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Edible Growth Medium: A New Window for Probiotic Research

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Abstract

Conventional growth media for microbes contains lots of non-edible components that are harmful to consume when the organism is ready to intake—like probiotics growing in chemical media (*Lactobacillus* grown in MRS media). The study was conducted to develop an edible and low-cost growth media that supports the growth of probiotics Lactic Acid bacteria for the enhancement of probiotic research. The 04 isolates of *Lactobacillus* (*L. plantarum*, *L. rhamnosus*, *L. ferciminis* and *L. bifarmentans)* and 02 *Bifidobacterium (B. infantis* and *B. Bifidum)* were used for the evaluation of medium efficacy. To formulate the edible culture media, 04 vegetables and 02 pulses were used. The media was formulated in different formulations. For *Lactobacillus*, maximum growth was observed at MM-2 media formulation that was about 15.62 log CFU/ml for *L. plantarum* and compared to MRS media that was 11.78 log CFU/ml. *Bifidobaterium* showed the highest viability at MW-1 edible media formulation which was about 12.72 log CFU/ml whereas in Bifidobacteria selective media the cell viability was 12.48 log CFU/ml. The edible media has no toxic or unhealthy effect on mice while trailing in an animal model and shows excellent results in encapsulation with alginate. In comparison with the performance of traditional chemical media, the formulated media were found to be cost-effective and safe for human consumption.

Keywords

Edible Media, *Lactobacillus*, Probiotic, Low Cost, Mass Culture

1. Introduction

A growth medium or culture medium is a solid, liquid or semi-solid designed to

support the growth of microorganisms [1]. Culture media contains all the elements that most bacteria require for growth and are not selective, so they are used for the general cultivation and maintenance of bacteria kept in laboratory culture collections [2]. An undefined medium (also known as a basal or complex medium) contains a carbon source such as glucose, various salts, a source of amino acids and nitrogen like beef and yeast extract [3] [4].

Growth media provides minimized cell damage, suitable for screening and maximum viability of cells. The cost of media is also an important consideration. Commercially available media are not only costly but also non-consumable [5]. Peptones are the most widely used source of nitrogen in microbial media [6]. Some are made by cooking milk or meat products (beef, pork) in acid, but most are made by incubating milk or meat with trypsin, pepsin, or other proteolytic enzymes to digest the protein to a mixture of amino acids, peptides, and polypeptides [7] [8] [9]. On the other hand, these microbial growth media are highly demanded in developing microbiological research. The most important application of these media is in the production of commercially available fermented food products, especially dairy products [10] [11]. Before starting the fermentation of food products, the required microbial cells are subjected to a pre-culture cultivation [12]. Then the organisms are separated from media and introduced into fermentation. For this reason, the constituents of the media are very important as, if it contains any component that is harmful to human, it may come across the fermented food through the starter culture. Lactobacillus are often used as probiotics, in dairy food products that are grown in mainly De Man Rogosa and Sharpe Agar (MRS agar) which contains manganese sulfate and magnesium sulfate. These components can cause irritation in skin, respiratory system and in long term repeat it may cause organ damage [13].

Probiotics are defined as live microorganisms that provide health benefits when consumed [14]. Probiotics have to be alive when administered. These microbes are thought to help restore the natural balance of bacteria in the gut (including stomach and intestines) when it has been disrupted by an illness or treatment. But, these probiotics are not easy to produce on a large scale [1] [15]. It needs several steps in recovery stages such as centrifugation, filtration, formulation, drying etc. which increase the production cost. Moreover, the viability of bacteria is often reduced during these recovery steps [16] [17]. So, it was a little attempt to prevent the loss of cell count.

Meanwhile, the numbers of viable micro-organisms are recommended for the aptitude of probiotic foods and for this reason, the challenges of maintaining viability and activity of probiotic cultures in foods to the end of shelf-life are two important criteria that must be fulfilled to provide effective probiotic food products for general consumption [18]. Dried concentrated probiotic cultures are the most suitable form for inoculation into functional foods [19], given the ease of storage, handling and transport, especially for shelf-stable functional products. The challenges associated with the introduction and maintenance of

high numbers of viable probiotic cultures into foods include the form of the probiotic inoculum used, process conditions, reconstitution conditions, ability of the probiotic culture to grow and retain viability in the food environment and maintenance of probiotic characteristics in the food product through to the time of consumption [20] [21].

All these problems can be reduced if the probiotics are grown in human consumable media [22]. This study was about to develop an edible media that can support the growth of both probiotics and other non-fastidious bacteria at a low cost. Probiotics, grown in this media, do not need to go through that conventional method of recovery. Instead of these, viable cells with edible broth can be directly inoculated into raw material. Moreover, this media has been formulated with inexpensive vegetables as the source of carbohydrates and minerals and pulses as the source of nitrogen [23].

Pulse 1 and pulse 2 are the very good source of protein (nitrogen source) and carbohydrate (carbon source). Pluse 1 contains 32.98% carbohydrate and 33.33% protein in dry conditions. Pulse 2 is a good source of protein, but it is also a great source of carbohydrates [24]. It has 68.8% carbohydrate and 9.82% protein in a dry state. These nitrogen and carbon sources were replaced with peptone, beef extracts and glucose in chemically defined media [6].

Vegetables are a good source of minerals and vitamins need to grow bacteria. These vegetables contain mostly protein, carbohydrate, vitamins (vitamin C, thiamin, riboflavin, niacin, vitamin B6, folate) as well as minerals such as sodium, potassium, magnesium, phosphorus etc. [25]. A high content of potassium present in veg 2 & veg 3. Veg 1 and veg 4 provide a large amount of phosphorus. Veg 2 and veg 3 are a great source of sodium and so on. All these ingredients are available at the local fresh market.

2. Materials and Method

2.1. Materials

The microorganisms of *Lactobacillus* (*L. plantarum*, *L. rhamnosus*, *L. ferciminis* and *L. bifarmentans)* and *Bifidobacterium (B. infantis* and *B. Bifidum)* were taken from Industrial Microbiology Laboratory, Institute of Food Science and Technology, Bangladesh council of Scientific and Industrial Research, Dhaka. All the raw materials were collected from the local market. The dehydrated culture media and other chemicals were purchased from Himedia (India), Merck (Germany) and Sigma (USA). The experiments were conducted in Industrial Microbiology Laboratory, IFST, BCSIR, Dhaka from Jan/2018 to Dec/2018 repeatedly. The animal test was performed in animal house research section, IFST.

2.2. Methods

2.2.1. Raw Material Processing

Fresh four vegetables were sliced and blanched at 85˚C for 15 minutes. The veg-

etables and pulses were then dried at 72˚C using a hot air oven. All these ingredients blended with electric blender separately and sieved to get a fine powder with diameter of 180 - 330 μm.

2.2.2. Formulation of the Base Medium for Mass Culture

Respective amount of vegetable and pulses powder was mixed thoroughly to develop a basal medium in comparison to synthetic commercial media compositions. There are two types of formulations 1) with minerals (MM) and 2) without minerals (MW) and the ingredients were added in different proportions to formulate the edible medium.

The process is given below in a flow chart.

Base media powder + water
↓
Boiled at 100˚C for 25 - 30 mins
↓
Homogenized for 5 mins
↓
Filtrated
↓
Add mineral salts (optional)
↓
Mixed by homogenization and autoclave at 121˚C at 15 psi for 15 mins

Sodium acetate, dipotassium hydrogen phosphate, ammonium citrate minerals were used for media formulation. Sodium Acetate and Ammonium Citrate inhibits *Streptococci*, molds, and other oral microbial flora and restrict swarming [26]. Dipotassium Hydrogen Phosphate is the buffering agent.

2.2.3. Inoculation of Probiotic Bacteria into Media for Cell Count

All the freshly cultured isolates were inoculated into McCartney bottle with different edible broth formulations and incubated in the anaerobic jar (Oxiod) at 37˚C for 24 hrs. MRS broth for *Lactobacillus* and Hichrome Bifidobacterium broth for Bifidobacteria were used as a control. Each of the cultured media (1 ml) with probiotic bacteria was transferred to 9 ml of sterile Ringer's solution (solution of several salts dissolved in water for creating of isotonic solution) followed by serial dilution, plated and incubated at 37˚C in anaerobic condition for 48 hrs. Then cells were counted and compared with edible broth and commercial culture broth. The process was repeated for triplicate.

2.2.4. Measuring of Optical Density

All cultural broth after 24 hrs incubation was taken for the measurement of OD by spectrophotometer at 600 nm wavelength.

2.2.5. Mass Culture

MW (medium without minerals) and MM (medium with minerals) formulated

media were prepared according to the composition as previously described. 1% fresh culture of isolates inoculated into 1 L of edible broth and incubated at 37˚C for 24 hrs anaerobically.

2.2.6. Determining the Growth Curve of *Lactobacillus*

The growth curve of *L. plantarum* was observed to determine the log, lag and the stationary phase of the isolate. The overnight incubated fresh culture was inoculated into MRS broth at 37˚C anerobacally. The cell count was performed every hour with ringer's solution as a diluent and plated onto MRS agar. The plates were then incubated at 37˚C under the anaerobic condition for 24 hrs and then the number of colonies was counted. The graph was plotted cell count against time to observe the growth pattern.

2.2.7. Freeze Drying

The cultured isolates along with 7% skim milk and 0.3% sodium alginate were added and the suspension was kept in −20˚C overnight. The freeze-drying process was started to obtain a dry powder containing the probiotic bacteria using Alpha 1 - 2 LD Plus (Germany) freeze dryer. The bacterial cell was enumerated before and after the freeze-drying process and expressed as CFU/ml.

2.3. Animal Trials

Experimental protocols for animal trail approved by BCSIR institutional ethical review committee and followed while performing the research with mice. Eight healthy swiss albino male mice of 6 - 7 weeks of age were taken. They were divided into 2 groups:

- A control group that contains 3 mice.
- A sample group that contains 5 mice.

The mice were kept at 25˚C temperature.

2.3.1. Feeding

Each mouse was served 20 gm of regular feed. The control group was served only regular feed (total 60 gm). The sample group was served (total 100 gm) regular feed mixed with 100 mg sample powder to each mice (total 500 mg). Each gram of sample feed contained at least 8 log CFU of bacterial culture. This experiment was carried out for 10 days straight. During this trial, all mice were observed carefully by their locomotor behavior and physical parameters- stool, urine, water consumption, body temperature.

2.3.2. Bodyweight Measurement

Body weights of all these mice were taken before starting feeding. After 10 days trial, again their body weights were measured.

3. Results and Discussion

The nutritional value of dried raw materials was calculated from the nutritional value of these ingredients according to USDA National Nutrient Data [25] in

Table 1.

3.1. Viability of *Lactobacillus* Isolates in Edible Media

3.1.1. In the Case of Different Media Formulation

The isolates were cultured triplicate in MRS media and MM-1, MM-2, MM-3, MM-4 formulations. All four isolates showed more growth in edible media compared with MRS media selective for *Lactobacillus*. Among them, the viability of the isolates was most high in MM-2 media formulation. Then the viability has decreased linearly through the formulations of nutrients were increased. This was most likely for the reason of increased osmotic stress exacted by the carbon and nitrogen source of the media [27] [28]. All the isolates were not grown equally in edible media. *L. plantarum* and *L. bifermentum* were showed a high viability count. Among them, *L. rhamnosus* showed a slow growth and the least viability in edible media (Figure 1).

3.1.2. In the Case of Comparison of Growth

MRS medium was designed to favor the luxuriant growth selectively for *Lactobacilli* for lab study. Though the isolates grow at MM-2 formulation of edible media, the viability was compared with standard culture maintained in MRS broth [2] [29]. The results showed that edible media has a better ability to grow the isolates than in MRS media. Log numbers were estimated and it was observed

Table 1. An estimation of the nutritional value of the ingredient used in this experiment.

Raw materials/Ingredients	Pulse 1 (100 gm dw)	Pulse 2 (100 gm dw)	Veg 1 (100 gm dw)	Veg 2 (100 gm dw)	Veg 3 (100 gm dw)	Veg 4 (100 gm dw)
Carbohydrate, gm	32.98	68.8	81.26	79.16	62.12	72.5
Protein, gm	36.49	27.25	12.5	7.76	24.0	16.0
Sodium, mg	2.18	6.54	12.5	575	375.0	225.0
Potassium, mg	1964.78	738.28	4250.0	2666.6	3737.6	2125.0
Magnesium, mg	306.14	51.26	150.0	100.0	187.6	150.0
Phosphorus, mg	769.74	306.44	550.0	291.64	550.0	325.0

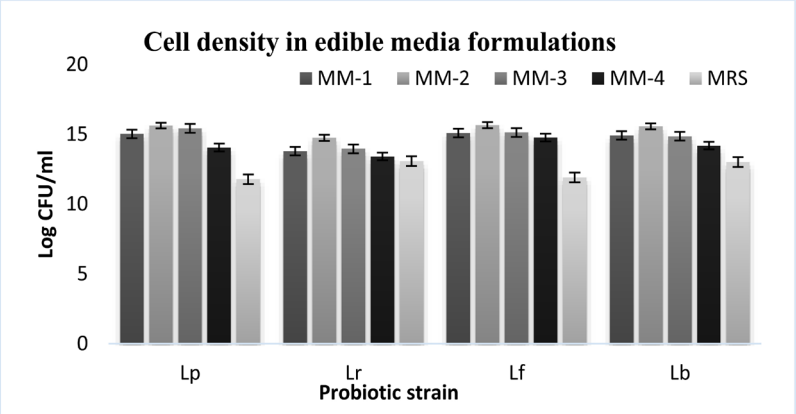

Figure 1. The log number of *Lactobacillus* in different media formulations.

that all the four *Lactobacillus* have more growth in edible media than that of traditional MRS media. In the edible media, *L. plantarum* exihited the highest growth followed by *L. ferciminis*. The other two isolates showed not so different. Pathak and Singhal [30], replaced beef extracts with germinated lentil seed powder in MRS media. The microbial growth of *L. lactis*, *L. casei*, *L. plantarum* had a positive effect on the in modified MRS media as compared to MRS [29]. The average growth of *Lactobacillus* in MM-2 edible media was more than 15 log CFU/ml where the average cell density in MRS media was 12.44 log CFU/ml (**Figure 1**).

3.2. Viability of *Bifidobacterium* Isolates in Edible Media

3.2.1. In the Case of Different Media Formulation

The isolates were cultured repeatedly in bifidobacterium selective broth and agar compared with formulated ediblemedium MW-1, MW-2, MW-3, MW-4. The two Bifidobacterium isolates showed more growth in edible media compared with Bifidobacterium selective medium selective for *Bifidobacterium*. Among them, the highest growth of the isolates was shown in MW-1 media formulation and with the increment of media ingredients the culture density was decreased followed by MW-4 (**Figure 2**).

3.2.2. In the Case of Comparison of Growth

Bifidobacterium selective medium was designed to favor the growth of *Bifidobacteria* for the lab study, though the isolates grew at MW-1 formulation of edible media at its highest. The viability was compared with standard culture maintained in Bifidobacterium selective media and the results showed that edible media has a better ability to grow the isolates than the commercial one. Log numbers of bacteria were estimated and observed that all the two isolates grew well with small count difference. The *B. infantis* isolate showed a little increased log number (12.56) in edible media than the log number (12.32) in bifidobacterium selective media. Pathak and Martirosyan [5] worked on *Bifidobacterium* strain 231, *Bifidobacterium* strain 234. They used their modified MRS media for the culture of these two bacteria. In their study, they found that growth was better in MRS media than in modified MRS media. The average growth of *Bifidobacterium* in MW-1 edible media was 12.60 log CFU/ml where the viability

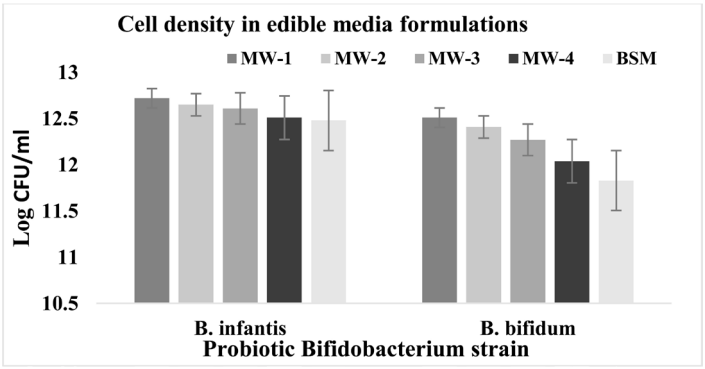

Figure 2. The log number of *Bifidobacteria* in different media formulation.

in bifidobacteria media was 12.48 log CFU/ml (Figure 2). Arulanantham *et al.* [6] carried out a study where they used cowpea, green gram, black gram and soya meat (processed soya bean) in different formulations for the growth of *E. coli*, *Bacillus* sp., *Klebsiella* sp., *Staphyllococcus* sp. and *Pseudomonas* sp. They found that *Staphyllococcus* sp. grew well in all the protein formulations and *Klebsiella* sp. grew least in the protein formulations tested. They concluded that soya meat agar (soya meat + agar) was an effective alternative culture media source next to nutrient agar to grow bacteria.

3.3. Measuring of OD for Lactobacillus and Bifidobacterium

Optical density (OD) or absorbance of a sample measured for estimating the bacterial cells density in a broth midium. As visible light passes through a cell suspension the light is scattered. The higher scatter indicates that more bacteria or other material is present. The amount of light scatter can be measured in a spectrophotometer. However, the absorbance of every isolate containing edible broth was recorded at 600 nm wavelength as it was considered as standard because at this wavelength maximum absorption was observed. A High OD was observed for *Lactobacillus* isolates at MM-2 media formulation than any other media formulation indicating that at this media formulation bacterial cell was most viable (Figure 3). *Bifidobacterium* showed the highest absorbance at MW-1 edible media formulation (Figure 4). Then the cell formulation has decreased

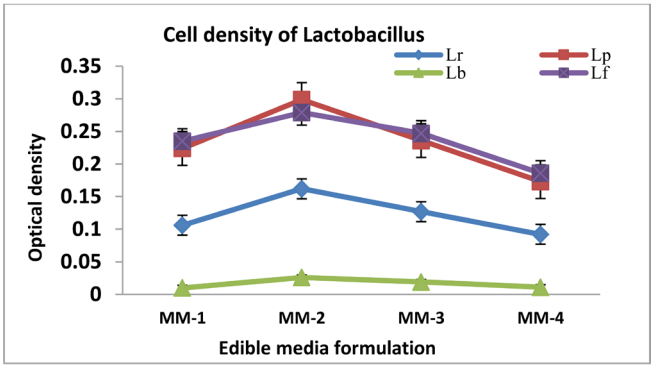

Figure 3. Optical density of *Lactobacillus* isolates in different media formulation.

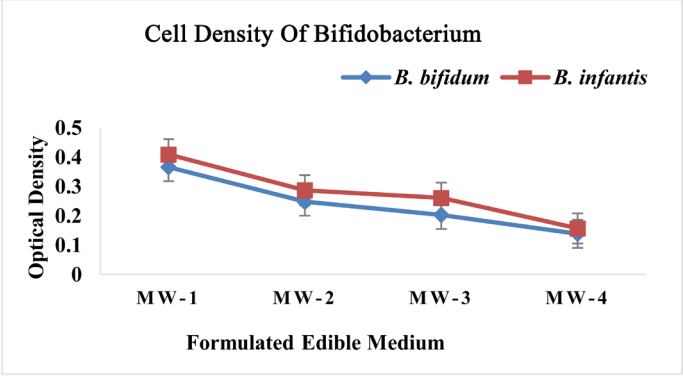

Figure 4. Optical density of *Bifidobacterium* isolates in different media formulations.

with increasing media formulations. The specific growth rates of all four isolates of *Bifidobacterium* decreased linearly as the concentration of dissolved solids increased in the medium. The higher sugar concentration in the medium probably exerts severe osmotic stress on the bacterial cells [4] [20].

3.4. Growth Curve of *Lactobacillus*

The performed test evaluated the growth dynamics of the isolate over a period of 32 hours. A better understanding of the growth patterns gives the idea of optimum timing for the maximum growth of cells when performing other tests on the isolate. From the results, it can be determined that between the hours of 0 - 8 was the lag phase. From hours 8 - 16 can be said to be the log phase where there is an exponential growth of the cells. From 20 - 32 hours the cells are in the stationary phase there is no significant net increase or decrease in cell number (**Figure 5**).

3.5. Survival of *Lactobacillus* before & after Freeze Drying

Freeze drying is a common method to incorporate probiotics into foods. However, during the processing, it affects the viability of cells. So, freeze-drying organisms are protected by adding cryoprotectants [20] [31]. In this experiment skim milk and sodium alginate was used as cryoprotectants and the loss of cells after freeze-drying was estimated. Cells were counted at 10^{-11} dilution in both situations. Before drying the count was 14.15 log and after drying it was decreased to 13.08 log count. Only 1 log of cell reduction was observed (**Figure 6**).

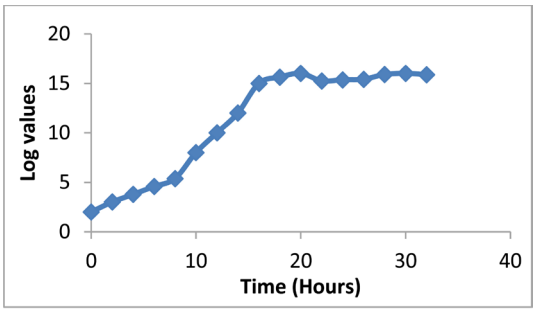

Figure 5. Growth curve of *L. plantarum*.

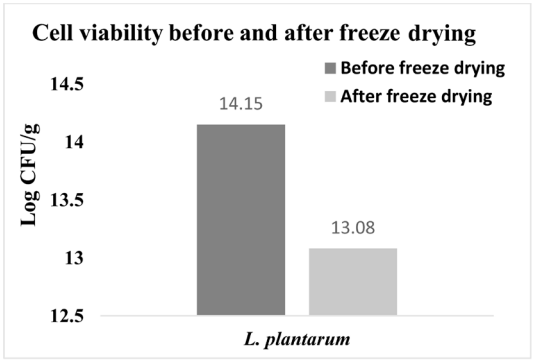

Figure 6. Survival of *Lactobacillus* in edible media before and after freeze-drying.

3.6. Field Trials

At the end of 10 days trial, the mice were weighed and compared with initial weight. During the trial, mice were kept in observation. It was seen that behavior, movements, body temperature, water intake, urination and stool of sample group members were as normal as control group members. The results showed that a higher rate of weight has gained by sample group than that of the control group (Table 2). The average weight gain in the control group was 5.67 gm where the average weight increased in the sample group was 13.20 gm. These results showed that the edible media with Lactobacillus culture in it does not contain any toxic or unhealthy compound that is harmful to health. It is clearly seen that the edible media is highly rich in nutrients that boosted the growth of sample group mice.

3.7. Costing of Edible Media

Different type's vegetables and grains were used to formulate the Edible media which is way cheaper than the commercially available media like MRS, M17, NA etc. which are continuously used for isolation, identification, enumeration of microorganisms in the laboratory for routine work. The Edible media was fabricated to replace these commercial media for daily experiments, which reduce the disbursement a lot (Table 3).

No expensive chemical was used only available and low-priced vegetables were used economical comparison showed this media is extremely economic

Table 2. Weight of the mice before & after feeding edible media with *Lactobacillus*.

		Initial weight (gm)	Final weight (gm)	Average weight increased
Control group	R1	23.8	27.9	4.934 gm
	R2	21.9	28.1	
	R3	21.8	26.3	
Sample group	R1	19.8	33.8	12.50 gm
	R2	21.5	34.1	
	R3	22.6	33.2	
	R4	22.8	34.7	
	R5	19.1	32.5	

Table 3. Cost comparison of the edible media and other commercial media.

Name of the media (Manufacturer)	Cost in BDT/500g
Bifidobacterium selective medium broth	23,000.00
MRS broth	9500.00
M17 broth	17,500.00
Nutrient broth	6500.00
Edible medium broth	1200.00

moreover industrial use of this media is also possible.

4. Conclusion

Edible growth media are those that are used to grow probiotics and can be consumed by a human with no harmful effects. Nowadays, only lactic acid bacteria especially *Lactobacillus* and *Bifidobacteria* are used as probiotics. These study results conducted that it is possible to formulate an edible media that can be used commercially in food processing industries for the production of probiotics. For example, the starter culture of *Lactobacillus* for yogurt should contain 6 - 7 log CFU/ml cells. But this edible media showed 11.3 - 13.26 log CFU/ml cells. It is clear that this media is more efficient in cell growth than conventional media. Industry requires that the organism grows faster, so that production time is reduced which is economical. Similarly, low-cost media is always pursuing to bring down production costs. Though it has formulated with the ingredients that are available at the cheap local market and are very reasonable in price (which are rich in nutrients required for microbes), this media can be a good alternative of conventional chemically defined media. Moreover, this media contains lots of vitamins, minerals and amino acids as it formulated with grains and vegetables. It can be added extra nutrition in food. Because of being so nutritive, a high rate of weight gain was observed in field trials. Another positive side of this media is—it supported the growth of not only *Lactobacillus and Bifidobacteria* but also gram-positive and gram-negative bacteria as comparing to conventional media. So this media can be used rather than nutrient media for laboratory purposes. It is recommended that more research curriculum should have carried out in the future by using other possible ingredients. Moreover, other bacteria use in food industries and also both fastidious, non-fastidious microbes for laboratory purposes should be included in further research.

Ethical Approval and Consent to Participate

Experimental protocols for animal trail approved by BCSIR institutional ethical review committee and followed while performing the research with mice.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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