

Optimization of an effective growth medium for culturing probiotic bacteria for applications in strict vegetarian food products

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ABSTRACT

Background: This study aimed to modify de Man Rogosa Sharpe culture medium (termed MRS) for selective cultivation of probiotics strain for the consumption by the strictly vegetarian human population. Vegetarian probiotic foods by definition must be free from all animal-derived ingredients. This not only includes the product ingredients but the probiotic inoculum as well. Probiotic starter cultures are traditionally grown and stored in media containing milk or meat-derived ingredients. The presence of these ingredients makes the probiotic cell concentrates unsuitable for use in vegetarian products and thus creates the need for a growth medium which is free from animal-derived ingredients. Present study investigated the growth of a strain of *Lactobacillus lactis* in MRS. The present invention relates in general to a bacterial culture media, and more specifically a complex microbial culture media, based on plant seed powder extract in place of animal extract for probiotic bacterial growth.

Methods: *Lactobacillus lactis*, a probiotic, was grown in standard MRS culture medium as well as in our various test media (TM) containing various vegetal source in place of beef extract, yeast extract and peptone as in case of MRS. The inoculated culture mediums were incubated at 37°C for 72 hours and growth of probiotic is recorded at regular intervals. The growth was recorded as Colony Forming Units (CFUs).

Results: The best growth of probiotic is observed in TM 2. TM 2 is the leguminous seed extract. Starter culture mediums for probiotics or other bacteria primarily contain protein from animal source. The possibility of using vegetal protein from TM 2 extract in place of peptones and meat extract for the nitrogen supplementation of culture media for the growth of lactic acid bacteria has been demonstrated.

Conclusion: The absolute vegetarian culture medium containing TM 2 is better than standard MRS for the growth of probiotics.

Abbreviations: de Man Rogosa Sharpe (MRS), Colony Forming Units (CFU), test media (TM), National Dairy Research Institute (NDRI), Tamarind seed powder (TSP), solid-state fermentation (SSF), *Lactobacillus casei* Shirota (LcS)

Keywords: probiotics, lactic acid bacteria, vegetarian.

INTRODUCTION:

Probiotics are dietary supplements of live bacteria or yeasts thought to be healthy for human consumption. Probiotic products aimed at improving health by modifying microbiota composition have already become widely available and acceptance of these products appears to be on the rise. According to the currently adopted definition by WHO/FAO, probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host” [1].

Current research proves that human gut microbiota composition/activity is a key factor when assessing a person’s risk for obesity and associated diseases such as: atherosclerosis, dyslipidemia, insulin resistance, diabetes, hepatic steatosis and steatohepatitis. [2-5] The market for probiotics continues to grow as awareness of their health benefits increases, in conjunction with the scientific data to prove this. Some benefits of probiotics include: immune stimulation, enhancement of bowel mobility, and reduction of inflammatory or allergic reactions. Recent research on the molecular biology and genomics of the probiotic bacteria *Lactobacillus* has focused on its interaction with the immune system, anti-cancer potential, its effect on adipocyte cell size and body fat, and its potential as a biotherapeutic agent in cases of antibiotic-associated diarrhea, travelers' diarrhea, pediatric diarrhea, inflammatory bowel diseases and irritable bowel syndrome [6,7]. Specifically, interest has increased in lactic acid bacteria because of its potential to improve cholesterol as well as its anticarcinogenic, antipathogenic, and antidiabetic properties [8-12]. According to the American College of Gastroenterology, there are 95m people in the US that suffer from digestive problems. Some 60m are thought to suffer from heartburn, and 50m from irritable bowel syndrome. In addition, it is estimated that around 20m people suffer from stomach ulcers. To date, the largest segment of the digestive health market - particularly within the functional food category - is taken up by products made with probiotics, or 'friendly bacteria'. *Lactobacilli* can even be found in sensitive household products such as infant foods and a variety of pharmaceutical concoctions [8, 13-14].

The study aimed to modify de Man Rogosa Sharpe culture medium (termed MRS) for the selective cultivation of probiotics strain for consumption by the strictly vegetarian human population. Vegetarian probiotic foods by definition must be free from all animal-derived ingredients. This not only includes the product ingredients but the probiotic inoculum as well. Probiotic starter cultures are traditionally grown and stored in media containing milk or meat-derived ingredients. The presence of these ingredients makes the probiotic cell concentrates unsuitable for use in vegetarian products and thus creates the need for a growth medium which is

free from animal-derived ingredients. This particular study investigated the growth of a strain of *Lactobacillus lactis* in MRS. The resulting invention relates in general to a bacterial culture media, and more specifically a complex microbial culture media, based on plant seed powder or extract in place of animal extract for probiotic bacterial growth [15].

The present invention proposes a probiotic starter culture growth medium comprised of vegetal nitrogen and carbon source. Animal extract has been replaced by plant seed powder. The growth of probiotics was much enhanced in our newly developed culture medium; TM 2 (which is lentil). When the experiments were carried to see the colony forming units (CFU), it was observed that growth of probiotic was much enhanced in our culture medium than standard MRS.

METHODS:

Pure culture of *Lactobacillus lactis*, a probiotic, was obtained from National Dairy Research Institute (NDRI), Karnal, India. This probiotic was grown in standard MRS culture medium as well as our test mediums containing various plant seed powders as vegetal nutrition source in place of beef extract, peptone, and yeast extract as in the case of MRS. Where TM 1, TM2, TM3, TM4, TM5, and TM6 were mung beans, lentil, chickpea, peanut, Bengal gram and wheat respectively germinated for 24, 48 and 72 hours. Sample seeds were soaked for 8 hours and then for germination it is germinated further for 24, 48, and 72 hours after soaking. Soaked and Germinated seeds were dried at 45°C and then powdered. Powder was used as respective sample. Pure culture of *Lactobacillus lactis* was used as inoculums as per instructions given on the culture. The inoculated culture mediums were incubated at 37°C for 72 hours and growth of probiotic is recorded at regular intervals. The growth was recorded as Colony Forming Units (CFUs) in various media. The experiments were carried out thrice to come to a conclusion.

The following ingredients were dissolved in 850ml of distilled water in Test media, and pH was adjusted to 6.5 for each of these:

10g respective seed powder

20g Glucose

1g Tween-80

2g K₂HPO₄

5g Na-acetate

2g (NH₄)₂ citrate

0.2g MgSO₄-7H₂O

0.05g MnSO₄-H₂O

1% Pure Agar

Test culture media were then autoclaved. Various serial dilutions were prepared.

RESULTS:

The results of the previously described experiments are depicted in the following figures. The tables shown below contain data for varying colony forming units.

Table-1: CFU for Probiotic, Lactobacillus Lactis in Absolute

	MRS	T.M-1	T.M-2	T.M-3	T.M-4	T.M-5	T.M-6
24 hrs	4	1	3	11	0	2	1
48 hrs	6	3	7	12	1	5	4
72 hrs	15	3	17	21	2	9	6

This table shows most undiluted CFU growth occurred at the 72 hour point. According to this table, T.M-2 and T.M-3 were most productive; with 17 and 21 units, respectively, formed at 72 hours.

Table 2. CFU for Probiotic, Lactobacillus Lactis in dilution up to 10^{-4}

	MRS	T.M-1	T.M-2	T.M-3	T.M-4	T.M-5	T.M-6
24 hrs	3	0	7	3	0	4	0
48 hrs	5	7	15	6	1	7	5
72 hrs	8	15	19	8	1	8	5

When diluted to 10^{-4} , T.M-1 and T.M-2 demonstrated the most successful growth of colony forming units. At 48 hours, T.M-1 had 7 formed units, and T.M-2 had 15. At 72 hours, the units in these media had increased to 15 and 19, respectively.

Table 3: CFU for Probiotic, Lactobacillus Lactis in dilution up to 10^{-5}

	MRS	T.M-1	T.M-2	T.M-3	T.M-4	T.M-5	T.M-6
24 hrs	11	0	4	1	0	5	1
48 hrs	13	2	9	3	0	6	3
72 hrs	15	2	17	3	0	8	5

Table 3 proves the consistent growth of CFU's was most fruitful in T.M-2.

Table 4: CFU for Probiotic, Lactobacillus Lactis in dilution up to 10^{-6}

	MRS	T.M-1	T.M-2	T.M-3	T.M-4	T.M-5	T.M-6
24 hrs	4	2	6	3	2	2	0
48 hrs	4	5	11	7	6	4	4
72 hrs	7	7	15	11	7	6	7

Based on Table 4, T.M-2 and T.M-3 were both found to be fertile when diluted to 10^{-6} . The final measurement at 72 hours shows 15 units in T.M-2 and 11 in T.M-3.

Table 5: CFU for Probiotic, Lactobacillus Lactis in dilution up to 10^{-7}

	MRS	T.M-1	T.M-2	T.M-3	T.M-4	T.M-5	T.M-6
24 hrs	10	1	6	2	1	3	0
48 hrs	11	3	11	7	3	5	1
72 hrs	13	7	13	7	7	6	3

Table 5 sustains the assumption that T.M-2 provided the most consistently productive growth at all three stages in all dilutions.

This series of experiments establishes the conclusion that T.M-2 proved to be the most productive test medium containing various plant seed powders. We also observed that T.M-2 was most effective when diluted to no more than 10^{-4} .

Figures 1-5, shown below, provide an alternate view of the growth which occurred in the various conditions created in our experiment.

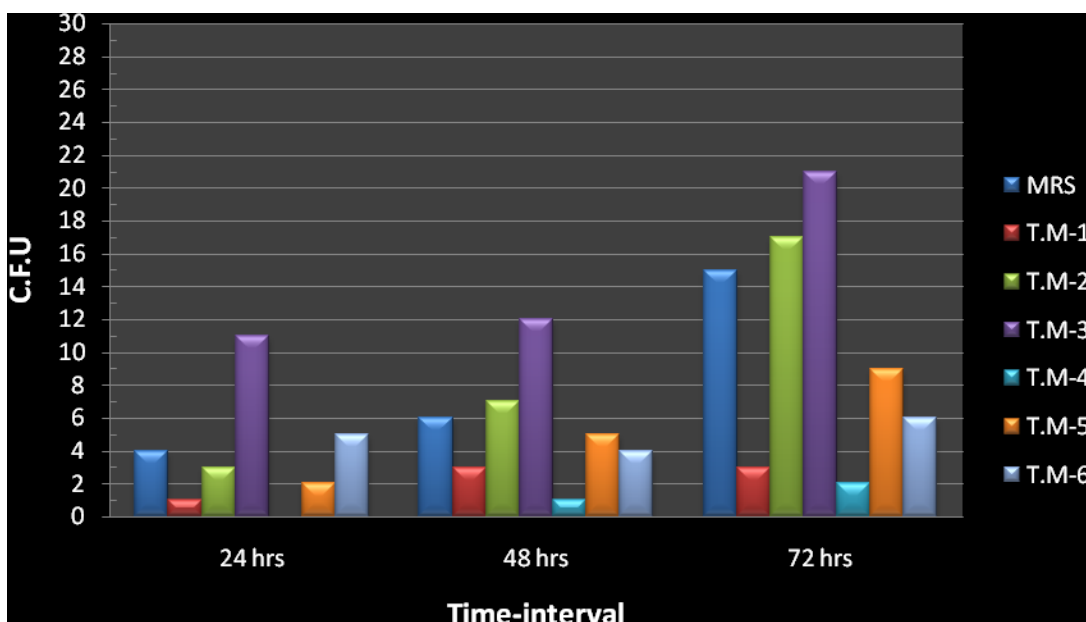


Figure-1: CFU for Probiotic, Lactobacillus Lactis in Absolute

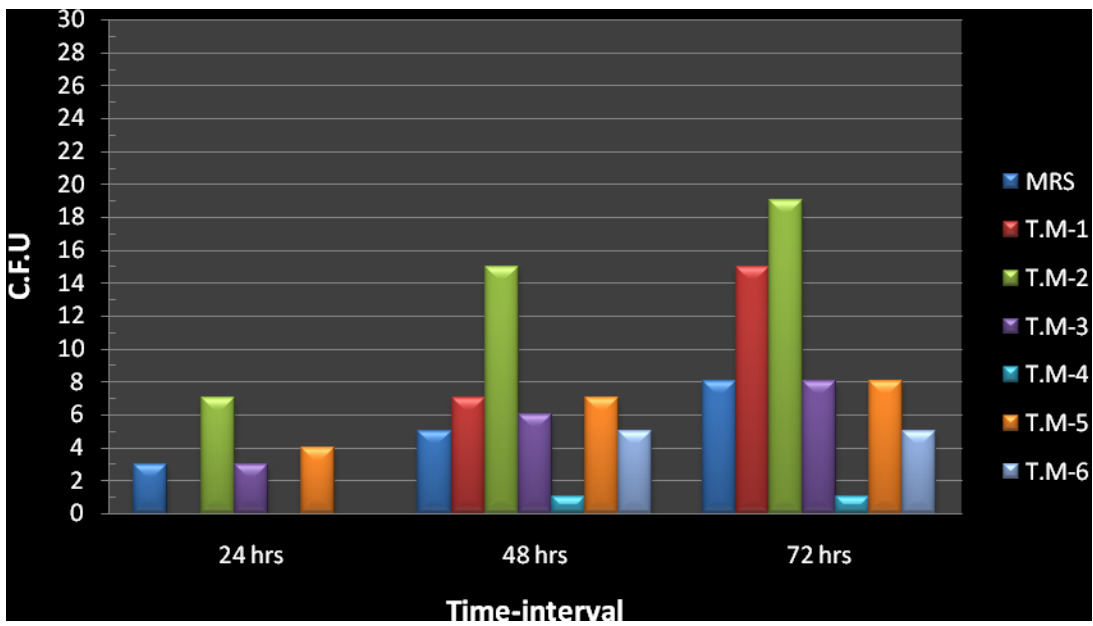


Figure-2: CFU for Probiotic, Lactobacillus Lactis in dilution up to 10^{-4}

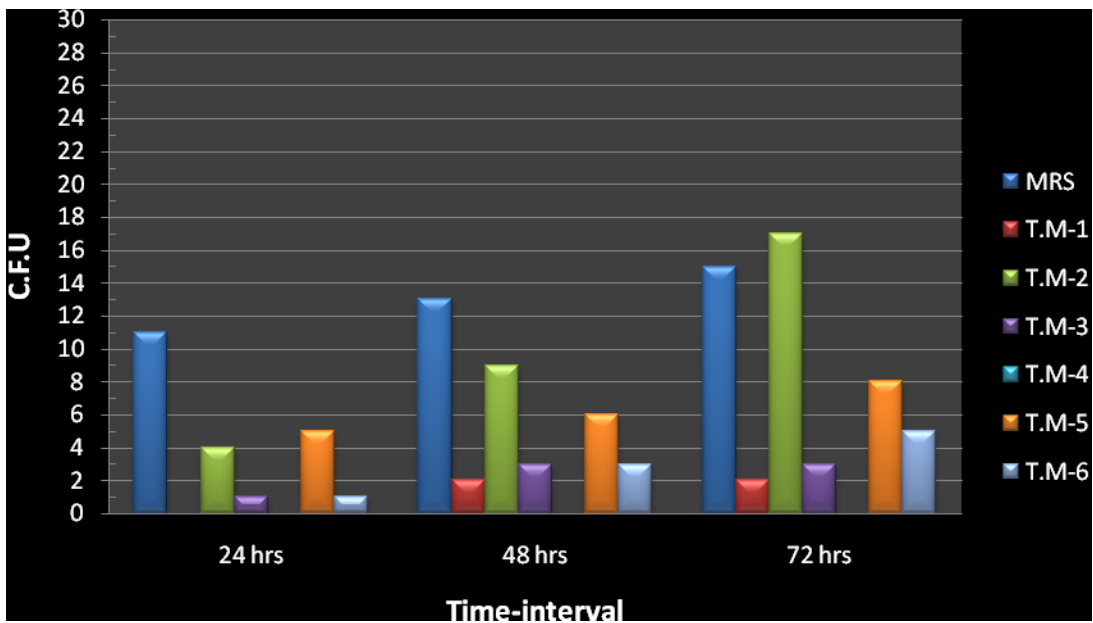


Figure-3: CFU for Probiotic, Lactobacillus Lactis in dilution up to 10^{-5}

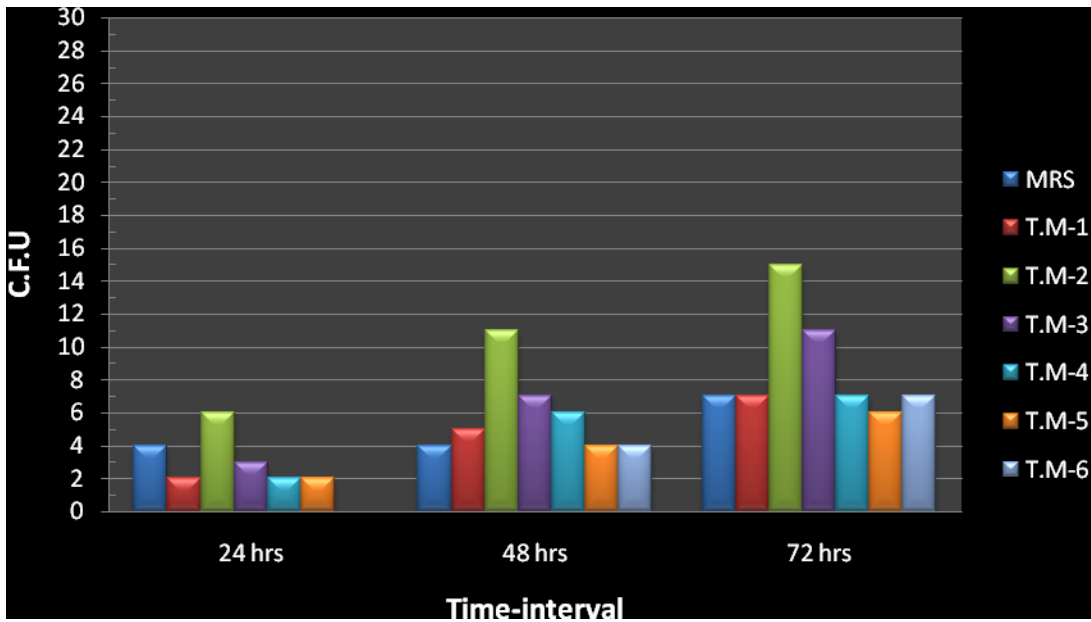


Figure-4: CFU for Probiotic, Lactobacillus Lactis in dilution up to 10^{-6}

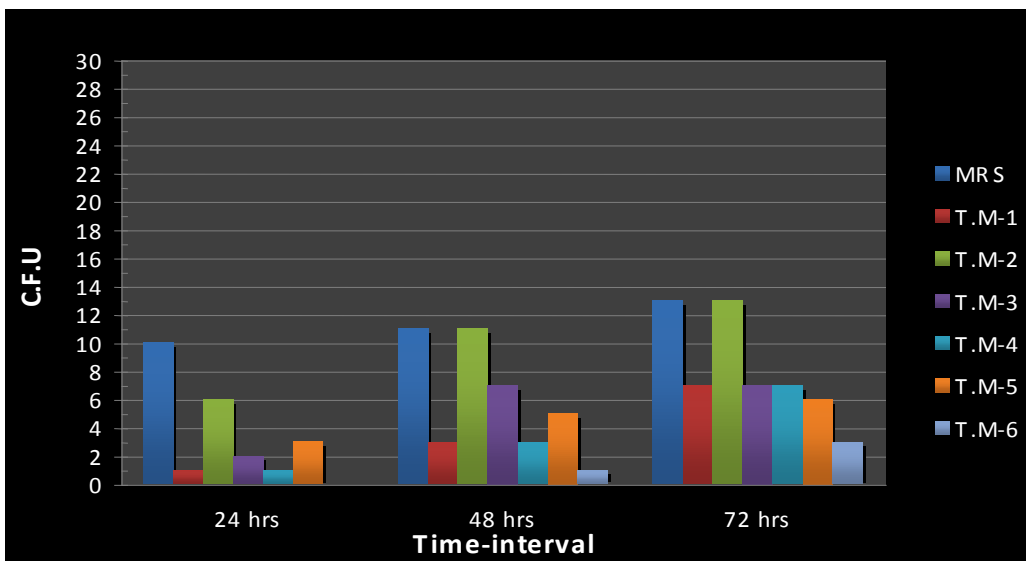


Figure-5: CFU for Probiotic, Lactobacillus Lactis in dilution up to 10^{-7}

The best growth of probiotic is observed in TM 2; seed germinated for 72 hours. Starter culture mediums for probiotics or other bacteria primarily contain nitrogen source from animals. This invention provides an effective vegetal culture medium.

DISCUSSION:

Use of plant seed powder-like jack fruit seed powder for the growth of the fungal culture of Monascus purpureus for pigment production has been reported by Babitha et al. (2006) [16]. The

use of plant seed powder for the microbial production of enzymes has been reported in few of the publications. Tamarind seed powder (TSP) was tested for the production of tannase under solid-state fermentation (SSF) using *Aspergillus niger* ATCC 16620 [17- 18]

Our study investigated the use of alternative nutrition sources containing a variety of plant seed powders to grow the probiotic *Lactobacillus lactis* alongside the standard de Man Rogosa Sharpe culture medium. According to the results of our experiments, our test medium containing lentil seed powder proved to be the most fertile source for the growth of colony forming units compared to the other media tested. In addition, the most effective length of germination appeared to be 72 hours. Our experiments showed the greatest number of colony forming units existing at 72 hours when the test media was diluted up to 10^{-4} .

Based on our experimental data, we can conclude that when diluted to 10^{-4} , TM 2 was the most productive medium at the 72 hour mark.

Another study aimed to develop a solid culture medium for differential isolation of the probiotic strain *Lactobacillus casei* Shirota (LcS) and for selective cultivation of lactobacilli present in oral samples. Type strains of lactobacilli and isolates from commercial probiotic products were inoculated onto modified de Man Rogosa Sharpe agar (termed 'LcS Select'), containing bromophenol blue pH indicator, vancomycin and reducing agent L-cysteine hydrochloride for differential colony morphology development. *L. casei* Shirota cultured on the novel medium produced distinctive colony morphologies, different from other lactobacilli tested [19].

Vegetarian probiotic foods by definition must be free from all animal-derived ingredients. This not only includes the product ingredients but the probiotic inoculum as well. Probiotic starter cultures are traditionally grown and stored in media containing milk or meat-derived ingredients. The presence of these ingredients makes the probiotic cell concentrates unsuitable for use in vegetarian products and thus creates the need for a growth medium free from animal-derived ingredients. The present invention proposes a probiotic starter culture growth medium comprised of vegetal nitrogen and carbon source.

The absolute vegetarian culture medium containing TM 2 is better than standard MRS for the growth of probiotics. In all experiments, alternative test media derived from plant seed powders demonstrated better CFU growth than in standard MRS culture medium in various dilution levels. This research provides an alternative and efficacious vegetal culture medium. The culture medium developed in the present invention will provide a pure vegetarian way to achieve pure vegetarian products for the strictly vegetarian human population. In countries like India, people of certain religion are seriously concerned about such issues. The developed culture medium will be a great relief for such communities.

Since CFU growth was still evident at the 72 hour point, further studies should be conducted to investigate the most effective germination period.

CONCLUSION:

1. In all experiments, alternative test media derived from plant seed powders demonstrated better growth of colony forming units (CFU) than in the standard de Man Rogosa Sharpe (MRS) culture medium.

2. Consistent growth occurred in all culture media, including the standard MRS culture medium as well as test media, at each dilution level.
3. In this series of experiments, the most productive germination period was 72 hours.
4. The test media containing lentil seed powder (TM-2) demonstrated the most consistent growth of colony forming units overall.

Competing interests: The authors declare that they have no competing interests.

Authors' contributions: Dr. Pathak is the principal investigator for the study, provided experiments, and was involved in the writing of this manuscript; Dr. Martirosyan provided additional analysis and discussion of the results, and assisted in writing this manuscript.

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