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An Effective Growth Medium for Probiotics for Strict Vegetarians

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Abstract: Development of MMRS (modified MRS Agar) culture medium; the absolute vegetarian culture medium containing plant seed powder in place of beef extract for the growth of probiotics. Vegetarian probiotic foods by definition must be free from all animal-derived ingredients. This research provides an alternative efficacious, cost effective vegetal culture medium. The culture medium developed in present invention will provide a pure vegetarian way to achieve pure vegetarian products for strictly vegetarian human population. In countries like India people of certain religion are seriously concerned about such issues. Present study investigated the growth of a pure culture strain of *Lactobacillus lactis*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Bifidobacterium* strain 231 and *Bifidobacterium* strain 234 provided by National Dairy Research Institute (NDRI), Karnal (Haryana, India), in MRS and MMRS. The developed culture medium is better than standard MRS and this will be a great relief for such communities.

Key words: Probiotics · MRS · Culture · Seed Powder · Vegetarian

INTRODUCTION

Probiotics are beneficial microorganisms that protect the host against diseases and defined as "live microbial feed supplements which beneficially affect the host by improving its intestinal microbial balance [1]. High level of cholesterol could be removed by *Lacobacillus species*. [2]. The market for probiotics continues to grow as awareness of their health benefits increases, together with their scientific backing. Some of the 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 the interaction with the immune system, anti-cancer potential and potential as a bio therapeutic agent in cases of antibiotic-associated diarrhoea, travellers' diarrhoea, pediatric diarrhoea, inflammatory bowl diseases and irritable bowl syndrome [3].

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. A study investigated the growth of two strains of *Lactobacillus acidophilus* (MJLA1 and La-5), *L. paracasei* ssp. *paracasei* (LCSH1 and 01) and *Bifidobacterium lactis* (BDBB2 and Bb-12) in five vegetal media. Media containing 25 g/L soy peptone, yeast extract and glucose monohydrate gave the most desirable results with the strains examined [4-5]. Selective media for specifically for *Bifidobacterium* was developed where lactose was the main carbon source [6].

A study evaluated the use of the media M-MRS, MRS-NNLP and RCPB pH5 in counting the number of *Bifidobacterium animalis* subsp. *lactis* in the presence of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus* after yoghurt fermentation [7]. Single and mixed cereal substrates were fermented with lactic acid bacteria to study and compare the effect of the media formulation on fermentation parameters. Three cereal flours namely malt, barley and barley mixed with malt (barley-malt) were selected and fermented with two probiotic strains: *Lactobacillus plantarum* (NCIMB 8826) and *Lactobacillus acidophilus* (NCIMB 8821). The effect of the single and mixed cereal flour suspensions on the fermentation of these two strains of lactic acid bacteria were studied at an incubation temperature of 30 °C for 28 h and was found that the growth was enhanced in media containing malt [8]. It was also observed that increased concentration of oregano had a positive effect on the growth of probiotic bacteria [9].

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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. This study aimed to modify de Man Rogosa Sharpe culture medium (MRS) so that it should be suitably used for strict vegetarian human populations. In this modified MRS (MMRS); animal extract has been replaced by plant seed powder. The seed used is *Lens culinaris,* 24 hour germinated, dried and then powdered, for selective cultivation of probiotics strain for the use of strictly vegetarian human population. The present invention proposes a probiotic starter culture growth medium comprising of vegetal nitrogen and carbon source. The present 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. The growth of probiotic was much of equivalent rate in our new developed culture medium MMRS.

MATERIALS AND METHODS

Pure culture strains of *Lactobacillus casei*, *Lactobacillus plantarum*, *Bifidobacterium* strain 231 and *Bifidobacterium* strain 234 provided by NDRI, Karnal, India; were cultured in MRS and in MMRS. For preparing 12 plates of MRS Agar: Dextrose 20g, Peptone 10 gms, Beef extract 8 gms, Yeast extract 4 gms,, Manganase Sulfate 0.05g, Magnesium Sulfate 0.2g, Sodium acetate 5g, Dipotassium phosphate 2g, Ammonium Citrate 2 g, Tween 80 1g, Bacteriological Agar 10 g; suspended in 1 liter of distilled water, mixed well and dissolved by heating with frequent agitation. Sterilized in Autoclave at 121°C for 12 minutes; cooled to 45° C, mixed well and then dispensed into plates. The plates were labelled as BLc, BLp, BBa, BBb, BLc8, BLp8, BBa8, BBb8, BLc24, BLp24, BBa24 and BBb24 for *L. casei*, *L. planatarum*, *Bifidobacterium* strain 231, *Bifidobacterium* strain 234, *L. casei* and 8 hr soaked seed sample, *L planatatum* and 8 hr soaked seed sample, *Bifidobacterium* strain 231 along with 8 hr germinated seeds sample, *Bifidobacterium* strain 234 along with 8 hr germinated seeds sample, *L. casei* along with 24 hr germinated seeds sample, *L. planatarum* along with 24 hr germinated seeds sample, *Bifidobacterium* strain 231 along with 24 hr germinated seeds sample, *Bifidobacterium* strain 234 along with 24 hr germinated seeds sample. For preparing 12 plates of MMRS beef extract, peptone and yeast extract were together replaced by 10 gms of 24 hr germinated seed powder and plates were labeled as CLc, CLp, CBa, CBb, CLc8, CLp8, CBa8, CBb8, CLc24, CLp24, CBa24 and CBb24. After solidification of the medium, 0.1ml of specific pure culture inoculums was spread through sterilized glass rod in case of Blc, Blp, Bba, Bbb, Clc, Clp, Cba, Cbb plates. In 0.1ml of specific pure culture inoculums added with 0.1 ml of 10^{-4} diluted 8 hours soaked seed powder was spread in case of BLc8, BLp8, BBa8, BBb8, CLc8, CLp8, CBa8, CBb8 and 0.1ml of specific pure culture inoculums added with 0.1 ml of 10^{-4} diluted 24 hours germinated seed powder was spread in case of BLc24, BLp24, Bba24, Bbb24, Clc24, Clp24, Cba24, Cbb24. Turned the plate 90 degrees and repeated the side to side, up and down streaking. Turned the plate 45 degrees and streaked for third time. Plates were incubated at 37ºC overnight for growth. Colony Forming Units (CFU) was counted for Day1, Day2 and Day3 and was used as the growth parameter.

RESULTS AND DISCUSSIONS

Germinated Seeds of Lentils Replace Beef Extract Effectively in MRS: The microbial growth was better observed in MMRS as compared to MRS. Observations clearly indicate that germinated lentil seed powder can be an effective alternative to beef extract, peptone and yeast extract (Table 1). This provides an opportunity to have strict vegetarian functional foods and nutraceuticals to strict vegetarian human society. The growing understanding between probiotics and health has increased the demand for probiotics. Use of probiotic microflora was promising areas for the development of functional foods. Probiotics in the form of substances containing *lactobacillus, bifidobacterium* and *acidophilus* cultures had been used for centuries to preserve food [10]. Individual microbial strains inhabit certain sections of GI tract, where they digest certain sugars, proteins or fats. Specific germination of 24 hrs of *Lens culinaris* (lentils) seeds contributes towards the growth of probiotics (Fig. 1 and Fig. 2). Organic wastes were used for production of bacteriocin using *Lactococus lactis* [11]. Cereal based probiotics beverages were produced using *L. acidophilus* and *B*. BB-12. [12]. A probiotically and prebiotically active pet food comprising a germinated seed or grain was developed. The pet food is preferably formulated as a dry baked biscuit or a semi-moist biscuit. It provided a nutritious pet food with prebiotic and probiotic benefits [13].

Table 1: Comparative CFU growth in Standard MRS and MMRS

	CFU/DAY1		CFU/DAY2		CFU/DAY3	
Bacterial Strain	MRS	MMRS	MRS	MMRS	MRS	MMRS
B^{Lc} / C^{Lc}	0	0	152	160	605	650
B^{Lc8} / C^{Lc8}	54	45	397	450	780	800
B^{Lc24}/ C^{Lc24}	150	190	1010	1500	2000	2500
B^{LP} / C^{Lp}	0	0	168	190	715	705
B^{LP8} / C^{LP8}	49	42	397	452	795	845
B^{LP24}/ ^{LP24}	145	205	950	1050	1800	2000
B^{Ba} / C^{Ba}	0	0	190	205	715	750
B^{Ba8} / C^{Ba8}	65	56	450	560	850	890
B^{Ba24} / ^{Ba24}	210	305	1100	1200	2500	2750
B^{Bb} / C^{Bb}	0	0	185	198	750	810
B^{Bb8} / C^{Bb8}	59	52	470	530	815	900
B^{Bb24} / ^{Bb24}	250	300	1200	1500	2350	2550

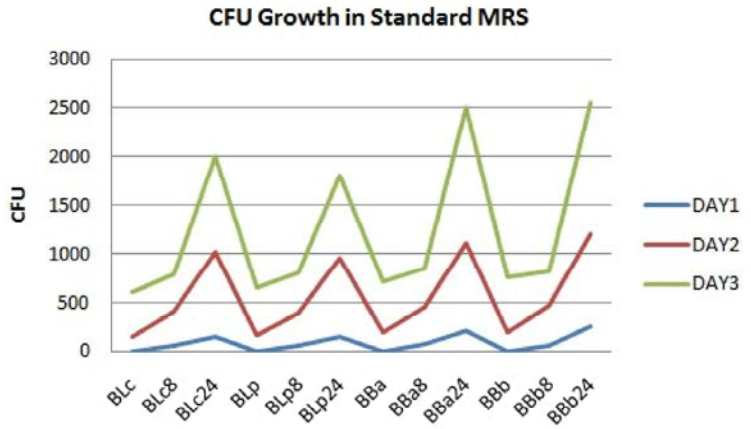

Fig. 1: Probiotic Bacteria Growth in Standard MRS Medium.

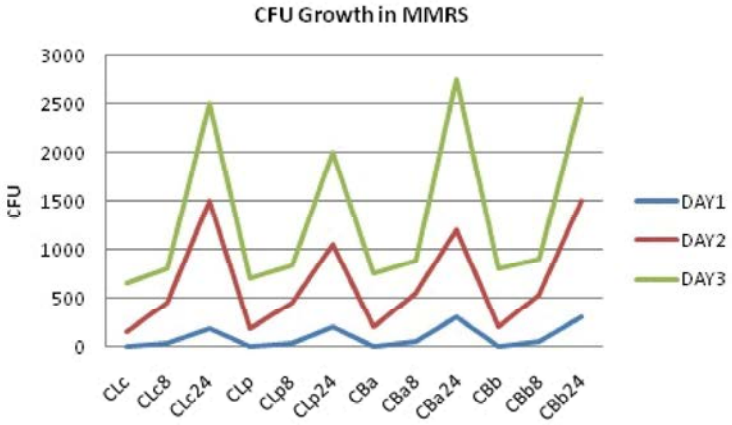

Fig. 2: Probiotic Bacteria Growth in MMRS Medium

Germinated *Lens culinaris* as Prebiotic Food Matrix: Factors affecting microbial viability are hydrogen peroxide, oxygen level, pH, post acidification and food matrix. Glucose, fructose and sucrose gradually increased during germination of *Lens culinaris* seeds [14]. Food matrix acts as prebiotics. Stachyose, maltose and raffinose of germinated *Lens culinaris* might help the probiotic growth apart from ROS and Hydrogen peroxide [15]. Prebiotics are the non digestible dietary components that stimulate the proliferation of probiotics.

The low molecular weight carbohydrates (LMWC) as maltose, glucose, sucrose and fructooligosaccharide (FOS) are available in germinating seeds. When the seeds imbibe water hydrolytic enzymes are activated. Alpha-amylase was released in embryo of the seeds during germination process and with the aid of an alpha-amylase; starch is broken down to produce a mixture of one-, two-, or three-monomer-long molecules (glucose, maltose and maltriose). This is a competitive advantage for the probiotic, if it is consumed with prebiotic. Prebiotic is one of the most promising functional foods as a component presented in foods. Prebiotics contribute health benefits by promoting the growth of beneficial bacteria (probiotics) [16].

Rise in Intracellular ROS in Germinating Seeds: Reactive oxygen species (ROS) are produced from embryogenesis to germination, i.e., in metabolically active cells. ROS accumulation can therefore be also beneficial for seed germination and seedling growth by regulating cellular growth, or controlling the cell redox status. Reactive oxygen species (ROS) derivate from the reduction of oxygen which gives rise to superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO^-) and singlet oxygen ($^1O_2$) [17]. The primary factor responsible for aerobic growth inhibition is proposed to be the production of hydrogen peroxide (H_2O_2) in the growth medium. A H_2O_2-forming NADH oxidase was purified from O_2-sensitive *Bifidobacterium bifidum* and was identified as a *b*-type dihydroorotate dehydrogenase. The kinetic parameters suggested that the enzyme could be involved in H_2O_2 production in highly aerated environments [18]. pH of H_2O_2 is about 6.2 thus it is compatible to the growth of probiotics. The rise in intracellular ROS in germinating *Lens culinaris* seeds might constitute an environment favorable to anaerobes. Since catalase is an enzyme commonly found in aerobes and facultative anaerobes but is absent in almost all obligate anaerobe including bifidobacteria [19, 20]. Eukaryotes have catalase and glutathione peroxidase (GPX) as H2O2 decomposing system to survive under aerobic condition (20, 21, 22). Little is known about GPX activity in prokaryotes and GPX gene and glutathione synthesize pathway have not been detected in obligate anaerobes including bifidobacteria (22, 23). NADH peroxidase was found in bifidobacteria and some types of peroxidases were predicted, including thiol peroxidase, alkyl hydroperoxide reductase and peptide methionine sulfoxide reductase. These findings indicate that germinated seeds can well be used as prebiotics and MMRS developed in this study can be a relief to strict vegetarians.

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