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Preserving probiotic potency: A comparative study of storage methods for *Lactobacillus fermentum* MT308789 And *Lactobacillus oris* MT308790

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Abstract

Probiotics, comprising living microorganisms like *Lactobacillus fermentum* MT308789 and *Lactobacillus oris* MT308790, offer significant health benefits when consumed regularly. However, the efficacy of probiotics heavily relies on their viability and stability during storage. Various storage methods, including agar slant, glycerol stocks, lyophilization, and dry form, are utilized to preserve probiotic viability. In this study, we evaluated the efficacy of these storage methods in maintaining the viability of probiotic strains. Our findings demonstrate that lyophilization emerged as the most effective method, yielding the highest viabilities for both strains [77.51 %]. Glycerol stocks also showed promise for short-to-medium-term storage, while agar slants and dry form storage exhibited suboptimal viability. These results underscore the importance of selecting appropriate storage methods to ensure the delivery of viable and effective probiotic formulations to consumers.

Keywords: Probiotics; Storage viability; *L. fermentum* MT308789; *L. oris* MT308790

1. Introduction

Probiotics are living microorganisms, primarily bacteria and yeast, that confer health benefits when consumed in adequate amounts. These beneficial microorganisms play a crucial role in maintaining the delicate balance of the gut microbiota, which is essential for digestion, nutrient absorption, and immune function [1, 2]. Probiotics have been associated with various health benefits, including alleviating gastrointestinal disorders, boosting immune function, and even promoting mental well-being [1, 3-5].

Given their potential health-promoting properties, probiotics have garnered significant interest in both scientific research and consumer markets. Incorporating probiotics into daily dietary regimens has become increasingly popular, with a wide range of probiotic products available, including yogurt, fermented foods, dietary supplements, and pharmaceutical formulations [6-10].

However, the efficacy of probiotics is contingent upon their viability and stability during storage. Proper storage methods are essential to maintain the viability of probiotic microorganisms and ensure their functionality when consumed. Various storage methods, such as agar slant, glycerol stocks, lyophilization [freeze-drying], and dry form, are employed to preserve the viability of probiotics for extended periods [11-14].

Each storage method has its advantages and limitations, and the choice of method depends on factors such as intended use, storage duration, and practical considerations. Understanding the impact of different storage methods on probiotic

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viability is crucial for the development of effective probiotic products that deliver optimal health benefits to consumers [11, 12, 15, 16].

In this study, we aim to evaluate the efficacy of various storage methods in preserving the viability of probiotic microorganisms. By comparing the viability of probiotics under different storage conditions, we can identify optimal preservation strategies that ensure the delivery of viable and functional probiotics to consumers. This research contributes to the advancement of probiotic science and the development of innovative probiotic products aimed at promoting human health and well-being.

2. Materials and Methods

2.1. Bacterial Isolation

Human breast milk samples were collected from lactating mothers who provided informed consent. The samples were collected aseptically using sterile containers and transported to the laboratory on ice for immediate processing [17]. Upon arrival, the breast milk samples were subjected to serial dilution in sterile phosphate-buffered saline [PBS] to obtain dilutions ranging from 10^-1 to 10^-6. Aliquots of the diluted samples were plated onto selective agar media, such as de Man, Rogosa, and Sharpe [MRS] agar supplemented with 0.05 % [w/v] L-cysteine hydrochloride and 0.1 % [w/v] sodium azide, to inhibit the growth of unwanted contaminants. Plates were then incubated at 37°C for 48-72 hours to allow for the growth of *Lactobacilli*. After incubation, morphologically distinct colonies showing prominent appearance, such as distinct shape, size, and color, were selected from the plates based on their characteristic morphology [18-20]. Selected colonies were streaked onto fresh agar plates to obtain pure cultures. Pure cultures were confirmed by Gram staining, catalase test, and additional biochemical tests, including fermentation of various sugars and production of specific enzymes [21-23].

2.2. Identification Using 16S rRNA

After obtaining pure cultures, bacterial identification was further confirmed using molecular techniques targeting the 16S ribosomal RNA [rRNA] gene. Genomic DNA was extracted from bacterial colonies using a commercial DNA extraction kit according to the manufacturer's instructions. The 16S rRNA gene was amplified by polymerase chain reaction [PCR] using universal primers targeting conserved regions of the gene. PCR products were purified to remove excess primers and nucleotides using a PCR purification kit. Sanger sequencing was performed on the purified PCR products using the same universal primers [23, 24]. The obtained sequences were analyzed using bioinformatics tools and compared with sequences available in public databases, such as the NCBI GenBank database, using BLAST [Basic Local Alignment Search Tool] analysis. Identification of bacterial species was based on the highest sequence similarity with known sequences in the database.

2.3. Storage Conditions

2.3.1. Agar Slant

Sterile agar slants were prepared by autoclaving MRS agar medium in test tubes at 121°C for 15 minutes. After autoclaving, the agar medium was cooled to approximately 50°C, and the tubes were tilted at an angle to solidify the medium, forming slants. Pure cultures of *L. fermentum* MT308789 and *L. oris* MT308790 were inoculated onto the surface of the agar slants using a sterile inoculating loop. The tubes were then sealed with sterile cotton plugs and incubated at 37 °C for 24-48 hours. Once growth was observed, the slants were stored at 4 °C for short-term storage or at -80 °C for long-term storage [11, 14].

2.3.2. Glycerol Stocks

Bacterial cultures were grown overnight in MRS broth at 37 °C. Overnight cultures were centrifuged at 3000 × g for 10 minutes at 4 °C to pellet the cells. The supernatant was discarded, and the cell pellets were resuspended in sterile 20 % [v/v] glycerol solution prepared in MRS broth. Aliquots of the cell-glycerol suspension were dispensed into cryovials, sealed tightly, and stored at -80 °C for long-term preservation [11, 25].

2.3.3. Freeze-Drying [Lyophilization]

Mid-log phase cultures of *L. fermentum* MT308789 and *L. oris* MT308790 were prepared by inoculating a single colony into MRS broth and incubating ally at 37°C until reaching an optical density [OD] of 0.6-0.8 at 600 nm. The cultures were then centrifuged at 3000 × g for 10 minutes at 4 °C to pellet the cells. The cell pellets were resuspended in sterile 10 % [w/v] skim milk solution as a cryoprotectant and transferred to pre-sterilized lyophilization vials. The vials were frozen

at -80 °C overnight and then loaded into the freeze dryer for lyophilization at -50 °C and 0.05 mbar for 24-48 hours. Once lyophilized, the vials were sealed under vacuum and stored at -20 °C until further use [19, 26-29].

2.3.4. Dry Form

Mid-log phase cultures of *L. fermentum* MT308789 and *L. oris* MT308790 were prepared as described above. The cultures were centrifuged, and the cell pellets were washed twice with sterile PBS to remove any residual media components. The washed cell pellets were then air-dried in a sterile laminar flow hood for 24-48 hours until completely dry. The dried bacterial samples were transferred to sterile containers and stored at room temperature in a desiccator until further use [7, 30, 31].

3. Results and Discussion

The viability and efficacy of probiotic strains, such as *Lactobacillus fermentum* MT308789 and *Lactobacillus oris* MT308790, are of paramount importance in the field of nutrition and health. Probiotics are living microorganisms that, when consumed in adequate amounts, confer health benefits beyond basic nutrition [2, 21, 32, 33]. They have been associated with a myriad of health-promoting effects, including improved digestive health, enhanced immune function, and potential contributions to mental well-being [4, 34-37]. As such, ensuring the viability and stability of probiotic strains during storage is essential for maintaining their efficacy and delivering optimal health benefits to consumers [36, 38, 39].

Various storage methods have been employed to preserve the viability of probiotic bacteria, each with its advantages and limitations. Agar slant storage, a conventional method widely used in microbiology laboratories, involves the growth of bacterial cultures on solid agar media in test tubes. However, this method has been associated with decreased viability over time, attributed to factors such as oxygen exposure, nutrient depletion, and potential oxidative stress within the agar slant environment [11, 14]. In our study, agar slant storage resulted in suboptimal viability for both *L. fermentum* MT308789 and *L. oris* MT308790, with viabilities of 40 % and 39 %, respectively, after lyophilization (table 1).

Table 1 Viability of Microorganisms under various storage conditions

***L. fermentum* MT308789 [No of cells X107]**				
Viability of cells	**Slant**	**Glycerol**	**Dry Form**	**Lyophilization**
Initial	11.25±0.68			
After lyophilization	4.50 ± 0.40	6.80 ± 0.50	7.20 ± 0.45	8.72±0.45
% viability	40 %	60 %	64 %	77.51 %
***L. oris* MT308790 [No of cells X107]**				
Viability of cells	**Slant**	**Glycerol**	**Dry Form**	**Lyophilization**
Initial	10.89±0.75			
After lyophilization	4.35 ± 0.70	6.80 ± 0.60	7.40 ± 0.50	8.44±0.45
% viability	39 %	62 %	68 %	77.51 %

In contrast, glycerol stocks, prepared with a cryoprotective glycerol solution, showed improved viability compared to agar slants for both strains. Glycerol serves as a protective agent during freezing and storage, minimizing cellular damage and enhancing viability [11, 29]. Viabilities of 60 % and 62 % after lyophilization for *L. fermentum* and *L. oris*, respectively, suggest that glycerol stocks offer promise as a short-to-medium-term storage option. However, while glycerol stocks provide enhanced preservation compared to agar slants, they still fall short of optimal levels for prolonged storage [11, 29].

Dry form storage, involving the air-drying of bacterial cultures, yielded intermediate viabilities for both strains in our study. This method offers simplicity and convenience, with the potential for long-term storage at room temperature [11, 14]. Viabilities of 64 % and 68 % after lyophilization for *L. fermentum* and *L. oris*, respectively, suggest that dry form storage may be suitable for short-to-medium-term storage. However, further optimization may be necessary to enhance

viability for extended storage durations, particularly considering the varying sensitivity of probiotic strains to desiccation and environmental stress.

Lyophilization, or freeze-drying, emerged as the most effective method for preserving probiotic viability in our study. With viabilities of 77.51 % after lyophilization, both *L. fermentum* and *L. oris* exhibited remarkable stability and longevity. Lyophilization involves the removal of water content from bacterial cells, thereby minimizing cellular damage and providing an optimal environment for long-term preservation [19, 27]. This method has been widely used in the pharmaceutical and food industries for its ability to maintain the viability and functionality of sensitive biological materials, including probiotic bacteria [26, 28, 29].

The findings of our study underscore the critical importance of selecting appropriate storage methods to maintain probiotic viability and efficacy. While agar slants and dry form storage may serve as viable options for short-to-medium-term storage, glycerol stocks and lyophilization offer superior viability and stability for long-term preservation. These results have significant implications for probiotic researchers and industry stakeholders involved in the formulation and production of probiotic products. By optimizing storage protocols, manufacturers can ensure the delivery of viable and effective probiotic formulations to consumers, thereby maximizing health benefits [40-42].

Furthermore, our study highlights the need for continued research and innovation in probiotic storage. Future studies could explore novel preservation techniques, such as encapsulation or microencapsulation, to further enhance probiotic viability and functionality [32, 40, 43, 44]. Additionally, investigating the impact of storage methods on probiotic functionality, including survival during gastrointestinal transit and health-promoting effects, would provide invaluable insights into overall product efficacy. By addressing these research gaps, we can advance our understanding of probiotic storage and contribute to the development of improved probiotic formulations aimed at enhancing human health and well-being.

4. Conclusions

In conclusion, the viability and stability of probiotic strains during storage are critical factors influencing their efficacy and health-promoting properties. Our study highlights the superior efficacy of lyophilization in preserving probiotic viability compared to other storage methods. Glycerol stocks also offer promise for short-to-medium-term storage, while agar slants and dry form storage present limitations in maintaining probiotic viability. These findings provide valuable insights for probiotic researchers and industry stakeholders, guiding the development of effective probiotic formulations that deliver optimal health benefits to consumers. Continued research and innovation in probiotic storage are essential to enhance our understanding and utilization of these beneficial microorganisms in promoting human health and well-being.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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