FORMULATION OF CULTURE MEDIUM FOR THE ENUMERATION OF LACTIC STREPTOCOCCI AND LACTOBACILLI FROM FERMENTEDMILK PRODUCTS

ABSTRACT

Aims: The present study is focused to formulate a single selective differential medium for the enumeration of mixed lactic flora such as lactic streptococci and lactobacilli in domestic and market fermented milk products.

Study design: In order to enumeratelactic streptococci and lactobacilli in a mixed flora of fermented milk, formulation of single selective medium was optimized using inhibitors and indicator instead of separate selective medium for lactic streptococci and lactobacilli.

Place and Duration of Study: The present study was conducted in the Department of dairy Microbiology, Dairy Science College, Karnataka Veterinary, Animal and Fisheries Sciences University(KVAFSU), Hebbal, Bengaluru-24, Karnataka from February 2020 to February 2021.

Methodology: Fermented milk samples like curd, yoghurt were collected, serially diluted and pour plated using Readymade M17 and MRS agar media and formulated selective yeast glucose agar medium. Lactic colonies obtained were enumerated, expressed as $\log_{10}$cfu/g and critical difference was calculated to declare significance and non-significance among the microbial parameters.

Results: Readymade M17 and MRS agar media gave better recovery of viable cells of lactic streptococci and lactobacilli from market samples of curd, yoghurt and domestic curd samples.The study helped to convert a general purpose medium yeast glucose agar as selective differential medium with addition of calcium propionate of 0.8 per cent to avoid *Bacillus* spore formers and sodium benzoate of 1.2 per cent to inhibit yeast, the common contaminants in fermented milk with 0.05 per cent BCP that led to formation of purple colonies of lactic streptococci and yellow colonies of lactobacilli.

Conclusion: The selective yeast glucose agar with 0.8 per cent and sodium benzoate of 1.2 per cent with 0.05 per cent BCP with purple colonies of lactic streptococci and yellow colonies of lactobacilli can be successfully used for the enumeration of mixed lactic flora in fermented milk products instead of M17 medium for lactic streptococci and MRS for lactobacilli separately.

Keywords: *Bacillus spore formers; Bromocresol purple; Lactic streptococci; Yeast; Yogurt*.

1. INTRODUCTION

Lactic acid bacteria are among the most important groups of microorganisms used in food fermentation. Lactic acid bacteria (LAB) are a group of Gram positive, non-spore forming cocci or rods, catalase negative and fastidious organism with high tolerance of low pH 5.5 to 5.8 [1]. Fermentation of milk, preserves its nutrients for long time and fermentation is made possible by inoculating the milk with lactic acid bacteria. Fermented milk products extend potential therapeutic benefits to the consumers. People have the concept of using fermented milk products as they are safe for consumption due to end products offermentation released by Lactic Acid Bacteria (LAB) due to their well-known status as GRAS (Generally Regarded As Safe). LAB include a variety of industrially important genera such as

*Lactococcus, Streptococcus, Leuconostoc, Pediococcus*and Lactobacillus. LAB play an important role as starter cultures for fermentation in dairy and food industries. Starters are selected strains of microorganisms (LAB) deliberately added to milk during conversion into dahi, yoghurt, cheese and other fermented dairy products. The lactic acid bacteria could be mainly divided into two groups based on the end-products formed during the fermentation of glucose. Homofermentative lactic acid bacteria such as *Lactococcus* spp., *Streptococcus thermophilus*, and some of the lactobacilli like *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lb. acidophilus* produce lactic acid as the major or sole end product of glucose fermentation. Where asheterofermentative produce lactic acid, other acids and even gases and include *Leuconostoc*spp*., Lb. brevis, Lb. fermentum* [2].

Enumeration of mixed lactic flora like lactic streptococci and lactobacilli may be difficult using a single culture medium though selective M17 and MRS agar or general purpose medium like yeast glucose agar or Elliker agar media present. Eight agar media and one control medium were analyzed to compare their proficiency in evaluating the *Lactococcus* population in raw milk: M17 Nal, Elliker, modified Elliker, PCA (plate count agar) + milk, turner agar modified KCA, modified Chalmers, Turner, FSDA. (fast slow differential agar). The M17 medium was used as reference. The KCA medium proved to be the most selective towards lactococci andit was not possible to separate the *Streptococcus* from the *Lactococcus* colonies on KCA. The "*Lactococcus*-like" population including these two genera was estimated at a mean level of 3.18 log(cfu)/mL and 4.14 log(cfu)/mL in cow and goat raw milk respectively[3]. A load of *Lactococcus* spp. was counted as 1.12×10^7, $8.01\times10^7$and 2.75×10^9 CFU/ml from raw cow's milk, cheese and yogurt, respectively, on M17 agar[4].

BRIGGS[5] a medium for streptococci and lactobacilli, LAE (Lactic-Agar-Elliker), were developed[6]. A number of lactobacilli strains did not grow well in any of these media, so a nonselective medium known as lactobacilli MRS that was able to support the growth of lactobacilli was developed[7]. Briggs agar was a medium frequently used for the cultivation of lactobacilli from milk and dairy products. Early observations suggested that typical flat greyish-brown and rough colonies ("B-colonies") appearing on Briggs or BL agar plates may be *L. acidophil*us. MRS medium had a similar composition to APT (All-purpose Tween 80 agar) and was developed primarily with the intention of substituting tomato juice by defined growth factors such as Mg^{2+} and Mn^{2+}. De Man Rogosa Sharpe containing bile (MRSB), Man-Rogosa Sharpe agar (MRS) containing nalidixic acid, paromomycin, neomycin sulphate and lithium chloride (MRS-NPNL), M17 and *L. casei*(LC) agar failed to select *Lactobacillus acidophilus, Bifidobacterium,* starter LAB and *L. casei*strains respectively. However, LC agar appears appropriate for *L. paracasei*and MRSB **f**or yoghurt starter bacteria in the absence of *L. reuteri*and *L. rhamnosus*Ashraf and Smith (2015)[8] The selective potential of culture media largely depended on target species[9]. MRS agar added with soya peptone of 0.02, glucose of 0.12, yeast extract of 0.2 and magnesium sulphate of 0.08per cents to normal composition and they found good growth with counts of 9 $\log_{10}$cfu/ml of lactobacilli. Both MRS and Acetate agar statistically showed significant difference in counts of lactobacilli from yogurt sample by serial dilution using PBS (phosphate buffered saline) as diluent with log counts of 7.41 and 4.59 cfu/g respectively when incubated the plates at 37°C in candle jar[10]. During the cultivation of milk samples during the first, third- and seventh-days, lowest number of lactobacilli colonies per day was recorded during the seventh day, at 3.7 ± 0.1 log cfu/mL in cow milk at 37 °C for 24–72 h using MRS medium[11].

It was found that MRS agar medium was not efficient in distinguishing between *Lactobacillus rhamnosus*LV108 and *Streptococcus thermophilus*grx02. In co-cultured milk (*Lb. rhamnosus*LV108+ *Str. thermophilus*grx02), the viable count of *Str. thermophilus*grx02 could be enumerated by controlling the culture time on LM17(Lactobacillus selective agar) up to 10^8cfu/ml. By restricting the carbon source and culture time, mixtures of *Lb. rhamnosus*LV108 and *Str. thermophilus*GRX02 could be enumerated rapidly and easily in 48 h (minimum 36 h), which could benefit the development, production and storage of probiotic dairy products containing these two species[12].

If only one lactic culture was used in fermented milks, the problem to enumerate would be less. But the problem encountered to enumerate in a mixture of lactic cultures used in the preparation was found to be difficult. The viability of each group of lactic culture might be a problem Tomato juice found to stimulate the growth of many LAB and it was included in Briggs agar[5]. The yogurt starters and commercial samples that grew on Elliker's lactic agar supplemented with 1% Tween 80 and 50 mg/ml of 2,3,5- triphenyltetrazolium chloride produced small, red *Streptococcus thermophilus* colonies and larger, white *Lactobacillus bulgaricus* colonies. The distinction was somewhat strain dependent but was satisfactory in most cases. Addition of 7 % skim milk (11% solids) to lactic agar in place of 2,3,5-triphenyltetrazolium chloride allowed good rod-coccus differentiation, regard- less of strain or yogurt brand[13]. A total of 10 sucuk (Turkish-type fermented sausage) samples, obtained from Denizli, had on an average of 8.34 log CFU/g TAMB, 8.91 log CFU/g LAB (at the MRS agar) and average 8.25 log CFU/g LAB (at the Elliker's lactic agar) both lactobacilli and lactic acid producing cocci[14].The plate count agar with bromocresol purple was recommended for the enumeration of lactic acid bacteria (LAB) in foods was good for enumeration but not for differentiation of each LAB in a mixed culture. Compared with PCABCP, mMRS produced larger colonies of various sizes in 2 days, making enumeration easier. However, various LAB produced colonies with the same white colour, made it difficult to differentiate each species. Compared with PCA-BCP and mMRS, mMRS-BPB could differentiate each species based on its characteristic pH change during growth. Because BPB changed colour within a range from pH 3 to 5, it was useful for detection of the pH change produced during fermentation of LAB[15]. The Fast-Slow Differential Agar (FSDA) medium was developed in 1984 and still remains the standard to rapidly differentiate fast and slow milk-coagulating lactic streptococci but unable to selectively isolate fast acid-producing strains due to the presence of a diverse microbiome including Non-Starter LAB and spoilage Gram-negative microbiota modified FSDA (mFSDA) with increased selectivity of nalidix acid (inhibit gram negative bacteria), ascorbic acid and yeast extract stimulate the growth of lactic streptococci and The pH indicator bromocresol purple enabled the chromogenic discrimination between LAB with different acid production capability[16] [17].

Sodium azidewas added to inhibit the contamination flora, including Gram negative flora and purple bromocresol allowed a direct selection of the Gram + and lactose + flora. For all samples medium M3 appeared to be the most appropriate for growth of Gram + and catalase – bacilli, owing to the high growth rates and the colony diameters ranging from 1-2 mm[18]. LC (*Lactobacillus casei*) agar appeared appropriate for *Lactobacillus paracasei*and MRSB (MRS with bile) for yoghurt starter bacteria *Latobacillusdelbrueckii*ssp. *bulgaricus*or *S. thermophilus* and LC agar could be used to target *Lb. paracasei*. Selective recovery of *Lb. delbrueckii*ssp*. bulgaricus* could be achieved using MRS 5.2 incubated at 45ºC anaerobic for 72 h. In general, MRS 5.2 at 45 °C for 72 h. supported the growth of almost all the *Lactobacillus* spp. with viable count of more than 9 log[8]. Yoghurt samples showed higher counts of lactic acid bacteria (LAB) that ranged from 1.0×10^6 to 5.6×10^7 cfu/mL and 2.2×10^7 to 5.4×10^8cfu/ both at aerobic and anaerobic condition using MRS agar (pH 6.2-6.6) and Rogosa agar (pH 5.2-5.6) for total LAB enumeration as well as lactobacilli isolation while YGLA (Yeast Glucose Lactic agar pH 7.0**)** for the isolation of streptococci incubated at 37 °C for 48-72 h both at aerobic and anaerobic conditions. In general, anaerobic LAB growth of 6.1×10^5 to 5.4×10^8 cfu/mL counted higher than the aerobic growth of 1.1×10^5 to 5.6×10^7cfu/mL[19]. The mRCM-aniline blue performed better than the conventional medium in culturing, enumerating, and differentiating *L. bulgaricus*. Therefore, mRCM-blue could be used as a selective medium to enhance the growth and differentiation of *L. bulgaricus*in order to meet the increasing demand for this beneficial species of bacteria[20].(Nwamaioha*et al.,* 2017). Commercial yogurt such as plain, apple, blue berry and yogurt prepared from heat treated raw milk were subjected for the viable count estimation using BCP (Bromocresol Purple) agar containing 0.5 % poly peptone, 0. 5 % yeast extract, 0.1 % glucose, 0.1 % Tween 80, 0.01 % L-cysteine and 0.006 % bromocresol purple with pH 7.0. The lactic counts of the yogurt samples were 3.7 $x10^8$, 2.7 $x10^8$, 6.7 $x10^8$

and 5.8 x10^8cfu/g for plain, apple, blue berry and yogurt prepared from raw milk, respectivelyNishino *et.al.* (2018)[21]. The modified Reinforced clostridial medium (RCM) was used to selectively enumerate and isolate *Lactobacillus delbrueckii*ssp. *bulgaricus,* by optimizing the addition of 0.5 % fructose, 0.5 % dextrose, 1 % maltose, and 0.25 % sodium pyruvate while replacing lactose as a carbohydrate source. The cell recovery and bacterial counts of *Lb. delbrueckii*ssp. *bulgaricus* in tested. products (pure *Lb. delbrueckii*ssp. *bulgaricus* strains, starter culture, probiotic supplements and yogurt) using mRCM with sodium pyruvate (mRCM-PYR) were significantly higher than de Man, Rogosa, and Sharpe (MRS) culture medium. The growth of other lactic acid bacteria (*Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus rhamnosus*, and *Lactobacillus reuteri*) and Bifidobacteria was retarded in this modified medium compared with their growth in MRS. The results thus suggested that mRCM-PYR could be recommended as a reliable alternative growth medium for the selective enumeration and isolation of *Lb. delbrueckii*ssp. *bulgaricus*in a mixed culture[22]

In this study, it was considered as a basis to define a selective medium, by adding a pH indicator and some inhibitory compounds to select the LAB flora from various dairy products like fermented milk products such as curd, yoghurt etc. Common microbial contaminants like aerobic bacterial spore formers and yeast if present in fermented milk products posed problem during enumeration and predominated over starter or lactic acid bacteria. There might be requirement of certain inhibitors to be incorporated to medium to overcome the contaminants [23, 24].Calcium propionate ($C_6H_{10}CaO_4$) which having MW of 186 was water soluble. The compound was effective at pH 5.5. It prevented *Bacillus*spp. from producing the energy as they were protonophores at 0.4-1 per cent in LAB media. Sodium benzoate (C_6H_5COONa) soluble in water with MW of 144 had its optimum activity at pH 5-6. The concentration of 0.1-0.3 per cent inhibited yeast and molds by leakage of PMF and thus ATP synthesis of fungi was affected[23, 24].If fermented milk had yeast as contaminant then yeast predominated over LAB. Once yeast sometimes even *Bacillus*spp. made colonies, then isolation of the LAB became difficult. Media might require addition of antimycotic (sodium benzoate) and anti-sporulating (calcium propionate) agents[25].

2. MATERIAL AND METHODS

Domestic and commercially available market samples of Dahi and Yoghurt were collected and enumeration of lactic streptococci like species of *Lactococcus, Streptococcus thermophilus* and *Lactobacillus* spp. was carried out by viable count through serial dilution technique and pour plate method [26]. The media used are M17 agar for lactic cocci and MRS agar for lactobacilli formulated (weighed each ingredient and prepared the medium[27]); ready made media (HiMedia) as well general purpose medium Yeast Glucose Agar (YGA). The plates of lactococci and both Streptococcus thermophilus as well lactobacilli were incubated at 30° C/48 h and 37 ^{0}C/48 h in anaerobic candle jar respectively. After the completion of the incubation period, the colonies were counted in countable plates ranging between 30 - 300 by colony counter and average count was expressed as $\log_{10}$cfu/g of the product. General purpose media such as yeast glucose agar (YGA) and Elliker's agar composition[26]were prepared and used for total lactic count. For differentiating lactic cocci and lactobacilli, the pH indicator Bromocresol purple (BCP) and Bromophenol blue (BPB) were added at various levels after sterilization used for plating. Out of two general purpose media, one medium that yielded good viable lactic counts was selected to make into selective or differential medium. Antimycotic (sodium benzoate) and sporulating (calcium propionate) agents were added at various levels to ready made M17, MRS and YGA media prior to pouring into petri dishes. The requirement of direct microscopic count of fermented milk products for comparison with viable count was also carried out[26].The optimized levels of BCP, calcium propionate and sodium benzoate to yeast glucose agar made it selective medium for the enumeration of lactic cocci and lactobacilli present in fermented milk products. For confirmation of the colonies for lactic nature, the selected colonies were inoculated into sterile skim milk (9 % total solids) and incubated at the optimum temperature for overnight

incubation and checked for curdling and determined acidity (% lactic acid) and smear prepared and looked for morphology of cells.

3. RESULTS AND DISCUSSION

The results of enumeration obtained for lactic streptococci and lactobacilli in fermented milk products, using readymade media and formulated media are discussed in this section.

3.1 Enumeration of lactic cocci and lactobacilli from commercial and domestic fermentedDairyproductsusingFormulatedmedia(FM)andReadymade media(RM):

Fermented products such as market samples of curd, yoghurt and domestic curd samples wereenumerated using Formulated (each ingredient wasweighed and medium prepared -FM) and Readymade (powder reconstituted - RM) media likeM17forlactococciaswellas*Streptococcusthermophilus* andMRSmediaforlactobacilli incubated in candle jar at 30ºC and 37ºC respectively for 48 h. One marketyogurtsampleshowedhighlacticacidof1.02percentwhileonesampleof domesticcurd hadloweracidity noticedof0.68percent.Theviablecountoflactococcionformulated M17 medium in fermented milk samples ranged from 7.53 to 8.88 log_{10}cfu/g whereashighestcountwasfoundinyoghurtofabout8.88log_{10}cfu/g whilemarketcurdsamplehadlow count of 7.53 log_{10}cfu/g. But when readymade M17 medium was used, lactococcicolonies ranged from 7.95 to 8.95 log_{10}cfu/g in fermented products in which more viablecount of8.95 was observed in one brand of market yogurt sample and lowviablecount of 7.95inone marketcurdsample. Viablecountoflactobacilliwasfrom5.85to7.91log_{10}cfu/g infermentedproducts on formulated MRS agar. In readymade MRSagar, counts ranged from 6.00 to 7.96 log_{10}cfu/g where highest count was found in one of the domestic curd sample of 7.96 log_{10}cfu/g while in one of the market yoghurtssample the viable count of lactobacill was 6.00 (Table 1).

Table 1:Enumerationoflacticacidbacteriafromcommercialanddomesticfermented dairy products using Formulated media (FM) and Readymade(RM)

Sample name	Code	TitratableAcidity	Viablecount			
			Lactococci/*Str. thermophilus* (incurd/yogurt)		Lactobacilli (incurd/ yogurt)	
			M17		MRS	
			FM	RM	FM	RM
		%Lacticacid	Viablecount (log_{10}cfu/g)			
Commercialfermenteddairyproductssamples						
Curd	MC1	0.76[a]	7.59[a]	8.00[a]	7.17[a]	7.60[a]
	MC2	0.80[a]	7.56[a]	7.95[a]	7.57[a]	7.77[a]
	MC3	0.81[a]	7.53[a]	8.00[a]	6.87[a]	7.00[a]
Yogurt	MY1	1.02[a]	8.88[a]	8.95[a]	5.85[a]	6.00[a]
	MY2	1.00[a]	8.80[a]	8.91[a]	7.00[a]	**7.**11[a]
Domesticsamples						
	HC1	0.68[a]	8.68[a]	8.88[a]	7.69[a]	7.89[a]

Curd	HC2	0.72[a]	8.65[a]	8.90[a]	7.76[a]	7.96[a]
	HC3	0.83[a]	8.50[a]	8.78[a]	7.91[a]	7.67[a]
CD(*P*=.05)		**2.53**	**2.61**	**2.59**	**2.30**	**2.45**

Note:

- CD–Criticaldifference
- For lactococci M17 medium (formulated and readymade media) was used withincubation at 30ºC /48 h and lactobacilli using MRS medium in candle jar at37ºC/48h
- Allthevalues areaverageof threetrials
- Same superscripts in the column indicate non-significance while differentsuperscriptsindicate significance difference

Among formulated and readymade mediausedforlactococci(M17agar)andlactobacilli(MRSagar)fromfermentedmilkproducts,readymade media gave better recovery of viable cells of lactic acid bacteria, which mightbeduetouseofpureformsofingredientsinthedehydratedmediacomparedtoformulatedones where each ingredient is weighed which was time consuming. Readymade media areeasy to prepare as it was just reconstitution and warming and do not require any pHadjustmentwhichwasnottrue withformulatedmedia.Hence inthe further studiesreadymadeM17 and MRS wereused.LAB is the most widely used starter culture in fermented foods, especially dairyproducts. LAB have the ability to adapt to different environments, which could explaintheirwideuseinthefermentationofdiversefoodproducts.BRIGGS[5], a medium for streptococci and lactobacilli, LAE (Lactic-Agar-Elliker)[6], were developed. A number of lactobacilli strains did not grow well in any of thesemedia, so a nonselective medium known as lactobacilli MRS[7] that was able to support thegrowth of lactobacilli was formulated. Many strains of LAB growwell at 42–43°C and give higher recoveries when enumerated using modified MRS media[28]. MRS and M17 were the most commonlyusedstandardmedia,exhibitingconsistentgrowthforlactobacilliandlactococci,respectively[29].

3.2 Effect of incorporation of calcium propionate and sodium benzoate to ready made M17 and MRS media on lacticcountsoffermented milk products:

Marketyogurtshowed moreacidityof1.10while domesticcurdsamplehadloweracidity of 0.68 per cent, with range of 0.68 to 1.10 per cent lactic acid in fermented milkproducts.Theviablecountoflactococciinmarketcurd,yogurtanddomesticcurdsampleson control M17 agar was 8.98, 8.70, 8.80 that showed reduced to 8.60, 8.38, 8.48 on M17with 1.5+1.5 per cents of calcium propionate and sodium benzoate both, which might beattributedto inhibition ofaerobicsporeformers aswell asyeast (Table 2).

Table2:EffectofincorporationofcalciumpropionateandsodiumbenzoateincorporatedtoM17andMRSonthelacticcountoffermented milk products

Name of thesample	Sample code	Titratable Acidity (%LA)	Control M17	Control MRS	Additionofcalciumpropionate+sodium benzoate %								
					0.50 + 0.50	0.75 + 0.75	1.0 + 1.0	1.5 + 1.5	0.50 + 0.50	0.75 + 0.75	1.0 + 1.0	1.5 + 1.5	2.0 + 2.0
					M17				MRS				
			Viablecount(log_{10}cfu/g)										
Marketcurd	MC1	0.72[a]	8.98[a]	8.30[a]	8.90[a]	8.86[a]	8.80[a]	8.60[a]	8.00[a]	7.95[a]	7.90[a]	7.84[a]	7.80[a]

Marketyogurt	MY1	1.10[a]	8.70[a]	7.90[a]	8.62[a]	8.58[a]	8.46[a]	8.38[a]	7.84[a]	7.78[a]	7.70[a]	7.60[a]	7.50[a]
Domestic curd	HC1	0.68[a]	8.80[a]	8.53[a]	8.68[a]	8.60[a]	8.51[a]	8.48[a]	8.43[a]	8.36[a]	8.20[a]	8.02[a]	8.00[a]
CD(*P*=.05)		**1.21**	**1.23**	**1.15**	**1.23**	**1.23**	**1.23**	**1.23**	**1.08**	**1.08**	**1.07**	**1.10**	**1.11**

ViablelactobacillionMRSagar(control)were8.30,7.90,8.88and8.53log_{10}cfu/g noticedinmarketcurd,yogurtanddomesticcurdsamples,respectively (Table 3).

Table 3:Effectofincorporationof optimized calciumpropionateandsodiumbenzoatetoM17and MRS media on lacticcount offermented milkproducts

Sample name	Sample code	Mediumusedforplatinglacticcocci and lactobacilli			
		M17 control	MRS control	M17 with 1.5 %calcium propionateand 1.5 % sodiumbenzoate	MRS with 2 %calcium propionateand 2 % sodiumbenzoate
		log_{10}cfu/g			
Marketcurd	MC1	8.60[a]	8.00[a]	8.34[a]	7.80[a]
	MC2	8.94[a]	8.58[a]	8.56[a]	8.34[a]
	MC3	8.96[a]	8.30[a]	8.60[a]	8.00[a]
Markety ogurt	MY1	8.70[a]	5.86[a]	8.44[a]	5.66[a]
	MY2	8.58a	8.10[a]	8.32[a]	7.86[a]
Domesticcurd	HC1	8.56[a]	8.80[a]	8.46[a]	8.60[a]
	HC2	8.59[a]	8.46[a]	8.40[a]	8.26[a]
	HC3	8.53[a]	8.80[a]	8.31[a]	8.62[a]
CD(*P*=.05)		**4.43**	**4.27**	**4.43**	**4.30**

Butafterincorporation of 2.0+2.0 per cent of calcium propionate and sodium benzoate the countswere 7.80, 7.50 and 8.00 in market curd, yogurt and domestic curdsamples respectively. Reduction in counts was noticed in both M17 and MRS agar afterincorporationofcalciumpropionateandsodiumbenzoateat1.5percenteachand2per cent each,respectivelyhelpedincontrolofbothaerobicsporeformersandyeaststhatappearedassurfacecolonieson boththemedia.Theeffectoftheinhibitorsonmicrobialcontaminants might be ascribed to leakage of proton motive force that affected ATPsynthesisofbothcontaminantsthatinturnledtodeathduetostarvation[23, 24].Thus,incorporationofcalciumpropionateandsodiumbenzoatecouldbesuccessfullypracticed inthelaboratoriesforthemicrobiologicalanalysisoffermentedmilkproductstogetactualcountsof lactococci and lactobacilli.

3.3 Enumeration of lactic cocci and lactobacilli from curd samples using yeast glucose agarandElliker'sagarwith pH indicators:

Inordertoformulateasinglemediumforbothlactococciandlactobacillionlycurdsamples obtained from market and home were tried. After optimization the standardizedmedium was used to obtain LAB present in other fermented milk products. In order toenumerate LAB present in market and domestic curd samples, yeast glucose agar andElliker's agar were used as such and also incorporated with pH indicators bromocresolpurple (0.005 per cent) and bromophenol blue (0.002 per cent). Yeast glucose agar andEllikeragarmediaconsideredasgeneral-purposemediaforlacticacidbacteriawhenused forplatingofmarketand domesticcurdsamples,hadviablecountsof8.38and8.42while

Elliker'sagarexpressedlessercountsoftotallacticacidbacteriaof8.16and7.84 log_{10}cfu/g, respectively (Table 3).Similarmodewasnoticedin Bromocresolpurpleat0.005percent andBromophenolblueof0.002percentincorporatedyeastglucoseagarandElliker'sagarseparately,whereEllik er agarwithbromophenol bluefailedtorecoverlacticacidbacteriato fuller extent when compared with yeast glucose agar with boromocresol purple as thecountsobtained on Ellikeragarwas comparablylesser (Table 4).

Table4:Enumerationoflactic cocci and lactobacillifromcurdsampleusingYeastGlucoseAgar(YGA)andEllikerAgar(EA)withBrom ocresolpurple(BCP) and Bromophenol blue(BPB)

Samplename	Mediumusedforplatinglacticcocci an lactobacilli					
	YGA	EA	Incorporation ofBCPat0.005 % to		Incorporation ofBPB at0.002 % to	
			YGA	EA	YGA	EA
	Viablecount(log_{10}cfu/g)					
Marketcurd (MC1)	8.38[a]	8.16[a]	8.30[a]	8.00[a]	8.10[a]	7.80[a]
Domesticcurd(HC1)	8.42[a]	7.84[b]	8.30[a]	7.70[a]	8.00[a]	7.50[b]
CD(*P*=.05)	**0.57**	**0.24**	**0.73**	**0.74**	**0.48**	**0.17**

Onthecontrary,Bromophenolblue(BPB-0.002percent)insteadofBromocresolpurple(BCP-0.005percent) was recommendedinMRSagarwhencomparedwith Plate count agar as MRS-BPB that led to maximum recovery of probiotic cultures inyogurtdrinkof1x10^8comparedtoPCA-BCPenumerationofLAB(2.5x10^7)withblue-colouredlactobacillicoloniesanditalloweddifferentiationofeachLABinamixedculture[15].

3.3.1 Enumerationoflactic cocci and lactobacilli fromcurdsamplesusingyeastglucoseagarandEllikeragarwithpHindicatorscomparedwithdirectmicroscopic count:

YeastglucoseagarandElliker'sagarwereusedassuchandalsoincorporatedwithpHindicatorsbromocresolpurple(0.005percent)andbromophenolblue(0.002percent).Thedirectmicroscopiccountsofbothcurdsampleswerearound8logcountforcocci,rods and yeasts of market and domestic curd samples respectively (Table 5).

Table5:Enumeration oflactic cocci and lactobacillifromcurdsampleusingYeastGlucoseAgar(YGA)andEllikerAgar(EA)withoptimized levels of Bromocresolpurple (BCP)andBromophenol blue(BPB) compared with direct microscopic count.

Sample name	Typeofcells			Mediumusedforplatinglacticcocci and lactobacilli							
	Cocci	Rods	Yeast	YGA	EA	IncorporationofBC at0.005 %			EA	Incorporation of BPB at0.002 %	
						YGA				YGA	EA
						Colonytype					
						S	Y	P			

	DMC(log_{10}cells/g)			Viablecount(log_{10}cfu/g)							
Marketcurd (MC1)	8.69[a]	8.48[a]	7.98[a]	8.38[a]	8.16[a]	7.88[a]	8.30[a]	8.60[a]	8.00[a]	8.10[a]	7.80[a]
Domesticcurd(HC1)	8.98[b]	8.52[a]	7.80[a]	8.42[a]	7.84[b]	7.69[a]	8.42[a]	8.80[a]	7.70[a]	8.00[a]	7.50[b]
CD(*P*=.05)	**0.24**	**0.62**	**0.36**	**0.58**	**0.24**	**0.24**	**0.44**	**0.27**	**0.47**	**0.73**	**0.24**

Note:*S–surface colony;Y–Yellow colony;P–Purplecolony*

The viable counts on yeastglucoseagarwerebetterandnearlysameasDMCbutEllikeragarshowedlowercountsoflactic acid bacteria when market and domestic curd samples were plated. Bromocresolpurplewasaddedat0.005percenttoyeastglucoseagar,marketanddomesticcurdsampleswere plated three types of colonies obtained were surface, yellow and purple colonies inmarket & domestic curd samples. Bromophenol blue of 0.002 per cent in yeast glucoseagarandEllikeragarwhenusedforplatingdidnotshowcolonydifferentiationwithrespectto colour, Hence, use of yeast glucose agar and Elliker agar with boromophenol blue wasstopped in further studies. Yeast glucose agar with Bromocresol Purple (BCP) was usedwhich showed better viable counts of lactic acid bacteria than Elliker agar with BCP. Thetwo pH indicators bromocresol purple and bromophenol blue, bromocresol purple werecompared bromocresol purple worked better with colony differentiation regard to colourslikeyellow and purple. Amediumforstreptococciandlactobacilli,LAE(Lactic-Agar-Elliker),wasdeveloped[6] that contained tryptone, yeast extract, gelatin, glucose,sucrose,lactosesodium chloride,sodium acetate,ascorbic acidwith pH 6.8. While 50mg/mlof2,3,5-triphenyltetrazolium chloride with 1 percentTween80 supplemented to Elliker'slacticagarhelped to produce small, red *Streptococcus thermophilus* coloniesand larger, white *Lactobacillus bulgaricus* colonies. Addition of 7 per cent skim milk(11 per cent solids) to lactic agar in place of 2,3,5- triphenyltetrazolium chloride allowedgood rod-coccus differentiation, regardless of strain or yogurt brand. On this medium,called yogurt lactic agar, *L. bulgaricus* appeared as large white colonies surrounded by acloudy zone and *S. thermophilu*s as smaller white colonies devoid of a surrounding halo[13].Yogurtlacticagarcomparedfavorablywith*S.thermophilus*andLactobaciIlusagarmediaforthe recovery of*S. thermophilus* and*L. bulgaricus* insingle and mixed culture. A total of 10 sucuk (Turkish-type fermented sausage) samples, obtained fromDenizli, had on an average of 8.34 log CFU/g TAMB, 8.91 log CFU/g LAB (at the MRSagar) and average 8.25 log CFU/g LAB (at the Elliker's lactic agar) both lactobacilli andlacticacid producing cocci[14]. Even HiMedia[27] also had developed and recommended for cultivation oflactobacilliand streptococci of importancein dairyindustry.

3.3.2 Effect of addition of bromocresol purple (BCP) to yeast glucose agar on thedifferentiation of colonies oflactic cocci and lactobacilli:

Bromcresol purple is a pH indicator that differentiated the colonies of lactic cocci and bacilli present in fermented milk products. With this background, 0.005, 0.05and 0.5 per cent of BCP was added to yeast glucose agar and poured to plated market and domestic curd samples (Table 6).

Table6:Optimizationofadditionofbromocresolpurple(BCP)toyeastglucoseagarfortheenumeration of lacticcolonies in curdsample

Name of thesample	Yeastglucoseagar withBCP (%)								
	0.005			0.05			0.5		
	Typeof colony								
	S	Y	P	S	Y	P	S	Y	P
	Viablecount(log_{10}cfu/g)								
Marketcurd (MC1)	7.88[a]	8.30[a]	8.60[a]	7.70[a]	8.32[a]	8.56[a]	7.50[a]	8.38[a]	8.58[a]
Domesticcurd(HC1)	7.69[a]	8.42[a]	8.80[a]	7.60[a]	8.44[a]	8.78[a]	7.40[a]	8.40[a]	8.72[a]

CD(*P*=.05)	0.30	0.51	0.44	0.51	0.48	0.32	0.47	0.64	0.38

Note:*S–surface colony;Y–Yellow colony;P–Purplecolony*

Viable counts of surface, yellow and purple colonies on yeastglucoseagarcontainingBCPof0.005,0.05and0.5percentsdidnotshowmuchdifference,BCP added at 0.005 per cent faded the colour of colonies on 2nd day and became difficultto take the counts while 0.05 per cent the colour of the colonies remained the samethroughout the incubation period. BCP with 0.5 per cent led to dark colouration and againthe colonies were difficult to count. Yeast glucose agar with 0.05 per cent bromocresolpurplewas used toobtainviablecountsof lacticacidbacteria in further studies.

3.3.3 Optimizationofmediumfortheenumerationofmixedfloraoflactic cocci and lactobacilli fromcurdsamplesusingyeastglucoseagarwithBCP,calciumpropionateandsodiumbenzoate:

Addition of calcium propionate at 0.2, 0.4, 0.6 and 0.8 per cents to yeast glucoseagar with 0.05 per cent BCP when used for lactic counts in market and domestic curdsamples, thesurfacecoloniesmeansaerobicsporeformingbacterialcoloniesreducedto 0.00 while counts of both yellow indicated lactobacilli and purple colonies indicatedlactococci were more than 8.50 $\log_{10}$cfu/g (Table 7).

Table7:Enumerationoflactic cocci and lactobacillifromcurdsampleusingYeastGlucoseAgar(YGA)withBromocresolpurple(BCP),calcium propionateand sodiumbenzoate

Sample name	Mediumusedforplatinglacticacid bacteria								
	YGA + 0.05 %BCP	Colony Type	YGA+ 0.05% BCP							
			Calciumpropionate(%)				Sodiumbenzoate(%)			
			0.2	0.4	0.6	0.8	0.3	0.6	0.9	1.2
		$\log_{10}$cfu/g								
Marketcurd (MC1)	8.48[a]	S	7.20[a]	5.02[a]	3.26[a]	0.00[a]	7.10[a]	5.00[a]	3.00[a]	0.00[a]
		Y	8.60[a]	8.70[b]	8.78[b]	8.88[b]	8.40[a]	8.60[b]	8.70[b]	8.80[b]
		P	8.80[b]	8.88[c]	8.92[ca]	8.98[ca]	8.80[a]	8.84[ca]	8.90[ca]	8.98[ca]
Domesticcurd (HC1)	8.65[a]	S	7.90[a]	6.80[d]	3.62[ab]	0.00[ab]	7.60[a]	5.40[ab]	3.20[ab]	0.00[ab]
		Y	8.62[a]	8.45[e]	8.82[da]	8.62[da]	8.60[a]	8.76[da]	8.86[da]	8.98[da]
		P	8.72[a]	8.80[f]	8.88[ea]	8.92[ea]	8.78[b]	8.90[ea]	8.96[ea]	9.00[ea]
CD(*P*=.05)	**0.27**		**1.55**	**1.56**	**1.69**	**1.71**	**1.51**	**1.65**	**1.69**	**1.70**

Note: *S–surface colony;Y–Yellow colony;P–Purplecolony*

Sodium benzoate when added at0.3, 0.6, 0.9and1.2percentstoyeastglucoseagarwith0.05percentBCPsametrendwasnoticedwithrespecttosurfacecoloniesthatdrasticallycametonilwhereasyellowcolonies(lactobacilli)and purplecolonies(lactococci)increasedintheirnumbers.Overall reduction of surface colonies and increase in lactic acid bacterial colonies were observedwhen0.8percentcalciumpropionatethatinhibitedsporeformingbacteriaand1.2percentsodium benzoate inhibited yeast incorporated in yeast glucose agar containing 0.05 percentBCP.

3.4 Use of selective yeast glucose agar with0.05percentBCPand optimized levels of calciumpropionateaswellassodiumbenzoate for the enumeration of lactic cocci and lactobacilli in fermented milkproducts:

Marketsamplesofcurd,yogurt aswelldomesticcurdweresubjected for enumeration of lactic acid bacteria present, selective yeast glucose agar wasusedthatcontainedyeastglucoseagarwith0.05percentofBCP,calciumpropionateof 0.8 per cent and sodium benzoate of 1.2 per cent. Market curd (MC1) and yogurt (MY1) samplesshowedhigherlacticcountofmorethan8log(8.84,8.85 log_{10}cfu/g), yellow colonies that indicated lactobaciili (through microscopicexamination) of 8.16 log count on an average while purple colonies of lactococci of 8.51log_{10}cfu/g were noticed in higher total lactic counts among fermented milk products. Two market curd (MC2, MC3), domestic curd (HC1, HC3) as well market yogurt (MY2)samplesexhibitednearly 7.72logonanaverageoftotallacticcountonyeastglucoseagarwith0.05percentBCP,yellowcoloniesoflactobacilliof7.20logandnearlysamecounts of purple colonies were noticed (Table 8).

Table 8: Enumeration of lactic cocci and lactobacilli from fermented dairy products using selective Yeast GlucoseAgar(YGA)with optimized levels of Bromocresolpurple(BCP)withbothcalciumpropionateandsodiumbenzoate

Samplename	Sample code	Mediumusedforplatinglacticacid bacteria		
		YGA with 0.05 %Bromocresolpurple (control)	YGA+ 0.05 % BCP+ 0.8 % Calcium propionate +1.2 %Sodium benzoate (selective YGA)	
			Colonytype	
			Yellow	Purple
		log_{10}cfu/g		
Marketcurd	MC1	8.84[a]	8.16[a]	8.56[a]
	MC2	7.77[a]	7.30[a]	6.95[a]
	MC3	7.60[a]	7.00[a]	6.95[a]
Marketyogurt	MY1	8.85[a]	8.00[a]	7.27[a]
	MY2	7.47[a]	8.47[a]	7.32[a]
Domesticcurd	HC1	7.90[a]	7.20[a]	7.50[a]
	HC2	8.50[a]	8.20[a]	8.48[a]
	HC3	7.60[a]	7.27[a]	7.47[a]
CD(*P*=.05)		**2.54**	**2.59**	**2.57**

Thus, yeast glucose agar with BCP of 0.05 per cent + calcium propionate of 0.8per cent and sodium benzoate of 1.2 per cent could be used as selective media for totallacticcountinfermentedmilkproductsthatalsodifferentiatesyellowcolonyaslactobacilliandpurple colony for lactococci, an added advantageof themedium.On par with the present study, 50mg/mlof2,3,5-triphenyltetrazoliumchloride,aredoxindicator was added to Elliker's lactic agar toproducesmall,red*Streptococcusthermophilus*coloniesandlarger,white*Lactobacillusbulgaricus*colonies from commercial yogurt samples[13]. On the contrary MRS with Bromo phenol blue showedadvantagesinenumerationofLABduetoincubationtimethanPlate count agar with Bromocresol purple,that alloweddifferentiationofeach LAB in amixedculture[15].M1agar,containingtwochromogenicsubstrates(β-galactosidase&β-glucosidase),allowedselective enumeration *of Lactobacillus rhamnosus*, two strains of *Lactobacillus paracasei*ssp. *paracasei*and *Streptococcus thermophilus* based on differential β -galactosidase andβ-

glucosidaseactivitiesandincubationat37°Cor44°Ctoincreaseselectivity.Asecondagar medium, M2, containing one chromogenic substrate (β-galactosidase) was used toselectivelyenumerateβ-galactosidaseproducing*Lactobacillusdelbrueckii*ssp.*bulgaricus*at 47 °C. By contrast with the usual culture media, the chromogenic method allowedunambiguous enumeration of each species, including discrimination between the two *L.paracasei*,up to 10^9CFU/g offermented milk[30].

3.5 Activityoflacticcolonies appearedon selectiveyeastglucoseagar

Theyellowsubmergedcoloniesthatappearedonyeastglucoseagarcontaining0.05per cent BCP with 0.8 per cent calcium propionate and 1.2 per cent sodium benzoateobtainedfromcurdsamplemicroscopicallyrevealedrodswheninoculatedtosterileskim milk set the curd in 8 h had 0.63 to 0.68 per cent lactic acid. Purple colonies that appearedon selective yeast glucose agar when curd or yogurt was plated demonstrated cocci withacidityof0.63to0.66percentlacticacidthatsetthemilkin8hafterinoculationofcolony.Both the colonies revealed catalase positive and acid, reduction and coagulation in litmusmilk (Table 9).

Table9:Activity oflacticcoloniesappeared onselectiveyeastglucoseagar

Name of thesample	Selectiveyeastglucose agar (YGA + 0.05 % BCP + 0.8 % Calcium propionate +1.2 %Sodium benzoate)			
	Colonymorphology	Cellmorphology	Setting Time(h)	TitratableAcidity (%Lacticacid)
Market curd(MC1) /Domesticcurd(DC1)	Yellowsubmerged	Rods	8	0.63[a]
	Purplesubmerged	Cocci	8	0.66[a]
Marketyogurt (MC1)	Yellowsubmerged	Rods	8	0.68[a]
	Purplesubmerged	Cocci	8	0.63[a]
CD(*P*=.05)				**1.78**

The confirmation of rods and cocci which might be lactobacilli and lactic cocci thatappeared on yeast glucose agar with 0.05 per cent BCP + 0.8 per cent calcium propionateand 1.2 per cent sodium benzoate might be a prime medium for fermented milk productsto get total lactic counts as lactic cocci and lactobacilli instead of using separate media forlactococci(M17 agar)and lactobacilli (MRS agar). LAB strains (13) were identified on the basis of phenotype out of whichseven isolates (53.8 per cent) were found to belong to the genus Lactobacillus while theremaining six isolates (46.2 per cent) were under the genus Streptococcus. IdentifiedLactobacillusspeciesincluded*L.delbrueckii,L.hilgardii*,and*L.plantarum*while*Streptococcus* species were identified as S. *faecalis, S. lactis, S. thermophilus* and *S.faecium*. Among the identified all isolates, *S. lactis, L. delbrueckii* produced the highestacid (w/v) 0.46 per cent after 72 h. of incubation period. The isolates were obtained fromtheyoghurtsampleswithhighercountsof lactic acid bacteria (LAB) thatrangedfrom1.0 $\times10^6$to5.6×10^7cfu/mLand2.2×10^7to5.4×10^8cfu/mLonMRSagar(pH6.2-6.6)andRogosaagar(pH5.2-5.6)fortotalLABenumerationaswellaslactobacilliisolationwhileYGLA(pH7.0)fortheisolationofstreptococciincubatedat37°Cfor48-72hatanaerobic conditions[19]. On par with the present study, the formulated selective Yeast glucose agar with 0.8 and 1.2 per cents of calcium propionate and sodium benzoate, respectively to control aerobic spores and yeast (common contaminants of fermented milk products) along with bromocresol purple of 0.05 per cent helped to form purple coloured colonies of for lactic streptococci from market curd, yogurt and domestic curd samples[31].

3.6 Comparison oflacticcountsoffermentedmilkproductsonselectiveYGA

Comparison of viable counts on M17 and MRS with calcium propionate andsodium benzoate with selective yeast glucose agar containing bromocresol purple withcalcium propionate and sodium benzoate was carried out. Selective M17 (1.5 % calciumpropionate + 1.5 % sodium benzoate) and selective YGA (BCP 0.05 % 0.8 calciumpropionate + sodium benzoate 1.2 % - purple colonies as lactococci. had around 8 logviable count of lactococci in fermented milk products. Selective MRS and YGA (yellowcolonies as lactobacilli) media also showed similar counts of lactobacilli. The correlationcoefficient, r was 0.78 between selective M17 of lactococciand selectiveyeast glucoseagarforpurplecolonyoflacticcocci (thismightincludelactococci,streptococci,leuconostoc or pediococci) while r was 0.67 (Table 10) when MRS lactobacilli count was comparedwithyellowcoloniesoflactobacillionselectiveyeastglucoseagarindicatinggood relationship between both the types of selective agar media and better relationship wasbetweenselectiveM17agarandselective (differential) yeastglucoseagarwithrespecttopurplecoloniesoflacticcocci.

Table10:Comparisonoflacticcountsoffermentedmilkproductsonselectiveyeast glucose agar

Samplename	Sample code	Mediumusedforplatinglacticacidbacteria			
		M17+ 1.5 % CalciumPropionate (CP) + 1.5 % Sodium Benzoate(SB) (lacticcocci)	YGA+ BCP 0.05% + CP0.8%+ SB 1.2% (selective YGA) Purple colonies (lacticcocci)	MRS+ CP 2 % + SB 2 % (lactobacilli)	YGA+BCP0.05 % + CP0.8 % + SB 1.2 % (selective YGA) Yellow colonies (lactobacilli)
		log_{10}cfu/g			
Marketcurd	MC1	8.10	8.26	8.00	8.16
Marketyogurt	MY1	8.08	8.10	7.80	8.00
Domesticcurd	HC1	8.18	8.20	8.00	8.20
R(correlation coefficient)		**0.78**		**0.67**	

4. CONCLUSION

The readymade medium for lactic streptococci M17 agar medium gave better results when compared to formulated medium. The formulated medium with inhibitory agents like calcium propionate (0.8 %) and sodium benzoate (1.2 %) helped to curb the common contaminants like aerobic bacterial spore formers and yeast, respectively in the fermented milk products. Instead of using two selective media such as M17 and MRS agar media for lactic cocci and lactobacilii, respectively, in this study an attempt was made to convert general purpose medium yeast glucose agar (YGA) to selective and differential medium for bo the microflora which can be identified by colony colour differentiation. The calcium propionate and sodium benzoate along with pH indicator bromocrseol purple incorporation to yeast glucose agar medium, a general purpose medium can be easily converted to differential medium. The viable lactic streptococci formed purple colonies while yellow colonies of lactobacilli as a measure of differentiation based on acid production in case of mixed lactic flora in fermented milk products containing both lactic cocci and lactobacilli, the inhibitors calcium propionate and sodium benzoate inhibited the most common microbial contaminants of fermented milk products, aerobic spore formers and yeasts, respectively.

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