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Impact of Stability Environment on Anti-*E. coli* Efficacy of Probiotic  
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## ABSTRACT

Probiotic therapy has been shown to provide various health benefits; however, the impact of storage environment on the efficacy of probiotics remains limited. This study evaluated the effect of different storage conditions on the survivability and associated antimicrobial efficacy of live *Lactobacillus rhamnosus* at various shelf-life stages. *L. rhamnosus* was procured and stored at -20°C, 2–8°C, 25°C, and 40°C. The study followed two protocols. At 0, 3, 6, and 12 months, samples from each storage condition were evaluated for survivability and associated antimicrobial efficacy against *Escherichia coli* in vitro (Protocol 1). Additionally, representative samples from each storage condition were collected at 0, 3, 6, and 12 months and stored at -20°C to preserve their composition. After collecting the 12-month samples, all were collectively analyzed for survivability and antimicrobial efficacy (Protocol 2). A steady decline in the viability of *L. rhamnosus* was observed with increasing storage temperatures and relative humidity. Similarly, its efficacy in inhibiting *E. coli* growth in vitro declined as storage temperature increased. Both protocols showed comparable results. At month 12, Protocol 1 samples showed *E. coli* growth inhibition of 97.35%, 89.34%, 61.32%, and 28.45% for storage at -20°C, 2–8°C, 25°C, and 40°C, respectively, compared to baseline values. Protocol 2 samples showed growth inhibition of 98.97%, 91.60%, 63.21%, and 27.20% under the same conditions after 12 months. This study underscores the importance of optimal shelf-life storage environment for maintaining probiotic viability and associated efficacy. The study also proposes a novel methodology for evaluating probiotic efficacy which is useful for studying shelf-life environment associated impact.

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## INTRODUCTION:

The human gut is the home for numerous microbial habitats that plays important role in overall well-being of the gastrointestinal tract (GIT).<sup>1,2</sup> Numerous studies have highlighted that gut dysbiosis have a central role in various acute and chronic disease conditions.<sup>2-5</sup> Probiotics are live

microorganisms that can provide health benefits if consumed in adequate amounts.<sup>6,7</sup> Probiotics have multi-modal mechanisms of action, including prevention of pathogens binding to mucosal surfaces, production of antimicrobial compounds and secondary metabolites that can influence systemic activity, supports intestinal barrier structure and activity, immunomodulatory activity, and affecting the activity of various signalling pathways at the cellular and molecular level.<sup>8,9</sup>

Although clinical studies has demonstrated beneficial effects, the probiotics are very vulnerable to their environment, making their storage conditions one of the important parameters for maintaining their effectiveness throughout shelf life.<sup>10-12</sup> Therefore, it is important to evaluate the impact of storage conditions on the survivability of probiotics in order to ensure optimal desired

efficacy throughout shelf life period. However, studies have not evaluated the effect of storage conditions on probiotics efficacy throughout the shelf-life period. The current study aimed to evaluate the effect of storage conditions on the survivability of live *Lactobacillus rhamnosus* and its antimicrobial efficacy.

## **MATERIALS AND METHODS:**

### **Micro-organisms procurement and storage:**

The probiotic *L. rhamnosus* strain was provided by Sundyota Numandis Probiocuticals Pvt. Ltd. (Ahmedabad, Gujarat). The manufacturing process, briefly, involved the growth of *L. rhamnosus* seed culture in appropriate medium followed by batch fermentation to produce the probiotic biomass. The obtained biomass was then centrifuged and lyophilized. The obtained powder was live *L. rhamnosus* probiotic and stored in aluminium bags at -20°C. The probiotics were divided into four groups and stored at -20°C, 2-8°C, 25°C ± 60% relative humidity (RH), and 40°C ± 75% RH, respectively, for 12 months.

In the current study, the effect of storage environment on viability and antimicrobial efficacy of probiotic was evaluated by two different protocols with the processes being performed independent to each other. As per protocol 1, probiotic was evaluated for viability and antimicrobial efficacy at baseline condition. Similarly, at months 3, 6, and 12 after storage, fixed amount of probiotics were taken from each storage condition and evaluated for viability and antimicrobial efficacy using same methodology. For protocol 2, a fixed amount of probiotic from each storage condition was taken at month 0 (baseline), 3, 6, and 12, respectively. These samples served as the representative samples for individual storage condition and duration. After withdrawing, the representative samples were stored at 2-8°C for 24 hours and then transferred to -20°C for entire study duration. After collection of all probiotic representative samples for all storage condition, the antimicrobial efficacy of all samples were evaluated in a single experiment.

### ***Lactobacillus rhamnosus* powder strength evaluation:**

The strength of the live probiotic was evaluated by the colony counting method. Briefly, 1 gm probiotic sample was transferred in a sterile flask along with 100 ml sterile MRS broth. The solution was stored at 37°C incubation for approx. 40 minutes with appropriate shaking at regular intervals to ensure proper probiotic dissolution. The solution prepared is the “primary solution” with the strength of 10<sup>-2</sup>. Using sterile 0.1% peptone/MRS broth solution, serial dilutions were performed

using 1 ml of primary solution and 9 ml of the diluent to form a solution with a strength of 10<sup>-3</sup>. Similar serial dilutions were performed to form a series of dilutions till the final 10<sup>-10</sup> strength. After each dilution, the solution was vortexed to ensure proper mixing of the solution. In a separate sterile flask, 1 ml of sterile 1% cysteine HCl solution was added to 100 ml of molten MRS agar to form the cysteine/agar solution. In separate sterile petri plates, 1 ml of each serial dilution (10<sup>-2</sup> to 10<sup>-10</sup>) and 15 ml of cysteine/agar solution were added, swirled, and kept at room temperature for solidification. Finally, the petri plates were placed in anaerobic chamber at 37°C for 48-72 hours for incubation. After incubation, the number of colonies formed were counted for each serial dilution and using the dilution factor, the total strength of probiotic was identified. The complete process was conducted in triplicate, and the average of the counts were considered.

### ***E. coli* zone of inhibition study:**

The *Escherichia coli* (*E. coli*) strain ATCC8739 was used for evaluating the antimicrobial efficacy of live and attenuated *L. rhamnosus* was evaluated using *in vitro* agar well growth inhibition method. Briefly, *E. coli* pathogen cells were incubated 37°C and 120 rpm for overnight. Simultaneously, the Muller Hinton agar & Tryptic soya agar were used for preparing agar plates. Incubated *E. coli* (100 µl) were transferred to prepared agar plates and spread across the plate using sterile cotton swabs. Using sterile tips, 7 wells were created on the agar plate (each with a 1 ml capacity) for sample incorporation. The probiotic sample was prepared by serial dilution method to reach the final concentration of 3\*10<sup>6</sup> CFU per 100 µl solution of the baseline probiotic material. The same serial dilution was performed on the subsequent evaluation at 3, 6, and 12 months. The prepared probiotic sample (100 µl) was transferred to the plate well. The probiotic samples from T1 to T4 were termed S1, S2, S3, and S4, respectively. Sterile water was used as a negative control (NC), diluent used for serial dilution along with maltodextrin as placebo was used as a positive control (PC), while ciprofloxacin at a strength of 67 µg/ml was used as a standard control (SC). After loading all the samples (NC, PC, SC, and S1-S4) in the respective petri plate wells, the plates incubated at 2-8°C for 15-30 minutes to ensure proper sample diffusion in the agar medium, followed by keeping the plate at room temperature and then incubating the plate at 37°C for 18-24 hours. The entire process was performed in duplicate, and the *E. coli* zone of inhibition for each sample was measured using a calibrated scale.

**Statistical analysis:**

The live and heat attenuated probiotic strengths at different storage conditions were estimated as CFU per gm of product, respectively. The antimicrobial *in vitro* growth inhibition efficacy of was measured in millimetres (mm). Data values were presented as mean  $\pm$  SD. Microsoft excel was used for conducting descriptive analysis. GraphPad Prism was used for conducting the statistical analysis using one-way ANOVA with Tukey test.

**RESULTS:****Effect of storage condition on probiotic viability and physical attributes:**

As per the hypothesis, an increase in the storage temperature and relative humidity resulted in a decline in the live probiotic survivability. As presented in **Table 1**, a progressive decline in the live probiotic survivability is observed with increasing storage temperature. Compared to baseline, probiotics stored at  $-20^{\circ}\text{C}$  showed no change in viability throughout the storage period, while a progressive decline was observed in the viability with increasing temperature. The largest decline was observed at  $25^{\circ}\text{C}$  and  $40^{\circ}\text{C}$  conditions, in which at 12 months, the viability decline was found to be 64.5% and 100%, respectively, compared to baseline values, while it was just 3.2% and 19.4% at  $-20^{\circ}\text{C}$  and  $2-8^{\circ}\text{C}$  conditions, respectively.

**Table 1. Effect of storage temperature on live probiotics survivability**

	Baseline	Month 3	Month 6	Month 12
$-20^{\circ}\text{C}$	310	320 (+3.2%)	310 (0%)	300 (-3.2%)
$2-8^{\circ}\text{C}$	310	280 (-9.7%)	260 (-16.1%)	250 (-19.4%)

**Table 2. Anti-*E. coli* efficacy of probiotics evaluated using protocol 1 method.**

	Baseline	Month 3	Month 6	Month 12
Negative control	0.00	0.00	0.00	0.00
Positive control	0.00	0.00	0.00	0.00
Standard control (Ciprofloxacin)	22.73	21.53	21.20	21.93
$-20^{\circ}\text{C}$	16.37	16.73	15.83	15.93
$2-8^{\circ}\text{C}$	16.27	14.67*	14.30*	14.53*
$25^{\circ}\text{C} \pm 60\% \text{ RH}$	16.20	12.53*	11.00* <sup>^</sup>	9.93* <sup>^</sup>
$40^{\circ}\text{C} \pm 75\% \text{ RH}$	16.17	8.23*	5.67* <sup>^</sup>	4.60* <sup>^</sup>

Data presented as zone of inhibition (in mm). \* $p < 0.05$  v/s baseline storage condition value. <sup>^</sup> $p < 0.05$  v/s month 3 storage condition value.

**Table 3. Anti-*E. coli* efficacy of probiotics evaluated using protocol 2 method.**

	Baseline	Month 3	Month 6	Month 12
Negative control	0.00	0.00	0.00	0.00
Positive control	0.00	0.00	0.00	0.00
Standard control (Ciprofloxacin)	22.77	22.17	21.67	22.03
$-20^{\circ}\text{C}$	16.13	16.07	15.83	15.97
$2-8^{\circ}\text{C}$	15.87	14.80*	14.87*	14.53*
$25^{\circ}\text{C} \pm 60\% \text{ RH}$	15.77	13.50*	10.77* <sup>^</sup>	9.97* <sup>^</sup>
$40^{\circ}\text{C} \pm 75\% \text{ RH}$	15.93	8.20*	5.77* <sup>^</sup>	4.33* <sup>^</sup> #

$25^{\circ}\text{C} \pm 60\% \text{ RH}$	310	250 (-19.4%)	190 (-38.7%)	110 (-64.5%)
$40^{\circ}\text{C} \pm 75\% \text{ RH}$	310	80 (-74.2%)	0 (-100%)	0 (-100%)

\*Data presented as mean CFU/gm probiotic powder (%change in survival rate compared to baseline value). #Data presented as mean number of heat-attenuated cells/gm probiotic powder (%change in number of intact cells compared to baseline value). CFU: Colony forming units; RH: Relative humidity.

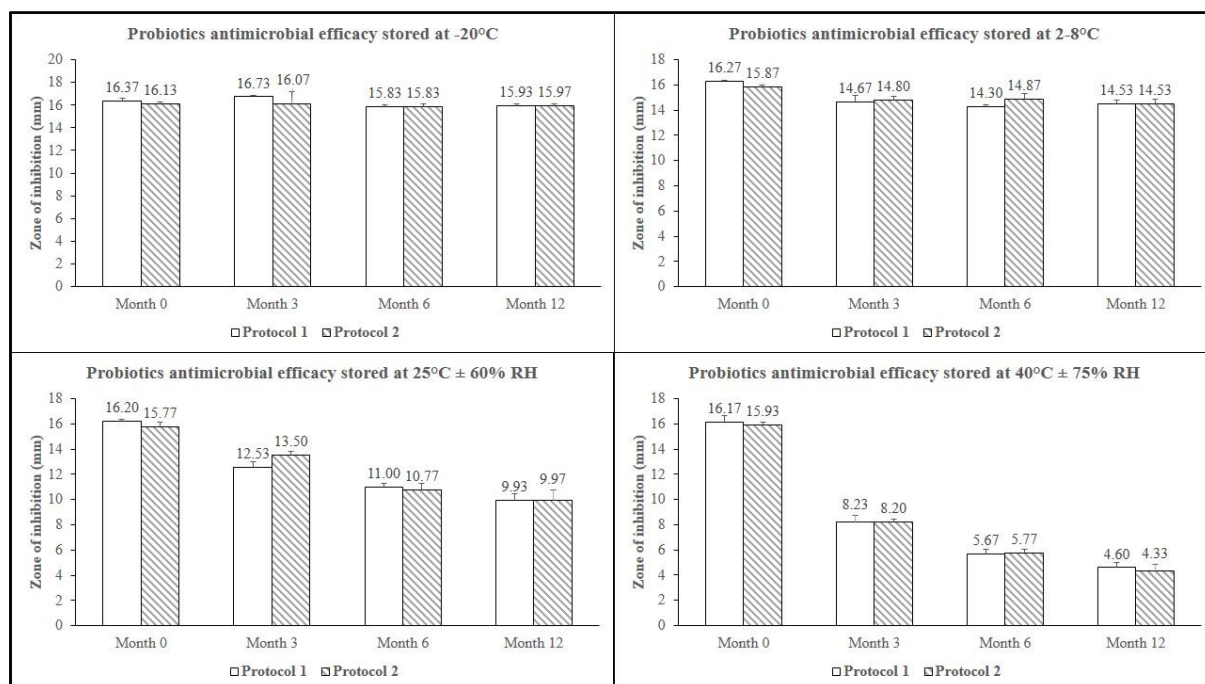
**Effect of storage condition on anti-*E. coli* efficacy:**

The effect of storage conditions on the antimicrobial efficacy of live probiotics was evaluated using *in vitro* inhibition of *E. coli* growth. As presented in **Table 2**, a progressive decline was observed in the antimicrobial efficacy of live probiotic with respect to increasing storage condition as evaluated by protocol 1 method. At 12 months of storage, the antimicrobial efficacy of live *L. rhamnosus* evaluated using protocol 1 method was declined by 2.6%, 10.7%, 38.7%, and 71.5% for the  $-20^{\circ}\text{C}$ ,  $2-8^{\circ}\text{C}$ ,  $25^{\circ}\text{C} \pm 60\% \text{ RH}$ , and  $40^{\circ}\text{C} \pm 75\% \text{ RH}$  conditions, respectively, compared to baseline values. Similar results were observed for probiotics evaluated using protocol 2 method. As presented in **Table 3**, the antimicrobial efficacy of probiotics evaluated using protocol 2 method was declined by 1.0%, 8.4%, 36.8%, and 72.8%  $-20^{\circ}\text{C}$ ,  $2-8^{\circ}\text{C}$ ,  $25^{\circ}\text{C} \pm 60\% \text{ RH}$ , and  $40^{\circ}\text{C} \pm 75\% \text{ RH}$  conditions, respectively, compared to baseline values.

Data presented as zone of inhibition (in mm). \* $p < 0.05$  v/s baseline storage condition value. ^ $p < 0.05$  v/s month 3 storage condition value. # $p < 0.05$  v/s month 6 storage condition value.

Based on the findings of the study, it was observed that both the methodologies (protocol 1 and protocol 2) showed similar antimicrobial efficacy of probiotics at different storage conditions. As presented in **Figure 1**, no significant difference

between the antimicrobial efficacies of probiotics evaluated using protocol 1 and protocol 2 methodologies was observed for all the storage conditions.



**Figure 1.** Comparison of probiotics' antimicrobial efficacies stored at different storage conditions and duration evaluated using protocol 1 and protocol 2 methodologies.

## DISCUSSION:

Numerous studies have majorly highlighted the clinical benefits of probiotics, but limited studies have evaluated and underscored the importance of storage conditions on the survivability and overall efficacy of probiotics. The present study aimed to evaluate the effect of different storage temperatures on the viability and antimicrobial efficacy of live *L. rhamnosus* probiotic. The results are in line with the hypothesis that storage conditions have a huge impact on the survivability and efficacy of probiotics.

Probiotics therapy have gained widespread attention in both scientific research and public health over recent decades.<sup>2,13</sup> Initially popularized for their role in gut health, probiotics are now recognized for their potential therapeutic effects on a wide range of physiological processes.<sup>13</sup> These beneficial microbes are found in fermented foods, dietary supplements, and certain pharmaceutical formulations, with strains of *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces* being among the most widely studied.<sup>13</sup> The human microbiota plays a critical role in maintaining homeostasis and

influencing overall health. Disruptions to this delicate balance, due to factors such as antibiotic use, diet, or stress, can lead to a variety of disease conditions.<sup>13</sup> In this context, probiotics are widely used to restore or support a healthy microbiome and modulate immune responses, metabolism, and gastrointestinal functions.<sup>13</sup> The effectiveness of probiotics as therapeutic agents is largely dependent upon their viability throughout their shelf life. However, ensuring probiotic viability is a complex challenge due to high sensitivity of probiotics to environmental factors like temperature, humidity, light, and oxygen that can significantly impact the survival of probiotics, ultimately affecting their health benefits and therapeutic efficacy as well.<sup>11</sup> The current study highlights that with increasing storage temperature, there was a progressive decline in the survivability of the live *L. rhamnosus* probiotic. This decline in viability was associated with a progressive decline in the probiotic antimicrobial efficacy to inhibit *E. coli* growth as well. Optimal storage conditions play an important role in preserving the viability and potency of probiotics. Improper storage, such as exposure to high temperatures, moisture, or

direct sunlight, can lead to the degradation of probiotic strains, reducing their ability to confer health benefits. Similarly, probiotic product manufacturers must carefully consider storage conditions during production, distribution, and storage at the point of sale to ensure that the products containing live probiotics maintain their viability and thereby their full therapeutic potential.

The current study presents a novel method for evaluating the efficacy of probiotics. In the current study, the antimicrobial efficacy of probiotics was evaluated using two different test protocols. As per protocol 1, the efficacy of probiotics was evaluated individually during the shelf life storage condition duration, while in protocol 2, representative samples of probiotics were collected and stored at -20°C to arrest their viability loss. After collecting all the probiotic representative samples from all the storage conditions and duration, the antimicrobial efficacy of the samples were evaluated in a single experiment. Based on the results, it was observed that there was no significant difference between the results of both the methodologies (**Figure 1**). This observation is novel and can be utilized for other probiotics study as well. While protocol 1 follows the conventional method of analysis, the protocol 2 method provides a more reliable method of probiotics efficacy analysis. This method ensures significant reduction in repetitive analytical labour and resource usage, while additionally reducing the variability observed in repetitive measurements. Overall this process ensures better study outcomes with reduced analytical effort and overall experimental costs.

The current study has various strengths. Firstly, the current study is novel in that it has evaluated the effect of different storage temperatures on survivability of live *L. rhamnosus* and associated efficacy during the shelf life period. Based on the observed results and in line with the hypothesis, the number of live probiotics was progressively reduced with increasing storage temperature during the shelf life duration. Secondly, in line with the hypothesis, the reduction in the number of live probiotics was associated with a reduction in their antimicrobial efficacy to prevent *E. coli in vitro* growth. Additionally, the current study successfully evaluated a new approach to investigate the antimicrobial efficacy of probiotics and showed similarity with the conventional probiotics analysis approach. The current study has certain limitations as well, including the use of an *in vitro* antimicrobial test instead of an *in vivo* experimental evaluation approach, the utilization of *L. rhamnosus* probiotic only while many well-documented probiotics are currently available in the market, and lastly, the inability to understand

the molecular effects associated with increased storage temperature on the probiotics leading to reduced effectiveness.

#### **CONCLUSION:**

Use of live probiotics for improving the health of the gut microbiome and thereby providing other health benefits. The current study underscores the potential impact of storage temperature on the overall viability and efficacy of live probiotics during the shelf life period. Considering the strengths and limitations, the current study is promising and can potentially help design future research studies. Additionally, the results of the current study can help the manufacturers to understand the importance of optimal storage conditions for probiotic-containing products. In addition, the study introduces an innovative approach for assessing the efficacy of probiotics. This newly proposed methodology is particularly valuable for understanding how storage environmental conditions encountered during shelf life duration can influence the viability and efficacy of probiotics.

#### **CONFLICT OF INTEREST:**

The authors declare no conflict of interest.

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