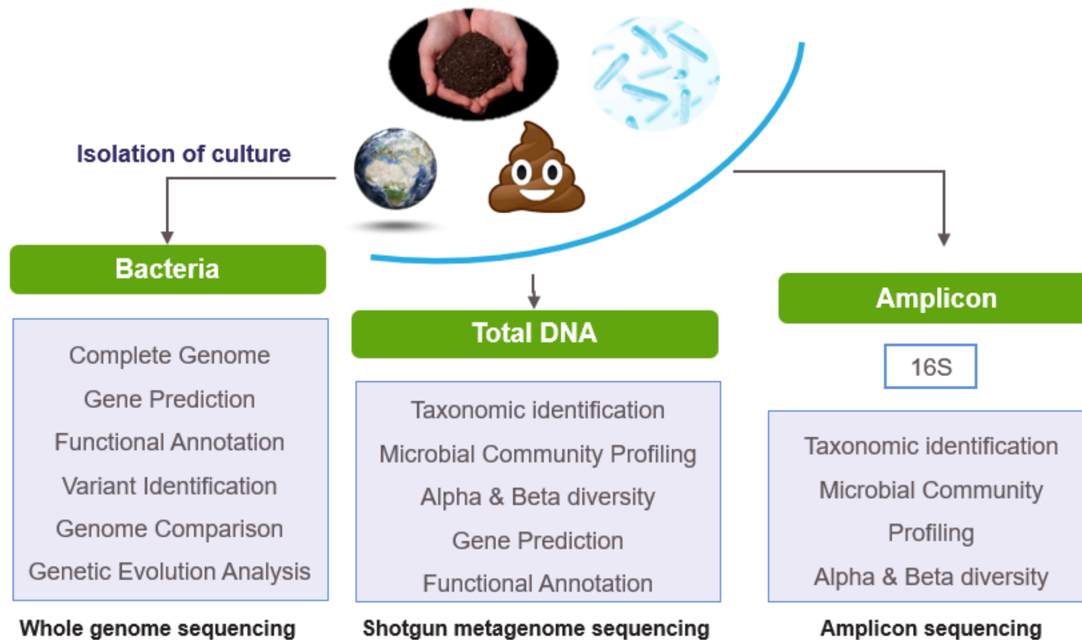


Figure 2. Applications of different sequencing methods.

Overall, the identification of potential probiotic strains by NGS can provide valuable insights of the microbial communities present in a given source.

16S amplicon sequencing

Sequence-based identification of bacteria utilizing 16S ribosomal RNA (rRNA) gene sequencing, which does not require culture, offers a way to circumvent the major drawbacks of culture-based identification.

Since 16S rRNA gene sequence is highly conserved across different bacterial species [97, 98], making it an ideal target for PCR amplification and sequencing. In addition, 16S rRNA gene is large enough, with sufficient interspecific polymorphisms of 16S rRNA gene, to provide distinguishing and statistically valid measurements. The 16S rRNA gene sequence is about 1,550 bp and is composed of both variable and conserved regions which can be divided into 9 hypervariable regions, also known as variable regions (V1-V9).

Previous studies show that no one region adequately differentiates all bacteria [99], and sequencing of select hypervariable regions can yield differing data interpretation [99–101]. For example, the region V1 best differentiated among *Staphylococcus aureus* and coagulase negative *Staphylococcus* sp. V2 and V3 were most suitable for distinguishing all bacterial species to the genus level except for closely related Enterobacteriaceae. V2 best distinguished among Mycobacterial species and V3 among Haemophilus species while V6 can distinguish most bacterial species except Enterobacteriaceae. V6 was also noteworthy for being able to differentiate among all CDC-defined select agents including Bacillus anthracis, which differed from *B. cereus* by a single polymorphism. V4, V5, V7 and V8 were less useful targets for genus or species-specific probes [99]. In general, amplification of certain hypervariable regions may bias results, leading to under- or overrepresentation of taxa, but may also be advantageous for distinguishing between certain species within a genus [102]. Sequencing only a portion of the 16S rRNA gene, instead of the full-length gene reduces phylogenetic resolution, that may not have enough information to accurately distinguish between closely related taxa, which can result in over-representation of some taxa and under-representation of others. [102]For example, the V1–V2 region performed poorly in classifying sequences

belonging to the phylum Proteobacteria, whereas the V3–V5 region performed poorly at classifying sequences belonging to the phylum Actinobacteria [103]. Similar trends were seen at the genus level for taxa of potential medical relevance. This can result in incorrect classification of some taxa and lead to biased conclusions about the composition and diversity of the microbial community.

PacBio and Oxford Nanopore have introduced third generation sequencing (TGS) technologies [104, 105] which are capable of routinely producing reads more than 1,500 bp. Thus, high-throughput sequencing of the full-length 16S rRNA gene is becoming increasingly prevalent. TGS widens the genetic field of view measured by amplicon sequencing, offering the promise of greatly increased resolution in taxonomic profiling applications and measurement of complete functional genes. One of the benefits of using PacBio Circular Consensus Sequencing (CCS) for 16S rRNA gene sequencing is the highest accuracy of the long reads generated. PacBio CCS reads has a self-correction character which produces HiFi reads quality higher than Q30. This allows the detection of rare and novel bacteria, as well as the ability to resolve complex microbial communities. Also, the previous research [104] concludes sequencing the entire 16S gene provides real and significant advantages over sequencing commonly targeted variable regions.

Overall, the 16S rRNA gene sequences allows differentiation between organisms at the genus level across all major phyla of bacteria, species and subspecies level. It provides a comprehensive view of microbial diversity and allows for accurate identification and classification of microorganisms, making it a valuable tool for studying microbial communities and their interactions. Additionally, it enables the detection of rare microorganisms, which may be missed with shorter sequencing fragments.

Analysis of 16S rRNA sequences

The analysis of full-length 16S rRNA sequences involves the following bioinformatics steps:

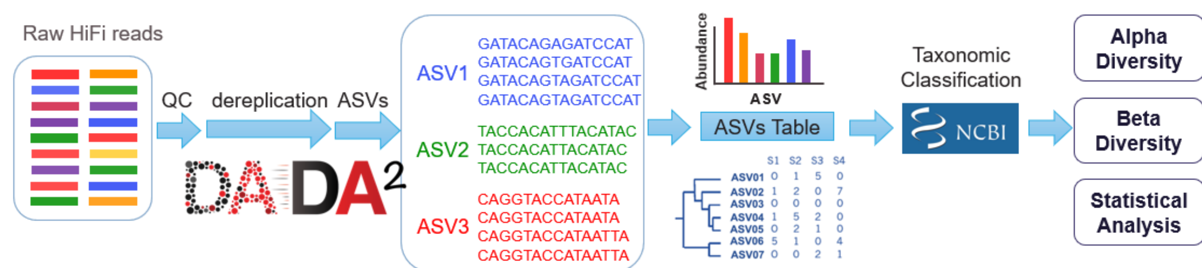


Figure 3. Procedures of bioinformatics analysis of full-length 16S rRNA sequences.

- Quality control & amplicon sequence variants (ASVs) generation: The HiFi reads are further processed with DADA2 (version 1.20) to obtain amplicons with single-nucleotide resolution [106]. DADA2 workflow includes quality filtering, dereplication, learning the dataset-specific error model, ASV inference and chimera removal. The trimming and filtering were performed with a maximum of two expected errors per read (maxEE = 2). DADA2 algorithm resolves exact ASV with single-nucleotide resolution from the full-length 16S rRNA gene with a near-zero error rate.

- Taxonomic classification: For each representative sequence, the feature-classifier [107] and classify-consensus-vsearch [108] algorithm in QIIME2 [109] was employed to annotate taxonomy classification based on the information retrieved from the NCBI database. Analysis using QIIME2 is mainly used for amplicon for some variable regions, V3-V4 among nine 16S rRNA variable regions in the case of Illumina's Miseq platform, or full-length 16S rRNA data generated using PacBio HiFi read. It is an integrated analysis package that includes various types of analysis functions. The analysis is performed in the Command Line Interface (CLI) environment, and visualization of the generated results can be checked through a web browser with graphical user interface (GUI). To analyze the sequence similarities among different ASVs, multiple sequence alignment was conducted by using the QIIME2 alignment MAFFT [110] against the NCBI database [111–113]. The entire process of analysis is carried out through the CLI environment, and the configuration and parameter settings for each command are complicated. Therefore, in the case of researchers who do not have much experience in performing analysis in the CLI environment, the accessibility of analysis using this platform is very low. However, GUI version of QIIME2 is provided by the Galaxy system, and users familiar with the Galaxy server can effectively use this platform.
  QIIME2 [114] (https://qiime2.org)
  If an analysis using CLI environment such as QIIME2 is difficult, researchers can utilize an online-based metagenome analysis server system that can perform GUI-based analysis. One of the most well-known analysis platforms is MG-RAST. MG-RAST is an open-source metagenomic analysis server that can perform analysis through a web browser and can conveniently use GUI-based analysis for NGS data produced for various types of libraries.
  MG-RAST [115] (https://www.mg-rast.org/)

- Diversity analysis: Various diversity analysis metrics can be calculated to understand the community composition and structure. These include alpha and beta diversity metrics.

  - Alpha diversity was indicative of the species complexity within individual samples based on 7 different criteria output, including observed-species, Menhinick's Richness, Margalef's richness, Shannon, Simpson, PD whole tree, and Good-coverage [116]. Beta diversity analysis was used to evaluate the differences among samples in terms of species complexity.

  - Two beta diversity parameters, the weighted and unweighted UniFrac [117, 118], were calculated by using the phyloseq packages in R software. A cluster analysis was preceded by a Principal Component Analysis (PCA), which was applied to reduce the dimensions of the multiple variables using the factoextra and ggplot2 package in R software. Principal Coordinate Analysis (PCoA) was performed using the distance matrix to acquire principal coordinates for

visualization of sophisticated and multidimensional data [119]. A distance matrix of weighted, unweighted UniFrac and Bray-Curtis dissimilarity among samples obtained previously was transformed into a new set of orthogonal axes, by which the most influential variable was represented by the first principal coordinate, and the second most influential one by the second principal coordinate, and so on. Non-Metric Multidimensional Scaling (NMDS) analysis was performed to fit the nonlinear model in ecological datasets [120]. To further increase the group distinction, the supervised Partial-Least-Squares Discriminant Analysis (PLS-DA) was used to evaluate and visualize variance based on ASVs level of gut microbiota composition among the groups. Unweighted Pair-group Method with Arithmetic Means (UPGMA) a hierarchical clustering was performed to interpret the arithmetic distances based on the average linkage algorithm.

- Statistical Analysis: Finally, statistical analyses can be performed to identify significant differences in microbial community composition between different samples or treatments. This can be done using various methods, including metagenomeSeq [121], welch's t-test [122], LEfSe [123], ANCOM [124], ALDEx2 [125], ANOSim, MRPP, or PERMANOVA.

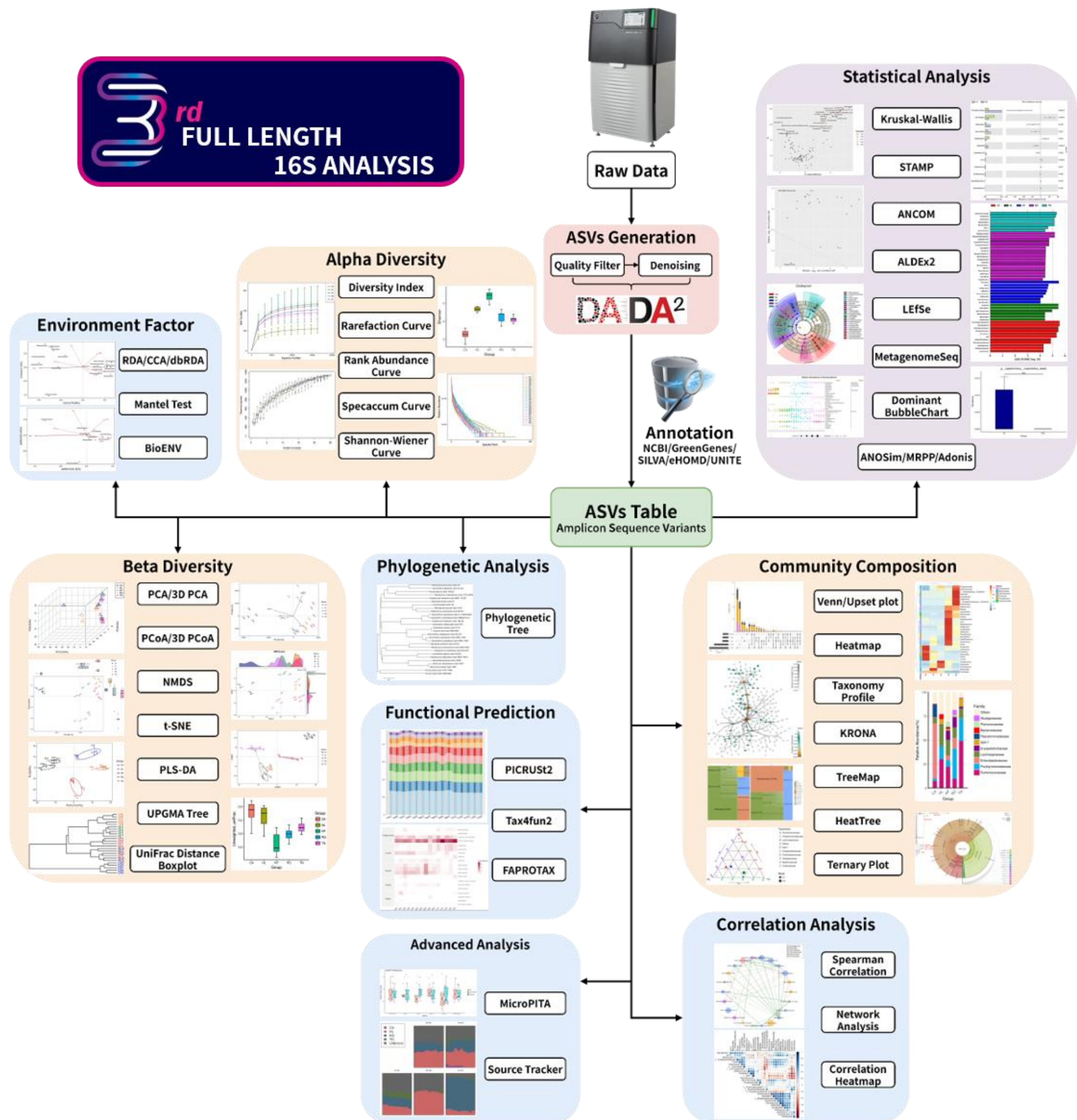


Figure 4.State-of-the-art full-length 16S analysis pipeline.

Shotgun Metagenome Sequencing

Shotgun metagenomic is a powerful tool for studying microbial communities by sequencing all DNA fragments in a mixed sample, allowing detection and identification of the microbial species present that may be difficult or impossible to culture on agar medium. The DNA in a sample is extracted and randomly fragmented into small pieces. These small fragments are sequenced using high-throughput DNA sequencing technologies, such as Illumina sequencer. Because the DNA is fragmented randomly, the sequenced data contains information about all the microorganisms in the sample, rather than just a few that were targeted for amplification and sequencing [126]. Then the sequenced data will be analyzed using bioinformatics tools to identify the genetic sequences of different microorganisms in the sample.

Shotgun metagenomic sequencing provides high resolution when it is paired with advanced bioinformatic techniques such as assembly, binning, and annotation. These methods can help in reconstructing the genomes of individual microbes, which can be used to identify the species present in the sample [127–129]. The bioinformatics workflow for shotgun metagenomics involves several steps, including quality control, de novo assembly, gene prediction, binning, taxonomic classification, and functional annotation.

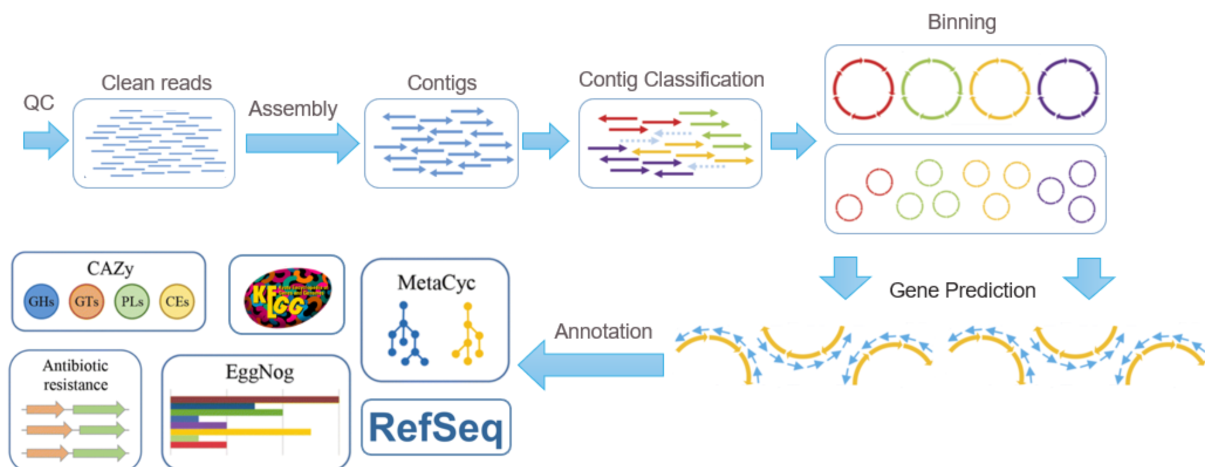


Figure 5. General workflow of shotgun metagenomic analysis.

Overview of the workflow for shotgun metagenomic analysis is described below:

- Quality control: The first step in the bioinformatics workflow is to perform quality control on the raw sequence data are filtered by Trimmomatic [130] to remove low-quality reads and trim sequencing adapters. Bowtie2 [131] is used to remove host contamination.
- Assembly: The high-quality reads are assembled into contigs using assembly tools [132–134] such as MEGAHIT [135], SPAdes [136, 137], or metaSPAdes [138].
- Gene prediction and annotation: Prodigal [139] is used to predict the open reading frames (ORFs) from the assembled contigs. A non-redundant gene catalog is constructed using CD-HIT [140, 141] with 95% identity.
- Binning: The resulting contigs or scaffolds are binned into individual genomes or groups of related genomes using binning tools such as CAGs [142], MetaBAT [143, 144], MaxBin [145, 146], or CONCOCT [147].
- Taxonomic classification: The binned genomes or Unigenes are taxonomically classified from NCBI RefSeq [148] using DIAMOND software [149]. The genome percentage completeness and contamination of all bins are assessed using CheckM [150].

- Functional annotation: The predicted protein-coding genes can be mapped to metabolic pathways database (KEGG) [151–154], functional category database (COG) [155] and eggNOG [156, 157], nitrogen cycling genes database (NCyc) [158], carbohydrate active enzymes database (dbCAN2) [159, 160], virulence factors database (VFDB) [161–163], antibiotic-resistance genes database (CARD) [164–168], antibacterial biocide- and metal0resistance genes database (BacMet) [169], and pathogen host interactions database (PHIbase) [170–174].

	Amplicon Sequencing	Shotgun Metagenome Sequencing	
	Full length / v3v4 16S	Mapping-based	Assembly-based
PROS	• Less expensive • non-host contamination • Full length 16S confers species-level resolution	• Fast, scales to large datasets • Less sequencing depth • Sub-species level resolution • Gene function	• Unmatched for identifying novel genomic and gene diversity • Profiling unknown/novel species • Gene function
CONS	• v3v4 resolution limited to genus level • Can't see "within genomes" (only sequence one gene) • PCR amplification	• Limited to identifying sequences like those that have been seen before (unknown species) • Host contamination	• Can't assemble a sequence unless it is well-covered in the community • Extremely resource-intensive • Host contamination • High Cost

Figure 6.Comparison of microbial sequencing methods.

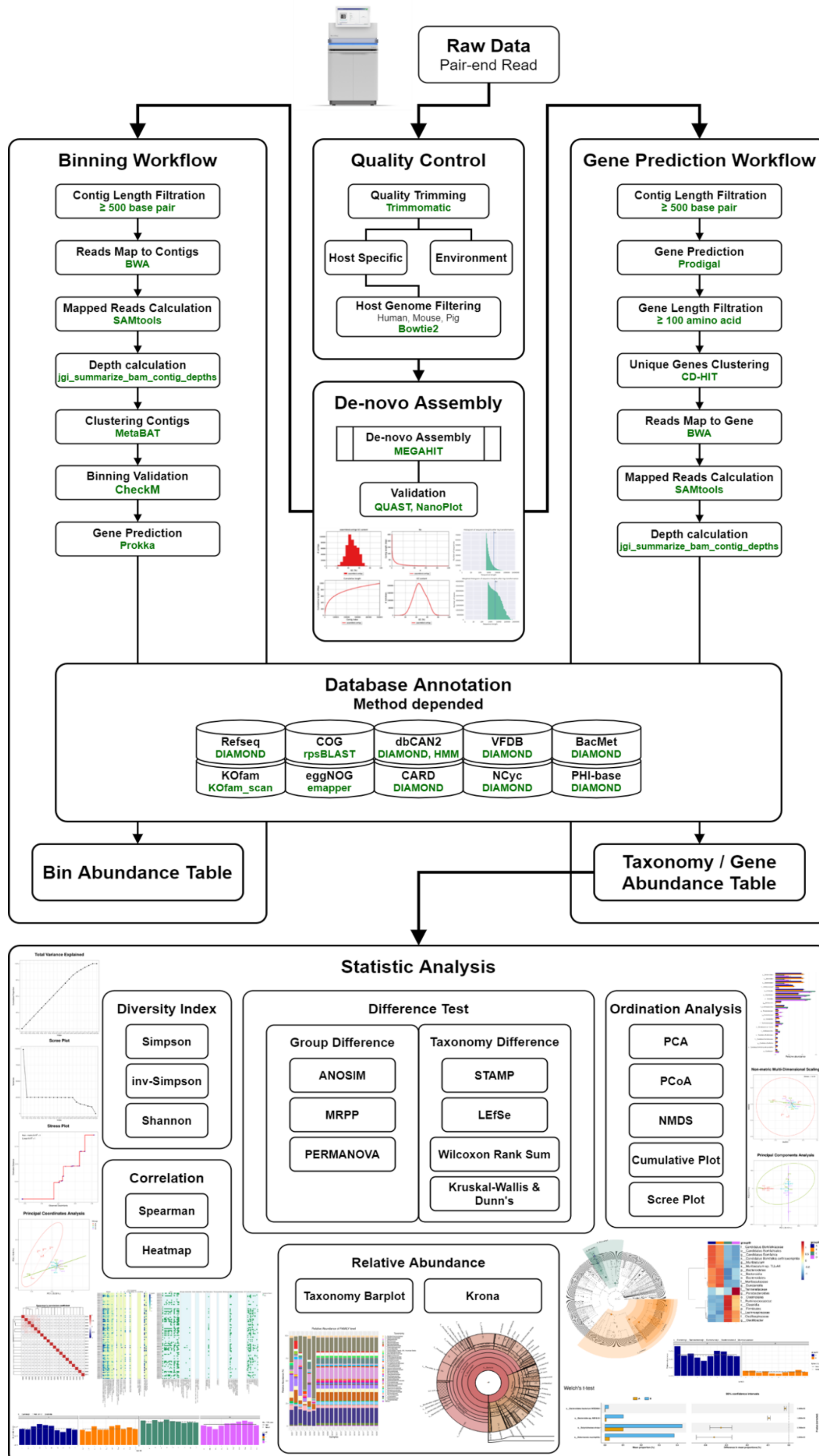


Figure 7.State-of-the-art shotgun metagenomic analysis pipeline.

Other programs for shotgun metagenomic analysis Kraken2 is an analysis tool that identifies the species of microorganisms included in the produced data using a unique K-mer combination for each species' genome. This tool can be used for 16S rRNA data as well as

shotgun metagenome sequencing. It works only in the CLI environment, so it can be difficult for users who are not familiar with CLI. Filtering is also essential for analysis using Kraken2 to get rid of the errors and avoid false positive. In addition, subsequent analysis programs such as Kraken-HLL or Braken can be useful to generate a more accurate taxonomic profile.

Kraken2 [175] (**https://ccb.jhu.edu/software/kraken2**)

Metaphlan4 is a tool that can check the composition of Bacteria, Archaea, and Eukaryotes in shotgun metagenome sequencing data. It identifies the species and microbial load of microorganisms based on the gene profile in whole meta-shotgun sequencing data. As an open-source program, it is used in the CLI environment. The accuracy of this tool for species identification is higher than K-mer based algorithm but the performance was not satisfactory as reported in a probiotic testing previously [176].

MetaPhlAn4 [177] (**https://huttenhower.sph.harvard.edu/metaphlan/**)

Megahit is an analysis program that can be used to assemble high-accuracy shotgun metagenome sequencing data produced through short-read sequencing such as the Illumina system. This tool can be freely used for research purposes through the GPLv3 License. The program is very intuitive and easy to use. However, like most analysis programs, this analysis is performed in a CLI environment rather than a GUI. But the installation of the analysis tool and usage is very simple, so researchers who are not familiar with CLI environment can also simply use this analysis tool. However, metagenome assembly requires large physical memory for computing systems. Therefore, a workstation that has at least 100 GB RAM is recommended.

Megahit [178] (**https://github.com/voutcn/megahit**)

Flye, a representative long-read assembly analysis tool, can also perform metagenome assembly on shotgun metagenome sequencing data using the internal module called metaFlye. It can be used freely under the BSD-3-Clause license, and by combining with polishing tools for each manufacturer such as gcpp or Medaka. Same as megahit, metagenome assembly needs large physical memory in the computing server, so proper servers have to be prepared before analysis.

metaFlye (**https://github.com/fenderglass/Flye**)

Whole Genome Sequencing

Whole genome sequencing involves analysis of a bacterium's complete genome and comparing it to a reference genome. It allows the high-resolution study of a bacterium's characteristics on antibiotic resistance, virulence factors, genetic variation from a group of bacteria at multi-levels. Readers may also refer to Gautam et al. [179] for a simplified step-by-step guide for WGS. This section simplifies the overall workflow of WGS analysis.

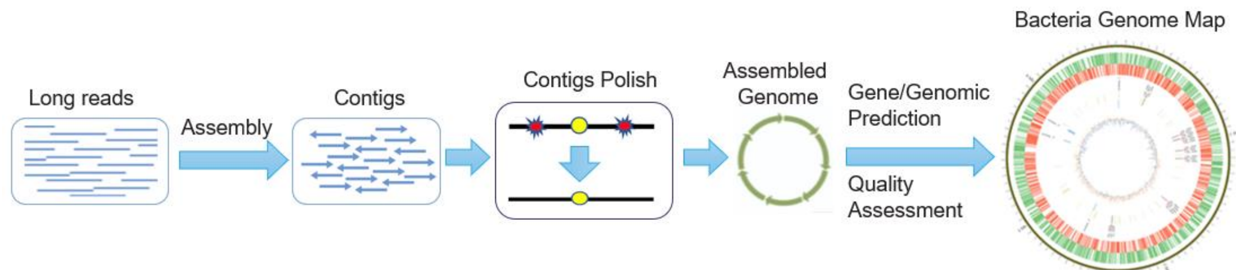


Figure 8. General workflow of WGS analysis.

Overview of the workflow for WGS analysis is described below:

- Long Read Assembly: Reads with average quality above Q7 were considered as "pass" reads for subsequent analysis. Sequencing result checked by NanoPack [180] to validate the read length profile. After that, raw read was assembled by Flye [181] or Canu [182] to obtain the primary contigs.

- Flye is an open-source assembler that can perform the assembly of both PacBio and Nanopore data. However, this tool is not able to circularize the genome. Although this tool is not available on GUI, it is performed in the CLI environment, and the command is simple. Hence, it is suitable for everyone, even beginners.
  Flye [181] can be accessed at: **https://github.com/fenderglass/Flye**

  Canu, an open-source platform that runs in a CLI environment can also be used for both PacBio and Nanopore data. Compared to Flye, Canu is slower and uses more memory. However, it provides circularization information.
  CANU [182] can be accessed at: **https://github.com/marbl/canu**

  In addition, SMRTLink is a comprehensive analysis tool provided by PacBio, which can be freely used for research purposes and is specialized in PacBio data analysis. After the initial installation is done through the command line interface (CLI), all analysis and processes are composed of a web browser-based GUI that is easy to use. There are various analyses available in SMRTLink. For instance, HGAP assembler can be used for probiotics genome assembly and to circularize the assembled genome.
  SMRTLink – Hierarchical Genome Assembly Process (HGAP) assembly can be accessed at: **https://www.pacb.com/support/software-downloads/**

- Contigs Polish: In general, the data generated by long-read sequencing devices such as PacBio and Nanopore have high potential to carry errors, even in the assembled output. For higher accuracy, hybrid polishing method performed using additional Illumina short read has been widely used. However, this method is very expensive. More recently, several correction algorithms have been developed to solve this problem. For example, gcpp is a polishing tool provided by PacBio and it has been optimized for base sequence correction in sequence assembly. It is included in the SMRTLink package and an open-source program that can be freely used for analysis purposes.

gcpp can be accessed at : **https://github.com/PacificBiosciences/pbbioconda**

For Nanopore technologies, Medaka has been used to correct the error base sequence within the assembly. In the case of Nanopore data, the selection of the base-calling model and pore version is very important. So proper error correction model has to be selected for accurate correction. Medaka is an open-source program and can be freely used for analysis purposes.

Medaka can be accessed at: **https://github.com/nanoporetech/medaka**

- Finally, for tools that are performed in CLI environment, Homopolish can be used. This tool can run the polishing process using the public genome of the same species. For long read sequencing data that contains errors, base sequence correction can be done based on the consensus of 20 genomes of the same species in the NCBI database. This is very useful for most of the probiotics species, as many high-quality genomes are already available in the database and the erorrs can be easily corrected. However, as this tool is dependent on the sequences in the database, sometimes specific mutation of the individual strains can be recognized as errors and removed. Hence, it is recommended to use it in combination with other existing polishing tools.

Homopolish [183] can be accessed at **https://github.com/ythuang0522/homopolish**.

- On the other hand, the contigs can also be polished by Racon [184] with the read alignment result constructed by Minimap2 [185], then processed the result with Medaka to acquire model correction. The quality of the consensus can also be achieved by aligning the Illumina reads to the model corrected contigs with BWA-MEM [175] and polishing consensus by pilon [186] d. Finally, contigs were automatically corrected via the homologous sequences extracted from closely related genomes by homopolish [183].

- Quality Assessment: Fully polished contigs were analysis by QUAST [187] and BUSCO [188] to evaluate the quality of assembly and the completeness of genome respectively.

- Gene Prediction & Annotation: Prokka [189] with default setting was used to predict open reading frame (ORF) and search for tRNA and rRNA region. Gene annotation was conducted by sequence align against the Refseq, COG, eggnog, KOfam, VFDB, CARD, NCycDB, dbCAN2 and PHIBase [148, 151–156, 158–166, 169–172, 174] database using DIAMOND [149], HMMER [190] and another database specified annotator.

- Genomic Prediction: For annotation of genomic features, several prediction tools were employed to predicted with default setting. RepeatMasker [191] was used to locate the repeat sequence such as SINE, LINE, LTR, etc. in genome. ProphET [192] predicted the prophage region by searching phage-like gene cluster in genome. Alien hunter [193] was used to predict the genomic island and CRISPRCasFinder [194, 195] was applied to annotate the CRISPR-Cas cluster.

- ResFinder EFSA is one of the analysis platforms included in the Center for Genomic Epidemiology (CGE) and it is a program used as a standard for antibiotic resistance gene analysis by the European Food Safety Authority (EFSA). It is an open-source program, and can be performed on both raw data generated from short-read NGS systems and assembly results. By providing a web browser-based GUI, even those

who have no experience in bioinformatics can perform analysis. A CLI version of the standalone program is also provided for large-capacity analysis.
ResFinder EFSA [196] (**https://cge.food.dtu.dk/services/ResFinder-EFSA2022/**)

RGI is an analysis program that uses the Comprehensive Antibiotic Resistance Database (CARD). Like ResFinder, it provides a web browser-based GUI environment and can be used freely for non-commercial purposes. The CARD database used by RGI contains the largest antibiotic resistance genes, so analysis using RGI can provide the most diverse result about known antibiotic resistance genes. However, unlike ResFinder, it requires assembled sequence rather than raw data as input, so single or metagenome assembly must be conducted. In the case of GUI version, GUI version is not designed for metagenome assembly, so the size of the query file is limited to 20 Mb. Therefore, analysis must be performed separately if the analyzed sample has several types of probiotics strains.
Resistance Gene Identifier – RGI [167] (**https://card.mcmaster.ca/analyze/rgi**)

AMRFinderPlus is a tool for Antimicrobial Resistance (AMR) Gene analysis provided by NCBI. It is an open-source tool and it can be used freely for research purposes. It uses its own AMR database built by NCBI However, it only works in CLI environment, so unfamiliar users with CLI may have difficulty in executing this analysis tool. Like RGI, since this tool was designed for a single assembled genome, analysis cannot be performed directly using shotgun metagenome sequencing data produced by NGS, and genome assembly is essential before analysis.

NCBI AMRFinderPLUS [197] (**https://github.com/ncbi/amr**)

VirulenceFinder is an analysis program provided by CGE along with Resfinder, AMR gene analysis tool. It is an open-source program that analyzes assembly or raw data through GUI. There are four available target species, and only the Enterococcus family among known probiotic species can be analyzed.
⊙ Virulence Finnder [198] (**https://cge.food.dtu.dk/services/VirulenceFinder/**)

VFAnalyzer is an analysis tool provided by the Virulence Factor Database (VFDB) that can perform analysis based on VF genes sequences deposited in the VFDB. Besides Enterococcus family, VFAnalyzer can analyze VF genes for additional probiotics species such as Streptococcus. However, since this platform is impossible to analyze raw NGS data, it is essential to conduct assembly before analysis. It is convenient to use via GUI environment and can be used freely for research purposes.

VFAnalyzer[199](**http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=VFanalyzer**)

Overall, the analysis workflow for WGS is complex and involves many steps. However, with the help of powerful bioinformatics tools, it is possible to obtain a detailed understanding of the structure, function, and evolution of bacterial genomes.

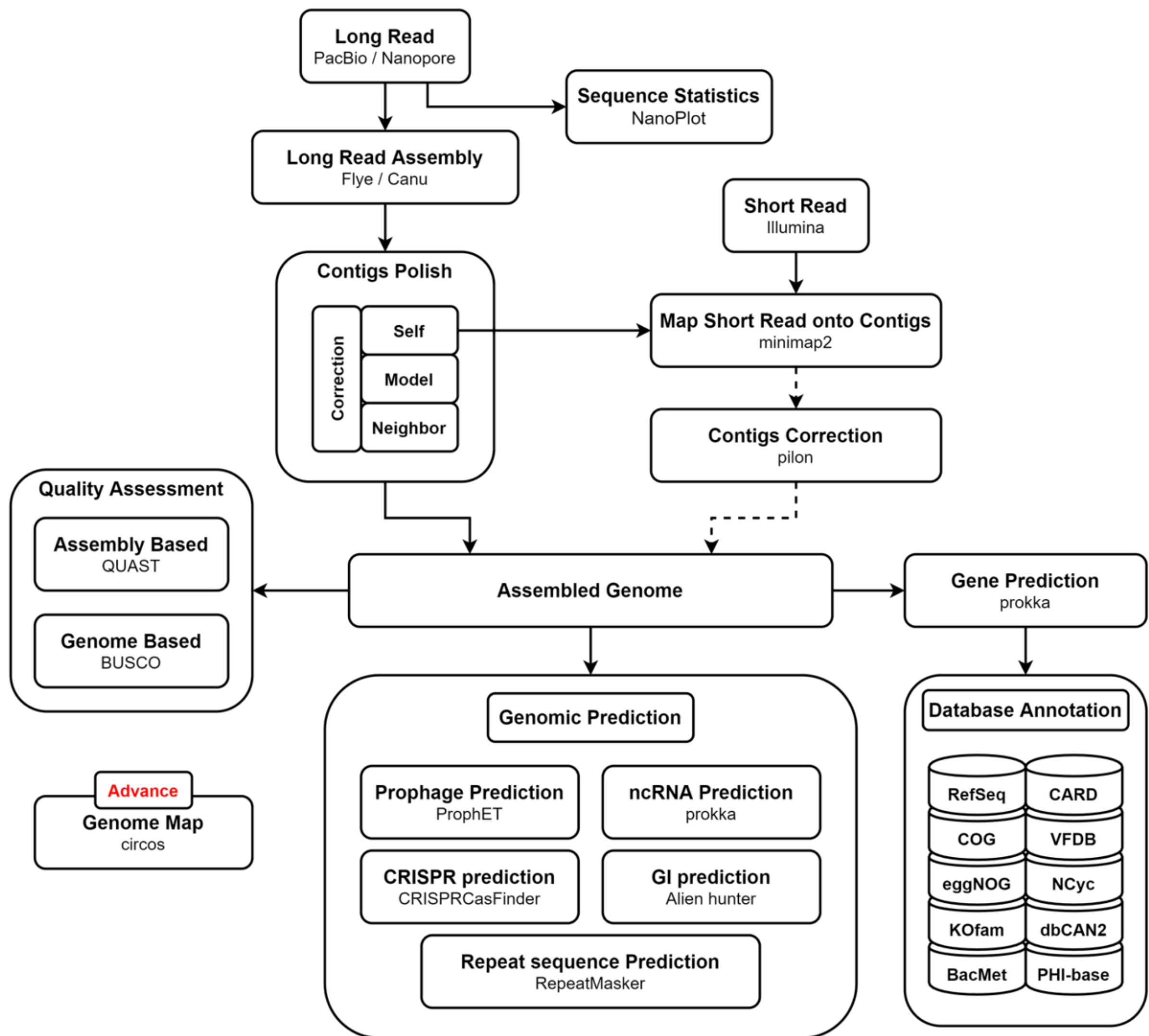


Figure 9. State-of-the-art bacterial genome analysis pipeline.

Choosing an appropriate sequencing method and a suitable sequencing platform

We have discussed multiple methods in the previous handbook sections. After choosing the most appropriate sequencing methods, you will need to select the most appropriate sequencing platforms depending on the criteria you set, including:

- Sequencing objectives,
- Financial resources,
- Sequencing time,
- Throughput, and
- Accuracy

Short-read or long-read sequencing?
Selecting the appropriate sequencing platform is a pivotal step in any sequencing experiment, prompting the consideration of short-read or long-read sequencing. Short-read sequencing yields shorter read lengths, while long-read sequencing generates longer fragments. Short-read sequencing produces fragments ranging from approximately 50 to 500 base pairs (bp), whereas long-read sequencing generates fragments spanning 5,000 to 30,000 bp.

Short-read sequencing offers a quick turnaround time and is cost-effective compared to long-read sequencing. This sequencing approach has played a crucial role in genomics, evolutionary studies, disease investigations, genome assembly, and gene function analysis. It delivers high-quality reads, allowing researchers to achieve extensive genome or target coverage, facilitating accurate identification of single nucleotide polymorphisms (SNPs) and mutations with confidence. Notable platforms driving this technology include Illumina and ThermoFisher (Ion Torrent). However, short-read sequencing results often exhibit fragmentation, and the amplification steps introduce potential sample biases. Moreover, generating sufficient overlapping DNA fragments can be challenging.

Conversely, long-read sequencing produces longer reads, aids in detecting insertions, deletions, translocations, inversions, repeats, and duplications. Additionally, long-read sequencing facilitates direct haplotyping [200], contributing to its appeal. Nonetheless, this platform is recognized for its lower throughput, higher error rates, and higher operational costs. Accuracy per read is typically lower compared to short-read sequencing, largely attributed to difficulties in controlling the speed of DNA molecules through the pore. Prominent platforms for long-read sequencing encompass Oxford Nanopore Technologies (ONT) and Pacific Biosciences.

To determine the most suitable sequencing approach for probiotics testing, the focus rests on strain identification, safety assessment of probiotic strains, and detection of contaminants (any microorganisms that other than the intended probiotics). Short read sequencing is often chosen for analyzing microbial composition and identifying microbial contaminants in a product. This method targets specific markers such as the 16S rRNA gene for bacterial identification, obviating the need for bacterial cultivation. Given its cost-effectiveness, high throughput, and speedy results, short-read sequencing excels at revealing microbial composition. Nevertheless, it may fall short in enabling strain-level identification.

For evaluating the safety of probiotic strains, encompassing the presence of antimicrobial resistance genes and virulence factors, long-read sequencing is more suitable than short-read sequencing. Long-read sequencing furnishes more comprehensive genetic insights into bacteria through longer reads, yielding more complete genome information and accurately capturing genetic variations, insertions, deletions, or translocations within the genome. This

technology enhances precision in strain identification, necessitating prior bacterial cultivation and DNA isolation before initiating the sequencing process.

While long-read sequencing boasts these advantages, it also presents challenges such as higher operational costs, lower throughput, and a demand for specialized expertise in bioinformatic analysis pipelines. Regulatory applications require stable methods that offer accurate results, study of antimicrobial resistance genes, virulence factors, and confirming the viability of probiotics via cultivation, making long-read sequencing favorable. Conversely, quality assurance, operational costs, turnaround time, and output might take precedence. Thus, short-read sequencing proves more suitable for determining overall microbial composition and contamination, vital for quality assurance.

In conclusion, selecting the appropriate sequencing approach hinges on specific probiotics testing objectives, available financial resources, and expertise in executing bioinformatic analysis.

International Application of Next Generation Sequencing in Probiotics Testing

China Risk Assessment Center for Food Safety, People's Republic of China

NGS has been applied at the China Risk Assessment Center for Food Safety (CFSA) to assess the safety use of new probiotic strains: two *Lactobacillus rhamnosus* (coded with L1 and L2) and one *Lactobacillus paracasei* (coded with L3). All these three strains were evaluated for their safety according to the following procedures.

Antimicrobial resistance susceptibility and animal testing
Three strains of probiotics were tested for their antimicrobial resistance susceptible to ampicillin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol and the results were expressed as the minimal inhibitory concentration (MIC). Meanwhile, the pathogenicity of strains was also tested by oral administration in mice. All three strains were not resistant to any of the antimicrobials tested. No abnormal manifestations and deaths related to administration of these three probiotics were found.

Genomic analyses
Genomic DNA of three probiotic strains were extracted and purified using the Bacterial DNA Kit (OMEGA, USA) following the procedures recommended by the manufacturer. WGS was carried out using both the Pacific Biosciences RS II platform (Pacific Biosciences, USA. SMRTbell Template Prep Kit, Sequel binding and Internal Control Kit, SMRT Cell, Sequel Sequencing Kit were used) and Illumina NovaSeq PE150 platform (Illumina, USA. NEBNext® UltraTM DNA Library Prep Kit was used). *De novo* assembly of the reads was performed using a hybrid assembly algorithm implemented in Allpaths-LG software (v44620; http://www.broadinstitute.org/software/allpaths-lg/blog/) [201]. SpeciesFinder 2.0 was used to identify the species [202]. The virulence factor database (VFDB, http://www.mgc.ac.cn/VFs/main.htm) was employed to predict the presence of any known virulence factors in the genomes of the sequenced strains with BLASTP and filtered with 80% identity and 95% match length [203]. The antimicrobial resistance genes were predicted referring ResFinder database (ResFinder v4.1, [204]) with 80% identity and match length. The genomes were annotated with Prokka 1.14.5 [189]. Roary v3.11.233 was used to compare the core genome sequence of all three sequenced strains together with reference genomes downloaded from the NCBI database [205]. According to the core genome alignment, FastTree v2.1.10 was used to analyse the evolutionary relationship and construct the

phylogenetic tree [206]. Figtree v1.4.4 was employed to visualize trees [207].

The strains of L1 and L2 were identified as *L. rhamnosus* while *L. paracasei* for strain L3, based on the whole genome data. Analysis of genomic sequences showed that strains of *L. rhamnosus* L1 and L2 consisted of a single circular chromosome, whereas a single circular chromosome as well as a circular plasmid were identified for *L. paracasei* L3. Under the analysis conditions stated above, any known virulence factors and antimicrobial resistance genes were not found on either chromosomes or plasmid of these three probiotic strains. Comparison and evolutionary analysis based on the core and pan genomes revealed that *L. rhamnosus* L1 and L2 were highly similar to *L. rhamnosus* ATCC 53103, and strain *L. paracasei* L3 was closely related to type strain of *L. paracei* (shown in Fig. 1 and 2, respectively).

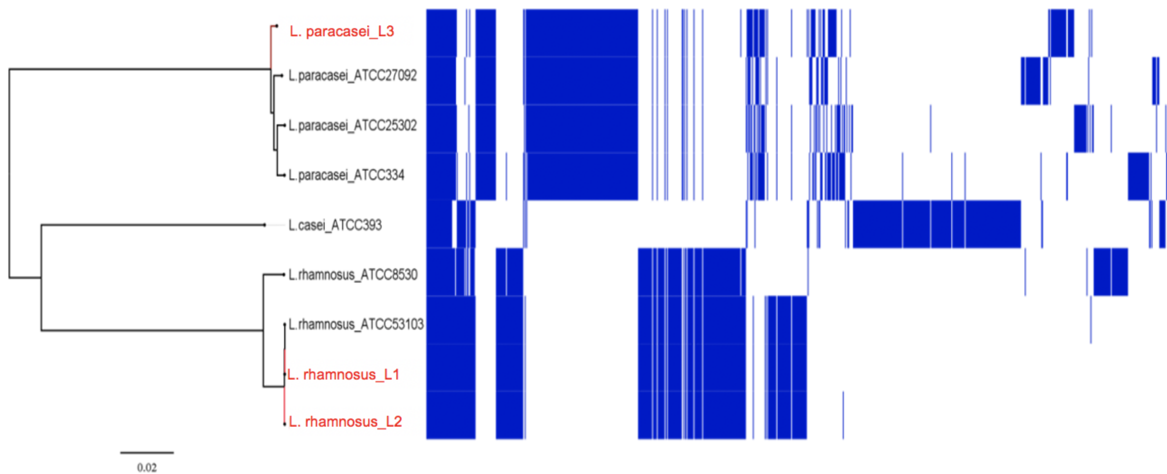


Figure 10.Comparison of core genome sequence between strains tested with corresponding type strains based on Roary.

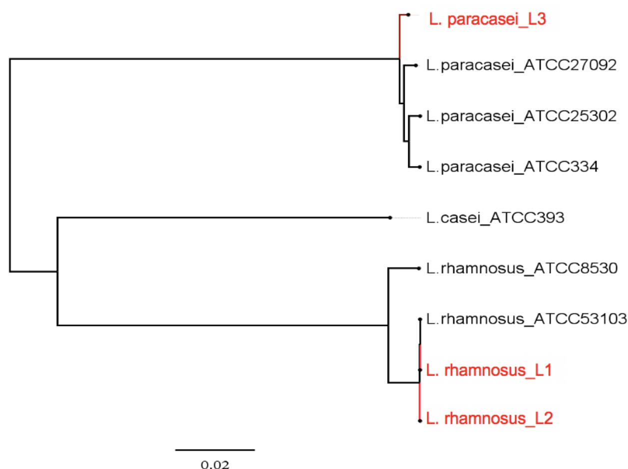


Figure 11. Phylogenetic tree analysis showing the relationships between three probiotic strains and type strains based on Figtree.

Ministry of Food and Drug Safety, Republic of Korea

As discussed in section 2.1, there are 19 bacterial species permitted (FFC Code). Among these approved bacterial species, 2 species of Enterococcus must obtain prior approval from MFDA for the absence of antibiotic resistance factors in the genome for individual strains

before use. The absence of antibiotic resistance factors in Enterococcus strains has to be demonstrated through the construction of complete genomes using WGS based on NGS and bioinformatics analysis.

MFDS conducts random sampling of probiotics products available in the market to inspect:
1. whether the labelled strains are present in the products, and/ or,
2. whether the viable cell counts are maintained at least at 10^8 CFU/g during the indicated shelf-life.

The inclusion of labelled strains is checked using the NGS: Shotgun Metagenome sequencing-based strain discrimination program [176] developed by MFDS, and multiple products are quickly tested through NGS multiplexing. The shotgun metagenome sequencing-based analysis method of MFDS uses a mapping coverage-based algorithm and the detailed algorithms are described in the paper "Accurate and strict identification of probiotic species based on coverage of whole-metagenome shotgun sequencing data"[176].

If a specific product is expected to have a problem in the shotgun metagenome sequencing-based method, additional meta community analysis based on amplicon sequencing, such as PacBio 16S full length HiFi read method or 16S Sanger sequencing based on the bacterial culture isolated for confirming the analysis result.

Measurement of more than 10^8 CFU / g of viable probiotics maintained during the shelf life is calculated based on the plate count method using De Man Rogosa Sharpe (MRS) or BL medium.

Food and Drug Administration, The United States

Use of genomic-scale technology has been applied to probiotics and cultured food products in the US. The first uses of genomics for regulatory needs has traditionally focused on contaminants and, specifically foodborne pathogens (GenomeTrakr Network | FDA) including for public health purposes (PulseNet | PulseNet | CDC) and has extended to nosocomial pathogens [208]. However, there was a recognized need to address live microbial products given the investments in tens of thousands of pathogen genomes that have been sequenced to date and deposited in the public repositories such as the National Center for Biotechnology Information's (NCBI) whole genome sequence database and sequence read archive (SRA) for storing raw sequence datasets. The objective in establishing such infrastructure is primarily focused on delimiting the scope of outbreaks and interrupting further transmission. These efforts have led to the identification of potential reservoirs of foodborne and nosocomial pathogens that could not have been achieved with the standard low resolution and targeted molecular techniques that have disadvantages when new strains with key signatures emerge that require retooling. It was recognized early in the sequencing revolution that using highly granular and replete/discreet datasets that genome sequencing affords have the ability to lead, direct, and/or complement aspects of relevant epidemiological analyses for public health. Additionally, the principles around public release and availability of such data is important as a resource (and distributed effort) for comparative genomic analysis in establishing/exploring species diversities, economy surveillance and associated trends, and context for single nucleotide polymorphism (SNP) differences for determining outbreak inclusivity/exclusivity. Regardless, a rationale was developed for the need to apply such powerful technology live microbial products. These efforts were intended to bring balance to an otherwise counterintuitive paucity of available data for such products including genus species that lack public genome sequence availability but nonetheless are intentionally consumed, in many cases, at the level of billions of bacteria multiple times daily.

Regulatory development: Before the advent of next generation sequencing technology, genomic-scale analysis of bacteria was heavily dependent on high density microarrays capable of encoding millions of strain-specific probes targeting whole genome genetic repertoire of multiple organisms in parallel. While these have been increasingly eclipsed by rapid and high throughput sequencing technology, their utility is still relevant with human diagnostics and research.

FDA "GutProbe" microarray: US FDA invested heavily in this technology and applied its use with strains frequently found in dietary supplements, foods, and the gut microbiome. The array consisted of five *Bacillus*, 18 *Bacteroides*, eight *Bifidobacteriums*, 13 *Clostridia*, two *Enterococci*, 24 *Lactobacilli*, one *Lactococcus*, two *Leuconostoc*, and two *Pediococcus* species representing in toto 92 whole genomes and 229 plasmids over 2·5 million probes [209]. The design and applied utility of the array was intended to assess DNA preparations directly from product and was successfully able to identify microbial constituents in single and multiple strain blends from eight probiotics found on the US market in one assay platform. Along with typical validation and determining limits of detection, array profiles were able to provide some relative quantification of constituents in multiple strain blends and, interestingly, was quite exquisite in determining lot-to-lot variations. Herein, it was able to demonstrably detect a change in formulation of one product which would prove useful for GMP-type applications [209]. Given the design elements, additional application was intended for use with cultured and/or microbially fortified foods and, with forward visioning, for needed safety and toxicology studies to measure relevant gut community impacts especially with anticipated next generation probiotic strain development.

While the array designs can be modified, generally this type of approach suffers from typical constraints of "solid state" molecular genetic platforms that require additional efforts to modify designs based on changing microbial landscapes and product diversity. However (and although traditional arrays have become technologically passé), there is value in transforming such proof-of-concept to currently available multiplexed strategies and panels to provide rapid assessments that eliminate the need for back-end bioinformatic analysis required with genome sequencing datasets. Regardless, this platform was indeed a powerful, first of its kind, metagenomic-scale toolbox intended to advance safety and manufacturing of products along with providing an additional resource for advancing gut community analysis and associated impacts.

Metagenomic sequencing: More recently (and currently ongoing), FDA has invested in the use of next-generation sequencing for live microbial dietary supplement products initially intended to assess and validate labeling information but with a view towards use for potential post-market surveillance. Similar to the approach with microarray analysis, the initial attempt involved a culture independent, direct from product application. The analytical power was developed from an in-house species-specific k-mer counting method that could achieve some stable, quantifiable relative abundances with constituents >1.5% of the final blend. Testing of ten top-selling products revealed several mislabeling issues mostly related to incorrect or outdated taxonomy and some issues in low confidence in the presence of certain constituents that were identified at or slightly below the limits of detection. Relatedly, there were some instances of labeled strains that were simply not detected in the analysis and at least one case of a potential contaminant, Enterococcus faecium, that was subsequently confirmed through culturing. Additionally, lot-to-lot variation using products implicated/detected with the GutProbe array were also confirmed by sequencing.

Since this initial published study, sequencing analysis has been successfully conducted on 123 live microbial dietary supplements of which labeling compliance was achieved for 80 products while discrepancies were identified from 43 products (35%) involving either species identified but not on the label, conversely on the label but undetected, or both (unpublished

data, Dr Carmen Tartera, FDA CFSAN). While this approach was the first of its kind for marketed live dietary supplement products, other studies have been published subsequently from several other economies including People's Republic of China, India, and Europe [210–212]. Interestingly for the study of products sold in the mainland People's Republic of China market, approximately 24-30% exhibited labeling discrepancies identified by sequencing or culturing techniques – similar in principle to the experience documented in the US. Furthermore, Lugli et al.[212] identified another potential important consideration involving genome evolution and stability detected with *Bifidobacterium animalis* subsp. *lactis* BB-12 when comparing product-derived sequence to sequence generated from the same strain many years prior revealing nearly 200 nucleotide polymorphisms. A similar comparative assessment in this study with another genus species, *Lactobacillus acidophilus* La-14, exhibiting lower levels of time-associated sequence polymorphisms (<10), highlights important considerations of genus and species stabilities and need for databasing depth (including temporally) to document such differences.

Collectively, these studies highlight some limitations of direct from product metagenomic sequencing for detecting and distinguishing low-level constituents and contaminants. Before application to products, much of the initial sequencing work at FDA involved mixing of known species in mock communities but could only achieve modest levels of detection. To address this need, some recent developmental work with bacteriophages and/or associated lysin specific for *Lactobacillus*, *Streptococcus* spp., *Bacillus subtilis*, and *Bifidobacterium* has been undertaken to reduce or eliminate the major strains in respective product formulations and effectively increase assay sensitivity for low-level constituents and contaminants. Some success has been achieved using contrived samples spiked with *Escherichia coli* (unpublished data, Dr Carmen Tartera, FDA CFSAN).

Database development: For better context and robust evaluation of culture-independent metagenomic sequencing efforts, standard microbial culture analysis of products was conducted in parallel as a gold standard reference of ground truth. Single colony isolated from probiotic products were obtained, banked, sequenced, and hence, the goals were expanded to build out culture-dependent use of NGS in this regard. With a recognized need for increased genomic depth for the species and strains typically found in US products, the culture work was leveraged for the creation of a first-of-its-kind public genomic database of microbes commonly found in dietary supplements to support regulatory needs, post-market surveillance, and enhance industry quality standards and capabilities (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA336518) amassing nearly 7000 entries to date. Subsequently, an additional database was established for fungal strains found in foods (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA482816) which have been implicated in some cultured food products (for example, see [213]) and noting, for one systemic review at least, fungemia as the most commonly observed adverse condition from probiotic consumption [214]. These efforts have proven to be an important nexus between cultured foods and dietary supplements.

Public health: FDA and CDC work together to resolve outbreaks and have pivoted quite extensively to genome sequencing in recent years. However, the cases with probiotics are relatively limited but generally seem to relate to use in at risk populations. Most notable is prophylactic use in preterm infants at risk of developing necrotizing enterocolitis. One systemic review in this regard cites 32 cases of adverse events (including some from the US) with the most commonly identified organisms in descending order of occurrence being Bifidobacterium, Lactobacillus, and Saccharomyces. Of these, 25 involved genomic analysis that implicated the administered probiotic [215]. Further specific examples in this area (albeit from other economies) have had demonstrable use of genome sequencing for connecting associated product to Bifidobacterium longum bacteremia [216] and a relevant, noteworthy observation from another study involving Bifidobacterium breve and the increased time

required if using a culture-only approach given related fastidious anaerobic requirements of such organisms that are outliers in typical clinical laboratory workflows [217]. An additional systemic review also highlights use and associated mortality with those over the age of 60, Clostridioides difficile colitis, antibiotic use, and interestingly Saccharomyces infection [214]. This review also noted that infectious complications predominately involved Saccharomyces and, further expanded the above list associated with preterm infants, to also include *Bacillus*, *Pedioccoccus*, and *Escherichia*.

As an extension to "at risk" populations, increased attention has been given to probiotics to mitigate antibiotic use and relatedly for prevention of antibiotic-associated diarrhea and *C. difficile* infection. To help inform clinical guidance and practice in the US and quantify inpatient probiotic utilization, CDC conducted a descriptive study surveying prevalence of probiotic use in a sample of 145 hospitals. Herein, probiotics were used in 2.6% of hospitalizations covering 96% of this hospital sample with *Saccharomyces* and *Lactobacillus* the most common genera employed [218]. With respect to limiting antibiotic use or as adjuvant to minimize impact of antibiotics on beneficial gut communities and colonization resistance, it is important to also pull from deep experiential literature to understand carriage and colonization with multidrug resistant pathogens to help inform related guidance. Interestingly and mostly recently, CDC and FDA convened a virtual public workshop in August 2022 to address drug development for prevention of multidrug resistant organism infections through decolonization and pathogen reduction strategies (Drug Development Considerations for the Prevention of Healthcare-Associated Infections—Virtual Public Workshop Co-Sponsored by the FDA and CDC (rescueagency.com). In this workshop, several presentations addressed pathogen colonization parameters that extend on the timeframe of months up to a year or more which, from a natural history perspective, sets a stage for long term study of live microbe supplementation and utility. Discussion of decolonization agents, as a potential new regulatory product class and evidentiary basis, would potentially include traditional drug agents (like mupirocin) but also involve live biotherapeutics with use of probiotics and phages to achieve pathogen reduction and/or decolonization.

Lastly, the typical genomic analysis with outbreaks and adverse events is focused on inclusivity and exclusivity in relation to offending clinical culture. In the case of Bertelli et al. [216] mentioned above, such analysis unequivocally connected bacteriemia isolates and product isolates with <10 nucleotide polymorphisms. From extensive experience with pathogen investigations, it is clear that such thresholds are organism-dependent and will require deep species analysis to gain perspective on expected diversity indices. Moreover, extensive work with the *Escherichia coli* genomic landscape conducted at FDA has been instructive in understanding species boundaries and taxonomic relationships. In this example (and through a genomic lens), *Shigella* spp. have arisen multiple independent times and are well-intercalated throughout the *E. coli* landscape, thereby arguing for their inclusion per se rather than a separate taxonomic designation. As an extension, use of genomics and related distributed efforts with databasing for improving genomic depth are important in redefining taxonomies which have significant implications for correct product identification, labeling, and relevant regulatory review, safety, and efficacy studies. Notable examples include recent changes involving union of Lactobacillaceae and Leuconostocaceae that could only be achieved by moving beyond traditional phenotypic traits and limited 16S-based discrimination for their identification [219]. Relatedly, noted confusion with *Bifidobacterium* taxonomy has implications in attributing the relevant species, subspecies, and strain characteristics especially for comparing across product trials but also for quality control [220].

SECTION C: NEXT GENERATION SEQUENCING APPLICATIONS IN PROBIOTICS AND MICROBIOME RESEARCH

Next generation probiotics

Next Generation Probiotics (NGPs) or Live Biotherapeutics (LBP) describe potential probiotics that have not been used as probiotics to promote health and may be delivered as drug [221]. While traditional probiotics consist of a few select strains of live bacteria or yeast that can colonize the gut and promote overall digestive and immune health [221].

Given the benefit of sequencing technology in microbe studies, it has also been used in commercial product development. In other words, using these technologies as an examination tool is qualified for strict human health product validation regulations. For instance, a next-generation healthy bacteria company (The Akkermansia Company™) founded by Professor Willem M. de Vos (Wageningen University, Netherlands), who discovered *Akkermansia muciniphila* in 2004, and Professor Patrice D. Cani (UCLouvain, Bruxelles, Belgium), who discovered the beneficial effects of the bacteria. The bacteria were first found in the human intestine by combining culture and 16S rRNA sequencing. For further investigation, a pure isolate was obtained by culturing with the anaerobic soft agar. The 16S rRNA gene sequence shows the isolate was part of the division *Verrucomicrobia*, but 99% similarity to three uncultured colonic bacteria, representing that the finding of this bacteria might be a novel bacterium, now known as *Akkermansia muciniphila* [222]. After seven years, *Van Passel* et al. have published the first complete genome sequence of Akkermansia muciniphila by performing 37 GI metagenomes and assembling those bacterial genomes that have >95% identical to Akkermansia muciniphila's 16s rRNA sequences [223]. In the following two decades, several diseases (metabolic disorders to inflammatory diseases, neurodegenerative diseases, and some cancers) have been linked to these bacteria, moving from correlation studies to proof-of-concept, all the corresponding mechanisms and beneficial effects are also studied [224]. With the accumulation of clinical positive effect evidence, in 2022, the bacteria pasteurized strain was developed as a novel food for overweight and obese individuals [225]. To clarify the identity of the novel food for regulations, the isolated bacteria's whole genome was sequenced by PacBio (TGS) and Illumina (NGS) shotgun genome sequence analysis [226].

NGPs represent an exciting new frontier in probiotic research, with the potential to revolutionize the way we treat and prevent a range of diseases. However, more research is needed to fully understand the safety and effectiveness of these new probiotics, and to ensure that they are developed and regulated in a responsible manner.

Application of Whole Genome Sequencing in cell structure prediction

The health benefits of probiotics depend on their bioactive molecules in which are available in their genetic information. Bacterial components have immune-stimulatory activities that affect host immunity. In this case, they act as ligands for innate immune receptors such as Toll-like receptors (TLRs), but the strength of the stimulation is known to depend on subtle differences in the structure of the bacterial molecules. For example, lipopolysaccharides (LPS) are major bacterial components that function as ligands for TLR4, but their activity varies depending on subtle structural differences in Lipid A, the active center of LPS [227].

The structural differences provide a potential means for diverse metabolic pathways; however, this could be easily predicted based on a fully annotated genome. On the other hand, the components of bacterial cells could be affected during the manufacturing process. For instance, pili encoded by the spaCBA of *Lactobacillus rhamnosus* GG (LGG) is expressed on the bacterial surface for binding to intestinal epithelial cells. It was reported that mechanical stress caused by centrifugation or culture scaled up using large tanks resulted in the detachment of pili [228, 229]. Hence, it is important to consider the possible effects of pasteurization or other denaturation processes.

Metabolomics application in probiotics

When producing probiotics using live bacteria, the production of microbial metabolites should be taken into consideration. Well-known microbiota-derived metabolites are short-chain fatty acids, bile acids, as well as Indole and Indole-related compounds, but other useful metabolites are known. *Blautia wexlerae* was recently identified in a Japanese cohort study as being more common in non-overweight people. Using an animal model, *Blautia wexlerae* exerted a preventive effect on obesity. Metabolomic analysis revealed that *Blautia wexlerae* produced larger amounts of S-adenosylmethionine and L-ornithine, which are known to promote metabolism, compared to other bacteria [230]. With the presence of *Blautia wexlerae*, the gut microbiota increases the steady-state plasma levels of high-antioxidant molecules, reactive sulfur species (RSS), such as hydrogen sulfide and cysteine persulfide (CysSSH), in the host and protects against oxidative stress.

Gut bacteria belonging to the Lachnospiraceae and Ruminococcaceae families have a high capacity to produce RSS [231]. Moreover, D-Trp can inhibit the growth of enteric pathogens and colitogenic pathobionts and suppress microbe-induced colitis, Gut microbiota, such as Firmicutes, that produce at least 12 free D-AAs including D-Trp, as well as *Bifidobacterium* and *Lactobacillus* which known to produce D-Trp, have the potential to create a healthy gut environment to prevent gut inflammation [232]. Since these metabolites can change according to the surrounding environment, the type of product form in which probiotics are provided is also an important aspect to consider.

The identification of effective molecules that exert probiotic effects and the establishment of a mechanism of action (MoA)-based evaluation system are equally important as genomic analysis.

NGS can be utilized for gene analysis of gut microbiota to identify potential probiotic strains. By analyzing the genomes of these bacteria, their metabolic pathways and potential health benefits could be identified. This helps in identifying strains that may be useful in treating specific health conditions. Furthermore, NGS can also be an aid in the study of the interaction between probiotics and host cells, providing insights into how they modulate host physiology and contribute to overall gut health. However, functional analysis by *in vivo* and *in vitro* models will be necessary to reveal the real effects of the probiotics.

Application of Metagenomic Sequencing Aid in Colorectal Neoplasms and Investigation of Gut Microbiome in Mediating Cancer Risk Factors

The human gut microbiome plays an essential role in health and disease. The alterations in gut microbiota are associated with various pathological conditions, including cancers such as colorectal cancer (CRC). CRC development is thought to result from interactions between genetic and environmental factors, and recent research has shown that changes in the gut microbiome may play a role in carcinogenesis.

Recent advances in NGS technologies and metagenomics have provided new insights into the gut microbial ecology and helped link gut microbiota to CRC. Metagenomic sequencing involves the analysis of genetic materials obtained directly from samples, such as stool or tissue samples. This approach allows researchers to study the entire microbial community in a sample. Metagenomic sequencing can provide information on the taxonomic composition of the microbiome, as well as functional role of genes and metabolic pathways present in the microbial population.

Studies using metagenomic sequencing have revealed differences in the gut microbiome between those with CRC and healthy individuals. For example, metagenomic studies have pinpointed certain gut bacteria, such as *Fusobacterium nucleatum*, enterotoxigenic *Bacteroides fragilis*, and colibactin-producing *Escherichia coli*, as they are over-abundant in CRC patients [233, 234]. Many subsequent target sequencing and functional studies have confirmed their roles in CRC. Apart from identifying bacteria in diseases, metagenomic sequencing can also provide insights into the non-bacterial microbiome and functional pathways in individuals with CRC [234, 235]. By sequencing all the DNA present in a sample, including that from bacteria, viruses, fungi, and other organisms, metagenomic sequencing can provide a comprehensive view of the metabolic pathways that are active in the microbiome.

Metagenomic sequencing also has promising applications to identify biomarkers for CRC. By identifying the specific microbial species and functional genes associated with cancer, metagenomic sequencing can provide a non-invasive and highly sensitive diagnostic tool. One potential approach is to use metagenomic sequencing to identify specific microbial markers present in the fecal microbiome of patients, followed by target amplification of differentially abundant markers [233, 236]. Previous studies have demonstrated satisfactory results of using metagenomic markers as diagnostic classifiers, as a new generation of fecal microbial marker test was being developed [237–241].

In conclusion, metagenomic sequencing is a powerful tool that can provide valuable insights into biology and translational research in cancer. By studying the entire microbial community in a sample, rather than just a single species, metagenomic sequencing can reveal differences in the taxonomic composition and functional pathways that are associated with CRC. This information can be used to develop new diagnostic and therapeutic strategies and to improve our understanding of the role of the gut microbiome in the development and progression of CRC.

SECTION D: CONCLUSIONS AND WAY FORWARD

Recommendations and Best Practices

Recommendations and best practices shared among the economies:

Recommendation 1: Establish a Harmonized Standard Method
To promote the widespread adoption of Next-Generation Sequencing (NGS) for probiotic testing, it is imperative to establish a universally accepted standard method. The governing bodies, in conjunction with relevant stakeholders from academia, industry, and regulatory agencies, should take a leadership role in this endeavor. By creating a harmonized standard, we can significantly reduce the discrepancies and uncertainties that often arise from using different standards in probiotic testing. This will provide a clear and consistent framework for conducting NGS-based probiotic testing across the globe.

Recommendation 2: Focus on Adapting Existing Methods
Recognizing the challenges in reaching a consensus on a new standard, we propose a pragmatic approach. Instead of solely focusing on creating a new method, we should concentrate on adapting and improving existing techniques. Culturing methods, which are fundamental for downstream work in probiotic testing, should be a key area of focus. By modifying and enhancing current methods, we can ensure that they align with the requirements of NGS-based probiotic testing. This approach allows for quicker implementation and can provide immediate benefits.

Recommendation 3: Facilitate Continuous Open Dialogue
To drive the adoption of NGS in probiotic testing, fostering an open and ongoing dialogue among all stakeholders is critical. This includes representatives from the probiotics industry, regulatory bodies, research institutions, and consumer advocacy groups. These discussions should aim to identify priority issues and challenges in probiotic testing. By engaging in continuous dialogue, we can collectively address these issues and work towards solutions that benefit both the industry and consumers. Public-private partnerships should guide the direction of these discussions to ensure alignment with real-world needs.

Recommendation 4: Strain Identification and Genetic Characterization
Probiotic strains exhibit specific health benefits, making accurate strain identification essential. Currently, culturing methods primarily enable genus-level identification, while biochemical analysis and PCR are used for species-level identification. However, there's no single method or standard that allows for simultaneous enumeration and identification. It's crucial to adapt existing methods to bridge this gap. Additionally, ongoing debates about "strain definition" highlight the need for a more nuanced approach, considering that genetic differences may not always correlate with distinct phenotypic characteristics or health benefits.

Recommendation 5: Culturing Method as the Gold Standard
While NGS, particularly Whole Genome Sequencing (WGS), offers valuable insights into probiotic testing, the culturing method remains the gold standard. However, it's important to recognize the complementary role of NGS. When using WGS, obtaining pure bacterial isolates is crucial. For metagenomic, shotgun, or shallow shotgun sequencing, cultivation may not be necessary unless specific verification is required. This recommendation emphasizes the synergy between traditional culturing methods and advanced NGS techniques.

Recommendation 6: Fit-for-Purpose NGS Lab Setup
Establishing an NGS laboratory for probiotic testing requires careful planning. Factors such as budget, research objectives, and available resources should guide the selection of equipment, sequencing platforms, and bioinformatics pipelines. It's essential to tailor the NGS lab setup to the specific goals of probiotic testing. This ensures that the chosen methods align with the depth of sequencing required for accurate results.

Recommendation 7: Promote Cross-Border Collaboration
Cross-border collaboration is pivotal for advancing the implementation of NGS as a standard method in probiotic testing. Continuous dialogue and cooperation among international stakeholders, including researchers, industry experts, and regulatory bodies, are essential. Collaborative discussions should focus on standardizing practices and addressing common challenges. By pooling collective efforts and sharing knowledge, we can drive significant progress in the field of probiotic testing. This international collaboration will ultimately lead to enhanced quality and safety standards for probiotic products on a global scale.

Conclusions

In the ever-evolving landscape of biotechnology and healthcare, the field of probiotics has emerged as a beacon of hope, promising to redefine our approach to well-being. Probiotics, these microscopic superheroes, have shown remarkable potential in enhancing human health. As we look towards the future, we find ourselves standing at a juncture where the collaboration of science and innovation promises to unlock unprecedented possibilities in the realm of probiotics.

The journey we embarked upon, culminating in the creation of this handbook, was not merely a scientific endeavor but a collaborative global effort that transcended borders. It was an initiative powered by the Asia-Pacific Economic Cooperation (APEC) and brought together a consortium of experts in probiotics and Next-Generation Sequencing (NGS) applications. Representatives from APEC and non-APEC economies joined hands, uniting their knowledge and vision for the betterment of humanity.

Our handbook delves deep into the heart of probiotics, deciphering the intricate world of these beneficial microorganisms. We start by painting a comprehensive picture of the international probiotic regulatory landscape, a vital foundation for any probiotic-related research and development.

In addition to regulatory insights, our readers gain access to a treasure trove of NGS technology knowledge and technical expertise. From the basics of NGS to in-depth exploration of bioinformatics platforms, we leave no stone unturned in equipping our readers with the tools they need to navigate the rapidly advancing world of genomics.

While probiotics offer enormous potential, their safety and quality assurance are of paramount importance. Our handbook meticulously examines the current microbiological testing standards for probiotics. We dissect these standards, uncovering their strengths and exposing their limitations. By doing so, we pave the way for innovation and the adoption of NGS-based probiotic testing.

One of the highlights of our handbook is a detailed exploration of probiotic product specification testing, a critical element in ensuring quality assurance. In particular, we emphasize the significance of strain verification. We recognize that the authenticity of probiotic strains is non-negotiable, and NGS presents an unparalleled opportunity to guarantee the identity and purity of these strains.

As we look towards the horizon, we see a future where NGS-based probiotic testing is not just a possibility but a global standard. The recommendations that have emerged from our collaborative efforts are the pillars upon which this future rests.

A Shared Vision, A Unified Future

As we conclude this handbook, we envision a future where probiotics are not just a supplement but a vital component of healthcare. The adoption of NGS-based probiotic testing, guided by these recommendations, will revolutionize how we ensure the safety and quality of probiotic products globally. With continued collaboration, dialogue, and innovation, we are poised to take a giant leap forward in sequencing the future of probiotics, unlocking their full potential for the benefit of humanity.

The journey has only just begun, and together, we are shaping a healthier world through the power of science and technology.

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ANNEX

Survey on Probiotics Testing

Introduction

This survey serves as a tool to identify the laboratories that conduct probiotics testing in both APEC and non-APEC economies, as well as to scope generation information, such as probiotics testing methods, standards that the laboratories are adopting, and their experience and application of Next Generation Sequencing (NGS). The survey was distributed to potential respondents from the government, academic/ research institutions, private sectors laboratories, not restricted to APEC economies that are:

- regulating probiotic products as food, medical food, pharmaceutical, live biotherapeutics, or supplements, excluding postbiotics,
- testing probiotics products as the category mentioned above,
- develop testing methods for probiotic products,
- establishing standards for probiotic products testing,
- producing probiotic products, and
- promoting standards development in probiotics testing.

The questionnaire consists of 4 sections, section A) background information, section B) operation and testing in respective laboratory, section C) regulation status for probiotic products, and section D) awareness and application of Next Generation Sequencing (NGS). Respondents were given options to fill in the questions that are relevant to them.

Respondents

Due to the confidentiality and privacy of the respondents, the identity of the respondents remains anonymous. The survey outcome is summarized and discussed below-

#	Economy of residence / work	Category of institution/laboratory
1	Viet Nam	Government lab
2	Singapore	Government lab
3	Indonesia	Government lab
4	Singapore	Commercial lab
5	Viet Nam	Government lab
6	Non-APEC economy: Germany	In-house (QC or R&D) lab
7	India	In-house (QC or R&D) lab
8	India	Government lab
9	Singapore	Commercial lab
10	Malaysia	Government lab
11	Non-APEC economy: Switzerland	In-house (QC or R&D) lab

12	The United States	In-house (QC or R&D) lab
13	Chinese Taipei	In-house (QC or R&D) lab
14	Chinese Taipei	Commercial lab
15	Japan	Commercial lab
16	Chinese Taipei	Research or academic lab
17	Thailand	Government lab
18	New Zealand	Commercial lab
19	People’s Republic of China	Research or academic lab
20	Malaysia	Commercial lab
21	People’s Republic of China	Research or academic lab
22	People’s Republic of China	Government lab
23	Non-APEC economy: The Netherlands	In-house (QC or R&D) lab
24	People’s Republic of China	Research or academic lab
25	People’s Republic of China	In-house (QC or R&D) lab
26	Japan	In-house (QC or R&D) lab
27	Viet Nam	Government lab
29	Republic of Korea	Government lab
30	Republic of Korea	In-house (QC or R&D) lab
31	Republic of Korea	Research or academic lab
32	The United States	Government lab
33	The United States	Commercial lab
34	The United States	Commercial lab
35	The United States	Commercial lab
36	The United States	Commercial lab
37	Non-APEC economy: Denmark	In-house (QC or R&D) lab

38	The United States	Commercial lab
39	People's Republic of China	In-house (QC or R&D) lab
40	Republic of Korea	Government Lab

Participating economies

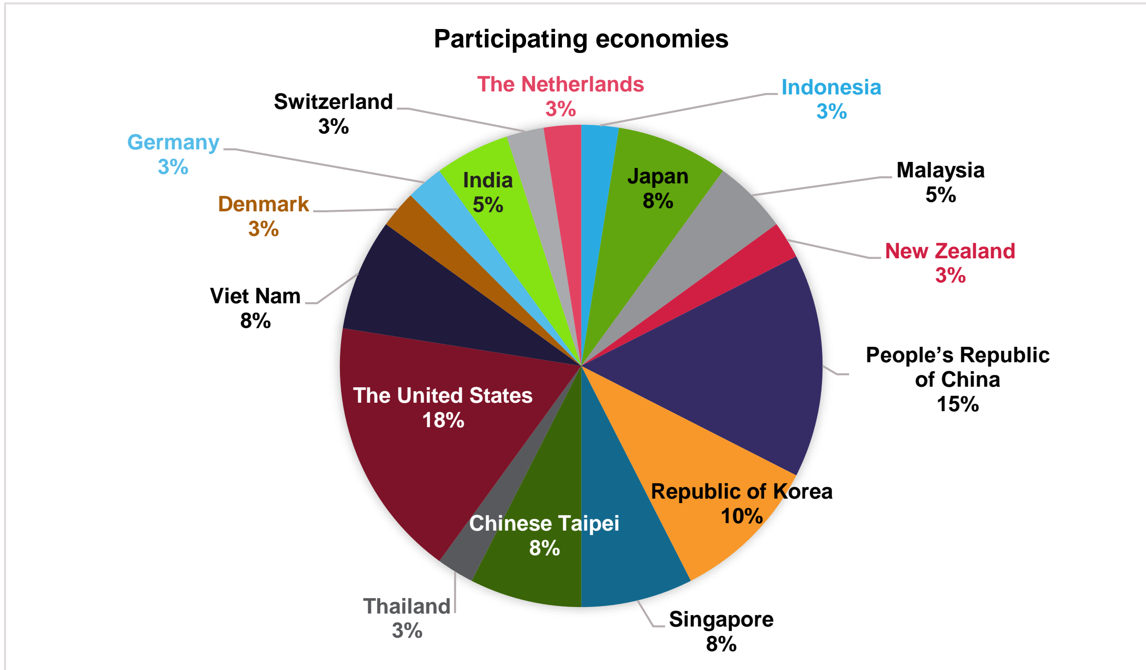

Figure 12. Economies that are participating at the Survey on Probiotics Testing and NGS Application.

The survey includes both APEC responses and non-APEC economies. A total of 40 responses from 16 economies which consists of 11 APEC economies and 5 non-APEC economies (Figure 12).

Operation and testing in respective institution/ laboratory

The participating laboratories (non-repetitive) comprises of 11 commercial labs, 11 government labs, 12 In-house (QC or R&D) labs and 6 research or academic labs.

Table 7. Capacity of the laboratory in supporting their organization.

Capacity of the labs in supporting their organization	Number of responses
Primary diagnostics	11
R&D	32
Service	22
Surveillance and emergency response	9

Of all the 40 participating labs, they support different capacities in their organization such as

R&D, service, primary diagnostics, and surveillance. Majority of these labs (32/ 40) serve their function as R&D and services (22/40) lab.

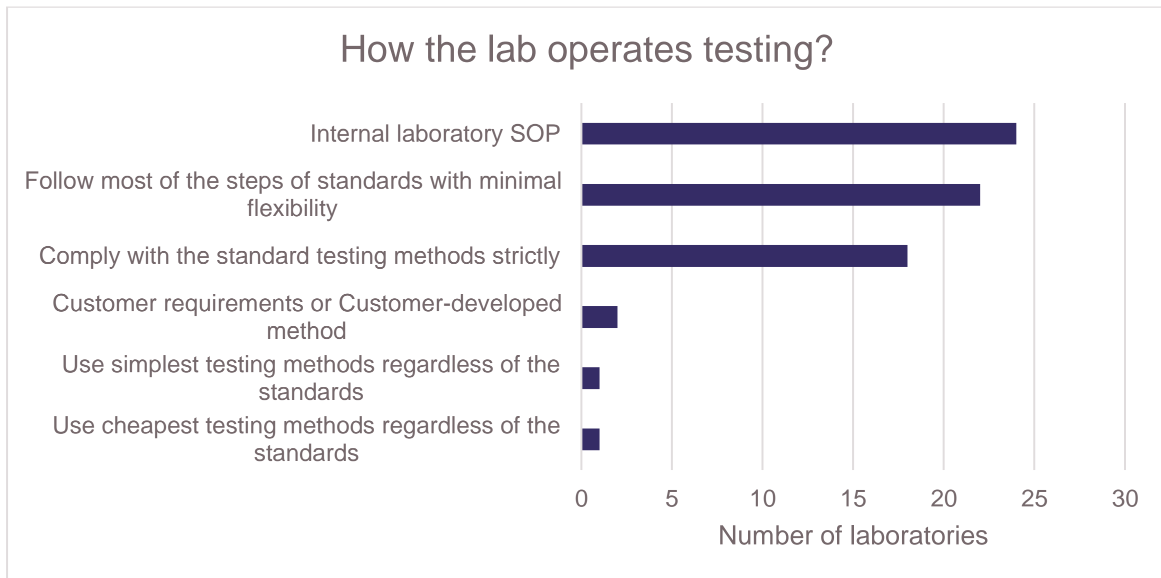

Figure 13. Laboratory testing operation. SOP, standard operating procedure

In the questions related to laboratory testing operation, the respondents were allowed to select more than one option which relates to their daily testing operation. In their day-to-day testing, 65% of the labs usually establish and comply their own internal laboratory standard operating procedure (SOP), while 59% of the labs comply strictly with the standard testing methods with minimal flexibility and 49% does not allow adjustment in standard methods. There were only two laboratories that claimed that they usually choose the simplest testing methods or cheapest testing methods regardless of the standards. On the other hand, the commercial labs will have to operate according to the customers' requirements or the customer-developed method.

Factors affecting choices of method

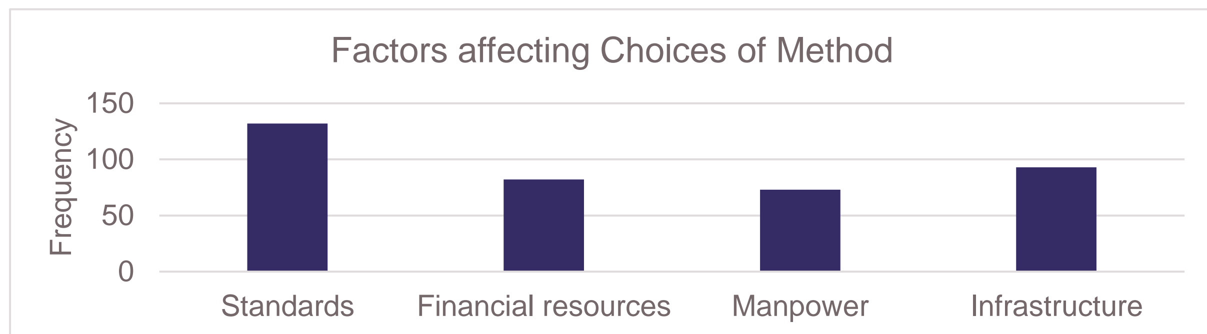

Figure 14. Factors affecting choices of methods.

According to the respondents, the absence of harmonized standards was identified as the foremost factor influencing their method selection. In fact, 86% of the labs have encountered challenges in lacking harmonized standards. There were also other challenges raised by the respondents, as listed below:

- Distinction from other coexisting strains is difficult.
- DNA or RNA extraction from dairy products and consistency of results between different methods.
- Food safety monitoring of Probiotics is difficult-Recovery of low level of Salmonella or Cronobacter in the presence of high-level competing organisms **(Concern on contaminants)
- High variability in probiotic enumeration method.
- Lack of experience with new probiotic organism.

Probiotic products testing

Figure 15. (A) Probiotics testing methods adopted by the participating laboratories. (B) PCR Methods that the laboratories are using to assess probiotics products. (C) NGS methods adopted.

Among the responses, 36 laboratories perform probiotics testing (10 commercial labs, 10 government labs, 10 in-house (QC/R&D) labs and 6 Research/academics lab), however, only 34 labs shared the methods that they used to test probiotics.

The methods discussed for probiotics testing in this context address key considerations such as cell viability, probiotic strain identification, and contamination issues. These include enumeration of probiotics in products, identification of probiotics, and detection of potential contamination. Figure 15 summarizes the methods used by the participating labs in probiotics

testing. The majority of the labs (91%) are applying culturally dependent methods due to the economy regulation and customer requirements, e.g. viable microbial count of 106-8 CFU/ml (g) as stated in their economy standards. Other than culturing methods, PCR is a common method used in probiotics testing for identification or enumeration, whether at species or strain level. For the labs that have experience in applying NGS for probiotics testing (Figure 16(C)) n=19, all of them have used WGS.

The probiotics testing volume of the participating labs ranges from 1-40 tests per month. There is also an in-house QC lab that performs >1000 probiotics testing per month. The lab with >1000 probiotics testing does not apply NGS for probiotics testing but has NGS for research purposes and has bioinformatics pipeline.

Regulation status for probiotics / probiotic products

Not all economies have specific regulations for probiotics and probiotic products. Probiotics could be categorized as food, medical food, functional food & supplement or live biotherapeutics. For the economies that are regulating probiotics, the requirement for minimum number of viable cells varies from 10^6 to 10^8 cfu/g (ml).

Based on the survey results, the industry in-house labs usually adopt the economy regulation and the standards such as ISO, USP, IDF, GB Method, while the commercial labs adopt the customer's requirements, the industry lab (R&D/ Quality lab) usually uses internal methods as these methods are optimized based on their own products.

Awareness and application of Next Generation Sequencing (NGS)

Among the responses, 25 respondents have experience in NGS application, whether in probiotics testing diagnostic, testing, service, research, or surveillance, but mostly of these labs applied NGS for research. Below is their experience in NGS experience:

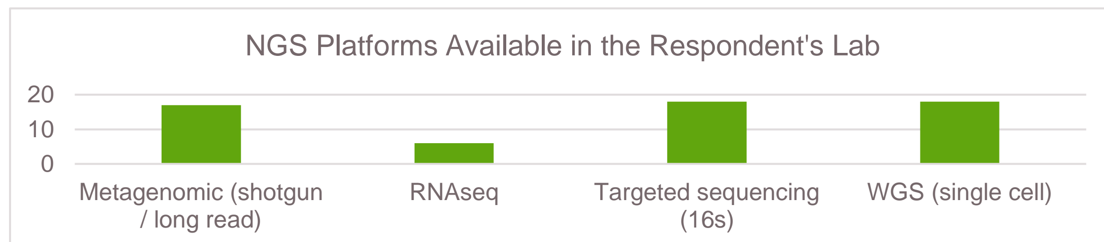

Figure 16. NGS platforms available in the labs.

The two most common sequencing platforms that the labs utilized are: 1) Illumina, 2) Oxford Nanopore.

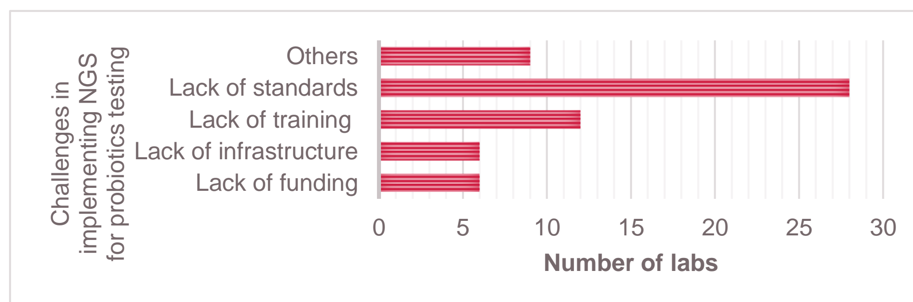

Figure 17. Challenges in implementing NGS for probiotics testing.

The respondents suggested that 'lack of guideline' in NGS as the most challenging situation they face when setting up the application of NGS for probiotics testing, same for 'lacking database'. Almost all the laboratories with NGS have properly developed bioinformatics pipelines.

Some of the respondents commented that application of NGS will be useful to the lab, not just in identification of probiotics strains, but it can be used for contamination detection as well. A traditional microbiologist also suggests that the NGS could possibly overcome the inhibition or challenges that the lab experienced when applying traditional methods. They also agreed that there is high variability among different NGS platforms, thus having a standard in place is critical.

On the other hand, the labs that do not apply NGS are in view that the demand of testing is low, concerning the financial returns will be slow, lack of infrastructure, database and bioinformatic pipeline. Some of them also raised concerns on the sensitivity and viability of cells when applying NGS. Several respondents agreed that it is necessary to establish a global standard for NGS to support safety evaluation of probiotics.

Summary

Based on the survey results, lacking standards for probiotic products testing has seen to be the most challenging factor and it suggests an urgent need to establish the guideline. Almost all the probiotics testing labs that are performing primary diagnostics, surveillance and service functions are adopting culture-dependent methods, mainly due to the economy regulation. While NGS is applied in R&D.

Biographical Sketches of the Committee Members and Authors

Cindy Shuan Ju Teh *(Project Overseer)* is an associate professor at the Universiti Malaya, Malaysia. Dr Teh joins the Department of Medical Microbiology, Faculty of Medicine since 2013, where she started to play a leading role in antimicrobial resistance study in Malaysia. Her research then extended from the molecular epidemiology of multidrug-resistant organisms (MDRO) to the better understanding of MDRO-microbiomes interaction and one health approach in combating AMR. She also developed rapid detection assays for human pathogens and the outbreak tracings of MDRO. Her research team is currently focused on research into the mechanisms of resistance to antibiotics and disinfectants, developing assays which rapidly identify the MDRO from patient samples, and investigating the role of microbiome in preventing or promoting the development of antibiotic resistance.
Dr Teh's work has been recognized domestically and internationally. She has published more than 110 publications and accumulated more than USD 2 million grants to date. Dr Teh is one of the academic editors of Peer J and member of the editorial board for Frontiers in Microbiology. She is a member of Young Scientists Network-Academy of Sciences Malaysia (YSN-ASM). Her outstanding achievement in science has won her the Institut Mérieux – MSIDC Young Investigator Award in 2018, and the Loreal-UNESCO For Women in Science in 2021. She received her PhD from the Universiti Malaya, Malaysia, in 2011.

Lay Ching Chai *(Co-Project Overseer)* is a microbiologist and a Pro-Vice Chancellor (Academic) at the Sunway University. She is the Chair of the Young Scientists Network-Academy of Sciences Malaysia (YSN-ASM) that represents top young researchers in Malaysia who are committed to contribute to the economy ecosystem beyond their individual research interests. Part of her role includes engaging young academics in Malaysia to rethink their role in shaping higher education for the future. She was serving at the University of Malaya as an associate professor prior to her role at Sunway University. Prof Chai's research focuses on infectious microorganisms, food safety and microbiological risk assessment. She served as the Vice Chair of the Technical Working Group of Microbiology under the Food Analysis Committee (JKAM) chaired by the Department of Chemistry Malaysia from 2016-2020; and is listed as the Malaysian expert on microbial risk assessment in the ASEAN Risk Assessor Directory. Her active contribution and involvement in microbiological safety research has won her the Malaspina International Award by the International Life Science Institute (ILSI) in 2017; and she is currently serving as the scientific advisor of ILSI Southeast Asia Region providing guidance and advice on food safety risk assessment. Prof Chai is also involved in promoting biosafety and biosecurity in Malaysia. Her research and leadership in promoting research integrity, inclusivity and excellence has been recognized through multiple awards such as the L'Oréal-UNESCO Women in Science Award in 2018, Marie Claire's Amazing Woman in Malaysia 2019, Prestige's 40 under 40 Malaysia 2019 and the Asian Women Entrepreneurs Leadership Award 2019. She received her PhD in Food Safety from Universiti Putra Malaysia.

Ming-Ju Chen is a distinguished professor at the University of National Taiwan University (NTU), Chinese Taipei. Her research interests now include isolation and identification of new bacteria and yeasts from different resources and application of these strains in human food and animal feed. She also involves the development of a new platform to evaluate the functionality of probiotics and study the possible mechanism and pathway using multi-omics. Dr Chen has published over 100 papers in areas such as dairy science, microbiology, food science and functional food. She also contributes more than 10 book chapters. Currently, she serves as General Secretary of the Asian Federation of Lactic Acid bacteria. She is also in charge of Food Safety Committee of International Life Sciences Institute (ILSI) Taiwan. She was Presidents of Taiwan Association of Lactic Acid Bacteria (2016-2019) and President of the Taiwanese Association of Animal Science (2015-2018), At NTU, she has served as the director of Center for International Agricultural Education and Academic Exchanges. She earned the doctorate in Food Science and Technology at the Ohio State University and a master's degree in animal science at National Taiwan University.

Patricia Conway is Visiting Prof at Singapore Centre for Environmental Life Sciences Engineering (SCELSE) at Nanyang Technological University, Singapore, Adjunct Professor at UNSW Australia and Chief Scientist and Founder of PC Biome Pte Ltd, Singapore. She has successfully combined basic research and translational applications for over 30 years by being affiliated with university for supervision of doctoral students and the basic research while being industry employed or by working closely with industry while being university employed.

Prof Conway's research interests are gastrointestinal microbiology, probiotics and prebiotics with particular emphasis on bacterial colonization and function within the gastrointestinal tract. She has focused on mechanisms of bacterial adhesion, pathogen inhibition and immune modulation in animals and humans and the impact of the gut microbiota on health in particular health conditions linked to inflammation, infection and metabolic disorders and developing intervention strategies for improving health, especially in infants and the elderly. Prof Conway received her BSc and MSc at the University of Queensland and obtained her PhD at the University of New South Wales, Australia.

Yinping Dong is an associate research fellow in China National Center for Food Safety Risk Assessment (CFSA), People's Republic of China. She is a member of ISO TC34/SC9/WG11, WG26 and four professional committees in China. Dr Dong has been engaged in food microbiology research for more than 10 years and has participated in domestic and international foodborne disease related emergency response for many times. In recent years, she received a grant from National Natural Science Foundation of China, two projects from Beijing Natural Science Foundation, a sub-project of key research and development plan from Ministry of Science and four National Food Safety Standard revision projects. Dr Dong won the first prize of the Science and Technology Award of China National Food Industry Association in 2018-2019, and the third prize of the Chinese Preventive Medical Association in 2021. Her major study field is food microbiology, especially in anaerobic microorganisms (*Clostridium* spp. and probiotics), there are many better research results. These include isolation, identification, subtyping, pathogenicity, drug resistance, WGS and bioinformatics analysis of bacteria in

food. In addition, she was responsible for revising four National Food Safety Standards related to probiotics. Dr Dong graduated from University College Dublin for PhD in Public Health.

Christopher A. Elkins is chief of the Clinical and Environmental Microbiology Branch of the Division of Healthcare Quality Promotion in the National Center for Emerging and Zoonotic Infectious Diseases (NCEZID) at the Center of Disease and Control (CDC), the United States. Prior to this appointment, he served as Director of the Division of Molecular Biology at the Center for Food Safety and Applied Nutrition (CFSAN), Food and Drug Administration. While there, he established genomic research programs for analysis of probiotics and enteric foodborne pathogens including metagenomic analysis of food and gut microbiomes to advance new lines of research in molecular toxicology and nutrition. His current primary research interests at the CDC have leveraged this expertise applied to the nosocomial environment and associated transmission of pathogens and antimicrobial resistance mechanisms. He was a postdoctoral fellow in the Department of Molecular and Cell Biology at the University of California at Berkeley studying intrinsic bacterial resistance mechanisms. He is a member of the American Society for Microbiology for more than 20 years and currently serves as Editor for Applied and Environmental Microbiology. He served in several deployments for the pandemic response as Lead and Co-Lead for the Laboratory and Testing Task Force. Dr Elkins received a Bachelor of Arts degree in Biology and History at Case Western Reserve University and a PhD degree in Microbiology from the University of Tennessee.

Yun-Gi Kim, is a distinguished professor at the Research Center for Drug Discovery, Faculty of Pharmacy, Keio University. Throughout his academic career, Prof Kim has held various positions, including assistant professor at the Department of Immunology, Faculty of Medicine, University of Tsukuba, and research investigator at the Department of Pathology, University of Michigan Medical School. He also served as an associate professor at the Division of Biochemistry, Faculty of Pharmacy, Keio University prior to his current role.

In addition to his academic pursuits, Prof Kim has made notable contributions in the corporate sector. He worked as a Senior Scientist at Vedanta Biosciences, Inc., where he played a pivotal role in the development of a Live biotherapeutic product for Clostridioides difficile infection (CDI). His groundbreaking research led to the identification of a gut microbial consortium that effectively prevents CDI, with successful Phase II clinical studies. His research interest specializes in the field of innate and mucosal immunity, with a focus on the role of gut microbiota in host defense and inflammation against enteric pathogens. Prof Kim's current research endeavors revolve around unraveling the molecular mechanisms underlying the influence of gut microbiota on infectious, inflammatory, and metabolic diseases. By utilizing mouse disease models, such as colitis, enteric infection, food allergy, and obesity, he investigates key gut microbes and their metabolites that contribute to disease prevention and progression. Notably, his research delves into the differential modulation of gut microbiota composition and metabolites by indigestible carbohydrates. He obtained his Bachelor's, Master's, and PhD degrees from Kitasato University in Tokyo, Japan. Following his doctoral studies, He conducted postdoctoral research at the University of Michigan Medical School.

Jun Kunisawa is director of Center for Vaccine and Adjuvant Research (CVAR) and Collaborative Research Center for Health and Medicine (CRCHM), National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN), Japan, and also serves as adjunct professor at Osaka University, Kobe University, The University of Tokyo, Hiroshima University and Waseda University. He received postdoctoral training at University of California, Berkeley after his graduation and was recruited by The University of Tokyo in 2004. He spent 9 years in Tokyo as Assistant and Associate Professor and then moved to NIBIOHN to establish a new laboratory. In 2019, he was promoted to his current position. Prof Kunisawa also serve as a director of CRCHM since 2022. His research has been focusing on the immune regulation by gut environment (e.g., diets and commensal bacteria) and its association with immune diseases and human health. He is also doing translational research for the development of vaccines, medicines, and functional foods. Prof Kunisawa was awarded his PhD from Osaka University in 2001.

Woori Kwak is a senior lecturer of the Department of Medical and Biological Sciences at the Catholic University of Korea. He is currently the chairperson of the academic section of the Korean Society for Lactic Acid Bacteria and Probiotics (KSLABP). As an expert in bioinformatics, Dr Kwak has conducted various microbial-related studies using Next Generation Sequencing (NGS) and has conducted and supported research required by academia, industry, and institutions. He developed a whole metagenome shotgun sequencing-based probiotics test method, which is currently being used as a standard method for probiotics product testing by the Ministry of Food and Drug Safety in Republic of Korea (KFDA). Dr Kwak also developed the cost-effective intestinal microbiome analysis method commercialized in the local hospitals of Republic of Korea. He is currently interested in research and development related to analysis methods that can raise the analysis resolution of existing probiotics analysis methods to the strain level and diagnostic test kits that can effectively and rapidly test various kinds of infectious diseases using NGS. He received his bachelor's degree in animal biotechnology and his MS and PhD degrees in bioinformatics from Seoul National University, Republic of Korea.

Yuan Kun Lee is a University Fellow of the National University of Singapore, Yong Loo Lin School of Medicine. He Served as President for the Singapore Society of Microbiology and Biotechnology (SSMB), Asian Federation of Society for Lactic Acid Bacteria (AFSLAB), and International Union of Microbiological Societies (IUMS). Prof Lee's research interests include effects of diet on crosstalk between gastrointestinal microbiome and the host, and microbial fermentation processes in production of food and pharmaceuticals. He received research grants from economy research foundations and private agencies, supervised 36 PhD graduates. Prof Lee published 255 papers in international peer reviewed journals, delivered 111 conference papers, contributed 52 chapters in books, and authored and edited 14 monographs. In addition, he has 3 patents to his name. Prof Lee received his PhD from the University of London (Queen Elizabeth College), United Kingdom in 1979.

Fengqin Li is a director of the laboratory of microbiology, China National Center for Food Safety Risk Assessment. Internationally, she serves as a member of Joint FAO/WHO Expert Committee on Food Additives, Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment, Joint FAO/WHO Expert Meetings on Foodborne Antimicrobial Resistance (AMR). As a member of JEMRA, Dr Li involved in drafting documents entitled Microbiological Risk Assessment Guidance for Food, and Risk Profile: Group B *Streptococcus* (GBS) *Streptococcus agalactiae* Sequence Type (ST) 283 in Freshwater Fish. As a member of Chinese delegate of CAC, Dr Li engaged in drafting Code of Practice to Minimize and Contain Antimicrobial Resistance as well as Guidelines on Integrated Surveillance of Antimicrobial Resistance. Nationally, she is chair of Biohazard Sub-Committee of National Expert Committee on Food Safety Risk Assessment of China, vice chair of Microbiological Examination Sub-committee of National Food Safety Standard Review Committee, president of Food Hygiene Sub-Committee of Chinese Preventive Medicine Association. Dr Li's major study field is microbiology and natural toxins. These include detection, identification, phenotypically and genetically subtyping pathogens transmission of antimicrobial resistance bacteria or genes based on WGS, detection and genotyping of foodborne virus (especially norovirus, sapovirus, hepatitis E etc.), natural occurrence of mycotoxins and diversity of toxin-producing fungi in foods, risk assessment and standard development for microbial agents in foods. Besides, Dr Li has also be involved in the development of safety evaluation guidelines and detection methods for probiotics as well as other microorganisms intended for use in foods. She graduated from Ehime University of Japan for PhD degree in Biochemistry and Food Science in 2001.

Sunny Wong is an associate professor at the Lee Kong Chian School of Medicine, Nanyang Technological University Singapore. He completed his clinical training in internal medicine and gastroenterology in the Prince of Wales Hospital Hong Kong, China. His main research interest is on gut microbiome, investigating the host-microbe interaction in digestive and metabolic diseases, and exploring this for discovery of novel biomarkers and therapeutics. He has won several awards, including the Asia Pacific Digestive Week Emerging Leaders Lectureship (2021), the Hong Kong College of Physicians Sir David Todd Lectureship (2020) and the Croucher Foundation Award (2014). He has published over 180 peer-reviewed articles including papers in *Nature Genetics*, *Nature Communications*, *Gastroenterology* and *Gut*, and is currently an Associate Editor for the *Journal of Gastroenterology and Hepatology*. Dr Wong received his MBChB at the Chinese University of Hong Kong, and his PhD in University of Oxford on genetics of bacterial infections.

Dr Clare Narrod is the Director of the Risk Analysis Training program at Joint Institute for Food Safety and Applied Nutrition (JIFSAN) at University of Maryland. At JIFSAN she leads the monitoring and impact effort associated with the evaluation of JIFSAN's capacity building efforts. Prior to coming to JIFSAN she worked at the International Food Policy Research Institute, the United States Department of Agriculture, and at the Food and Agriculture Organization. From 1998-2000 she served as an American Association for the Advancement of Science Risk Analysis Fellow at USDA. She received her PhD in Energy Management and

Environmental Policy in 1997 and a Master's Degree in International Development and Appropriate Technology both from the University of Pennsylvania. She has conducted research in Bangladesh, Brazil, Chile, People's Republic of China, Costa Rica, Ethiopia, Ghana, India, Indonesia, Kenya, Mali, Mexico, Nigeria, Thailand, Viet Nam, and Zambia. She has taught in Bangladesh, Colombia, People's Republic of China, Egypt, India, Jamaica, Malaysia, Russia, and the United States.

Yu-Ting Wang is a technical specialist at the Division of Research and Analysis, Taiwan Food and Drug Administration. She is responsible for developing and validating testing methods for food products containing probiotic microorganismfs and genetically modified organisms. Her research primarily focuses on food science, including development of high-throughput platform for rapid detection of probiotic bacteria and food-borne pathogens, improvement of selective enumeration for probiotic species, and risk evaluation of food products in transmitting antibiotic resistance. Dr Wang received her PhD from National Taiwan University, Chinese Taipei.

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