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A Better and Cheaper Culture Medium for Isolation of Lactic Acid Bacteria from Milk and its Products

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Milk and its products contain large number of *Lactic acid bacteria* (LAB) that are difficult to culture under laboratory conditions because of their complexity and diversity under dynamic ecosystem. MRS (de Man, Rogosa and Sharpe) medium was used to culture of LAB from milk and its products under aerobic and anaerobic conditions separately. The same samples were used to culture LAB on new Soybean culture medium (per litre) containing extract of 20 g soya nutri nuggets, 5 g yeast powder extract, 20 mL coconut milk and agar 20 g. The samples on both media were incubated at 37±1! for 48-72 h and examined for growth of different LAB. The cost of MRS medium available in the market ranges from U$ 50 to 100 per 500 g of which 50-60 g is used to prepare 1 litre of culture medium depending upon the manufacturer. The minimum cost of 1 litre MRS comes to U$ 5 while the cost of ingredients used in Soyanutri Jaggery Agar (SJA) medium is only 50 cents *i.e.* ten times cheaper than MRS and the number of colonies isolated from milk and its products were 8-10x higher than MRS medium. Jaggery (concentrated raw sugarcane juice) is available at the 5 Kg for 1U$ and easily available. 500 g of Soyanutri cost only U$ 1. Further researches are being conducted in our laboratories and it is suggested that Soyanutri Jaggery Agar medium may be used for routine culture of LAB in laboratories for the demonstration and practice for the students and also for screening of samples on large scale.

Keywords: MRS medium, Lactic acid Bacteria, Soyanutri Jaggery Agar medium, Cheaper and Better culture medium for LAB, Milk and its products**.**

Lactic acid bacteria (LAB) are well-known for their significant contributions to the food industry (fermentative capabilities) and for being naturally present in the digestive tracts both human and animal where they help in management of good health controlling obesity, inflammatory bowel disease, immunity, digestion of food including mental health.[1] *LAB* are members of heterogenous group of Gram-positive, non-sporing, non-respiring cocci or rods that possess various probiotic attributes[2-3] and provides numerous health advantages for humans and animals, including the abilities to generate antibacterial compounds and reduce the pH of the growth environment.[4]

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A nutrient-rich medium and an appropriate bacterial growth environment are necessary to cultivate *LAB* in the laboratory and at industrial level. Carbon, nitrogen, non-metal elements like sulphur and phosphorus, metal elements like Ca, Zn, Na, K, Cu, Mn, Mg, and Fe, vitamins, water, and energy are among the nutrients that are required for good growth and development of LAB.[5-7]

Lactic Acid Bacteria (LAB) are commonly cultivated on MRS broth and agar,[8] which is enriched and selective culture medium, first created by De Man, Rogosa, and Sharpe in 1960 for selective culture of species of *Lactobacillus.* MRS medium has been the industry standard for separating and preserving *LAB* from a variety of environmental sources, such as fermented foods, human and animal microbiota, and clinical specimens, since its inception. MRS medium contains a rich blend of nutrients that support the growth of *LAB*.[9]

The key components of MRS includes peptone which provide essential nitrogen and amino acids for bacterial growth, glucose act as carbon source that is easily fermented by *LAB*, producing lactic acid as a primary metabolic product, phosphate that act as buffers to maintain pH stability during fermentation, which is crucial as *LAB* produce lactic acid, lowering the pH, magnesium and manganese salts that are essential for enzyme activation and overall microbial metabolism and Tween 80 which act as a surfactant that helps to emulsify lipids and supports the growth of certain *LAB* species. *LABs* are highly specialized and sensitive to artificial media.[10] *LAB* such as *lactobacillus* has been well known for their wide range of utilization during fermentation in different food.[11-13]

MRS is regarded as a costly medium since it uses meat and yeast extract as its nitrogen source. 500 g of MRS agar medium normally costs about U$ 50 to 125 depending upon the quality and brand name of the manufacturer and an amount of 50 to 60 g is required to be added to make 1 Litre of the culture medium. Additional preparation and handling procedures are needed for bulk media, and the starting culture preparation process has to involve qualified personnel. Achieving a high cell density and using extra quality control measures to guarantee that *LAB* reaches its maximal development are other problems associated with this medium.[14-15] The literature reveals significant research is aimed at developing new media or finding alternative low cost ingredients to lower costs or to obtain higher cell density.

The soybean's macronutrient composition is significantly different from that of other legumes since it has significantly more fat, a moderate amount of protein, and a significantly smaller amount of carbohydrates. The quality of soy proteins is superior to other plant proteins and comparable to animal proteins, is another noteworthy feature of soybeans.[16] Many traditional Asian soyfoods are low in this macronutrient (30g) due to the soybean's low carbohydrate content. The fat composition of soybeans is made up of around 10% to 15% saturated, 19% to 41% monounsaturated, and 46% to 62% polyunsaturated fatty acids (PUFA) (roughly 2.9, 4.4, and 11.3 g saturated, monounsaturated, and PUFA per 100 g of soybeans, respectively).[17] A number of vitamins and minerals, including potassium, iron are abundant in soybeans.[18-20] In soybeans, isoflavones are virtually entirely found as glycosides.[20]

Coconut water is a nutritious beverage that is fat-free and low in calories and sugars. A number of inorganic ions including several electrolytes like sodium, potassium, magnesium, phosphorus and calcium, vitamins, auxins, cytokinins, gibberellins and other substances are found in coconut that contribute to its high nutritional and medicinal properties.[21-24] Raw yeast powder is cheap source of complex nutrients while Jaggery, another cheap source carbohydrate, prepared from the concentrated juice of sugarcane, is highly rich in several minerals and trace elements.

A combination of extract of soya nutri nuggets + raw yeast powder along with coconut water and Jaggery were evaluated to isolate and culture LAB from different sources and compared with commonly used MRS medium in terms of their qualitative and quantitative growth.

MATERIALS AND METHODS

MRS broth and agar media were purchased from Hi Media. 55.15 g of dry MRS medium was used to prepare 1 L of culture medium. It contained (g/L) – peptone 10 g, yeast extract 4 g, meat extract 8 g, glucose 20 g, tween 80- 1 g, potassium phosphate (dibasic) 2 g, sodium acetate hydrated 5

g, tris-ammonium citrate 2 g, magnesium sulphate 0.2 g and manganese sulphate 0.04 g with 12 g agar was dissolved in boiling distilled water and adjusted to pH when cooled. It was then autoclaved at 15 p.s.i. for 15 min.

Soyanutri Jaggery agar (SJA) medium was prepared by boiling 20 g Soyabean nutri chunks (Nutrela Company) + 5 g raw yeast powder and 500 mL of distilled water for 5 min which were filtered through 4 layers of muslin cloth to prepare Soya nutri yeast extract. 5 g Jaggery and 20 mL of coconut water were added to this extract along with 20 g agar and the volume was made to 1000 mL. It was autoclaved at 15 p.s.i. for 15 min and the agar was omitted in broth medium.

Adequate number of plates and flasks were prepared aseptically separately for MRS and Soyanutri Jaggery Agar medium for isolation of LAB from different sources.

Isolation of Lactobacilli

Lactobacilli were isolated from commercial samples of yogurt, curd, cheese and milk which were collected from the local market in Meerut, India. 1 mL of sample was suspended in 100mL sterile peptone water from which 10^{-1} to 10^{-4} dilutions were prepared. An aliquot of 100µl for each dilution was aseptically spread over the MRS agar and Soyanutri Jaggery Agar plates separately with the help of sterile L- shaped spreader and allowed to settle the culture. These plates were then incubated at 37 ±1! for 24-48 h. The discrete colonies appeared after 24 h of incubation and on the basis of texture, size, edges, colony color, Gram's staining reaction, catalase, oxidase and sugar fermentation tests, the colonies were identified as LAB as described earlier.[25-26] The colonies were counted with the help Colony Counter on the basis of the different morphology appeared on the surface of the different agar plates. CFU/mL was calculated using following equation by considering the number of colonies of a species found in the highest dilution of the test substance.

$$\text{CFU/mL} = \frac{\textit{No. of Colonies} \times \textit{Dilution Factor}}{\text{Volume of sample inoculated}}$$

RESULTS

Standard staining procedure revealed that all isolates were Gram's positive, rods or cocci having different arrangements. The *Streptococcus* species formed chains whereas the *Pediococccus* species showed tetrads in arrangements. The *Bifidobacterium* species were examined as short irregular rods. All isolates were evaluated for sugar fermentation by incubating them at 37 ±1!

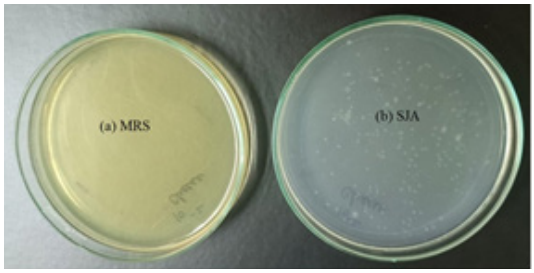

Fig. 1. Colonies of LAB from cheese

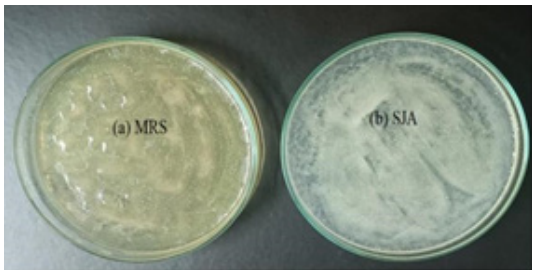

Fig. 2. Colonies of LAB from milk

Fig. 3. Colonies of LAB from curd

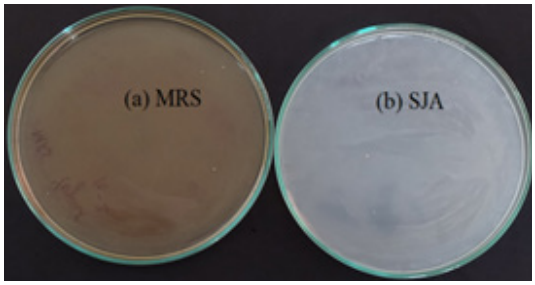

Fig. 4. Colonies of LAB from yogurt

Table 1. LAB isolated from milk and its products on MRS and SJA plates (each figure is an average of 3 independent replicates)

Genus	Raw Milk		Cheese		Yogurt		Curd	
	MRS	SJA	MRS	SJA	MRS	SJA	MRS	SJA
Lactobacillus spp.	6	7	3	3	3	5	4	5
Lactococcus spp.	4	5	4	5	4	4	5	5
Streptococcus spp.	4	5	2	4	1	1	2	3
Pediococcus spp.	2	2	0	1	0	0	1	1
Bifidobacterium spp.	3	4	1	2	1	2	2	3

Abbreviations used: MRS- de Man. Rogosa and Sharpe Agar medium, SJA-Soyanutri Jaggery Agar Medium.

Table 2. Number of bacterial colonies (CFU/mL) isolated from different samples of milk and its products on MRS and SJA media

Genus	DF	Colony forming unit (CFU/ml)							
		Milk		Cheese		Yoghurt		Curd	
		MRS	SJA	MRS	SJA	MRS	SJA	MRS	SJA
Lactobacillus spp	10^4	5.8×10^6	9.1×10^7	4.9×10^6	5.3×10^6	2.4×10^6	3.8×10^6	3.5×10^6	6.5×10^6
Lactococci spp		3.1×10^5	4.6×10^6	5.2×10^6	7.3×10^6	1.3×10^5	2.7×10^6	2.8×10^5	3.8×10^6
Bifidobacterium spp.		4.6×10^6	8.9×10^6	1.7×10^5	2.9×10^6	1.05×10^6	3.4×10^6	2.1×10^5	4.05×10^5

for 48 h. It was revealed that all 67 isolates of LAB cultured on MRS or SJA fermented one or more sugars and based on the sugar fermented these were tentatively identified as *Lactobacillus delbrueckii subsp. delbrueckii, L. salivarius subsp. salivarius, L. kalixensis, L. naeglii* from milk samples while from curd the isolated LAB were classified as *Lactobacillus fermentum, Lactobacillus casei, L. acidophilus, L. delbrueckii.* It was further found that *L. delbrueckii* fermented all sugars that included glucose, fructose, mannitol, maltose, lactose, starch and sucrose. The isolates of LAB from the yogurt were identified as *Lactobacillus acidophilus, L. casei, L. delbrueckii subsp. bulgaricus.* It was found that *L. delbrueckii subsp. bulgaricus* fermented fructose, glucose and lactose. The isolates of LAB from cheese were tentatively identified as *L. plantarum, L. brevis, L. helveticus, L. lactis. S*ome of the species of L*actobacillus* could not be identified. But our results showed that SJA medium is selective for isolation of LAB and is capable of isolation of greater number of species / isolates (Table 1, 2; Fig 1-4) than MRS.

Our result revealed that a total number of 52 isolates were isolated on the MRS plates while total 67 isolates of LAB were isolated on the Soyanutri Jaggery agar (SJA) medium from the same samples under similar conditions of culture (Figure 1- 4).

Isolation of number of colonies of LAB from milk and its products

The bacterial load from different milk and its products was examined using dilution plate method. *Lactobacillus* species were found highest in buffalo milk where 5.8×10^6 CFU/mL on the MRS medium plates were isolated while the same samples when inoculated on SJA medium, the number of colonies were much higher that accounted $9.1 \times 10^{7.}$ CFU/mL. *Lactobacilli* isolated, from cheese, yogurt and curd on MRS medium accounted 4.9×10^6, 2.4×10^6, and 3.5×10^6 CFU/mL respectively whereas on the SJA medium plates the same samples yielded 5.3×10^6, 3.8×10^6, and 6.5×10^6 CFU/mL respectively (Table 2). It clearly suggest that SJA medium yielded higher number of colonies from the same samples than on MRS medium. Similarly, *Lactococci* isolated on MRS medium were 3.1×10^5, 5.2×10^6, 1.3×10^5, and 2.8×10^5 CFU/ml from milk, cheese yoghurt and curd respectively, whereas from the same samples, the number of colonies on SJA medium were 4.6×10^6, 7.3×10^6, 2.7×10^6, and

3.8× 10^6 CFU/mL which were much higher (Table 2). *Bifidobacterium* species isolated from milk, cheese, yogurt and curd on MRS plates were 4.6×10^6, 1.7×10^5, 1.05×10^6, and 2.1×10^5 CFU/mL while on SJA medium the same samples yielded 8.9×10^6, 2.9×10^6, 3.4×10^6, and 4.05×10^5 CFU/mL respectively(Table 2). Our results clearly revealed that the CFU/mL of the isolates different LAB were much higher on SJA medium than MRS medium under the same cultural conditions (Table 2)

DISCUSSION

During our present study we isolated different species of *Lactobacillus, Lactococcus, Streptococcus, Bifidobacterium,* and *Pediococcus* from milk and its products using MRS and SJA media. The isolated species are well known as LAB. Six genera of LAB were also reported in traditional fermented milk by Savadogo and his co-workers[27-28] that included *Leuconostoc, Lactococcus, Lactobacillus, Enterococcus, Streptococcus*, and *Pediococcus* species. Similarly, five distinct taxa namely *Aerococcus, Streptococcus, Enterococci, Leuconostoc* and *Lactobacillus* of LAB were found by Gawad and his collegues from rayeb milk.[29] Additionally, Abdullah and Osman, observed a similar finding where *Lactobacillus* predominated in all analyzed samples, followed by *Lactococcus* and *Pediococcus*.[30] On comparison of qualitative isolation of species of LAB on MRS and SJA, it was found that 7, 5 and 5 species of *Lactobacillus* were isolated on SJA medium from raw milk, yogurt and curd respectively while the same samples on MRS allowed the isolation of 6, 3 and 4 species of *Lactobacillus* (Table 1). Similarly, greater number of species of *Lactococcus*, *Streptococcus* and *Bifidobacterium* were isolated from SJA medium than MRS (Table 1). The higher number of isolation of species of LAB on SJA than MRS may probably be because of diversified nutritive value of the former medium as the ingredients of SJA contain wide variety of organic and inorganic nutrients.

The soybean's is known to contain fats, moderate amount of proteins, but significantly smaller amount of carbohydrates, therefore, Jaggery was added into the SJA medium to provide the carbon source. The quality of soy proteins is superior to other plant proteins and comparable to animal proteins that are used in MRS medium.[16] Soybeans is made up of 10 to 15% saturated, 19 to 41% monounsaturated, and 46 to 62% polyunsaturated fatty acids (PUFA)[17] that provided reserve nutrients to the SJA medium which were utilized by LAB as and when required. A number of vitamins and minerals, including potassium, iron are present in soybeans[18-20] that are required by LAB during their growth and development. Isoflavones as glycosides are found exclusively in soybeans that provided additional nutrients to LAB.[20] Thus soybeans were used as substitute for meat extract used in MRS medium that reduced the cost of SJA medium.Coconut water is fat-free and contain variety of sugars along with several electrolytes like sodium, potassium, magnesium, phosphorus and calcium, vitamins, auxins, cytokinins, gibberellins and other substances that contributed to its high nutritional properties[21-23] for better growth and development of LAB. Raw yeast powder is a cheap source of complex nutrients that supplemented yeast extract used in MRS. It may be concluded that the expenses components of MRS medium were replaced by cheaper substrate that provided the equivalent nutrients, which drastically reduced the cost of SJA medium without compromising the quality of nutrients. Jaggery is a product of sugarcane juice and contains variety of natural sugars that helps LAB to ferment. Besides, high content of sugar, jiggery contain proteins, vitamins, phenolic acids and other essential nutrients including a variety of minerals.[31-33] Jaggery provides a good source of carbohydrates for the growth of microbes and our laboratories, we have replaced dextrose with jaggery where only 50% of its quantity provide sufficient carbohydrates for culture of *Azotobacter* and *Serendipita*.[34]

The major cost of MRS medium is based on the use of peptone, yeast and meat extract and sodium acetate. In SJA medium, we replaced peptone and meat extract with very cheap and easily available soynutri and the cost of yeast extract was reduced using yeast powder collected from local market. The cost of dextrose was reduced by jaggery which acted as buffer and provided all other nutrients including minerals.

Lactobacillus spp. are known to thrive well in extremely acidic environments with pH values of 4 to 5 or even lower, and these are

Lactobacilli that cause fermentation of the milk and its products. *Lactobacillus* spp. can endure under low pH conditions.[35] SJA medium allows the buffering of the medium and provide all nutrients that are available in MRS. Its composition is stable at acidic pH values that are preferred by LAB to grow and develop.

CONCLUSIONS

The cost of readymade powdered form of MRS medium available in the market ranges from U$ 50 to 100 per 500 g depending upon the brand and the name of the company. 50-60 g is used to prepare 1litre of culture medium that costs about 5 to 10 U$ per litre while the cost of 1litre SJA comes 50 cents only (ten times lower) and the number of species and their colonies isolated on SJA medium are much higher. However, further researches are being conducted in our laboratories with respect to other bacteria.

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Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

Author's contributions

Amar P Garg- Conceptualized, designed and developed the SJA medium, and finally corrected the manuscript; Anchal Bamal and Rashmi Goley (both Ph.D. research scholars)- conducted all experimental work, analysed data, collected literature and drafted first draft of the paper as part of their Ph.D. thesis.

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