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An Improvement to the Effective Growth Medium for Probiotics in Strict Vegetarians

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ABSTRACT:
Growth culture medium development and optimization; the absolute vegetarian culture medium employing seed powder of plant seed powder in place of beef extracts for probiotic growth. Vegetarian probiotic medications must be devoid of any animal-derived components by definition. This study suggests a more effective and cost-efficient vegetable growth media. The culture media established in this innovation will give method of producing vegetarian items for human population those are a purely vegetarian. People of specific religions in nations such as India are deeply worried about such concerns. The created culture medium outperforms regular MRS, which would be a great relief for these communities.

Keywords: optimization, Growth medium, Probiotics, Lactobacillus, Vegetarian

I. INTRODUCTION

There are several well-known probiotic bacteria in the Lactobacillus casei group, notably L. casei shirota [1] & L. rhamnosus GG [2]. Lactobacillus strains belonging to this category (L. paracasei, L. casei, L. zeae and L. rhamnosus) must be identified for both fundamental research & food industry uses. The majority of these strains share similar physiological features and dietary requirements, as well as growing in similar environments. Traditional Lactobacillus identifying phenotypic testing can be hard to interpret. The methods are also time expensive, and the outcomes are frequently imprecise. **[3].**

Although fermentation characteristics can easily differentiate this grouping of Lactobacillus from different members of the Lactobacillus genus [4], fermentation profiles cannot be used to unambiguously distinguish just four species. They belong to the same taxonomic family. With growing knowledge of genomic architecture and phylogenic interactions amongst Lactobacillus species, the classification of this group has altered significantly in recent years. **[5].** It's critical to characterize available commercial prebiotics to taxonomic and strain levels because they're being generated and promoted for human use on a massive scale. **[6].**

The human gastrointestinal tract's bacterial communities form a complex ecosystem [7]. A single subject's faeces included almost 400 different bacterial species [8]. Microorganisms are known to have active clearance mechanisms in the gastrointestinal tract, making it more difficult to introduce novel strains of bacteria into this environment [9]. Individual species, however, have host precision in colonisation; for example, L. fermentum, L. acidophilus, and L.plantarum are frequently found in the human faeces; even so, L. delb. spp. bulgaricus, which is used in mixture with S. thermophilus to create yoghurt, has been unable to repopulate the bucket and is not isolated in the faeces [10]. It can be useful to change the intestinal microbiota by adding lactobacilli in a variety of situations [11]. Increased nutritional utilisation, reduction of intolerance of lactose, therapy of hepatic cirrhosis and intestinal illnesses, and suppression of bacterially produced carcinogen production in the digestive tract are all reported benefits of bacteria supplementation [12]. The large intestine's balance is dynamic and is influenced by composition of diet, age, and other external conditions. Despite the importance of these elements, maintaining the intestinal microflora, which is primarily composed of good species such as probiotic bacteria, might improve our health. Microorganisms are known to have active clearance mechanisms in the human digestive tract, making it challenging to bring new strains of bacteria into this environment. **[13-14]**.

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II. METHODOLOGY

Isolation of bacteria.

Milk was used to isolate ten Lactobacillus casei strains. At 37 degrees Celsius, they were grown in MRS media. Without the usage of anaerobic environments, all strains developed effectively. Bacteria were isolated through serial dilution. The identification of the strain was carried out through performing the biochemical tests by following the Bergy's manual **[15].**

Lactobacillus spp. may survive in acidic environments:

To test the survivability of ten Lactobacillus strains in acidic conditions, active culture aliquots produced in broth of MRS for 20 hours at 37°C were regulated to pH 1.5, 2.0, 2.5, 3.0 3 with 5 N HCl and kept for 3 hours at 37°C. Pour plates counts of all samples were performed empolying 10-fold serial dilutions made in 0.1 percent normal saline, and the viable number of Lactobacillus was determined by colony counter of all samples. The research and evaluation were done in three different ways **[16].**

Lactobacillus spp. survival in the existence of bile salts:

To test the survivability of 10 L. casei strains in the influence of bile salts, dilutions of active cultures cultured in MRS broth for 20 hours at 37°C , pH was adjusted 4.5 with sterilized 1 N HCl or 1 N NaOH, based on the culture's ultimate pH after 20 hours. Furthermore, strong bile solution is prepared by mixing powder bile extract (Oxoid). The bile solutions was then filter sterilised and applied to two of the cultures to attain final concentrations of 1.0 and 1.5 percent, respectively, with the third culture serving as a control sample. The culture was grown for 3 hours at 37°C. Before administering the bile solutions, specimens were obtained, and every hour for 3 hours. Pour plate count of all specimens using 10-fold dilution series made in 0.1 percent normal saline were used to assess viable counts of L. casei strains. The test and evaluation were done in three different ways **[17].**

Lactobacillus spp. Enumeration:

The counting of Lactobacillus species were done on (MRS) agar (Oxoid). In 0.1 percent sterile normal saline, cultures were serially decreased from 10^{-1} to 10^{-8}. MRS agar was pour plate with one millilitre aliquots of the dilutions and cultured aerobic capacity at 37℃ for 72 hours. All colonies per gramme of culture were enumerated and reported as colony forming units (CFU) **[18].**

Lactobacilli deconjugate sodium taurocholate (STC) and sodium glycocholate (SGC):

The deconjugation ability of selected Lactobacillus strains with high acid & bile resistance was investigated. 6 mM sodium glycocholate, Sodium taurocholate, or a complex of sodium taurocholate and sodium glycocholate at 1.2 and 2.8 mM were added to ten-milliliter portions of MRS broth. Separate bile salts were supplied at 6 mM each to mimic quantities in the humans small intestine, whereas bile mixes included 1.2 mM STC and 2.8 mM SGC to mimic the molar ratio of two salts and humans bile **[19].** Each strain was injected at 1% and maintained aerobic capacity for 20 hours at 370°C. The ability to deconjugate bile salts was predicated on the production of deconjugated bile, and the quantity of free cholic acid produced by the microorganisms was measured using a modified method developed. After the incubation period, each organism's 10 mL culture was achieved at ph 7.0 using NaOH (1 N). The culture was grown for 3 hours at 37°C.

` Before administering the bile solutions, specimens were obtained, and every hour for 3 hours. Pour plate count of all specimens using 10-fold dilution series made in 0.1 percent normal saline were used to assess viable counts of L. casei strains. The test and evaluation were done in three different ways **[19].**

Freeze-drying:

All seven strains' pure cultures were moved from freezing culture broth into MRS solution was added and maintained at 37°C for 24 hours, with three transfers in total. The culture then were spun for 15 minutes at 5000rpm at 4°C. The cell pellet was re centrifuged (2714g at 4℃ for 15 minutes) after being suspended in20 millilitres of sterile 0.1M Na2HPO4 buffer (pH 6.8). The cell pellet was placed in 20 mL of sodium phosphate buffer containing 2 percent (wt/vol) UnipectineTM RS 150 as a cryoprotectant. The mixture was then aseptic conditions put onto petri dishe, sealed with paraffin and aluminium foil, and frozen overnight at -18℃, followed by 48 hours of freeze-drying in a freeze-dryer **[20].**

Lactobacilli freeze-dried enumeration:

At 0, 1, 2, 4, 6, 9, and 12 months of preservation, all seven strains were counted in duplicate. The viable number of Lactobacilli was counted using the pour plate method. For counting, MRS agar was employed, and the plate were kept

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at 37 ℃ for 72 hours. Colony forming units (CFU) per millilitre were used to calculate the counts **[20].**

Activity of proteolytic enzymes in reconstitute skim milk:

In MRS broth, one gramme of each freeze-dried Lactobacillus strain was cultured overnight at 37℃. To reduce free amino acid carryover after injection, 5 mL of cells were rinsed and re-suspended in sterile 0.32 mM sodium phosphate, pH 7.2, to the original volume. The cells were injected (1%) into RSM 12 percent (w/v) and incubated for 6 hours at 37°C. RSM that had not been injected served as a control. To make a final concentration of 0.47 M (7.7%) TCA, a 2.5 mL specimen of each cultured RSM was combined with 10 mL of 0.75M (7.7%) trichloroacetic acid (TCA) and 1 ml distilled concentrated to 5 mL of sample. After a 10-minute incubation period at room temp (22°C), the specimens were collected using Whatman number 4A filter paper and frozen until analysis. The percentage of primary amino groups in the filtrate was determined using the o-phthaldialdehyde (OPA) method. Spectrophotometer (Pharmacia, Biotech, Uppsala, Sweden) was used to analyse triplicate aliquots from each TCA filtrate at 340 nm **[21]**.

Formulation:

Milk was used to create a pure culture of L. casei, a probiotic. This probiotic was produced in both regular MRS culture environment and experimental test mediums, which included diverse plant seed powder as a vegetal nutritional source instead of beef and yeast extract, peptone, as in MRS. Mung beans, lentils, chickpeas, peanuts, Bengal gramme, and wheat were sprouted for 72, 48, and 42 hours, respectively, in TM6, TM5, TM4, TM3, TM2, and TM1. Samples seeds were immersed for 8 hours and then sprouted for 24, 48, and 72 hours following soaking for germination. Seeds were placed and germinated, then dried at 45°C and pulverised. As a representative sample, powdered was used. As inoculums, pure L. casei culture was employed according to the culture's instructions. The probiotic growth was measured at periodic intervals after the injected culture medium were maintained at 37°C for 72 hours. In different media, the density was measured in Colony Forming Units (CFUs). To get a conclusion, the trials were repeated three times.

In Test media, the following components were dissolved in 850mL distilled water, and the pH was adjusted to 6.5 for each: 10 grammes of seed powder Glucose (glucose) (glucose) (glucos 1 gramme of Tween-80 K2HPO4 (K2HPO4) (K2H 5 g sodium acetate 2 g (NH4) MgSO4-7H2O 2 citrate 0.2g MnSO4-H2O, 0.05g 1% of total After that, the pure Agar Test culture medium were autoclaved. Several serial dilutions were made.

III. RESULTS AND DISCUSSIONS

The Lactobacillus strains were successfully isolated from the milk sample by serial dilution and spread plates methods. While 10 different types of pure cultures were isolated from the milk samples. Then these cultures were identified by performing the biochemical characterization following bergy's manual.

These isolates were termed as the Lactobacillus. Initially these strains were analysed for their growth at low pH medium and found that the seven strains showing good growth as shown in figure 1-4. The increment in the CFU count of lactobacillu strains were obtained as the pH of the medium increased.

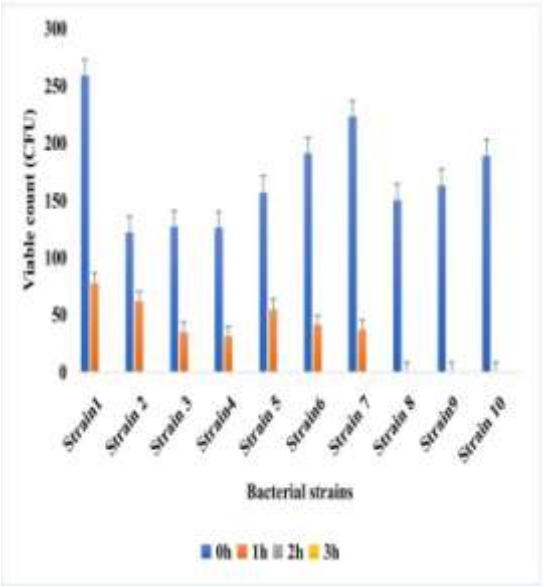

Figure 1: Survival of 10 three hours at pH 1.5 in HCl solution with several Lactobacillus strains.

International Journal of Pharmaceutical Research and Applications
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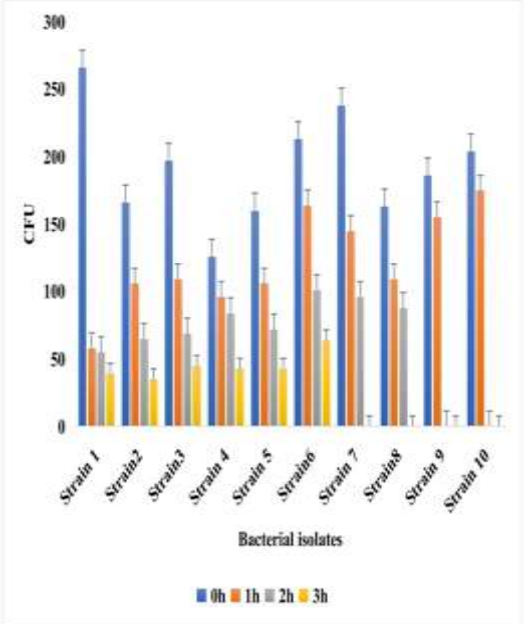

Figure 2: Lactobacillus isolates survive 3 hours in HCl at pH 2.0. solution

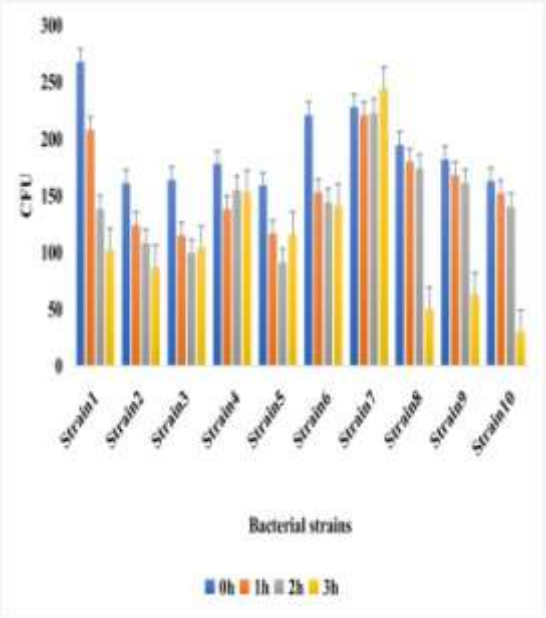

Figure 3: Bacterial strains at pH 2.5 at different time intervals

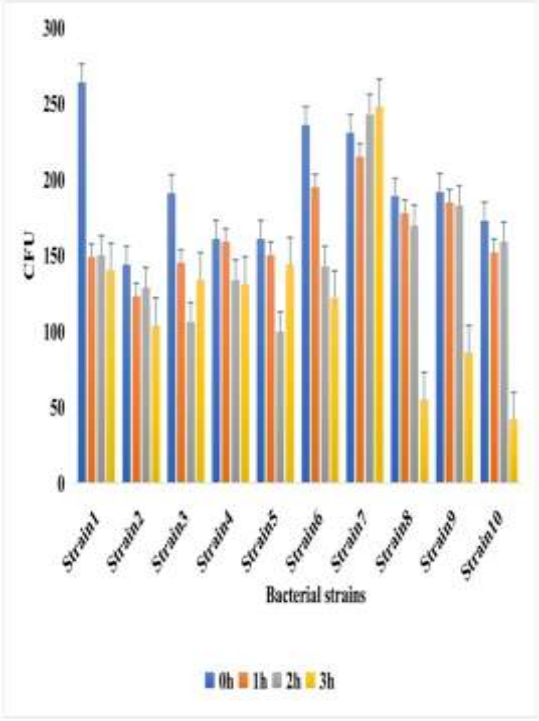

Figure 4: Lactobacillus isolates survive 3 hours in HCl at pH 3.0.

While increasing bile salt concentration it was found that the growth of the strains decreased as the CFU minimizes. Similarly, as the time duration of the cultures in the bile salt-containing medium increases the CFU counts decrease. As explained in Figures 5-7. Hence the seven cultures shows their survivability so they were selected for the further analysis.

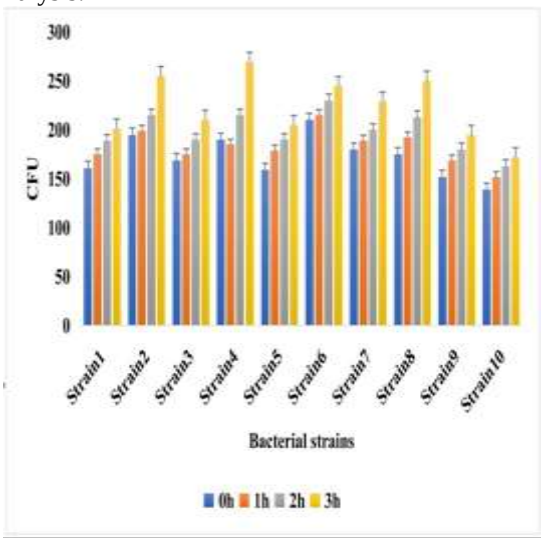

Figure 5: Studies on the survival of Lactobacillus strains at 0.0% bile solution

International Journal of Pharmaceutical Research and Applications
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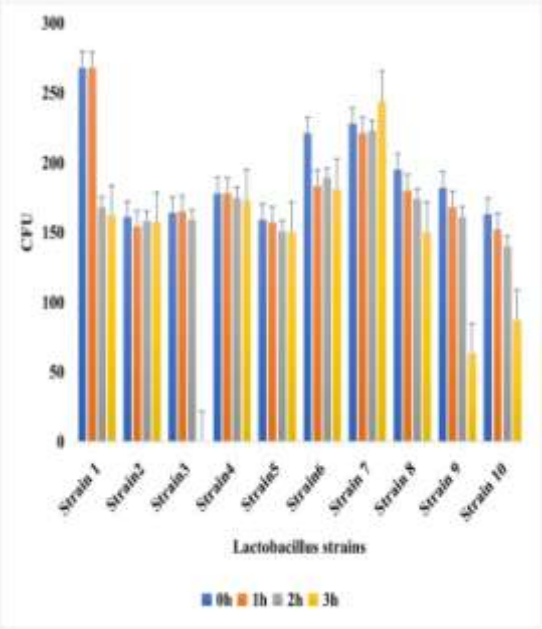

Figure 6: In the presence of 1.0 percent bile solution, 10 distinct Lactobacillus cultures survived for 3 hours.

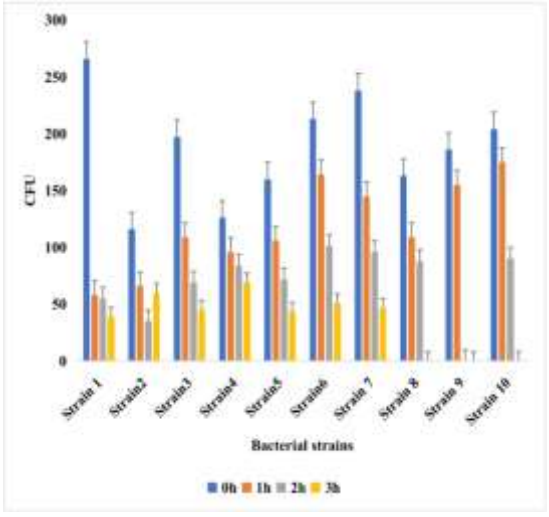

Figure 7: Graphical studies of bacterial growth at 1.5% bile salt concentration during different hours.

Table 1: Deconjugation of STC and SGC by Lactobacillus strains.

Strains	Released cholic acid (mM)		
	SGC	STC	SGC+STC
Strain 1	1.80 ± 0.11	1.41 ± 0.15	1.35 ± 0.38
Strain2	3.41 ± 0.18	2.45 ± 0.14	2.11 ± 0.30
Strain3	$1.92 \pm 0.50^{c, A}$	$1.20 \pm 0.14^{b, A}$	$1.68 \pm 0.14^{ac, A}$
Strain4	$2.30 \pm 0.40^{bc, A}$	$1.34 \pm 0.13^{b, A}$	$2.05 \pm 0.33^{ac, A}$
Strain5	$1.58 \pm 0.14^{c, A}$	$1.29 \pm 0.11^{b, A}$	$1.16 \pm 0.09^{c, A}$
Strain6	$4.70 \pm 0.44^{a, A}$	$3.29 \pm 0.38^{a, AB}$	$2.71 \pm 033^{ab, A}$
Strain 7	$1.85 \pm 0.33^{c, A}$	$1.45 \pm 0.12^{b, A}$	$1.92 \pm 0.19^{ac, A}$

According to the findings of our investigation, all twenty-two Lactobacillus spp. strains demonstrated good tolerance to acid and bile concentrations. Strains 1, 2, 3, 4, 5, 6, and 7 have demonstrated high acid and bile resistance. As a result, we chose Table shows the deconjugation capabilities of all seven strong Lactobacillus strains in addition further to prove their tolerance to bile in order to study their therapeutic effects in this project.

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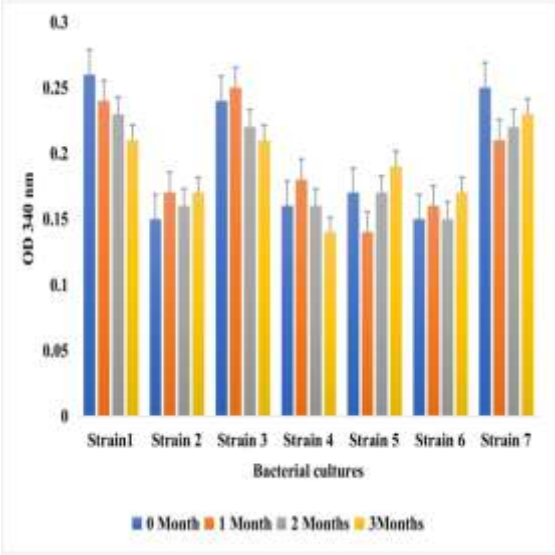

Figure 8: Proteolytic activity of Lactobacillus bacterial strains

The proteolytic activity of the shortlisted seven strain were studied and found that the stain 1, 3, 5, and 7 shows maximum activity. Hence the viable microbial count at different temperatures were studied for three months. Strain 1 and 3 shows almost similar activity while strain 5 and 7 also expresses the same results with minute differences as given in figure 9-11.

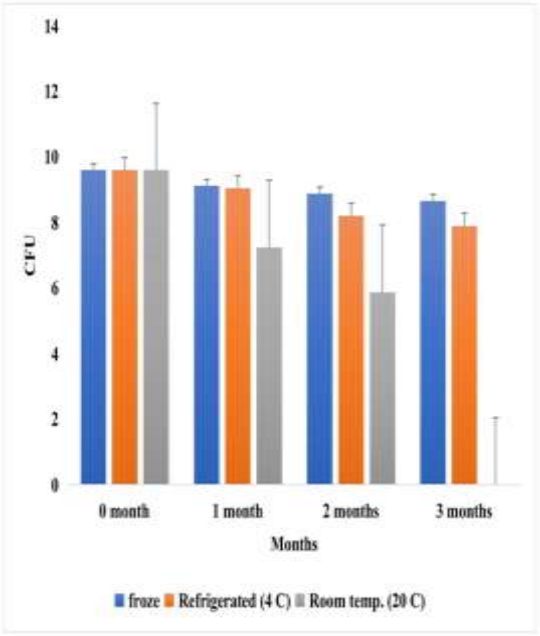

Figure 9: Viable microbial count of freeze dried strain 1 and 3 stored for 3 months

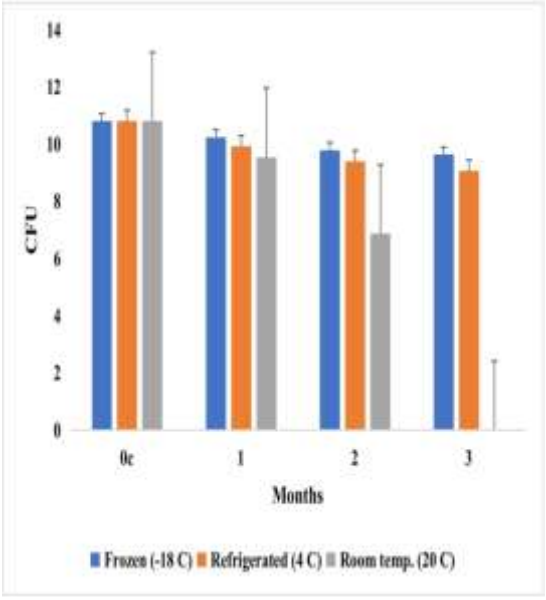

Figure 10: Viable microbial count of freeze dried strain5

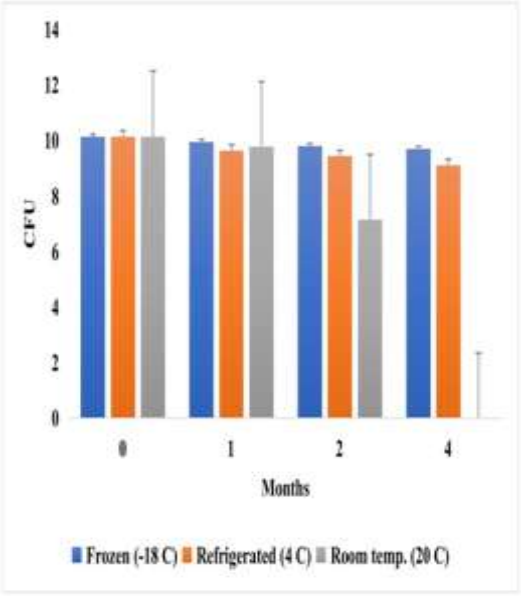

Figure 11: Viable microbial count of freeze dried strain7

The temp of -180C was shown to be optimal for long-term storage of Lactobacillus strains in this investigation. All four Lactobacillus strains were found to have the highest levels of viability and proteolytic ability. At cold temperatures (40C), bacterial counts decreased, and at 200C, the viable count decreased dramatically. Likewise, all Lactobacillus cultures maintained at -180C showed increased proteolytic action after

International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 417-425 www.ijprajournal.com ISSN: 2456-4494

three months. These findings point to the possibility of Lactobacillus strains being used commercially in pharmaceutical or cultured milk items in the future. The CFU count was tested at different dilutions as well as the growth was analysed up to 72 hours as indicated in table 2, with strain 1 showing the best result across the four strains.

Table 14: CFU for Probiotic, Lactobacillus strain in Absolute

Time	TM1	TM2	TM3	TM 4	TM 5	TM 6	MRS
Absolute sample							
24 hrs.	1	4	3	10	0	2	4
48 hrs.	3	8	7	11	1	5	6
72 hrs.	4	18	7	20	2	10	15
10^{-4} dilution							
24 hrs.	1	4	3	10	0	2	4
48 hrs.	3	8	7	11	1	5	6
72 hrs.	4	18	7	20	2	10	15
10^{-5} dilution							
24 hrs.	1	0	2	10	0	5	11
48 hrs.	3	3	9	11	0	7	13
72 hrs.	4	3	17	20	0	12	15
10^{-6} dilution							
24 hrs.	2	7	2	10	2	2	4
48 hrs.	3	12	8	11	7	4	4
72 hrs.	7	16	10	20	7	6	7
10^{-7} dilution							
24 hrs.	2	7	2	10	3	2	12

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48 hrs.	6	12	8	11	4	5	13
72 hrs.	7	16	10	20	5	6	16

The majority of undiluted CFU growth happened at the 72-hour mark, as seen in this table. T.M-2 & T.M-4 were the most productive, as per this data, with 17 - 20 units generated after 72 hours, respectively. T.M-1 and T.M-2 showed the highest effective colony - forminunits' unit development when diluted to 10-4. T.M-1 had seven created units after 48 hours, while T.M-2 had fifteen. The units in these medium had increased to 15 and 19, respectively, after 72 hours. The notion that T.M-2 generated the most continuously productive growing at all three phases in all dilutions is supported by the table, which shows that consistent CFU development was most fruitful in T.M-4. T.M-2 demonstrated to be the most successful test medium including diverse plant seed powder, according to this set of trials. T.M-2 was likewise found to be most efficient when diluted to a concentration of no more than 10-4. The best probiotic development was seen in TM 2, which was germinated for 72 hours. Animal nitrogen is largely used in starter culture media for probiotics and other microbes. This innovation provides a useful medium for vegetative culture.

IV. CONCLUSION:

1. Alternatives test medium made from plants seed powders grew more CFU than the conventional MRS growth media in all studies.
2. At every dilution grade, steady growth was seen in every culture media, comprising the regular MRS culture medium and also test media.
3. Effective germination period in this set of trials was 72 hours.
4. Overall, the lentil seed powder (TM-2) test media showed the greatest consistent development of colony forming units.

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[10]. There is, however, host specificity in colonization by individual species; for example, Lactobacillus acidophilus, L. fermentum and L. plantarum are commonly found in the feces of humans; whereas L. delb. spp. bulgaricus, the organism used in combination with Streptococcus thermophilus to make yoghurt, is unable to colonise the bowl and is not isolated in the

feces **[**

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