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Advances in Probiotics: From Definitions to Manufacturing to Applications

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Abstract

The field of probiotics has witnessed significant evolution since the coining of the term in 1953 by Werner Kollath. Initially defined as "active substances essential for a healthy development of life," the definition of probiotics expanded in 1965 to include "substances secreted by one organism that stimulate the growth of another." Finally, in 2016, the term was refined as "alive microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance." This evolving definition reflects the growing understanding and utilization of probiotics in various fields. Probiotics encompass diverse strains, each with distinctive functional activities. While commonly associated with species like Lactobacillus, Bifidobacterium, Enterococcus, and Streptococcus, the range of microbes employed in probiotics has expanded to meet diverse demands. Regular updates on microbial flora and research are vital to stay updated in this dynamic field. Bulk production of probiotics, particularly lactic acid bacteria (LAB) and Bifidobacteria, involves a meticulous manufacturing process to ensure robustness and stability. The definition of an "adequate amount" varies, with some regions requiring a minimum of 109 colony forming units (CFU). Quality control is essential to maintain high-performance standards. Probiotic strains are subjected to various stress factors during drying and storage, impacting their viability. Strategies such as adding antioxidants and storing at lower temperatures enhance shelf life. Selecting probiotic strains based on their resistance to stress is essential, but it doesn't guarantee functionality. The complexity of strain selection should consider various criteria for optimal bioactivity. Additionally,

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diversity among strains and their adaptation to specific environments play a crucial role. The long-term effects and individual variations in probiotic efficacy pose challenges, demanding further research and clinical trials. Ethical considerations, safety, and side effects are essential aspects of probiotic development, requiring rigorous assessment. Taxonomic identity, strain specificity, and disease specificity must be accurately determined to avoid misidentification and ensure safety. In conclusion, the field of probiotics has evolved significantly, with expanding applications and manufacturing processes. The challenges of strain selection, diversity, and long-term effects necessitate ongoing research and adherence to safety guidelines. Probiotics hold great promise but require careful evaluation and consideration of various factors to maximize their potential benefits.

Keywords:
Probiotics, Production, Lactobacillus, Bifidobacterium, dairy, Fermentation

Introduction:

Over history, diverse cultures discovered the art of making fermented drinks through trial and error and careful observation.The word fermentation is derived from the Latin word “fervere” meaning “to boil” **(Ozen and Dinleyici, 2015).** Microorganisms appeared 3.8 billion years ago much earlier than the homo genus appeared. Because of this, bacteria have more time to change and adapt to the survival strategies that allow them to survive even in the most hostile environment **(Tegegne and Kebede, 2022).**

Ancient civilizations like the Greeks and Romans used fermented dairy products to maintain good health whereas, in 76 BC Roman historians recommended the use of milk fermentation products to cure gastroenteritis **(Tegegne and Kebede, 2022).** Pasteur and those who followed him had a profound influence on microbiology.They found the concept of utilizing beneficial bacteria which has gained popularity alongside the progress in microbiology, notably at the Pasteur Institute **(Ozen and Dinleyici, 2015).** However, in the majority of scientific papers, Elie Metchnikoy has always been considered the father of the idea that probiotics have beneficial effects on human health **(Ozen and Dinleyici, 2015).**

The word probiotic was introduced in 1953 by the German scientist Werner Kollath which represents the “active substances that are essential for a healthy development of life”. But later in the year 1965, Lilly and Stillwell represented it as “substances secreted by one organism which stimulate the growth of another”. Finally Fuller has made a definition for probiotics as “alive microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance” **(Gasbarrini et al.!, 2016).**

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Review Article

Table 1 History of probiotics. (Source: Anusha et al., 2015)

Year	Author	Discoveries	References
1857	Pasteur	Lactic acid-producing bacteria	(Ozen and Dinleyici, 2015)
1889	Henry Tissier	*Bifidobacterium spp.*	
1892	Doderlein	The significant association between microorganisms and human health	(Santacroce et al., 2019)
1907	Ellie Metchnikoff	Identified the microorganisms responsible for fermentation from Bulgarian yogurt	(Anusha et al., 2015; Gasbarrini et al., 2016)
1965	Lilley and Stillwell	The first definition of probiotics was given	(Fontana et al., 2013)
1984	Hull	Introduced first probiotic species – *Lactobacillus acidophilus*	(Anusha et al., 2015; Santacroce et al., 2019)
1991	Henry Tissier	First to isolate a *Bifidobacterium*	
1991	Holocombh	Introduced it as probiotics	
1992	Fuller	Found that they help to improve the intestinal microbial balance	(Gasbarrini et al., 2016)

1.Probiotics and their Types

Different strains of probiotics have different potential even though they belong to the same species. Each of the strains is distinctive, portraying different functional activities **(Vandenberghe et al., 2010).** It encompasses a diverse range of microorganisms such as bacteria and yeast or fungi. Among them the most common varieties are Lactobacillus, Bifidobacterium, Enterococcus, and Streptococcus as shown clearly in Table 2. Similarly, certain fungal strains from the Saccharomyces genus including Saccharomyces boulardii are utilized with many of them recognized as natural inhabitants of the gut **(Santacroce et al., 2019).**

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Table 2 Some of the microorganisms used as probiotics. (Source: Anusha et al.,

Microorganisms	Varieties	References
Lactic acid-producing bacteria	*Lactobacillus acidophilus* *Lactobacillus plantarum* *Lactobacillus casei* *Lactobacillus fermentum* *Lactobacillus johnsonii* *Lactobacillus reuteri* *Lactobacillus rhamnosus GG*	(Fijan, 2014; Anusha et al., 2015)
	Enterococcus faecalis *Enterococcus faecium* *Escherichcia coli* *Streptococcus thermophiles*	(Fijan, 2014; Anusha et al., 2015)
	Bacillus cereus	
Bifidobacterium species	*Bifidobacterium adolescentis* *Bifidobacterium animalis* *Bifidobacterium bifidum* *Bifidobacterium infantis* *Bifidobacterium longum*	(Fijan, 2014; Anusha et al., 2015)
Non-pathogenic yeast	*Saccharomyces boulardii* *Saccharomyces bayanus*	(Fijan, 2014; Anusha et al., 2015)
Non-spore forming	*Coccobacills*	(Anusha et al., 2015)
Spore forming bacteria	*Bacillus subtilis* *Bacillus coagulans*	(Fijan, 2014)

The variety of microbes employed as probiotics has increased due to the growing demand in this field and the discovery of new microbes suitable for use as probiotics. It is advisable to periodically update microbial flora and stay abreast of research and published information on probiotics to enhance understanding and insights **(Amara and Shibl, 2015).**

Bulk Production of Probiotics

The most widely accepted definition of probiotics, as proposed by a FAO/WHO (2002) working group and later confirmed with minor grammatical changes by an ISAPP expert panel, states that probiotics are live microorganisms that, when administered in adequate amounts, bring about a health benefit in the host **(Hill et al., 2014).** When it comes to the types of microorganisms, our emphasis will be on lactic acid bacteria (LAB) and Bifidobacteria, which are the most commonly utilized probiotic genera. However, other genera like Bacillus and Saccharomyces are also employed as probiotics, each with unique growth requirements and stability characteristics **(Johnston et al., 2018).** As the viability of probiotics is crucial, they need to be manufactured in a manner that ensures their robustness and stability in sufficient quantities until the end of their shelf life. These probiotics should also be incorporated into consumer products that support their survival throughout their shelf life.

The definition does not specify what constitutes an "adequate amount." Nevertheless, in some regions, like Canada and Italy, regulators require a minimum dose of 109 colony forming units (CFU) **(Hill et al., 2014).** Additionally, the adequate amount is generally considered to be at least the dose that has been demonstrated to deliver the specific health benefit in question, with no evidence suggesting that a higher dose is harmful and, in some cases, it may even be advantageous **(Zhou et al., 2000; Morovic et al., 2017; Ouwehand et al., 2018).** Probiotics, particularly when included in dietary supplements, are often subject to transportation and storage at ambient temperatures and humidity levels. This can lead to a decrease in viability compared to refrigerated or frozen storage and handling. To ensure that the target dose is maintained until the end of the product's shelf life and to account for potential losses during storage and handling, an overage is typically included in the product **(Sreeja et al., 2013).**

While there is ample information available on identifying potential new probiotics and assessing their safety, as well as the health benefits of specific probiotic strains or combinations, this article does not cover those topics **(Sanders et al., 2014; Cremon et al., 2018).** Similarly, the discussion of the market potential and health economics of probiotics is directed to other sources **(Di Cerbo and Palmieri, 2015; Ouwehand, 2016).** Here, our focus is on the requirements for consistently and reliably producing high-quality, safe, and stable probiotics and ensuring their viability invarious delivery formats to provide effective probiotics and expanding their shelf-life.

Manufacturing Dairy Starter Cultures to Develop Probiotics

LAB and Bifidobacteria are manufactured on a commercial scale to meet the demand for probiotic dietary supplements and dairy starter cultures. From a manufacturing perspective, the goal is to produce a commercial product with the highest possible yield, consisting of viable and concentrated cells that remain stable and consistently perform in their intended applications. Customers expect these products to have a high cell count and a long shelf-life, capable of maintaining their stability under various temperature and humidity conditions. This is especially important for high-quality dietary supplements with established doses through clinical trials. In contrast, customers looking for dairy starter cultures desire rapid and consistent acidifying activity

activity in milk.

The manufacturing process for LAB and Bifidobacteria used in dietary supplements and dairy applications shares several common steps, as depicted in Figure 1. It begins with a carefully prepared frozen seed stock containing a pure strain, verified to be free of contaminants through quality control testing. This seed stock is used in a limited number of sequential seed fermentations to reach the desired inoculum volume, which is then transferred to the main fermentation vessel for growth. Alternatively, a frozen direct vat inoculation (DVI) material, containing a larger concentration of cells, can be directly added to the main fermentation vessel. Both approaches aim to minimize the number of generations from seed stock to final product, reducing the potential for genetic drift. The heat-treated medium used in the fermentation process contains a mixture of water, nitrogen sources, carbohydrates, salts, and micronutrients necessary for growth. The fermentation process is closely monitored, and once the main tank fermentation is complete, the cells are concentrated by separating them from the spent medium using centrifugation.

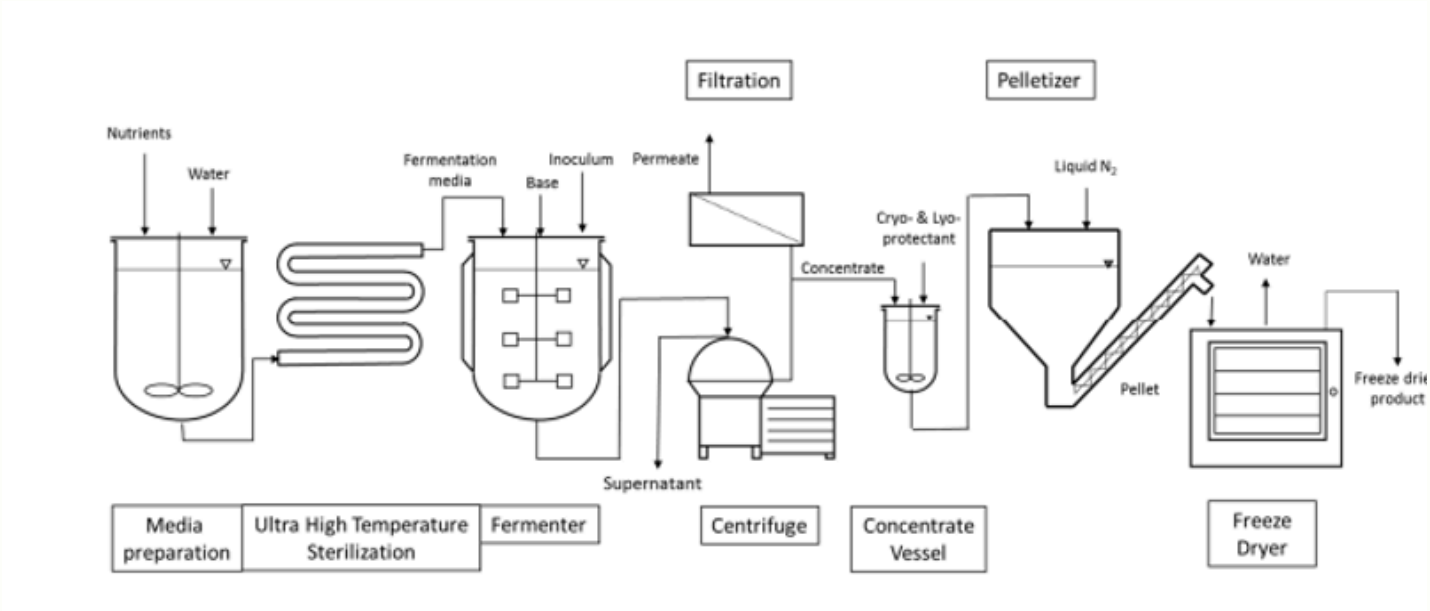

Fig. 1 A diagrammatic representation of production of dairy starter cultures for probiotics (Source: Fenster et al., 2019)

Depending on the final product application, stabilizer solutions (cryoprotectants to prevent cell damage during freezing and lyoprotectants to safeguard cells during freeze-drying) may be added to the cells before freezing. Cryoprotectants slow down ice formation by increasing solution viscosity, maintaining the amorphous ice structure close to the cells, while lyoprotectants stabilize the cell membrane's lipid bilayer in the absence of water (**Santivarangkna, Kulozik and Foerst, 2008).** Commonly used cryo- and lyoprotectants include carbohydrates and peptides, with skim milk powder being a choice in the dairy industry **(Carvalho et al., 2004).** After blending the probiotic concentrate with the cryoprotectant solution, various freezing methods can be employed. One method involves pouring the cryoprotected concentrate into cans and immersing sealed cans in a liquid nitrogen bath. The frozen cans can then be shipped to companies using probiotics in food

or beverages. Alternatively, a more efficient approach is to create pellets from the cryoprotected concentrate by dripping it into a liquid nitrogen bath. These pellets, typically 4–5 mm in diameter, are harvested and packed into bags, stored, and shipped at temperatures ranging from −45 to −55 ℃. Alternatively, frozen cell pellets can undergo freeze-drying (lyophilization) to produce a dried end-product. Frozen pellets are placed on trays that sit on temperature-controlled shelves, gradually heated after establishing a vacuum in the freeze-drying chamber. The freeze-drying process varies in length based on the strain, formulation, and cycle but generally takes several days to complete. Freeze-drying offers the advantage of maintaining probiotic cells at a low temperature, limiting damage to their structure and metabolites (Fonseca, Cenard and Passot, 2014).

Following removal from the dryer, the lyophilized material is milled into a powder with defined particle size and density. This milled material can then be blended with excipients (bulking agents), additional functional ingredients if needed, and flow aids, based on customer requirements. The resulting blend is used to create finished formats such as capsules, sachets, or tablets. Quality control testing is conducted on in-process samples and the final product to ensure it meets high-quality standards and is free from contamination.

Strain Production

In the course of development work, special attention is devoted to comprehending the production conditions related to the large-scale manufacturing of probiotics and assessing the performance of strains under conditions similar to those in a laboratory setting. Each step in the process is interdependent, and it is crucial to identify any sensitivities specific to the strains and to ensure the overall well-being of the cells throughout the process.

Scaling up can present considerable challenges because the processes for producing cells at a smaller, lab-scale level are inherently more tightly controlled and have shorter hold times at each step. For example, the commercial separation of cells from spent media through centrifugation may take hours due to the larger cell volume, in contrast to the lab scale with smaller volumes where this can be done in minutes. This leads to different stresses, such as heat and shear stress, compared to a lab-scale centrifuge (Crittenden, 2009). Additionally, several steps involving the pumping of cells are introduced during commercial-scale production, which typically do not occur during bench-scale development. Furthermore, cells in commercial production are exposed to varying pH and temperature conditions that are challenging to replicate precisely at the lab scale. Therefore, it's crucial to scale up to an intermediate volume in a pilot stage to evaluate and address these production conditions and stresses before progressing to full commercial production. Scaling up from the pilot stage to commercial scale may encounter similar challenges as scaling up from the lab scale.

As previously mentioned, hold times at different stages in the production process can significantly surpass those at the lab scale. To ensure the cells' robustness and their ability to meet shelf-life requirements, cells produced in pilot- and commercial-scale production are assessed for several hours beyond the typical hold times encountered during the various process steps. If adequate robustness is not demonstrated, and it's not easy to make adjustments to mitigate the sensitivity, the strain will return to the laboratory for further development before scaling up again.

Generally, if a strain is found to be particularly sensitive and challenging to develop at the lab scale due to strain-specific sensitivities, it is highly likely that additional challenges will be encountered during scale-up to the pilot stage and subsequent scale-up to commercial production. The same holds true for the robustness of the cells with respect to the hold times in the production process. Often, there are multiple iterations where the strain is returned to the laboratory for additional development work to address these identified sensitivities and robustness issues before scaling up again.

Similar to fermentation, freeze-drying must undergo evaluation at the bench-scale level before proceeding to commercial production. An optimal freeze-drying cycle is established through an iterative process involving adjustments to pressure, heating plate temperatures, and frozen pellet bed thickness until suitable water activity (Aw), cell count, and shelf-life stability are achieved **(Broeckx et al., 2016).** In addition, the cryoprotectant formula or dosage may be modified if cell survival is not satisfactory after this iterative process (**Viernstein, Raffalt and Polheim, 2005).** It's critical to assess whether the lab freeze-drier operation can be successfully scaled up to an industrial freeze drier. In particular, the capacity of the condenser, condensing rate, and heat transfer capabilities of the industrial freeze drier must be known and sufficient to remove the moisture released during the drying cycle.

Nutritional Requirements for the Strains

LAB and Bifidobacteria are demanding microorganisms coming to their nutritional requirements for growth and optimal performance. These microorganisms often lack the ability to produce some of the 20 essential amino acids on their own, necessitating the fulfilment of their nutritional needs from the external environment in order to thrive. The intricacies of these dependencies and nutrient requirements are typically influenced by the nutritional richness of the environment to which the microorganism is naturally adapted and sourced **(Hebert, Raya and de Giori, 2004).** For instance, Lactobacillus plantarum obtained from plant material has fewer nutritional dependencies and a greater ability to produce its own nutrients compared to Lactobacillus johnsonii, which is typically isolated from the human upper gastrointestinal tract, an environment rich in readily available nutrients like free amino acids, short peptides, and oligosaccharides **(Elli et al., 1999; Saguir and de Nadra, 2007).**

Understanding the specific nutritional demands of these microorganisms and developing a customized fermentation medium that supports their growth while enhancing their resilience to the stresses encountered during the manufacturing process is essential for achieving a high-performance end product. Identifying nutritional requirements that vary among strains necessitates a multidisciplinary approach that combines both knowledge-based and empirical methods. Employing techniques like genomics, transcriptomics, proteomics, and metabolomics to assess the genome, gene expression, protein expression, and metabolic processes of strains provides valuable insights into their nutritional needs and capabilities. This approach contributes to the development process and the overall performance of the final product **(Sieuwerts et al., 2010; Smokvina et al., 2013; Siragusa et al., 2014).**

Additionally, analysing the sterilized medium before inoculation and after fermentation (i.e., spent medium) yields practical data regarding nutritional requirements and limitations,

complementing and validating the information obtained through knowledge-based -omics techniques. Understanding the composition of complex raw ingredients, yeast extracts, yeast peptones, milk, and other nitrogen sources helps align critical components of fermentation media with the nutritional needs of the strain under development. This enables adjustments to the medium and the production process to achieve enhanced strain performance and more efficient management of manufacturing costs.

Furthermore, there is a wealth of empirical data that can be collected through these approaches, data that is not easily obtained or predicted using more knowledge-based -omics methods. With the right expertise and an innovative approach, these combined techniques are highly effective in comprehending strain-specific dependencies, sensitivities, nutritional needs, and limitations. This understanding is vital for successfully developing and manufacturing high-performance strains that meet the requirements of customers.

Production of Raw Materials

Considering the significance of the fermentation medium in the production of LAB and Bifidobacteria, alterations in the raw materials can significantly impact their growth and performance. These modifications to raw materials can arise from the supplier's initiatives, driven by factors such as cost reduction through process enhancements, changes in ingredient sourcing, or variations in the manufacturing process. Particularly, for complex ingredients like protein sources (such as yeast extract and milk), variations in composition are more evident compared to less complex ingredients like simple carbohydrates and salts.

The extent of variation in complex raw ingredients, which can include differences in amino acids, peptide size distribution, vitamins, nucleotides, salts, and carbohydrates, may not always be readily linked to the changes in strain performance. In some cases, these differences can be attributed to the presence or absence of other components that are less apparent or unidentified. For example, beet and cane molasses, used in the cultivation of baker's yeast for yeast extracts and peptones in food applications and fermentations, can introduce elements from the yeast cultivation process into the final products **(Tanguler and Erten, 2008).** These elements may either have no discernible impact on strain performance or can influence the performance of probiotic strains in a strain-specific manner. Furthermore, the source of cane and beet molasses can vary globally, affecting the performance and quality of yeast extracts and peptones, with potential consequences extending into fermentations utilizing these materials **(Nagodawithana and Wasileski, 1998).**

Production Consistency and Quality Control

Achieving consistent high performance in the manufacturing of LAB and Bifidobacteria hinges on the level of control maintained over the manufacturing process. It's not surprising that there exists substantial variability among strains, even those of the same species, in terms of their sensitivity and response to the manufacturing process, which ultimately influences their performance **(Barrangou et al., 2009; Milani et al., 2013).** These sensitivities are identified and addressed during the stages of lab-scale development, pilot-scale scale-up, and subsequent commercial-scale scale-up, ensuring that a state of consistent high performance is attained for each strain.

Once the process for each strain is established, it is imperative to maintain a consistent approach each time the process is executed. Controlling the manufacturing process encompasses several key aspects:

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1. Evaluation of raw material suppliers and assessment of raw materials to ensure high quality.
2. Establishment of meaningful and attainable ranges for process parameters and validation of the process's capability to consistently operate within those defined ranges.
3. Automation of the process to the extent possible to reduce inconsistencies associated with human error and manual control.
4. Ensuring that operators are well-trained and minimizing employee turnover.
5. Assessment of captured data from the process, utilizing Six Sigma approaches for continuous improvement, and ensuring the process is consistently replicated (Westgard and Westgard, 2017).
6. Ongoing evaluation of in-process samples and the final product to verify that the product meets high-performance standards and is free of contaminants.

In the manufacturing environment, the reality is that there will be variations in raw materials from one lot to another that may not become apparent until they are utilized in the manufacturing process. The production plant environment is dynamic, with new equipment installations and the implementation of new processes that can disrupt the plant's steady-state operation temporarily. Additionally, some aspects of the manufacturing process may involve more manual control and less automation. Shift changes for operators and employee turnover are common, and equipment can unexpectedly malfunction. Probes and sensors used to monitor various steps in the manufacturing process may experience malfunctions.

These factors present challenges that can affect the performance of the strains being manufactured, as process conditions and hold times may differ and potentially fall outside the range explored during strain development and scale-up work. Manufacturing experience has demonstrated that even seemingly minor and seemingly unimportant changes or differences in the process can have a significant impact on performance, which may vary but is often negative for performance. Even strains that are thought to be well-understood and reliably manufactured can exhibit unexpectedly poor performance if there are changes or differences in the process that deviate from what was established during development and scale-up. In some cases, attempts to troubleshoot and rectify process issues are complicated because the variations between production runs are multifaceted and not easily identifiable. This suggests that certain aspects of the process are not being adequately monitored and controlled to ensure consistently high strain performance.

The importance of process control cannot be emphasized enough, especially in the context of consistently manufacturing high-performance LAB and Bifidobacteria. This is particularly crucial given the strain-specific nutritional requirements, process sensitivities, and subsequent cell responses to the process steps that impact performance.

Quality Control and Assurance Evolution

Quality control (QC) and quality assurance (QA) share the common objective of ensuring the production of high-quality products for sale, but they diverge in their methodologies. Quality assurance primarily focuses on upholding quality systems within the facility to minimize product defects and errors. In contrast, quality control takes on the responsibility of conducting actual testing on raw materials, in-process samples, intermediate samples, and end-product samples, encompassing a wide range of examinations. While the core responsibility of QC remains the testing of end-products, what has evolved and expanded are the support programs associated with QC.

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In addition to GLP, metrology plays an equally crucial role in establishing a robust program for the qualification and calibration of laboratory equipment. Robust programs are essential to ensure that equipment, such as autoclaves, incubators, clean room environments, pipettes, etc., undergo continuous monitoring and maintenance, carried out by qualified external vendors and lab technicians. Metrology records are created, maintained, and retained to document these activities. The monitoring of air handling and water quality is also emphasized to prevent contamination downstream.

Documentation must encompass all observations, test results/raw data, and instances of deviations. Each production batch should be associated with a batch record, which includes all production and quality control documents. Quality assurance then reviews this file to ensure the presence of all relevant paperwork and compliance with regulatory standards. The retention period for batch records, metrology records, and other company paperwork, as well as the appropriate disposal procedures, depends on the regulatory guidance followed and company policies.

To avert quality issues stemming from subpar raw materials and packaging, vendors and their raw materials should undergo a qualification process in advance. This means that the following information should be verified and approved by the quality control (QC) team before use:

1. GMO status
2. Allergen status
3. Raw material purchasing specifications, covering chemical, physical, and microbiological aspects
4. Food-grade quality
5. Pesticide content
6. Irradiation status
7. Kosher rating
8. Raw material packaging type and size
9. Storage conditions and Shelf life
11. Review of the safety data sheet (SDS)
12. Review of a certificate of analysis (COA) from the vendor

It is also important to determine the type of inspection needed when an approved raw material arrives at the plant. This can involve checking its appearance, identification, chemical and physical properties, microbiological aspects, and reviewing the vendor's COA. Moreover, it is crucial to establish the frequency of QC checks, such as whether QC of raw and packaging materials should occur with every new batch or if a skip program can be established.

The competency of laboratory technicians should be monitored through a program that assesses the accuracy of quality tests against known controls. Maintaining the skills of laboratory technicians and retaining skilled personnel can help reduce discrepancies in results.

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A sampling plan must be developed to cover raw and packaging materials, in-process and intermediate stages, and end-products. Factors to consider when creating a representative sampling plan include determining the number of samples needed to accurately represent the batch. Statistical programs have been developed to ensure that the sample(s) provide confidence in the acceptability of the batch while avoiding excessive testing, which saves both technician time and costs. Additionally, if more than one sample is taken or if production runs include multiple sublots within a batch, it should be decided whether they need to be tested separately or if samples can be consolidated. The sample size should also be carefully considered.

Retained samples from each production lot should be stored at the recommended temperature specified for customers. The sample size should encompass material taken throughout the production run and allow for full QC testing.

With the established support systems, it's crucial to address questions related to the required end-product testing. The end-products consist of bacteria, but these bacteria do not undergo any further fermentation in the customer's end-product. Instead, customers directly consume the bacteria.

Several considerations need to be taken into account:

1. How will the bacteria be utilized in the customer's end-product?
2. What is the surrounding environment for the bacteria intended for consumers? For instance, will the bacteria be in a freeze-dried form, packaged in capsules, sachets, straws, or other forms, or will they remain in a frozen wet pellet form that can be added to liquids like juice?
3. What are the acidic or basic conditions of the customer's end-product, and how will these conditions affect the stability of the bacteria?
4. What type of consumer is the customer targeting? Infants, senior citizens, immunocompromised individuals, or a specific gender?

Examples of end-product testing are provided in Table 4. Future concerns revolve around efficiency and automation. There is a growing need for future methods that can be validated and accepted by customers. The QC lab, in collaboration with the RandD department, continually seeks ways to reduce release time and prevent the need for retesting. Additionally, customers are increasingly seeking more accurate identification, particularly when dealing with bacterial blends.

Table 4 Few end-product testing ideas. **(Source: Fenster et al., 2019)**

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S. No.	End-product Testing
1	Physical examination
2	Functionality refers to the requirement to demonstrate that the product will perform as expected by the customer. In the case of probiotic bacteria, this is achieved by establishing a label claim and consistently meeting that claim for every batch manufactured.
3	Absence of pathogens
4	Cross-contamination and hygiene concerns are on the rise, with an increasing number of customers seeking to minimize issues such as the presence of Yeast and Mold.
5	The process of identification has evolved from using microscopy and observable traits that provided a general distinction among probiotics to employing more precise genotypic assays through PCR technologies. In recent years, methods such as Riboprinting and 16S sequencing have become industry standards for identifying probiotics. These genetic-based techniques are capable of distinguishing probiotics at the species level and, in some instances, even beyond that level. Bacterial species often consist of multiple strains, and probiotics are typically marketed based on their specific strains. One way to define a strain is by identifying a difference of at least one base pair in a bacterium's genome. Strain-specific PCR assays can be developed to target unique genome sequences that distinguish the probiotic at the strain level. The significance of these assays has become increasingly evident, not only as new research highlights the impact of even a single base pair change but also as the probiotic industry continues to evolve.

Commercialization of these Probiotic Strains

After human intervention studies confirm that microorganisms meet the criteria for probiotics, the subsequent phase involves assessing if these strains can be scaled up for industrial production and effectively integrated into consumer products. Ideally, this stage of probiotic commercialization should run concurrently with clinical trials to prevent the investigation of a strain that cannot be commercialized.

Culturing microorganisms at an industrial scale and processing them in an industrial setting impose different demands on strains compared to laboratory-scale cultivation. Additionally, the medium requirements differ due to cost and other considerations. To maintain the consistent high quality of these strains, it is imperative to establish a robust quality control program that ensures uniform quality, from the ingredients used to the final product. A quality assurance program must also be in place to oversee dependable production processes, necessitating thorough documentation of procedures and outcomes.

Once high-quality probiotic bulk quantities have been manufactured, the strains must be incorporated into consumer products. These products have diverse requirements, encompassing shelf life, storage conditions, and product composition. Regardless, it is essential to deliver a minimal effective dose to consumers by the end of the product's shelf l

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Shelf-life of Packaged Probiotics

Probiotic bacteria, which are commonly used in animal nutrition and consumed by humans, are typically found in the form of dried biomass. Most of these probiotic bacteria belong to the group of lactic acid bacteria and have Generally Recognized as Safe (GRAS) status. Probiotic products are available in different formats, including capsules, suspensions, powders, and are incorporated into probiotic foods. However, all these forms face the common challenge of a loss of viability during processing and storage. For instance, fluid suspensions, while relatively easy to produce, are the least stable among probiotic forms and have a shorter shelf-life **(Santivarangkna, Kulozik and Foerst, 2007).** On the other hand, probiotics in solid forms like capsules and powders are more stable and can be stored for a longer duration. Their viability and shelf-life can be enhanced by adding protective substances or subjecting probiotic bacteria to stress factors before drying.

All probiotics contain live bacteria, mainly from the Lactobacillus and Bifidobacterium genera **(Niamah et al., 2021).** Clinical trials have shown the positive impact of probiotics on gastrointestinal diseases such as irritable bowel syndrome, diarrhoea, enteritis, and allergic conditions like atopic dermatitis. Probiotics are also known to boost the body's immune resistance through immunomodulation **(Gill and Prasad, 2008)**.

According to the World Health Organization (WHO) guidelines, for probiotic preparations to have a beneficial health effect, they should contain a minimum number of live bacteria (colony-forming units), typically at least 106 cfu/g. The quality of dried cells, including the number of live cells and their biological activity, often depends on the drying method used. These methods encompass spray drying, freeze drying, vacuum drying, and fluid bed drying. The resulting probiotic formulations can be applied in various innovative products such as nasal sprays, creams, and lotions **(Blanchet-Rethore et al., 2017; Jokicevic et al., 2021).** They are also used in food products to enhance their health benefits, and certain food items like ice cream are used to enhance the stability and shelf-life of probiotics **(dos Santos et al., 2017).**

Drying Methods

Cryopreservation is a widely employed method for long-term storage of live microorganism cultures, commonly used in microbiological laboratories. However, when viewed from a commercial perspective, this method has drawbacks, including its high energy consumption and the requirement to maintain and transport samples at sub-zero temperatures. Moreover, the freezing and thawing process can potentially harm the microorganisms. When producing significant quantities of probiotic cultures, it is preferable to employ alternative preservation methods, such as various drying techniques **(Broeckx et al., 2016).** The fundamental principles of these drying techniques are outlined in Figure 1. The tables display examples of the viability of specific probiotic strains after undergoing different drying methods (Table 5) and the protective effects of various materials used in the process (Table 6).

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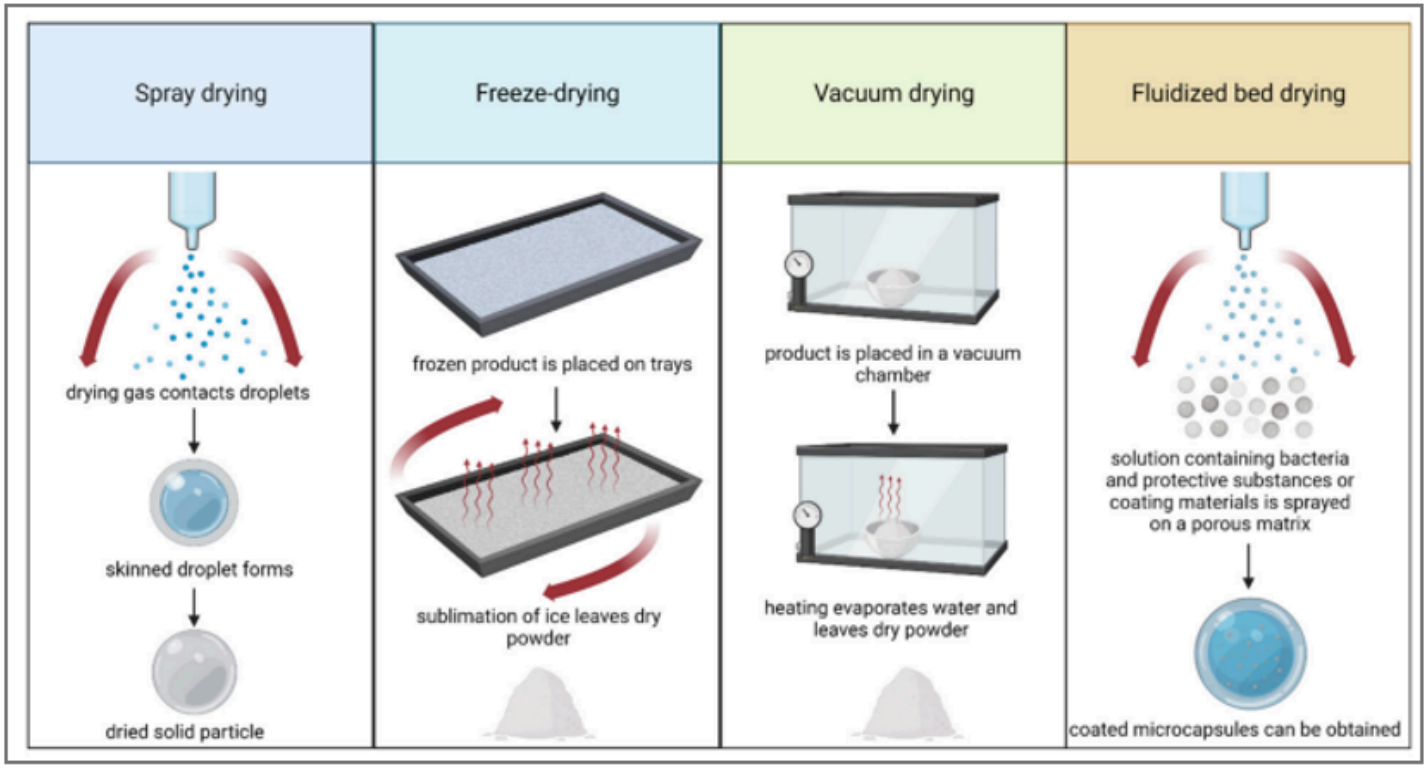

Fig. 1 Commonly applied drying techniques and their basic principles- A summary. (Source: Kieps and Dembczynski, 2022)

Table 5 Comparison of different drying methods and cell concentrations. (Source: Kieps and Dembczynski, 2022)

Reduction (log cfu/g)	Microorganism	Growth Parameters	Cell Concentration before Drying (log cfu/g)	Cell Concentration after Drying (log cfu/g)	Drying Method	Reference
<1	*Lactiplantibacillus plantarum 299v*	MRS Broth, 37°C	10.3	11.3	Freeze Drying	(Barbosa et al., 2015)
	Pediococcus acidilactici HA-6111-2	MRS Broth, 37°C	10.5	11.2	Freeze Drying	
	Pediococcus acidilactici HA-6111-2	MRS Broth, 37°C	9.0	9.4	Spray Drying	
	Lactobacillus kefir CIDCA 8348	MRS Broth, 30°C	8.8	8.2	Spray Drying	(Golowczyc et al., 2011)
	Lactobacillus plantarum CIDCA 83114	MRS Broth, 30°C	9.9	9.8	Spray Drying	
	Lactobacillus kefir CIDCA8321	MRS Broth, 30°C	8.4	8.1	Spray Drying	
	Lactobacillus rhamnosus LGG	MRS Broth, 37°C	11.0	10.2	Spray Drying	(Jokicevic et al., 2020)
>1	*Lactobacillus acidophilus NCDC016*	MRS Broth, 37°C	11.2	10.0	Spray Drying	(Arepally and Goswami, 2019)
	Escherichia Coli K12	TSB, 30 °C	10.7-10.9	7.9	Spray Drying	(Pispan, Hewitt and Stapley, 2013)
	Lactobacillus reuteri DSM 20016	MRS Broth, 37°C	8.7-9.7	7.7	Fluidized bed Drying	(Schell and Beermann, 2014)

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Table 6 Different materials and their Protective effects. (Source: Kieps and Dembczynski, 2022)

Reduction Post-drying (log cfu/g)	Microorganism	Drying Method	Protective Substances	Cell Concentrations before Drying (log cfu/g)	Cell Concentrations after Drying (log cfu/g)	Survivability (%)	Reference
<1	*Bifidobacterium bifidum*	Spray drying (double layered)	Gum arabic 9%, 1% β-cyclodextrin, 1% lecithin	6.93	6.18	89.22	(Arslan-Tontul and Erbas, 2017)
	Bifidobacterium bifidum	Spray drying (double layered)	Hydrogenated palm oil, 2% Tween 80	6.12	6.01	98.25	
	Saccharomyces cerevisiae var.	Spray Drying	Gelatin 10%	9.95	9.06	91.55	(Arslan et al., 2015)
	Saccharomyces cerevisiae var. boulardii	Spray Drying	Whey protein Concentrate 20%	9.65	8.86	91.81	
	Lactobacillus rhamnosus	Spray Drying	Native rice starch 10%	9.26	8.98	53.24	(Avila-Reyes et al., 2014)
	Lactobacillus rhamnosus	Spray Drying	Inulin 15%	9.18	8.91	53.55	
	Lactobacillus brevis WK12	Freeze Drying	Soy powder solution 10%	11.30	11.26	90.00	(Gwak et al., 2015)
	Lactococcus lactis WK11	Freeze Drying	Soy powder solution 10%	11.30	11.27	94.00	
	Bifidobacterium bifidum	Spray Drying	Gum arabic 9%, 1% β-cyclodextrin	10.12	7.57	74.81	(Arslan-Tontul and Erbas, 2017)
	Bifidobacterium bifidum	Spray Chilling	Hydrogenated palm oil, 2% Tween 80	9.51	8.25	86.79	
>1	*Saccharomyces cerevisiae var. boulardii*	Spray Drying	Modified starch 20%	9.65	8.64	89.53	(Arslan et al., 2015)
	Saccharomyces cerevisiae var. boulardii	Spray Drying	Maltodextrin 20%	9.65	8.61	89.24	
	Saccharomyces cerevisiae var. boulardii	Spray Drying	Pea protein isolate 10%	9.95	8.55	86.52	
	Saccharomyces cerevisiae var. boulardii	Spray Drying	Gum Arabic 20%	9.65	8.17	84.69	

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Factors affecting Viability of Probiotics

During the drying process, probiotic microorganisms face a range of stress factors, including excessive dehydration, thermal stress, mechanical stress, osmotic stress, and oxidative stress **(Santivarangkna, Kulozik and Foerst, 2008).** Probiotic microorganisms are categorized as having low thermal stability, but there exists a critical water content level that must be maintained. Dropping below this critical point can result in cellular dehydration and subsequent inactivation. Thermal stress and dehydration are recognized as the primary contributors to the loss of probiotic bacteria viability during spray drying. Stress factors impacting probiotic bacteria throughout various processing stages are illustrated in Figure 2.

Fig. 2 Different stages of preparation and administration affected by stress factors (Source: Kieps and Dembczynski, 2022)

Thermal stress, specifically the heat-induced inactivation of microorganisms, poses a significant risk during the latter phase of drying, as reported by multiple sources. In this stage, microorganisms can be exposed to the drying air's temperature, especially when dried particles often linger in the dryer until the entire process is finished. It's worth noting that not all bacteria are equally susceptible to thermal inactivation. For example, L. acidophilus has demonstrated superior survival rates under varying drying temperatures compared to E. coli K12. This discrepancy can be attributed to variations in cell wall thickness, with Gram-positive bacteria, such as L. acidophilus, having thicker cell walls. Furthermore, drying in a medium containing nutrient broth has yielded better survival rates than drying in a medium lacking broth component **(Pispa, Hewitt and Stapley, 2013).**

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Elevated temperatures can denature intracellular proteins and destabilize cell membranes, leading to cell death. However, higher temperatures simultaneously reduce the water activity of dried samples, resulting in enhanced storage stability. Consequently, when determining spray drying parameters for probiotic microorganisms, it is crucial to identify an optimal outlet air temperature. This temperature should be sufficiently high to yield low water activity in dried samples yet low enough to prevent cell damage **(Fu and Chen, 2011)**. Air temperature also significantly impacts the bulk density of dried probiotic powders. Higher temperatures increase evaporation rates, causing the powder to develop a more porous structure and become more susceptible to forming hollow particles **(Arepally and Goswami, 2019).**

Dehydration-induced inactivation of microorganisms often coincides with heat damage. During the drying process, water molecules are extracted from the cells, limiting chemical reactions and metabolic activity. As water is vital for stabilizing various cell components, its removal may lead to a loss of cell integrity, structural alterations, and damage to the enzyme system **(Broeckx et al., 2016).** This includes changes in the cell membrane's lipid bilayer, which can cause intracellular fluid leakage and, consequently, cell death **(Perdana et al., 2014).** Experiments with L. plantarum suspensions revealed that at an outlet air temperature below 45 ˚C, dehydration-induced inactivation was dominant, while above this temperature, both dehydration and temperature stress occurred simultaneously **(Perdana et al., 2013).** Additionally, the longer the drying time, the more cells underwent dehydrative inactivation.

Osmotic stress during drying arises from cells losing water to the environment, resulting in increased intracellular solution molarity and reduced cytoplasm volume. This leads to a loss of cellular turgor, plasmolysis, and, consequently, a decrease in viability **(Broeckx et al., 2016).**

Oxidative stress is induced by the presence of oxygen in the air and its dissolution in an aqueous suspension of microorganisms **(Ghandi et al., 2012).** The tolerance of probiotic bacteria to oxygen varies; for instance, many strains of lactic acid bacteria can tolerate oxygen, while most Bifidobacterium species require strict anaerobic conditions. Oxidative stress occurs when reactive oxygen particles interact with proteins, nucleic acids, and lipids, leading to protein denaturation, lipid oxidation, cell membrane damage, and cell death **(Broeckx et al., 2016).**

Shear forces during the spraying of the microorganism suspension into the dryer head can also deactivate probiotic microorganisms. Several studies have shown a correlation between the suspension pressure administered through atomizing nozzles and the survival of probiotic bacteria **(Ghandi et ai., 2012).**

In fluidized bed drying, like other drying methods, several factors can contribute to the loss of cell viability, mainly osmotic stress, excessive dehydration, and oxidative stress **(Fu and Chen, 2011).** The threat of thermal shock during fluidized bed drying is generally insignificant up to a material moisture level of 15% but increases as the water activity of the dried material decreases **(Broeckx et al., 2016).** Additionally, the pressure in the atomizing nozzle can influence cell viability. An increase in nozzle pressure above 1.5 bar has been observed to reduce the viability of Enterococcus faecium cells **(Stummer et al., 2012).**

Freeze-drying exposes microorganisms to various stress factors related to freezing and ice sublimation, resulting in cell changes such as deformation, mechanical damage caused by ice crystal formation, the loss of semipermeable properties of cell membranes, alterations in membrane lipids, and protein denaturation due to increased intracellular compound

concentration **(Broeckx et al., 2016).** Rapid freezing is recommended as it results in smaller ice crystals that do not damage microbial cells.

In vacuum drying, which occurs at a temperature between freeze drying and spray drying, cell damage is milder in terms of the effects of high or low temperature. Additionally, the absence of oxygen in the drying environment can reduce oxidative stress, making it suitable for drying oxygen-sensitive bacteria like Bifidobacteria **(Santivarangkna, Kulozik and Foerst, 2008).** However, dehydration stress remains a significant threat to cell viability during this process, primarily impacting the cell membrane **(Bauer, Kulozik and Foerst, 2013).**

These stress factors can be managed during the culturing stage to prevent the loss of viability during drying and storage. Research has shown that fermentation parameters, such as pH and temperature, can influence the stress resilience of specific strains during freeze-drying **(Hernandez et al., 2019).** Simultaneous exposure to mild heat (50 ˚C) and osmotic stress (0.6 M NaCl) has also been shown to enhance the storage stability of certain bacterial strains when compared to exposure to just one of these stress factors **(Nag and Das, 2013).**

Several factors can impact the viability and longevity of probiotic bacteria during storage. To extend the shelf life of dried probiotics, various protective strategies can be employed. These measures encompass the incorporation of antioxidants, such as 0.5% (w/w) vitamin E, which serves to shield the final formulation from oxidative stress **(Nag and Das, 2013)**. Moreover, storing probiotics at a lower temperature, specifically 4˚C, has shown to enhance the survival rate of dried probiotic powder compared to storage at higher temperatures like 22˚C or 35˚C **(Strasser et al., 2009).** You can find examples of shelf-life assessments and viability evaluations during storage in Table 7.

Table 7 Shelf-life of Probiotics. (Source: Kieps and Dembczynski, 2022)

Microorganism	Preparation Method and Matrix	Storage Conditions	Initial Cell Concentrations (log cfu/g)	Cell Concentrations after Storage (log cfu/g)	Monitored Parameters	Reference
Bacillus Coagulans	Fluid-bed-dried apple snacks	90 days at 25°C	7.89	6.78	viable cell counts, water activity and moisture content, enzyme activity, total phenolic content, antioxidant capacity, vitamin E concentration.	(Galvao, Rodrigues and Fernandes, 2020)
Lactiplantibacillus plantarum 299v	Spray drying in orange juice	12 months, 25 °C, aw = 0.03 hermetic glass flasks with silica gel	7.90	6.30	viable cell counts, water activity.	(Barbosa, Borges and Teixeira, 2016)
Pediococcus acidilactici HA-6111-2	Spray drying in orange juice	12 months, 25 °C, aw = 0.03 hermetic glass flasks with silica gel	8.70	8.00	viable cell counts, water activity.	
Lactiplantibacillus plantarum Lp 115-400b	coconut water oatmeal with inulin (1 g/100 mL)	4°C, 49 days	7.06 (9.12 at day 7)	7.23	viable cell counts, pH, lactic acid content, rheological parameters.	(Dharmasena et al., 2015)
Lactiplantibacillus plantarum Lp 115-400b	Coconut water oatmeal	4°C, 49 days	6.99 (9.01 at day 7)	6.41	viable cell counts, pH, lactic acid content, rheological parameters.	

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5. Challenges in ascertaining the taxonomic identity and bioactivity profile of probiotic strains

The basic requirements or challenges which has been encountered by the probiotic strains are discussed in Figure 3.

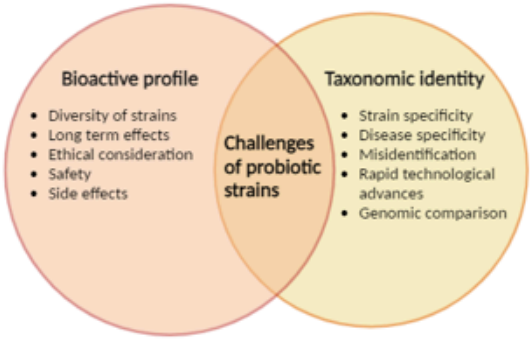

Fig. 3 Challenges of Probiotic strains

Bioactivity profile of probiotics

Firstly, for the probiotics strain selection to be contemporary, their ability to withstand different stresses, especially those encountered during industrial manufacturing processes or gastrointestinal transit, has been a crucial criterion. However, the tolerance to stress does not necessarily indicate functionality. It might be due to the lack of precise conceptualization **(Vinderola et al., 2017).**

Secondly, after ensuring safety, the selection of the probiotic strains have been included based on their resistance to low pH and bile salts to predict gastric resistance and their adherence to the mucus or cell lines as indications of "temporary gut colonization". Notably, certain strains that demonstrate health benefits in well-conducted clinical trials may not perform well in in-vitro stress tolerance assay **(Vinderola et al., 2017).** Taking this into consideration, the attributes of complexity of probiotic strain selection should be studied for its optimal effectiveness of bioactivities.

Diversity of strains

Based on a study, there has been an update in the criteria essential for the safety of probiotics based on the components of the intestinal barrier, risk of adhesion and translocation, and metabolic and other remote effects like gene toxicity and platelet aggregation. It is also problematic that mechanical understanding of probiotic activity incorrect identification of species and misunderstanding of labels **(Salvetti and O'Toole, 2017).** This is subject to debate, and there are no formal guidelines for the safety of assessment of probiotic bacteria **(Salvetti and O'Toole, 2017).**

Probiotic bacteria like Lactobacillus which is mostly found in nature have more than 200 recognized species and subspecies.However, the Bifidobacterium distribution is limited to the mammalian gastrointestinal tract. So, adaption to specific environments like the human intestine is limited because comparative genomics has shown a tendency to reduce genome size by adaptation **(Sun et al., 2015; Vinderola et al., 2017).**

Long term effects

Currently, the probiotics development is full of uncertainty because of their poor methodologic quality, the effects of some probiotics are uneven and vary between every individual. It is unpractical to accept that one-size-fits-all probiotics **(Guo et al., 2020).** For instance, in Clostridium species, there are a few challenges when it comes to their efficiencies in medical interference and animal husbandry. It includes

1. The strong adhesion to the intestinal surface which is essential in maintaining permanent and consistent benefits, so it is better to choose strains with high adhesion abilities **(Paredes-Sabja et al., 2014).**
2. It is urgent to identify the underlying mechanisms of Clostridium spp. sporulation and germination in more depth as it talks about their stress resistance **(Paredes-Sabja et al., 2014).**
3. Combining Clostridium and Bifidobacterium spp. for treating the diseases has positive results but more trials are required to confirm its efficacy **(Ibarra et al., 2018).**
4. The applicability of the species depends on individual differences like food habits, age, physiological state, and previous microbial community **(Martz et al., 2015).**
5. Each strain is different and to consider for further use as probiotics is hopeful but difficult **(Guo et al., 2020).**

Ethical consideration

It is crucial to emphasize the health benefits of probiotics as they are specific to individual strains **(Zavišić et al., 2023).** According to their use and effect, probiotic products are regulated by the Food and Drug Administration as nutritional supplements, food ingredients, or medicines. If the probiotics are mentioned under the category of curing, treating, or preventing human diseases then it must be classified as a drug and must meet a strict requirement. Although the requirements vary from country to country, the minimum criteria should be obtained with drug regulatory standards **(Cordaillat-Simmons et al., 2020).**

Based on the regulatory standards, probiotics are to be mentioned as a health claim with the following criteria defined probiotic strains, proper delivery of viable strains, and effective dose at the end of the product's shelf life **(Zavišić et al., 2023).** Due to European Health Restrictions Regulations (Regulation (EC) N ◦ 1924/2006, the use of the word 'probiotic' for advertising and food supplement packaging is not permitted, if there is no relevant scientific evidence then it is classified as unauthorized health reference **(Zavišić et al., 2023).**

Some of the probiotics that have been registered as medicine can also be found in the market leaving regulatory confusion, as probiotics are presented as drugs, nutritional supplements, and food for special medical purposes, functional foods, and food ingredients with different categorical designations, and advertisements **(Zavišić et al., 2023).** Mostly the data on individual strains are obtained by reviewing published documents, and based on this evidence the companies merely mention the species name and not the specific strain. This is the fundamental information for antimicrobial resistance (AMR) concern **(Zavišić et al., 2023).**

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Safety

For safety reasons, the FAO/WHO working group recommends certain criteria for assessing the probiotic strains. It includes checking on the toxic production capacity, antibiotic resistance, and haemolytic potential, determining its metabolic activity, and considering its effect on humans (i.e.) evaluating the side effects and the post-market surveillance aspects **(Sharifi-Rad et al., 2020).** For example: In particular, certain types of Clostridium species exert toxin plasmids with the commensal bacteria in the gut. So considering safety, this species should be detected strictly to avoid vertical and horizontal transmission of virulence factors **(Guo et al., 2020).**

Side effects

Probiotics may be theoretically responsible for four types of side effects **(Guo et al., 2020**), and it includes

1. Systemic infections
2. Deleterious metabolic activities
3. Excessive immune stimulation in susceptible individuals
4. Gene transfer

5.6.1Systemic infections

Some reports communicate the infection is caused by microorganisms that are considered to be probiotics including fungi, bacteria, sepsis, and endocarditis, and the cases are explained in Table 8.

Table 8 Systemic infections caused by probiotics. (Source: Doron and Snydman, 2015).

Microorganisms	Reports	Risk factors	References
Fungemia – *S. cerevisiae* or *S. boulardii* in blood cultures of patients	33	-	(Doron and Snydman, 2015; Sharifi-Rad et al., 2020)
Bacteremia – *L. acidophilus, L.casei* and *L. rhamnosus*	8	Immune-compromised state, weakened function of the intestinal barrier, and the use of central vein catheter. These are the risk factors involving mainly *Lactobacillus* species.	
Sepsis - *S. boulardii, L. rhamnosus, Bacillus subtilis, Bifidobacterium breve* or probiotic combinations	9		
Endocarditis – *Lactobacillus* and *Streptococcus*	2	-	

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Deleterious Metabolic activities

D-lactic acidosis is mainly associated with Lactobacillus and is found in patients with short bowel syndrome. It leads to diarrhea and gastrointestinal lesions, because of de-conjugation and dehydration of bile salts **(Munakata et al., 2010).** For example: In patients with terminal ileostomy, if they consume fermented dairy products with L. acidophilus and Bifidobacterium it will lead to the conversion of primary bile salts to toxic-free secondary substances. These secondary bile salts derived from intestinal bacteria in the body's secretion are considered to be dangerous. Because they may have carcinogenic potential by interacting with mucus-secreting cells, promoting cell proliferation, or they can act as carcinogenic stimulants **(Sanders et al., 2018).** In terms of the metabolism of intestinal carcinogens and toxic substances, bacterial enzymes like reductases and hydrolases are related **(Sharifi-Rad et al., 2020).**

Excessive Immune stimulation in Susceptible individuals' probiotics

Probiotics have been shown to affect both the innate and adaptive immune systems, with effects on cytokine secretion and the function of dendritic cells. Even concerns have been expressed about the potential to overstimulate the immune reaction in some individuals, causing autoimmune or inflammation phenomena. These are just theoretical concerns but not reported on human subjects **(Doron and Snydman, 2015).**

Gene transfer

It is found that probiotic strains are resistant to some antibiotics and can maintain the virulence factors that specifically transfer the elements and cause resistance. It has been theoretically stated that when probiotics are used, the possibility of latera transfer of genes occurs and may spread to new and further virulent bacteria. In addition, lactic acid bacteria get attached to the plasmids and certain antibiotic resistance determinants placed on plasmids including L. lactis and Enterococcus species **(Cui et al., 2015).** So, to control and prevent any antibiotic resistance cases it is necessary to establish both the presence and transfer of bacterial resistance. Meanwhile, the plasmid genes are responsible for functions like the production of bacteriocins, carbohydrate metabolism, and resistance to antibiotics **(Sharifi-Rad et al., 2020).** This condition is mainly observed in Enterococcus faecalis, and it confirmed the ability to transfer the genes to other bacteria such as Enterococcus, Staphylococcus, Clostridium, Lactobacillus, and Bacillus species. Likewise, many antibiotic resistance-related genes are listed amongst Lactobacilli such as L. acidophilus, and L. delbrukii subsp. Bulgaricus, L. johnsonii, L. reuteri and L. plantarum**(Doron and Snydman, 2015).**

Taxonomic identity

Strain specificity

A few challenges have been found when identifying the types of probiotic strains, including a lack of global standards for the identification of strains and incomplete identification of probiotic strains **(McFarland et al., 2018).** Mostly the strains are designated based on manufacturing codes, depository numbers, or by the person who isolated the strain. Sometimes genomic phenotyping has moved the microbes from one group to another or reclassified them into separate groups **(Khatri et al., 2017).**

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Table 9 represents the different types of probiotic products and their taxonomy which has changed over the years. Even the brand names get changed depending on the country or the formulation **(McFarland et al., 2018).**

Table 9 Taxonomic changes in the strains over time. (Source: McFarland et al., 2018).

Brand name	Older designations	Current designations
Protectis	*L.reuteri* DSM55730 or *L.reuteri* SD2112 or *L.reuteri* ATCC 55730	*L.reuteri* DSM17938 or ATCC7938 (daughter strain)
Yakult	*L.casei* YIT9029	*L.casei* Shirota
Protecflor	*B. longum* RW001 and *L. rhamnosus* R11 and *L. acidphilus* R52 and *S. boulardii*	*B. longum* R175 (CNCM I-755) and *L. rhamnosus* R11 (CNCM I-1720) and *L. helveticus* R52 (CNCM I-1722) and *S. cerevisiae boulardii* (CNCM I-1079)

Disease specificity

It is important to note that the same strain of probiotic or mixture of strains will be effective for one disease and may not be effective for other types of diseases **(McFarland et al., 2018).** Even the use of probiotics is diverse ranging from prevention of disease to preventing the side effects of the treatment of certain diseases and to the treatment of chronic disease conditions **(McFarland et al., 2018).**

Misidentification

Despite their significance, exploiting Lactobacilli has been very challenging due to certain reasons like unusual phenotypic and genotypic diversity, ambiguous species identities, and unclear relatedness between them and the commercially important lactic acid bacteria **(Zheng et al., 2015).** In considering food microbiology and human nutrition Lactobacillus is one of the most studied species. Out of that only 7-8% of this particular species has been officially studied and have been accepted as probiotics with health claims **(Salvetti and O'Toole, 2017).**

The lack of mechanical understanding of probiotic activity and the incorrect identification and misleading labeling of probiotic species are major disadvantages for predicting the safety of probiotic intervention and creating a comprehensive list of the criteria to be assessed **(Salvetti and O'Toole, 2017).** The genomic diversity of Lactobacillus and its prophylactic structure strongly suggest that it is necessary to reevaluate its taxonomic structure and separate its feasibility into more uniform generations **(Sun et al., 2015).** Doing this way it helps to prevent misidentification which is considered to be a major cause of the misdescription of probiotic foods **(Salvetti and O'Toole, 2017).**

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Rapid technological advances

Even though misidentification of lactobacilli happens 16S rRNA gene sequence analysis has some part in recognizing the database but still shortcomings of this method include low taxonomic resolution particularly when trying to separate the closely related species (i.e Lb. plantarum/Lb. paraplantarum/Lb. pentosus or Lb casei/Lb. paracasei/Lb. rhamnosus **(Salvetti and O'Toole, 2017).** To overcome this discrimination, different markers such as pheS, rpo A, and recA have been used as an alternative but the problem exists as it remains crucial to ensure the readability of identification results **(Salvetti and O'Toole, 2017).**

Genomic comparison

When the genomes of about 175 species of Lactobacillus were sequenced recently, the analysis showed the average Nucleotide Identity (ANI) and the phylogenetics showed the genus Lactobacillus is paraphyletic. It means that mixed with the other five species of Lactobacillus such as Pediococcus, Weissella, Leuconostoc, Oenococcus, and Fructobacillus exhibited a larger genomic diversity **(Sun et al., 2015).** As a result, the recently defined Lactobacillus genes have problems with strain and species distinction in the short phylogenetic range **(Salvetti and O'Toole, 2017).**

Conclusion

New possibilities for probiotics:

Probiotics have found diverse and innovative applications. It is essential not to limit the development of probiotic products solely to dietary supplements and dairy-based probiotic foods, which have been the primary focus of numerous research studies on probiotics. Emerging opportunities lie in areas such as cosmetics, functional foods, non-dairy probiotic products, nutraceuticals, and medical applications **(Min et al., 2019; Jokicevic et al., 2020; Reque and Brandelli, 2021).**

Target demographics:

Prioritizing alignment with global trends when identifying probiotic target demographics is crucial. For instance, the rising concern over allergies demands research into measures to alleviate or eliminate their effects. Probiotics have demonstrated their beneficial impact on conditions like rhinitis, asthma, and atopic dermatitis **(Lopez-Santamarina et al., 2021)**. Another prevailing global trend, particularly prominent in developed nations, is the aging population. Probiotics can enhance the immune response, which holds significant importance for the elderly. Moreover, they might enhance the efficacy of vaccinations **(Akatsu, 2021).**

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