Asia-Pacific
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NGS-based Microbial Testing for Probiotics Products: Guidelines Development and Laboratory Capacity Building

Final Summary Report

APEC Sub-Committee on Standards and Conformance

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APEC Project: SCSC 04 2021A

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Executive Summary

According to several probiotics market research, the global probiotic market is anticipated to reach a substantial value of USD75 billion in 2023, with an estimated annual growth rate (CAGR) of 7.53% projected from 2023 to 2028. The intense competition within the market has fueled a continuous stream of innovations in probiotic products, encompassing diverse forms such as food and beverages, infant formulas, and dietary supplements. The introduction of multi-strain probiotic products has compounded the complexities of laboratory testing for accurate product verification.

Probiotics have a long history of safe consumption and are generally recognized as safe. However, the potential risks associated with novel probiotic strains or uncommon strains, genetic mutations, and harmful microbe contamination necessitate stringent laboratory analyses to uphold product safety and quality. The current reliance on culture-dependent methods is notably time-consuming, expensive, and inefficient, especially when dealing with novel products featuring unique probiotic strains. The employment of various culturing methods further contributes to confusion and may not be suitable for the novel strains, leading to significant discrepancies in final test results. The escalating volume of probiotic trade underscores the urgent need for a more harmonized testing method.

Next Generation Sequencing (NGS) is an emerging tool to overcome the probiotics testing hurdles faced by many testing labs. However, many APEC developing economies face low NGS utilization due to limited capacity and facilities. Enhancing this capacity is vital for effective probiotics management in APEC economies, ensuring quality, safety, and competitiveness of their products in the global market. This endeavor seeks to promote sustainable growth and equitable development throughout the APEC region.

With a focus on addressing these issues, this project, APEC SCSC 04 2021A gather experts to identify best practices and establish protocols for NGS-based probiotic testing in labs. The project also seeks to build NGS-testing capabilities across APEC economies, addressing the need for capacity-helps to enhance market prospects for novel local products from these economies on a global scale. This initiative aligns with the development and capacity-building requirements of APEC developing economies, addressing health security priorities and food supply contamination concerns.

In conclusion, this final summary report will expound upon the research methodology, consolidate the research outcomes, and present the handbook published as a result of this project.

Introduction

Background

The global probiotics market is estimated to reach about USD75 billion annually and estimated to grow exponentially at the CAGR of 7.53% (2023-2028) [1–3]. Notably, among these growth trends, APEC economies, which encompass the Asia-Pacific and North America regions as per research findings, stand-out as both fastest growing and the largest market for probiotics [3]. This escalating global demand for probiotics is attributed to the health benefits of the probiotics. As the market experiences heightened competition, there is an evident surge in the incorporation of probiotic strains across different types of food products and in different forms, for instance, dietary supplements in powder, capsules format, yogurt, beverages, ice cream and even infant formula. Furthermore, there are also increased concerns on incorporation of multistrains probiotics in a single product, which also pose challenges in terms of laboratory testing for accurate strain verification.

Probiotics have been available for more than 10 decades. Although it has a long safe consumption history, a lot of unique probiotics strains have emerged in the market and claimed to provide specific health benefits. Therefore, it is ultimately critical to ensure the safety and quality of the products. Food safety authorities and laboratory scientists will need to have a rigorous and robust testing method to verify the safety of the products. NGS is an emerging tool to address the challenges related to the conventional culture methods.

To be considered as probiotics, it must fulfill the following criteria [4]:

1. contain live microorganisms; and
2. exhibit health benefits on host when adequate amount of probiotic bacteria is administered.

Therefore, it is important to ensure the testing method will be able to determine cell viability and quantify specific bacteria in the products.

In August 2022, the project, titled "SCSC 04 2021A: NGS-based Microbial Testing for Probiotics Products: Guidelines Development and Laboratory Capacity Building," received endorsement and support from the APEC SCSC. Spearheaded by University of Malaya, Malaysia through the Department of Standards Malaysia (JSM), this initiative is co-sponsored by Australia; People's Republic of China; Thailand; the United States; and Viet Nam.

Aims and Objectives

The core aim of this project is to tackle the challenges associated with probiotics testing and establish effective strategies for integrating NGS in this context. To realize the overarching aim, the following objectives are outlined:

1. Best practice sharing and make recommendations
   The primary objectives are to identify the challenges in current probiotics testing approaches and propose the relevant adaptation and facilitate the use of NGS-based testing in probiotics analysis. Therefore, the research outcomes delve on the sharing of best practices and recommendation for probiotics testing.

2. Capacity building
   A pivotal objective of the project is to promote the testing capacities of laboratories within the APEC economies. This will be achieved by providing specific lectures and specialized hands-on training in regard to NGS-based probiotics testing. By equipping the respective laboratory personnel with the necessary expertise, the project aims to elevate their proficiency and confidence in conducting NGS-based

probiotics testing.

3. Collaborative network establishment
   Fostering collaboration is a key objective that involves bringing together a diverse array of stakeholders, including experts, food safety authorities, researchers, and industry representatives. This project aims to facilitate the exchange of knowledge, insights, and experiences in the field of probiotics management. By nurturing cross-border cooperation, the project seeks to create a supporting network that promotes innovation and best practices.

An Expert Committee on Application of Next Generation Sequencing in Probiotics Testing for Quality and Safety Assurance was established in September 2022. Comprising experts from diverse fields including food microbiology, safety, NGS technology, probiotics, and bioinformatics, this committee bears the responsibility of:

1. Strategizing, designing, and conducting an online survey and focus group discussions (FGDs) to collect pertinent information.
2. Identifying suitable laboratories and contacts as survey respondents and FGD informants.
3. Curating and synthesizing outcomes from FGDs.
4. Developing a comprehensive handbook that outlines NGS-based guidelines for probiotics testing, drawing insights from FGDs, roundtable discussions, and panel sessions.

Anticipated Outcomes:

The project endeavors to deliver impactful outcomes that elevate probiotics testing and management practices:

1. A comprehensive survey on probiotics testing and the consolidation of findings from focus group discussions.
2. A Technical and Networking Workshop focused on NGS applications in probiotics testing, fostering knowledge dissemination and networking opportunities.
3. An online platform for knowledge-sharing and networking dedicated to probiotics testing.
4. The creation of a practical handbook titled "Sequencing the Future of Probiotics: A Practical Handbook to Next Generation Testing for Safety and Quality."

In summary, the "SCSC 04 2021A: NGS-based Microbial Testing for Probiotics Products" project aims to revolutionize probiotics testing by harnessing cutting-edge NGS technology. By establishing guidelines, enhancing laboratory capacities, and fostering collaborative networks, this initiative will significantly contribute to the safety, quality, and overall advancement of probiotic products across APEC economies and beyond.

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

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Research findings on testing approaches

Survey on probiotics testing and focus group discussions

The expert has drafted the survey on probiotics testing as a tool to 1) engage the relevant personnel in this field, 2) identify the laboratories that conduct probiotics testing, 3) scope generation information on 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 from all APEC economies. However, due to the limited responses from the APEC economies, we then extended the invitation to the non-APEC economies.

The laboratories participated in this survey must fulfil at least one of the roles listed below:

- 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/ or
- 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.

The foremost challenge encountered in probiotics testing has been the absence of harmonized standards. A majority of testing laboratories have been adopting internal laboratory methods for this purpose. Nonetheless, the application of multiple methods has led to disparities of up to 1 Log in Colony Forming Units (CFU) difference in results. Cultural-dependent techniques have been adopted for the enumeration of probiotics, predominantly because of the definition of probiotics in international and local regulations.

Approximately fifty percent (50%) of these laboratories have incorporated Next-Generation Sequencing (NGS) capabilities. However, these capabilities are primarily employed for research and development (R&D) objectives, owing to the absence of well-defined protocols or benchmarks for the utilization of NGS in probiotics testing.

Therefore, it is imperative to initiate the development of standards or guidelines. The survey questionnaire and the detailed report are accessible in the accompanying Annex.

This survey has successfully identified and engaged specific laboratories that are actively involved in probiotics testing. Among the pool of respondents, the committee has selected potential contributors for focus group discussions. However, the involvement of these contributors in the discussions was entirely voluntary.

During the FGDs, the contributors highlighted that the demand for NGS in probiotics testing remains low in the Asian context. This is primarily due to the fact that regulations do not necessitate the sequencing of probiotic strains. Additionally, manufacturers tend to favor internal testing methods despite potential variations in counts when compared to third-party laboratories. The contributors also emphasized the necessity for guidelines regarding verification and validation. They underscored the importance of setting reasonable limits and ensuring that these are aligned with industry standards for specific probiotic strains. It was further highlighted that the health benefits of probiotics are strain-specific; however, existing

cultural-dependent techniques only allow for identification up to the species level. This has highlighted a significant discrepancy in expectations. Furthermore, a consensus regarding the definition of 'strains' is lacking, and it was noted that genetic variation is not the sole determinant influencing the health benefits of a probiotic strain.

Summary of capacity building workshop

Technical and Networking Workshop on Next Generation Sequencing for Probiotics Testing

This is a three-days in-person workshop supported by APEC and University of Malaya, held from 6 June 2023 to 8 June 2023 in Kuala Lumpur, Malaysia. It brought together a total of 70 experts and participants (32% male and 68% female, from 12 economies (Australia; Chile; People's Republic of China; Indonesia; Japan; Republic of Korea; Malaysia; the Philippines; Singapore; Thailand; The United States; and Viet Nam) from the government agencies involved in setting up standards and conducting lab testing, research institutes, commercial testing labs, as well as probiotics producers. The workshop consists of presentations from the experts, NGS demo sessions and discussions. It aimed to:

- Provide an overview of current testing approaches,
- Share knowledge on NGS applications in probiotics testing with real case studies,
- Explore on how NGS can address gaps in current testing methods,
- Demonstrate NGS workflow in probiotics testing, and
- Discuss the opportunities in probiotics testing.

The Agenda, Workshop Photos, and Presentation Summaries are available in the Annex.

The diverse presentations related to probiotics testing and NGS spanned several key themes:

Day 1
The event kicked off with an overview of the project's progress, unveiling insights gathered from surveys and focus group discussions regarding probiotics testing. Regulatory variations across Asian economies were highlighted, and the potential of NGS to bridge existing gaps in testing was emphasized.

Day 2
Discussions delved into the safety and claims of probiotics, with emphasis on strain-specific identification due to functional characteristics. The use of metagenomics in identifying potential probiotics was explored, underscoring how advanced sequencing technologies have revolutionized the field, enabling the discovery of new probiotics and personalized probiotic interventions.

Day 3
This marked the application of NGS in strain-level probiotic identification. The challenges in identifying clonal strains, the current methods being used in the industry, and the potential of NGS were discussed in detail. Standardization efforts and issues, such as strain definition and consensus on using WGS or phenotypic differences, were also addressed.

The workshop's conclusion revolved around the pressing need for standardization of probiotics testing method. Establishing a consensus on strain identification and enumeration methods was highlighted as a crucial priority. The panelists underlined the necessity of open dialogue and collaboration among stakeholders in formulating standards, given the dynamic nature of the probiotics industry.

The workshop's comprehensive exploration of NGS's potential in probiotics testing underscored its promise in bridging current gaps, enhancing safety measures, and substantiating claims. However, it was acknowledged that achieving standardized NGS methods for probiotics testing would require ongoing discussions and collaboration among experts, regulatory bodies, and industry stakeholders. The event served as a pivotal step

toward harnessing NGS's potential for ensuring the safety, quality, and efficacy of probiotics products.

Best practices and recommendations

This project has entailed extensive discussions with representatives from various sectors and economies associated with probiotics testing. The best practices and recommendation are summarized as follows:

1. Establish a harmonized standard for NGS-based probiotic testing

   In order to foster the adoption of Next-Generation Sequencing (NGS) for probiotic testing, a universally accepted standard method must be established. Establishing a harmonized standard to be used internationally or in specific regions will greatly reduce the disparities and ambiguities of the results variations, thus providing a consistent framework for NGS-probiotics testing.

2. Focus on adapting current probiotic testing method

   Culturing methods are foundational to downstream probiotic testing stages. Recognizing that the development of a standard for NGS-based probiotic testing will require considerable effort from different sectors, adapting culturing methods should be one of the key areas of focus. Modifying culturing methods to align with NGS-based requirements can yield immediate benefits and expedite implementation.

3. Need for continuous open dialogue

   Through ongoing and sustained dialogue, we can collaboratively tackle these challenges and endeavor to formulate solutions that serve the best interests of both the industry and consumers alike. These discussions should be steered by public-private partnerships to guarantee their alignment with practical, real-world requirements and demands.

4. Strain identification and genetic characterization

   Health benefits demonstrated by the probiotic are strain-specific, emphasizing the necessity of precise strain identification. Currently, culturing method predominantly facilitate identification at genus-level, while species -level identification relies on biochemical analysis and polymerase chain reaction (PCR). Nevertheless, there is no single method that enables enumeration and identification simultaneously. Consequently, it is imperative to modify existing methods to address this disparity. Furthermore, ongoing discussions regarding “strain definition” underscore the requirement for a more subtle approach, recognizing that genetic disparities may not consistently correspond with discernible phenotypic traits or health benefits.

5. Culturing method is still the gold standard
   Even though WGS provides valuable insights, culturing remains the gold standard for testing probiotics. The synergistic role of NGS should be acknowledged. Unlike metagenomic, shotgun, or shallow shotgun sequencing, WGS requires pure bacterial isolates, whereas metagenomic, shotgun or shallow shotgun sequencing may not require cultivation unless further verification is essential. It is recommended that traditional culturing methods be combined with advanced NGS techniques to achieve synergistic results.

6. Tailored NGS lab setup

   Making decisions for the procurement of equipment, choosing sequencing platforms, and setting up bioinformatics pipelines for the Next-Generation Sequencing (NGS) laboratory should be guided by considerations such as budgetary limitations, study objectives, and resource availability. It is crucial to personalize the NGS laboratory setup to the specific objectives of probiotic testing since the latter ensures that the chosen methods are in accordance with the desired sequencing depth, ensuring precise and consistent results. Optimizing the effectiveness and efficiency of NGS-based analysis in such an instance requires thorough planning and coordination of the NGS facility setup with the special needs of probiotic testing.

7. Propagating Cross-Border Collaboration

   For NGS to become an established method for probiotic testing, international collaboration is essential. International stakeholders need consistently work together and communicate together, including scientists, industry representatives, and regulatory bodies. The primary objective of dialogues that collaborate ought to focus on solving common challenges while establishing harmonized standards. Through collaboration and knowledge exchange, probiotic testing can advance substantially. This worldwide endeavor will ultimately result in greater safety and quality standards for probiotic products spanning the economies.

Development of Handbook

Drawing upon the insights gathered from surveys, focus group discussions, and workshop deliberations, the collaborative endeavor of experts in probiotics and Next-Generation Sequencing (NGS) from both APEC and non-APEC economies has given rise to the "Sequencing the Future of Probiotics" handbook. This manual provides a comprehensive understanding of the evolving probiotics landscape, emphasizing NGS-based approaches for ensuring safety and quality.

The handbook's framework unfolds across four sections, each designed to provide a comprehensive understanding of the multifaceted probiotics domain:

- Section A: Probiotic Landscape

Readers will learn the fundamentals of probiotics in this first section, making it clear for beginner while providing useful information for experts in the area.

- Section B: Probiotics Testing

This section represents where the handbook's heart is. The second section provides readers with an in-depth review of probiotic testing methods, enabling readers to overcome hurdles.

- Section C: NGS Application in Probiotics and Microbiome Research

Readers are given the chance to learn about more extensive application of NGS technology in probiotics and microbiome studies.

- Section D: Conclusion and Way Forward

Readers will discover a summary of the key takeaways from this handbook in the last part, along with useful recommendations for further investigation and discussion.

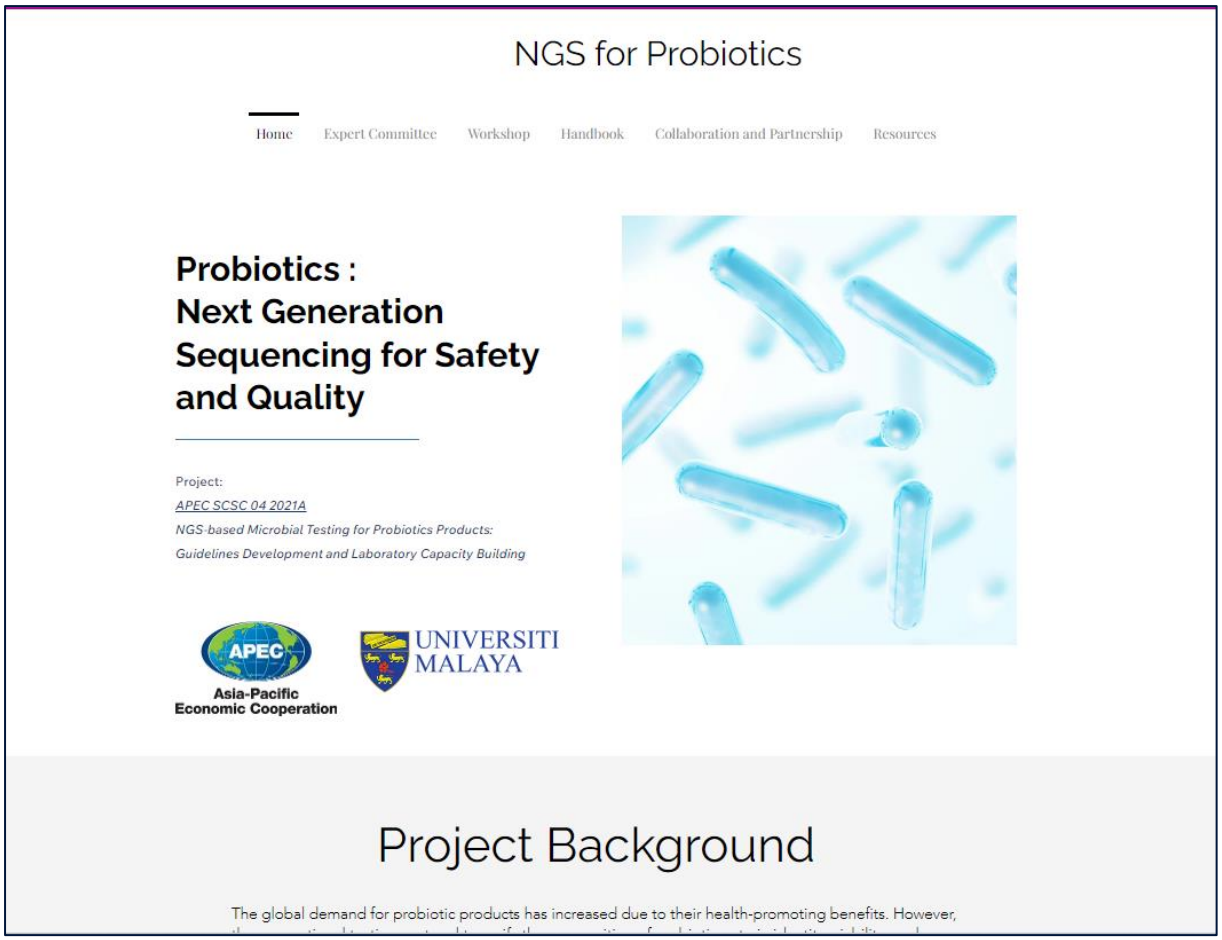
In summary, this handbook undertakes a comprehensive exploration of the probiotics landscape, contextualized both local and international regulations. It meticulously expounds upon the pivotal role of NGS technology in surmounting the challenges posed by conventional culturing methods. Moreover, it deliberates extensively on the recommendations pertaining to the establishment of NGS facilities, providing invaluable guidance to the stakeholders.

This handbook is made available on APEC Publication Database. For individuals or entities with a vested interest in this handbook, the contact information of the designated points of contact is readily available within this report.

Online Platform for Knowledge Sharing for Probiotics Testing and Networking

A satellite website https://www.ngsforprobioticstesting.com/ has been developed to share information and allow networking among labs, researchers and experts from APEC and non-APEC economies. This website comprises of the following sections:

- Introduction to Expert Committee
  The expert committee serves as the supporting network for on-going discussions relevant to probiotics testing.
- Workshop Materials
  Selected presentation slides are shared on this page for the audience' reference.
- Handbook
  Once the e-copy handbook is published, the website manager will upload it for public reference.
- Collaboration and Partnerships
  In case if there is/are interested party(s) to contact the project overseer team, they are welcome to reach out via this page.
- Resources
  This page contains links to useful reading materials and some other organizations that address topics of relevance.



Opportunities and way forward

The ever-evolving probiotic industry has fundamentally reshaped our approach to well-being. The nexus of science and innovation holds the key to unlocking boundless possibilities within the realm of probiotics. Through the concerted efforts of experts in the field, convened by the APEC SCSC project, we have embarked on a journey to delve deeper into the intricacies of probiotics testing and harness the immense potential of cutting-edge NGS technology to address the multifaceted challenges inherent in current probiotics testing.

The objective of our investigation is to create a more harmonized standard for NGS-based probiotics analysis, an endeavor that requires the collaboration of all parties involved. Achieving this goal will need regular interaction and a dedication. In addition to implementing NGS, stakeholders are encouraged to take a pragmatic strategy that entails modifying and standardizing current culturing methods to reduce results discrepancies, ultimately paving the way for more effective probiotics management across economies.

The continuation of open dialogue and the creation of public-private partnerships, both of which are essential to preserving the relevance and consensus of all stakeholders concerned, are crucial to the success of our mission.

While it is acknowledged that the culturing method remains as the gold standard, stakeholders need to recognize the complementary role that NGS serves in enhancing the safety and quality of probiotic products and minimizing trade barriers. We are in an ideal situation to make significant progress in the field of probiotics testing through promoting collaboration and the sharing of knowledge, which will culminate in the establishment of harmonized standards for both the safety and quality of probiotic products.

The discoveries of this study are merely the beginning of our journey towards addressing the challenges and embracing the opportunities that are brought together by the implementation of NGS in probiotics testing. The project overseer team is enthusiastic about pursuing dialogues and engagement in order to further work in this intriguing topic. We anticipate NGS-driven probiotic testing transcends possibility to become the widely accepted standard.

Post-workshop survey

At the end of the workshop, attendees were invited to provide feedback on various aspects of the event, including the workshop's objectives, topic relevance, gender equality, speakers, materials shared, allotted time, and interest in future workshops. Participation in providing feedback was voluntary, and a total of 10 participants kindly submitted their responses through the online form. The workshop received consistently positive feedback regarding its content and organization.

The first section of the feedback form asked participants to rate different aspects of the workshop on a scale of 1 (strongly agree) to 5 (strongly disagree). The following are the results of the evaluation:

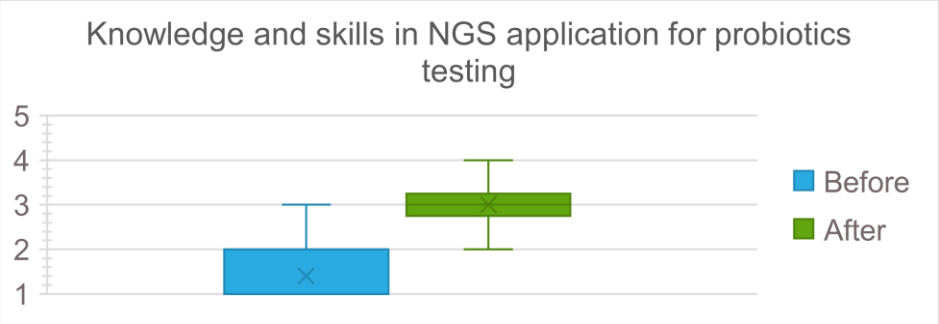
	Scope	Score
1	Workshop objectives were clearly defined.	1.2
2	Workshop content was well reflected and relevant.	1.2
3	Gender issues were sufficiently addressed.	1.8
4	Experts were well prepared and knowledgeable about the topic.	1.3
5	The materials shared were useful.	1.2
6	Time allotted was sufficient.	1.7
7	Interest in attending future workshops.	1.1

The speakers and content of the workshop received high praise from the participants. When asked about what they gained from the workshop, the following thoughts were shared:

- Increased knowledge on the application of NGS in probiotics testing.
- Understanding of the role of genetic information from microbes in diagnostics, treatment, and health supplementation, as well as the significance of safety and efficacy considerations for probiotics in the market.
- Awareness of other applications of NGS beyond probiotics testing.

These insights highlight the valuable information and perspectives that the participants acquired during the workshop.

In the evaluation section of the feedback form, participants were asked to rate their level of knowledge and skills in NGS for probiotics testing before and after the workshop on a scale of 1 (beginner) to 5 (advanced). It was observed that participants became more confident in their knowledge and skills in this area following the workshop.



The feedback form also sought participants' evaluations on various aspects of the program, including visuals, venue, and organization of the workshop. Most of these attributes received excellent ratings. Participants expressed their appreciation for the workshop's comprehensive coverage of diverse topics and the opportunity to gain insights from different perspectives through experience sharing. The ice-breaking session was particularly praised

for its engaging nature, facilitating effective communication during the roundtable discussions. Additionally, participants valued the networking opportunities that arose throughout the workshop. However, some attendees expressed a desire for more time to conduct hands-on experiments, as they believed it would have further enhanced their learning experience.

Regarding future activities, participants were asked to suggest preferred topics for upcoming discussions. The following topics were suggested:

- Technical training in probiotic testing, specifically focusing on the culture method for different strains of probiotics.
- Molecular techniques such as qPCR and NGS for identifying AMR genes or virulence genes.
- Hands-on bioinformatics analysis.
- Updates in probiotics testing guidelines.
- Hands-on NGS session, covering sample preparation to bioinformatics analysis.
- Quantification of probiotics at the species and strain levels.

These suggestions will be taken into consideration for planning future activities and ensuring the continued development of participants' knowledge and skills in probiotics testing.

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Annex

Survey Form

Survey on Probiotics Products Testing Guidelines and Application of Next Generation Sequencing (NGS)

Project Number:	**SCSC 04 2021A**
Project Title:	**NGS-based Microbial Testing for Probiotics Products: Guidelines Development and Laboratory Capacity Building**
Proposing Forum:	Committee on Trade and Investment / Sub-Committee on Standards and Conformance / Food Safety Cooperation Forum / Partnership Training Institute Network

This project, **SCSC 04 2021A: NGS-based Microbial Testing for Probiotics Products: Guidelines Development and Laboratory Capacity Building** is co-funded by APEC and the University of Malaya, Malaysia. An expert committee has been established, consisting of probiotics and microbiological testing experts to identify the relevant guidelines for probiotics testing. This survey aims to identify standards/ methods / guidelines for probiotics products testing and the application of NGS Technology in Probiotics Testing Laboratories from both APEC and non-APEC economies. The outcome of this survey will be published in a **Handbook of Best Practice Guidelines for NGS Probiotic Testing**.

The term "**Probiotic Products**" mentioned in this survey refers to probiotics, probiotics in foods, functional foods, medical foods, supplements, live biotherapeutics and pharmaceutical products. We seek your kind support in the completion of this survey and submit to Ms Lee Hui Key (huikeylee@ilsisea.org.sg).

Yours sincerely,
Expert committee of NGS for Probiotics Product Testing

This survey consists of 4 sections, background information, operation and testing in respective laboratory, regulation status for probiotic products, and awareness and application of Next Generation Sequencing. Please fill in all questions under Section (A) and fill in questions that are only relevant for you in Section (B), (C) and (D).

Section (A) Background Information

Please fill up / select the relevant option(s).

1. Name:
2. Email:
3. Gender:

	Male
	Female
	Prefer not to say

4. Education level:

	High School
	Diploma
	Bachelor
	Master
	PhD

5. Economy of residence/ work (Economy):
6. Job level:

	Executive
	Managerial
	Top Management

7. Are you currently engaged in food/ pharmaceutical products analysis and testing?

	Yes
	No

8. How long have you been engaged in food/ pharmaceutical products analysis and testing?

	Never
	less than 5 years
	5-10 years
	more than 10 years

Section (B) Operation and Testing in respective institution/ laboratory

1. Category of your institution/laboratory:

	Government lab
	Commercial lab
	In-house (QC or R&D) lab
	Research or academic lab

2. Capacity and Capability (You may select more than ONE)

	Primary diagnostic
	Service
	Surveillance and epidemic response
	Research and development

3. How do you operate the sample testing? Select the relevant option(s)

	Comply with the standard testing methods strictly
	Follow most of the steps of standards with minimal flexibility
	Use simplest testing methods regardless of the standards
	Use cheapest testing methods regardless of the standards
	Internal laboratory SOP
	Others, please specify:

4. Rank the factors that affect the choice of methods for testing,
   1(most important) to 4 (least important):

	Standards
	Financial resources
	Manpower
	Infrastructure

5. Have you ever encountered a situation of lack of validated testing methods during your inspection/ routine work?

	Yes
	No

6. Are there any probiotic products being tested in your institution / laboratory?

	Yes
	No

7. Is your institution / laboratory interested in testing probiotic products?

	Yes, any specific reason? _____
	No
	Not sure

If your answer is "No" or "Not sure", please skip the rest of the questions under section (B) and (C).

8. What is the method of testing the probiotic product? (You may select more than ONE)

	Cultural-dependent method
	Biochemical identification
	PCR, species level identification
	PCR, strain-level identification
	Gene-based detection
	Protein-based detection
	qPCR
	Automated system, eg: MALDI TOF, VITEK, etc.
	WGS
	Others, please specify:

9. How many test(s) do you perform for a mix?

_____

10. What are the challenges in probiotic product testing? You may select more than ONE.

	Lack of standards
	Lack of infrastructure
	Lack of funding
	Lack of training
	Others, please specify:

11. How many test(s) carried out within a month?

_____

Section (C) Regulation status for probiotics / probiotic products

1. Is there a regulation/ related documentaries for probiotic and probiotic products?

	Yes, please specify the guideline, decree or circular:_____
	No
	Not sure

2. Which category(s) does/do the probiotics products (viable cells only) falls under, you may select more than ONE:

	Food
	Medical food
	Functional food & supplements
	Live biotherapeutics

3. Which standard(s) does your institute/ laboratory adopt for probiotics testing? Please specify.

_____

4. Which agency/ department(s) is/are responsible for probiotic regulations?

_____

5. Is there a requirement for minimum number of probiotic microorganisms in probiotic products?

	Yes, please specify the microbial load:
	No
	Not sure

6. Is there a positive list of approved probiotics?

	Yes
	No
	Not sure

7. Is there a minimum threshold for contaminants in probiotic products?

	Yes
	No
	Not sure

Section (D) Awareness and application of Next Generation Sequencing (NGS)

1. Are you familiar with the NGS?

	Yes
	No
	Not sure

2. Do you have experience in performing NGS?

	Yes
	No

a. If your answer is yes, select the relevant option(s):

	WGS (single cell)
	RNAseq
	Metagenomic (shotgun / long read)
	Targeted sequencing (16s)

b. Which platform are you using?

_____

3. Does your lab have any bioinformatic pipeline?

	Yes
	No

4. In your laboratory, NGS has been used for:

	Diagnostic
	Testing
	Service
	Research
	Surveillance
	Not applicable

5. Do you think the application of NGS is valuable in food testing?

	Yes
	No
	Not sure

a. If your answer is yes, select the relevant options:

	identification of probiotics
	contaminants identification
	drug-resistance genes & virulence

6. What are the challenges of application of NGS in probiotics products testing? (You may select more than ONE)

	Lack of guideline
	Lack of database
	Lack of funding
	Lack of infrastructure
	Lack of training
	Others, please specify:

7. Do you wish to apply NGS technology in your institution / laboratory?

	Yes, any specific reason(s)? _____
	No

8. Rank the components of NGS Training based on your laboratory's need, 1(most important) to 4 (least important):

	DNA extraction
	Library preparation
	Bioinformatics pipeline
	Data management & security

9. Do you think it is necessary to develop a guideline for the application of NGS in testing of probiotic products?

	Yes
	No
	Not sure

10. Do you think it is necessary to develop a sequence database that can be accessed by regional institutions/laboratory?

	Yes
	No
	Not sure

11. For laboratories that do not apply NGS, if you wish to establish NGS, what are the challenges are you facing currently?

_____

12. Would you be outsourcing the sequencing service? Bioinformatics analysis?

_____

13. If you have other comments, please specify.

_____

------End------

Survey Report

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

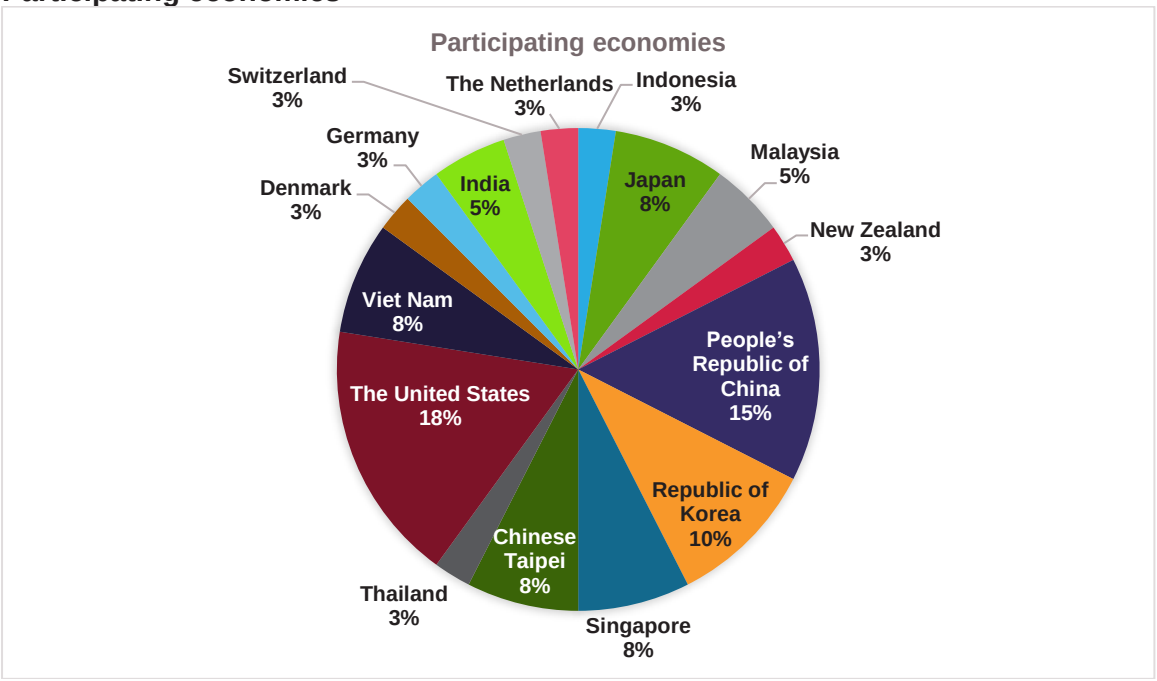


Figure 1. Economies that are participating at the Survey on Probiotics Testing and NGS Application.

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 1).

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 1. 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.

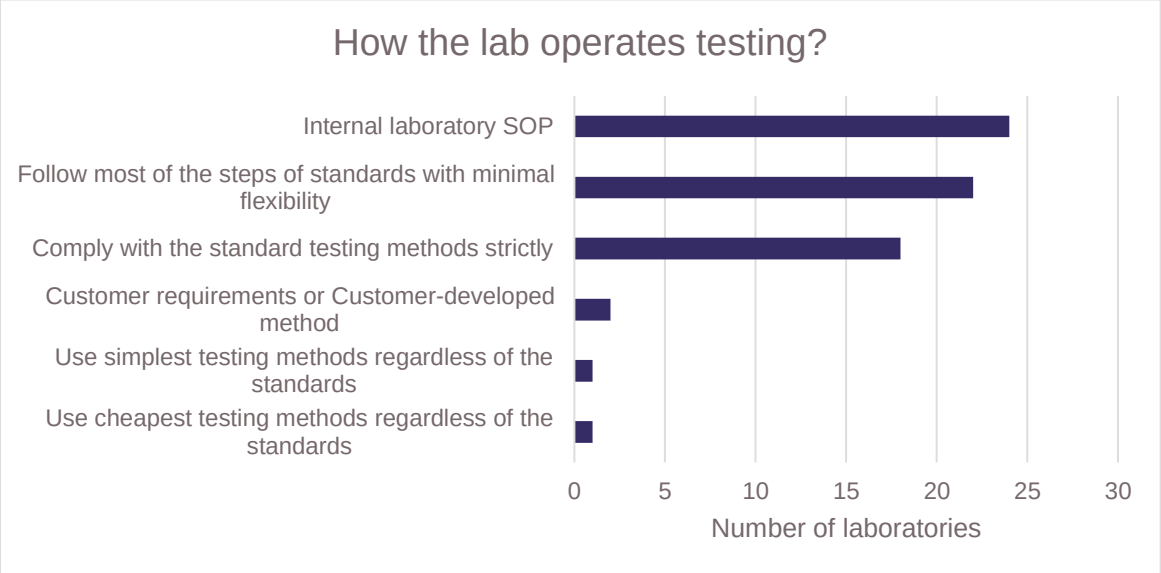


Figure 2. Laboratory testing operation. SOP, standard operating procedure

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 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

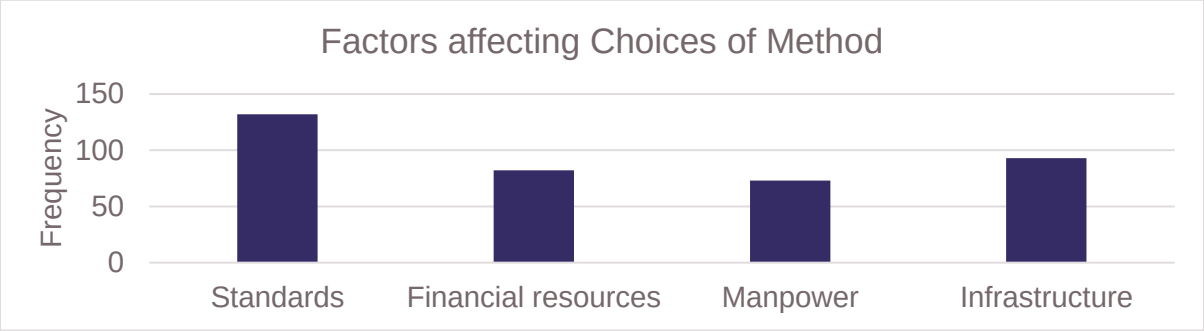


Figure 3. Factors affecting choices of methods.

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

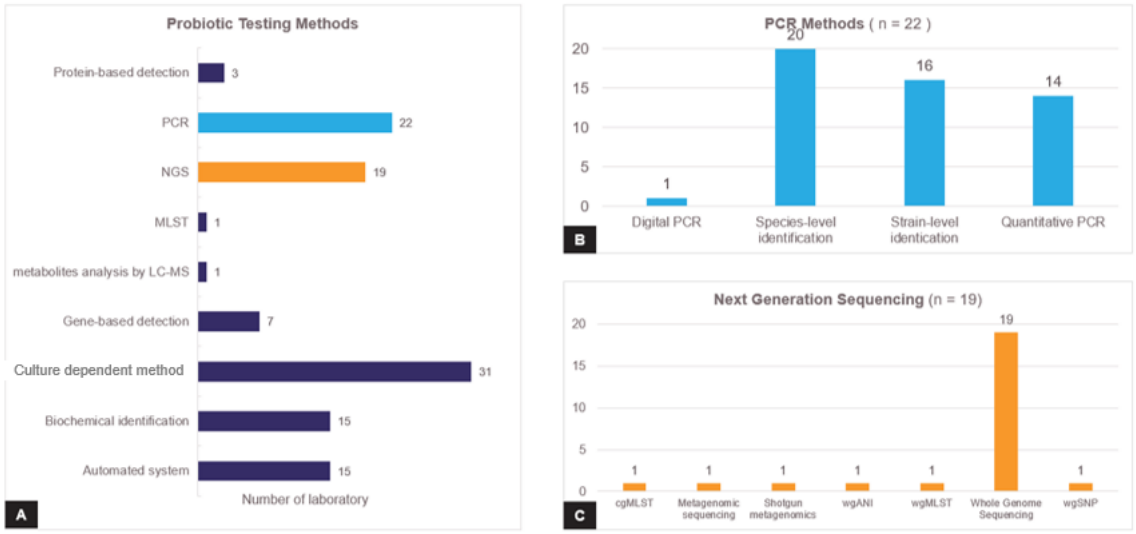


Figure 4. (A) Probiotics testing methods adopted by the participating laboratories. (B) PCR Methods that the laboratories are using to assess probiotics products. (C) NGS methods adopted.

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 4. 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 10^6-10^8 CFU/ml (g) as stated in their economies' 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 4(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 5. NGS platforms available in the labs.

The two most commonly used sequencing platforms are: 1) Illumina, 2) Oxford Nanopore.

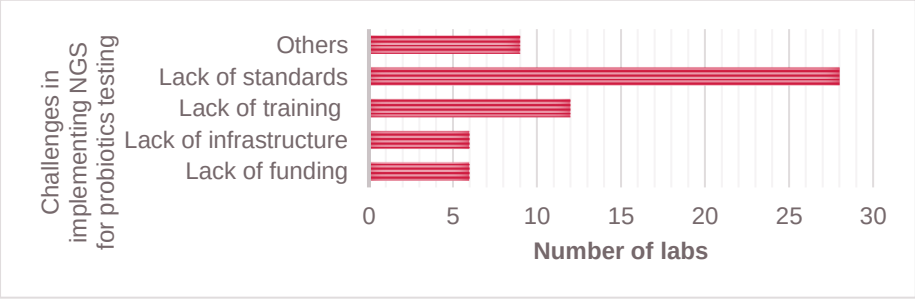


Figure 6. Challenges in implementing NGS for probiotics testing.

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.

Technical and Networking Workshop for Next Generation Sequencing Application for Probiotics Testing

Agenda

Day 1: 6 June 2023 at Pullman Bangsar, Kuala Lumpur

Time	Program	Speaker
8.30am – 9.00am	Registration	
9.00am – 9.20am	Welcome remark	UM
9.20am- 9.40am	Ice breaking	Prof Dr Lay Ching Chai, Sunway University
9.40am – 10.10am	SCSC 04 2021 Project Introduction and Findings	Prof Dr Lay Ching Chai, SU
10.10am – 11.10am	Presentation: Overview of probiotics testing and challenges	Prof Dr Yuan Kun Lee, National University of Singapore
11.10am – 11.30am	Break	
11.30am – 12.10pm	Discussion: How NGS address the gaps in the current practices	Prof Dr Patricia Conway, University of New South Wales
12.10pm – 1.00 pm	Presentation: NGS application in probiotics testing	Dr Yinping Dong, China National Centre for Food Safety Risk Assessment
1.00 pm – 2.00pm	Lunch	
2.00pm – 3.00pm	Presentation: Possibilities and limitations of NGS in probiotics testing	Dr Woori Kwak, The Catholic University of Korea
3.00pm – 4.00pm	Roundtable discussion	Prof Dr Lay Ching Chai, SU and other speakers
4.00pm	Break/ End of Day 1	

Day 2: 7 June 2023 at University of Malaya, Kuala Lumpur

Time	Program	Speaker
9.00am – 9.30am	Travel to UM	
9.30am – 10.00am	Presentation: Safety and claims of probiotics	Prof Dr Patricia Conway, UNSW
10.00am – 10.40am	Presentation: Functional analysis of probiotics- *Blautia wexlerae* as a probiotic candidate for weight control	Prof Dr Jun Kunisawa, National Institutes of Biomedical Innovation, Health and Nutrition
10.40am – 11.10am	Group photo session and break	
11.10am – 11.40am	Grouping and lab tour	UM High Impact Research Team
11.40am – 1.00pm	Lab session (in group) Isolation of organism from probiotic sample Assessment of DNA quality	Assoc Prof Dr Cindy Teh Shuan Ju, UM Jia Jie Woon (Group 1)

	Library Preparation Sequencing	Yee Qing Lee (Group 2) Zhi Xian Kong (Group 3) Min Yi Lau (Group 4)
1.00pm – 2.00pm	Lunch	
2.00pm – 3.00pm	Lab demo- Bioinformatics analysis	Dr Jacky Dwiyanto, UM
3.00pm – 3.30pm	Break	
3.30pm – 4.30pm	Panel Discussion: Bioinformatics analysis and pipeline development	Moderated by Assoc Prof Dr Chong Chun Wie, Monash University Panelists- Dr Woori Kwak, CU Dr Yinping Dong, CFSA Assoc Prof Sunny Wong Hei, NTU
7.00pm – 8.45pm	Dinner and Networking Session	

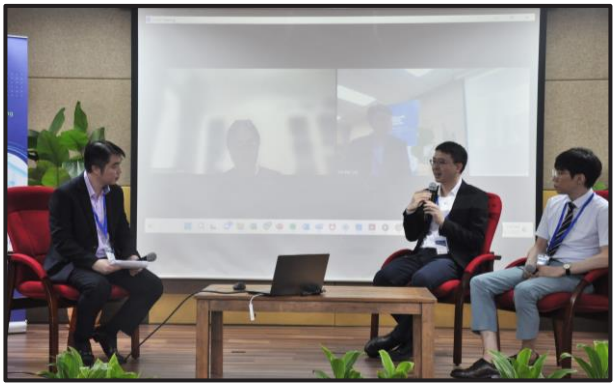
Day 3: 8 June 2023 at Pullman Bangsar, Kuala Lumpur

Time	Program	Speaker
9.00am – 9.40am	Best practice sharing: The role of next generation sequencing in strain level probiotic identification	Dr Anthony Kieffer, IFF
9.40am – 10.20am	Presentation: Method standardization in probiotics testing	Dr Adrianne Klijn, Nestlé
10.20am – 10.40am	Break	
10.40am – 11.20am	Presentation: Application of metagenomic sequencing in identifying new probiotics	Assoc Prof Sunny Wong Hei, Nanyang Technological University
11.20am – 12.00pm	Presentation: Beyond Probiotics and Prebiotics: The Versatile Applications of Next-Generation Sequencing in Pharmaceutics and Food Safety	Dr Chee-Onn Leong, AGTC Genomics
12.00pm – 12.30pm	Presentation: Development of National Standards and Testing Lab Accreditation	Mr. Mohd Hamzaini Hashim, Department of Standards Malaysia
12.30pm – 2.00pm	Lunch	
2.00pm – 3.00 pm	Panel discussion: Reflection and way forward	Lead: Prof Lee/ Prof Conway Prof Chai (Food safety) Dr Chong (Bioinformatics) Dr Klijn (Standards/Testing)
3.00pm – 3.30pm	Discussion and concluding remark Closing, tea break provided	Assoc Prof Dr Cindy Teh, UM

Workshop Photos

Group Photo

Roundtable Discussion



Panel discussion on bioinformatics analysis, one of the panelists called in from People's Republic of China.

Laboratory demo sessions for NGS

Lectures at the workshop.

Lecture-presentation by Prof Jun Kunisawa.

Final panel discussion by experts from different fields in relevance to probiotics testing.

Presentation Summaries

Day 1

SCSC 04 2021 Project Introduction and Findings

The workshop began with an introduction by Prof Chai Lay Ching, who provided an overview of the project SCSC 04 2021A and shared updates on its progress. Prof Chai highlighted key findings from the survey and focus group discussions (FGDs) conducted as part of the project. The survey and FGD outcomes revealed a lack of harmonized standards in probiotics testing, with most laboratories still relying on the conventional culturing method. This adherence to traditional methods is primarily driven by regulatory requirements in different economies. Prof Chai mentioned that the FGDs were conducted virtually, grouping participants based on their geographical regions: Asian and Western (United States and Europe). The challenges faced in implementing NGS-based testing for probiotics varied significantly between these two groups, depending on the market demand and regulatory landscape. Additionally, Prof Chai addressed the challenges posed by matrix complexity and the discrepancies between vertical methods and internal methods, which have further complicated the adoption of NGS technologies for probiotics testing. The concerns and issues raised through the survey and FGDs will be thoroughly discussed in the forthcoming handbook, which is being published as part of the SCSC 04 2021A project. The handbook aims to provide comprehensive insights and guidance on probiotics testing, taking into account the specific challenges and considerations identified during the project.

Overview of probiotics testing and challenges

Prof Lee Yuan Kun delivered a presentation on the regulatory landscape of probiotics in various Asian economies, this table will be published in the Handbook developed for this project. Prof Lee highlighted that the regulation of probiotic products is not harmonized in Asia. Many economies permit general health claims based on the ingredients, except Viet Nam which requires the proof of effects for the health claims on finished goods. One thing that is common among the Asian economies is that probiotics are considered as live microorganisms.

He emphasized that the culturing method remains the gold standard for probiotics testing according to these regulations. Prof Lee highlighted the strain-dependent nature of probiotic effects and pointed out the increasing prevalence of commercial multistrains probiotic products, which could potentially create regulatory loopholes. He stressed that the current culturing methods are limited in their ability to quantify probiotics only at the genus level. Without additional testing, it becomes challenging to substantiate the claims related to the

added strains in these products. He referenced a previous study that demonstrated how the effects of multistrains products can be hindered by steric hindrance [5]. To address the gaps in current testing methods, Prof Lee proposed the potential of NGS technologies. However, he emphasized the importance of standardized platforms and protocols to ensure reliable and consistent results in probiotics testing.

Discussion around limitations and challenges in implementing NGS for probiotics testing

This discussion was conducted prior to the hands-on session and more information on how NGS can be utilized is presented, moderated by Prof Patricia Conway. The discussion outcome is summarized in point form as below:

1) Culturing method is still the gold standard for regulatory compliance.
    - NGS cannot differentiate live/ dead cells
    - NGS is not able to quantify cells in CFU
2) No standard is available for NGS in probiotic testing and participants are not sure which method to use, e.g. *When to use whole genome sequencing, when to use metagenome sequencing, when to use shotgun sequencing?*
3) Quality control of the NGS is difficult due to
    a. Lack of positive control
    b. Lack of proficiency testing
4) Bioinformatics analysis pipeline is unclear, variable platform and softwares resulted in different results.
5) The diverse composition and the product matrix can impede the extraction process, requiring a standard protocol to overcome these challenges in DNA extraction.
6) Cost of investment (facility and capacity building) and cost per test.
7) Lab personnels have difficulty in adapting to the fast-paced technological advancement in NGS.

NGS application in probiotics testing

During the workshop, Dr Yinping Dong provided an overview of the regulatory developments for probiotic testing in China. She highlighted the recent revisions made to the National Food Safety Standards (GB), which extended the list of permitted bacterial and fungal species for use as probiotics in health foods. Dr Dong explained that enumeration of probiotics is carried out in China using plate counting method. However, for comprehensive characterization, additional identification methods are required, including phenotypic, genetic, and protein-based analyses. Notably, whole genome sequencing (WGS) is employed in China for genotypic characterization of probiotics.

WGS enables the analysis of various genetic attributes of the probiotic strain, including virulence genes, antibiotic resistance genes, and toxigenic genes. This comprehensive genotypic information aids in evaluating the safety and efficacy of probiotic products. To illustrate the application of WGS, Dr Dong shared a case study on how this technology was utilized in probiotic product surveillance in China. Dr Dong's presentation highlighted the importance of using advanced techniques like WGS for a thorough understanding of probiotic strains and ensuring compliance with regulatory requirements in China.

Possibilities and limitations of NGS in probiotics testing

Dr Woori Kwak emphasized that his presentation focused on testing methods rather than research purposes, citing accuracy differences. He discussed how Sanger sequencing is no longer suitable for studying mixed cultures of bacteria, while NGS allows comprehensive

analysis of microbial communities through multiplexing. However, he acknowledged limitations in accurately sequencing full-length 16S rRNA sequences with current NGS methods. Dr Kwak highlighted the use of PacBio HiFi sequencing and Oxford Nanopore, leveraging the rRNA operon as a phylogenetic marker **[6]** to generate high-accuracy full-length 16S rRNA sequences, compensating for the higher error rate of long read sequencing. He emphasized the importance of achieving 99% full-length sequences and 100% coverage of the V4 hypervariable region for accurate NGS results **[7]**.
The decreasing cost of sequencing has prompted a shift towards Whole Meta Shotgun (WMS) sequencing, which eliminates the need for Polymerase chain reaction (PCR) amplification by enabling direct sequencing after DNA extraction. WMS enables the study of gene contents related to safety and provides insights into microbial community functionality. Dr Kwak discussed the analysis concept of WMS, including Kranken 2 for species identification using K-mer and Metaplan 4 for species identification based on gene content. In Korea, probiotic products are regulated based on labeled probiotics and their ability to maintain a count of ≥ 10^8 CFU/g throughout the shelf life. The Ministry of Food and Drug Safety (MDFS) conducts post-market surveillance through random sampling and employs NGS, along with a developed bioinformatics pipeline, to verify probiotic content. Concerns were addressed prior to developing NGS standards, considering factors such as accuracy, affordability, compatibility with multiple sequencing platforms, and user-friendly analysis on personal devices. The developed method **[8]** successfully detected and quantified strains in single and multistrains probiotics, although WMS has limitations in detecting probiotic strains present in small amounts, potentially resulting in false negatives. WMS allows the study of gene contents related to safety, including antimicrobial resistance (AMR) and virulence factors (VF). Dr Kwak mentioned databases such as RGI **[9]**, ResFinder **[10]**, and AMRFinderPlus **[11]** for AMR analysis, cautioning about the completeness of the database or algorithm used. For VF factors, VFanalyser **[12]** with a modified pipeline is employed.
Dr Kwak concluded by highlighting the rapid pace of the probiotics market and the challenges of NGS analysis for strain identification due to errors and mutations. He emphasized the need to select appropriate NGS sequencing, and analysis methods based on specific circumstances and government regulations, particularly in the case of probiotic regulation.

Roundtable discussion
This roundtable discussion was moderated by Prof Dr Chai and summarized as below-

1. Are there any specific technical or operational hurdles that arise when implementing NGS for probiotics testing? How can these challenges be mitigated?
    - No standard / guideline, consistency of the results is not assured.
    - Low capability in data analysis (may be overcome by collaboration)
    - Limited demand (can be overcome by raising awareness, include trade facilitation department)
    - Lack of expertise (resource person)
    - Database availability
    - Facility (not that equipped and to maintain the server and system requires high cost)
2. What types of support, resources, or infrastructure are required from relevant parties (e.g., regulatory bodies, research institutions, industry stakeholders) to effectively implement NGS for probiotics testing?
    - Financial support (facilities, maintenance and capacity building)
    - Network
    - Reference laboratory
    - Communication among the people in field
    - Standard/ guidelines
    - Relevant parties to ensure the development and implementation
    - Certified materials needed to ensure consistency of the results

- Collaboration between relevant stakeholders

2. Are there any specific training or educational initiatives needed to equip professionals with the necessary skills and knowledge to implement NGS for probiotics testing?
   - Capacity building program is needed, particularly in sequencing and bioinformatics.
3. Who are the key stakeholders involved in the implementation of NGS for probiotics testing, both from a scientific and regulatory standpoint?
   - Probiotics producers, service provider, policy maker and regulatory body, scientists
4. Are there any gaps or areas where the involvement of additional stakeholders would be beneficial for the implementation of NGS in probiotics testing?
   - Lack of bioinformatician (gap between the university program and the industrial practice)

Day 2
Safety and claims of probiotics

Prof Patricia Conway emphasized the importance of testing probiotics and highlighted the need to identify probiotic strains at the strain level due to their strain-specific functional characteristics and potential safety concerns. In Australia, probiotics in food are regulated by Food Standards Australia New Zealand (FSANZ) without requiring disclosure of the number of probiotic bacteria, while the Therapeutic Goods Administration (TGA) regulates probiotics as complementary medicines, which must disclose the amount of active ingredients. Complementary medicines require evidence of efficacy, but this is not assessed by TGA-Listed. However, in 2018, TGA introduced Assessed Listed, which necessitates supporting evidence from high-quality scientific studies, and TGA conducts pre-market assessments on the efficacy evidence. To help applicants determine whether their product falls under the category of food or medicine, TGA developed the **Food-Medicine Interface Guidance Tool.** Prof Conway discussed health claims in food [13], distinguishing between general claims and high-level claims. General claims relate to the effect of nutrients or substances on health, supported by scientific evidence, while high-level claims pertain to the relationship between a nutrient and a serious disease or biomarker.

Prof Conway then explained how NGS is adopted to verify the probiotics strains claimed in multiple commercially available products [14] and reveal the presence of safety concern-factor in a study performed by Chris' research team [15]. Among the 10 products that were tested using metagenomic sequencing, they find that some probiotics strains labelled are not detected in the products, while some non-labelled strains are detected (8/10 samples). Wang et al uses WGS to evaluate the virulence factors, toxic metabolites, and its mobility (genes) in the probiotic strains recovered from some commercially available products. She concluded that NGS serves as a valuable tool for evaluating the safety of probiotic products, providing evidence for the claims, and assessing potential risks. If the mechanism is understood and there will be an opportunity to apply in supporting health benefit claims and functionality claims.

Functional analysis of probiotics- *Blautia wexlerae* as a probiotic candidate for weight control

Prof Kunisawa highlighted the crucial role of diet and commensal bacteria in our gut in maintaining overall health and the connection to various diseases. Diet and intestinal bacteria are regarded as potential sources for drug discovery and the healthcare industry,

including probiotics. These bacteria produce metabolites and nutrients derived from the diet, known as postbiotics. Therefore, the interaction between the host, commensal bacteria, and diet is of utmost importance.

Prof Kunisawa's research team employs a combination of NGS, bacterial cultures, germ-free gnotobiotic mice, and metabolome analysis to investigate dietary materials and their metabolites. To handle the large amount of data generated, the team utilizes bioinformatics. For cohort studies, multiple sites are established to collect human samples and information. Nearly 10,000 individuals have participated and provided data on their diet, lifestyle, exercise, sleep, and health conditions. Blood, stool, and saliva samples are collected for metabolomic and immune analyses. These data are incorporated into the NIBIOHN JMD database and the Microbiota and Phenotype Correlation Analysis Platform (MANTA) **[16]**. MANTA enables non-bioinformaticians to study the correlation between commensal bacteria and BMI, aiding in identifying bacteria and metabolites associated with disease control and diet/lifestyle habits.

Animal models were used to verify the results and identify bacteria and metabolites. *Blautia wexlerae*, abundant in non-obese individuals, was found to control weight gain and diabetic symptoms in a high-fat diet. Genetic and metabolomic analyses revealed unique pathways and metabolites produced by *B. wexlerae*, including S-adenosylmethione (SAM), Acetylcholine, L-ornithine, succinate, lactate, acetate, and Amylopectin. Incorporating *B. wexlerae* into high-fat diet gnotobiotic mice restored short-chain fatty acid production and prevented its decline (going back to the original production rate). These findings suggest *B. wexlerae* as a potential drug discovery application and probiotic. However, further safety and efficacy assessments are required in human samples. Prof Kunisawa emphasized the usefulness of NGS and whole-genome sequencing (WGS) for studying potential drug discovery and probiotics. The research team is also focused on personalized medicine and probiotic systems.

Lab demo sessions

NGS
During the workshop, lab demo sessions were conducted by Assoc Prof Dr Cindy's team. Participants were able to gain hands-on experience with the general workflow of whole genome sequencing (WGS) using the Illumina MiSeq platform. This provided them with practical knowledge of the sequencing process.

Bioinformatics
On the other hand, the bioinformatics analysis sessions were facilitated by Dr Jacky Dwiyanto. He guided participants through the steps involved in processing raw WGS data, including screening for multilocus sequence typing, antibiotic resistance genes, and plasmid groups. He also covered topics such as comparative genomics and demonstrated the use of statistical programming with R for data cleaning and visualization.

Panel Discussion
One of the highlights of the workshop was a panel discussion on bioinformatics analysis and strain identification in probiotic products. Dr Dong shared that the current practice relies on culturing methods and species-level identification in China. Molecular methods like PCR and qPCR (real time-PCR or quantitative PCR) have been explored to validate cultivation, and NGS is considered a potential tool for strain identification. However, defining strains based on the number of SNPs remains unresolved, requiring further consensus among stakeholders. Dr Kwak agreed and emphasized the need for communication and collaboration to address this issue.

During the discussion, Dr Chong suggested using NGS to predict metabolite production

based on genomic sequences. Dr Wong agreed in principle but highlighted the complexities of cross-species interactions. Dr Kwak supported the use of NGS for metabolite production prediction but noted that cross-species interactions may not be accurately predicted by NGS alone. The panelists agreed that DNA sequencing data, supplemented with meta-transcriptomics, could provide insights, but validation through bacterial culture is necessary.

Dr Kwak explained that NGS is used to address safety concerns by identifying potential risks such as antimicrobial resistance (AMR) genes and virulence factors. Enumeration and estimation of probiotic strains using NGS is challenging, requiring multiple complementary methods. Dr Wong focused on translational research in oncology, studying microbe-host interactions and animal models. Dr Dong highlighted the need for government regulation to control the commercialization of multistrain probiotics and ensure product quality.

The panelists discussed the individual-specific nature of the gut microbiome and the challenges of tracking the effects of probiotics, which work differently in different individuals. The topic of Average Nucleotide Identity (ANI) thresholds for species and strain levels was raised [17], but no consensus on strain-level ANI was mentioned. The panel discussion shed light on the complexities of strain identification and the potential of NGS in predicting metabolite production, emphasizing the need for collaboration and validation through bacterial culture.

Day 3

The role of next generation sequencing in strain level probiotic identification

Mr. Anthony Kieffer began his presentation by emphasizing the importance of clinically proven functional attributes associated with specific strains and doses of probiotics **[18, 19]**. Health claims are typically based on single strains or consortia, and advancements in whole genome sequencing (WGS) have made highly clonal strains more distinguishable. He also highlighted the increasing regulatory and standard-setting requirements in the field.
In terms of probiotic identification, Mr. Kieffer acknowledged the need for multiple techniques to ensure accurate strain identification. The table below summarizes the identification methods used in the industry, including API kits, 16S rRNA, RiboPrinter, MALDI-TOF, PCR assays, and NGS.

Current identification method	Level of distinction	Application
API Kits	Species level	Confirmation of external lab results Strain characterization
16S rRNA	Species/ Subspecies level	Confirmation of identity (quality testing) Identification of contaminants
RiboPrinter	Species/ Subspecies level	Confirmation of identity (quality testing) Identification of contaminants
MALDI -TOF	Species/ Strain level	Confirmation of identity (quality testing) Strain characterization
PCR Assays	Strain level	Confirmation of external lab results Identification of contaminants Single strain identification
NGS	Strain level	Whole genome sequencing (WGS) Methods under development Locked nucleic acid (LNA) primers/ probes, qPCR, dPCR, NGS

Regarding WGS adoption, Mr. Kieffer mentioned the use of two NGS platforms (Oxford

Nanopore and Illumina). Raw sequence data is assembled, and annotation is performed using different software tools to create comprehensive annotations. These annotated genomes are then utilized for genetic safety assessment, monitoring genetic drift, designing strain-specific PCR assays, and transcriptomics. For highly clonal strains, he acknowledged the challenges in suboptimal design conditions and mentioned the use of a combination of long and short read sequencing to improve whole genome sequence. They also employ imaging equipment and software to visualize band size, concentration, and the amplification quality of samples. NGS is also utilized to identify highly clonal strains in multi-strain blends, and a method using locked nucleic acid (LNA) primers to detect 1base pair differences is under development.

Mr. Kieffer discussed the hurdles for NGS implementation, including high testing costs and the need for skilled bioinformaticians to develop proper pipelines. Validation, resource-intensive processes are also required. In conclusion, strain-specific identification is an emerging requirement for probiotic products, enhancing quality and ensuring efficacy. Although NGS implementation for routine testing is still in development, it shows promise in overcoming challenges posed by highly clonal strains.

Method standardization in probiotics testing

Dr Klijn explained how probiotic identification by using the Tree of Life for taxonomic difference. Technologies like biochemical analysis, MALDI-TOF, Ribotyping, PFGE, 16S sequencing, WGS, and strain-specific PCR can be used for identification from the genus to strain level. Standards such as ISO 23418 (WGS), EN 17697 (PFGE), and CEN/TS 15790 (PCR) exist. Dr Klijn emphasized the need for considering subspecies identification and the limitations of different methods. Strain-specific PCR is preferred for strain-level identification. However, issues surrounding strain definition and consensus on using WGS or phenotypic differences must be addressed at the international level.

Enumeration methods for bacteria include culturing, flow cytometry, qPCR, droplet digital PCR, and NGS, with CFU (colony-forming units)/ culturing method being the gold standard. Enumeration is crucial for probiotic requirements, and several standards are available. However, the current methods often focused on lactic acid bacteria may not recover all probiotics. Considerations should be made for other genera that can grow on the same media. Individual standards for yeast and mold count exist but do not cover probiotics. Multiple species or strains in a probiotic product can lead to under-representation if genus-specific methods are used. Strain-specific enumeration methods have been developed to address this issue, but discrepancies can arise when different labs use different methods. Standardization efforts for enumeration methods should be done at the horizontal level, with clear reflection of method limitations in legislation. Detection or enumeration of contaminants other than probiotics is also important. Guidance from US Pharmacopoeia (USP) and International Dairy Federation (IDF) is available, but sample preparation modifications may be needed for probiotics testing. Little guidance exists for these modifications, requiring validation and verification. Horizontal standards are necessary to provide accurate detection and enumeration of contaminants in probiotic products.

In summary, there is a need for standardization in probiotic identification and enumeration methods. Addressing the limitations of current methods, developing horizontal standards, and providing guidance for detecting and enumerating contaminants in probiotic products are important considerations.

Application of metagenomic sequencing in identifying new probiotics

Associate Professor Dr Sunny Wong and his research team have focused their efforts on discovering and understanding the differences in microbial composition in the bodies of cancer patients. With the advent of advanced sequencing technologies, they are now able to

study the metagenome of the gut microbiome without the need for conventional culturing methods, which often fail to capture non-culturable bacteria. Dr Wong explained the use of metagenomic sequencing by comparing different sequencing technologies. He emphasized the term "dysbiosis," which refers to an imbalanced condition in the microbiome where pathobionts increase during disease while beneficial microbes are reduced. The depleted microbes are recognized as potential probiotics against diseases.

Studies have estimated that up to 20% of all cancers are linked to microbes **[20, 21]**, which is particularly relevant to prevalent Asian cancers. As the knowledge on cancer-related microbiomes expands, the focus has shifted to studying the ecological context of cancer **[22]**. Dr Wong shared the pioneering metagenomic discovery of colorectal cancer (CRC) in 2013, where researchers demonstrated that Fusobacterium nucleatum changes the tumor environment, leading to a more pro-inflammatory state and the progression of colorectal neoplasia **[23, 24]**. Subsequently, Dr Wong's team focused on studying the CRC metagenome in the Chinese population **[25]** and compared it to populations in the US and Europe **[26]**. They found that the same population of gut bacteria was consistently enriched in CRC patients, regardless of the population difference. Simultaneously, they examined the bacteria that were enriched or depleted in the CRC metagenome and proposed that the depleted bacteria could serve as potential probiotics. These probiotics have the potential to prevent or delay CRC and improve current therapies for CRC. Dr Wong provided several examples of how metagenomic studies are utilized to understand cancer ecology and explore translational applications, including the discovery of new probiotics and personalized probiotic approaches. Overall, Dr Wong's research showcases the power of metagenomic studies in unraveling the complex relationship between the microbiome, cancer, and the potential for probiotic interventions.

Final Panel Discussion

An interesting discussion on the future of probiotics testing took place at the end of the workshop, moderated by Prof Patricia Conway and Prof Lee Yuan Kun. The panel included Prof Chai Lay Ching (food safety), Dr Adrianne Klijn (standards and food testing), and Dr Chong Chun Wie (bioinformatics and gut microbiome).

Prof Conway expressed concerns about the errors and uncertainties arising from the use of different standards in probiotic testing. The panel agreed that establishing a standard method is crucial to reduce result variation and emphasized the need for regulatory bodies to collaborate with relevant stakeholders. Prof Chai added that there is currently **no harmonized standard** across economies or even within a single economy. Dr Klijn shared that discussions within the ISO working group have not resulted in a consensus due to a lack of willingness to compromise among stakeholders.

Given the difficulty in reaching a consensus, Prof Conway suggested focusing on achievable goals in a shorter timeframe, such as **adapting current methods**. Since culturing methods are necessary for downstream work, modifying existing methods was considered worthwhile. Dr Chong proposed the formation of a **working group to facilitate** the adaptation of standards and ensure constant updates and reviews.

Considering the rapid growth of the probiotics industry, Prof Lee questioned the possibility of establishing a simpler method to assure the quality and efficacy of probiotics, enabling consumers to make informed choices. Prof Chai and Dr Klijn agreed that an **open dialogue among stakeholders** is necessary to determine priority issues, and **public-private partnerships** should guide the direction of the work.

While the health benefits of probiotics are strain-specific, current culturing methods only allow for genus-level identification, supplemented by biochemical identification and/or PCR

for species-level identification. The panel agreed that **no single method or standard enables enumeration and identification simultaneously**, suggesting the adaptation of current methods. The ongoing discussion on "strain definition" remains inconclusive, as genetic differences based on SNPs cannot guarantee different phenotypic characteristics or health benefits to the host. **Biphasic identification** is currently accepted in the field.
In conclusion, the panel stressed the critical importance of establishing standards for probiotics testing. However, they acknowledged that it will take time and require communication among all relevant stakeholders to reach a consensus. Given the time constraint, there is an urgent need to adapt current methods and expand the scope of probiotics to ensure accurate claims and safety for consumption.

Conclusion for the workshop
The cultivation method remains the preferred standard for probiotics testing. NGS (Next-Generation Sequencing) can serve as a supplementary tool to verify or enhance certain processes in probiotics testing. When conducting Whole Genome Sequencing (WGS), pure bacterial isolates are necessary. However, for metagenome/ shotgun/ shallow shotgun sequencing, no cultivation process is required unless verification is needed.

The establishment of an NGS lab for probiotics testing highly depends on the budget, objectives, and available resources. The selection of appropriate equipment, platforms, and bioinformatics pipelines will largely depend on the specific goals of implementing the NGS method and the desired depth of sequencing results.

In conclusion, extensive discussions are necessary to reach a consensus on issues related to strain identification, species-level or strain-level labeling, and other relevant matters. All stakeholders should engage in discussions and collaborate before NGS can be implemented as one of the standard methods for probiotics testing.

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