Biological Forum – An International Journal **15(4): 632-636(2023)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Enumeration of Lactic streptococci from Fermented Milk Products using Differential Medium

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(Received: 17 February 2023; Revised: 12 March 2023; Accepted: 18 March 2023; Published: 20 April 2023)
(Published by Research Trend)

ABSTRACT: In order to formulate a single medium for the accurate enumeration of lactic streptococci in fermented milk products avoiding common microbial contaminants and to easily find the colonies an attempt has been made in this study. Market samples of curd, yoghurt probiotic drink and domestic curd samples were enumerated using formulated and readymade media like M17 for lactococci as well as *Streptococcus thermophilus* incubated in candle jar at 30ºC and 37ºC respectively for 48 h. The readymade media gave better recovery of viable cells of lactic streptococci. The incorporation of calcium propionate and sodium benzoate at 1.5 and 2 per cent each to M17 agar helped in control of both aerobic spore formers and yeasts. Yeast glucose agar with 0.05 per cent Bromocresol Purple (BCP) showed better viable counts of lactic streptococci. In order to reduce aerobic spore forming bacteria and yeasts, calcium propionate of 0.8 per cent and sodium benzoate of 1.2 per cent incorporation to yeast glucose agar with 0.05 per cent BCP led to formation of more than 8 log counts of purple colonies and staining of colony smear revealed presence of cocci in chains. The purple colonies when inoculated to sterile skim milk set the curd in 8 h with 0.65 per cent lactic acid on an average indicating the presence of lactic streptococci. The findings of the study led to development of selective medium for lactic streptococci with addition of calcium propionate of 0.8 per cent to avoid Bacillus spores and sodium benzoate of 1.2 per cent to inhibit yeast to yeast glucose agar medium which is considered as general purpose medium for lactic streptococci with 0.05 per cent BCP could be used one of the selective medium for the enumeration of lactic streptocci with formation of purple colonies.

Keywords: Lactic streptococci, Yogurt, Candle jar, Aerobic spore formers, Yeast.

INTRODUCTION

Fermentation is considered as safe and acceptable preservation technology of food and fermentation using LAB can be categorized into two groups based on the raw material used, non-dairy and dairy fermentation. Recently, there is a growing interest to develop a variety of fermented milk products for other beneficial purposes, particularly for health purposes and preventing of toxins produced by food borne pathogens and spoilage bacteria that enter human body. The beneficial effects of fermented milk products are produced by a variety of bioactive compounds of LAB. The most well-known characteristics of LAB related to preservative property is their ability to produce acid, which in turn exhibit antimicrobial activity. Acidification of the milk protects the milk against spoilage microorganisms and proliferation of pathogens. LAB also release antimicrobial metabolites so called bacteriocins. Both acids and bacteriocins have great potential, used in food preservation which are considered as safe natural preservatives. Lactic streptococci that include *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. lactis bv. *Diacetylactis* and *Streptococcus thermophilus*. Out of these starter bacteria all the three species of *Lactococcus* are mesophiles while *Streptococcus thermophilus* are thermophilic in nature and all the four lactic streptococci are homofermenters producing only lactic acid from glucose after hydrolysis of disaccharide lactose. Lactic acid bacteria are grouped based on growth temperature and type of fermentation. Based on optimum growth temperature, lactic cultures are classified as mesophilic and thermophiles as given by Lactic acid bacteria are chemotrophic, they find that energy is required for their entire metabolism from the

oxidation of chemical compounds (Delavenne *et al*., 2012; Andi *et al.,* 2014).

A number of different selective media were created for the isolation of certain groups of lactic acid bacteria from yoghurt and other fermented dairy products. These include M 17 and Tryptose-Proteose-Peptone-Yeast extract-Eriochrome T (TPPY) for lactococci and streptococci reported that the growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* was suppressed on pour plates, when the pH of the M17 medium was adjusted to 6.8 (Shankar and Davies 1977). Dave and Shah (1996) found ST (*Streptoccus thermophilus*) agar containing 10.0 g of tryptone, 10.0 g of sucrose, 5.0 g of yeast extract, and 2.0 g K_2HP0_4 in 1 L of distilled water with pH of 6.8 $\pm$ 0.1, and 6 ml of 0.5 per cent bromocresol purple and 12 g of agar, was suitable for the selective enumeration of *S. thermophilus* from a mixed culture containing *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and Bifdobacteria, *S. thermophilus* formed yellow colonies with viable count of 8.50 cfu/g of was obtained. The pH of ST agar is 6.8, which might have been crucial in the growth suppression of Lactobacilli and Bifidobacteria when the plates were incubated for 24 h under aerobic conditions. Numerous media have been used for the detection, isolation and enumeration of yeasts and molds in foods. Traditionally, acidified potato dextrose agar (pH 3.5), wort agar (pH 4.8), malt extract agar (pH 3.5) and other acidified media have been used because they inhibit colony formation by most bacteria. Food mycologists now recognize that such media often yield lower yeast counts than media containing antibiotics. Stressed yeasts may not resuscitate at pH 3.5 and lactic acid bacteria that form colonies may interfere with yeast colony development and enumeration. Proteins from food samples may precipitate in acidified media, making enumeration of yeasts difficult (Tharmaraj and Shah 2003).

Calcium propionate ($C_6H_{10}CaO_4$) which is having MW of 186, soluble in water. The compound is effective at pH 5.5. It prevents microbes from producing the energy as they are protonophores at 0.4-1 per cent that inhibited Bacillus spores as well yeast and molds in LAB media. Sodium benzoate (C_6H_5COONa) is having MW of 144. Optimum activity of this compound is observed at pH 5-6 as it is soluble in water. The concentration of 0.1-0.3 per cent inhibited yeast and molds by leakage of PMF and thus ATP synthesis of fungi is affected (Schillinger and Holzapfel 2003; Mann and Beachut 2008). If fermented milk has yeast as contaminant then yeast predominates over LAB. Sometimes even Bacillus spp. make colonies, both contaminants may create problems in the isolation of LAB. Hence media may require addition of antimycotic (sodium benzoate) and anti-sporulating (calcium propionate) agents. It is soluble at 28°C in methanol, poorly soluble in water but better solubility is observed in phosphate-citrate buffer at 5.7. Briggs (1953) noticed that if only one lactic culture is used in fermented milks, the problem to enumerate will be less. But the problem encountered will be in a mixture of lactic cultures used in the preparation. The viability of each group of lactic culture may be a problem Tomato juice was found to stimulate the growth of many LAB and it was included in Briggs agar. The addition of sodium azide to inhibit the contamination flora, including Gram negative flora and purple bromocresol allowed a direct selection of the Gram + and lactose + flora. An acidic pH (5.0) can also be helpfully considered after protein hydrolysis to avoid protein denaturation (Djeghri-Hocine *et al*., 2010). Viable count estimation from commercial yogurt using BCP agar containing 0.5 % polypeptone, 0. 5 % yeast extract, 0.1 % glucose, 0.1 % Tween 80, 0.01 % L-cysteine and 0.006 % bromocresol purple with pH 7.0, revealed lactic counts of 3.7×10^8, 2.7×10^8, 6.7×10^8 and 5.8×10^8 cfu/g for plain, apple, blue berry and yogurt prepared from raw milk, respectively (Nishino *et al.,* 2017). A load of *Lactococcus* spp. was counted as 1.12×10^7, 8.01×10^7 and 2.75×10^9 CFU/ml from raw cow's milk, cheese and yogurt, respectively, on M17 agar (Taye *et al.,* 2021). 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 (Guley *et al*., 2022; Guley *et al.,* 2022a).

MATERIALS 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* was carried out by direct Microscopic count and viable count through serial dilution technique and pour plate method as prescribed by Harrigan (1998). The media used are M17 agar formulated (weighed each ingredient and prepared the medium as per the composition of Himedia manual, 1998) and readymade media (HiMedia), yeast glucose agar (Harrigan, 1998). The plates of lactococci and *Streptococcus thermophilus,* were incubated at 30° C/48 h and 37°C/48 h in anaerobic candle jar. 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 cfu/g of the product. Antimycotic (sodium benzoate) and sporulating (calcium propionate) agents were added to media prior to pouring into petri dishes. The pH indicator bromocresol purple was added to medium after sterilization used for plating. 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.

RESULTS AND DISCUSSION

Enumeration of lactic acid bacteria from commercial and domestic fermented dairy products using Formulated medium (FM) and Readymade medium (RM):

One market yogurt sample showed high lactic acid of 1.02 per cent while one sample of domestic curd had lower acidity noticed of 0.68 per cent. The viable count of lactococci on formulated M17 medium in fermented milk samples ranged from 7.53 to 8.88 $\log_{10}$cfu/g whereas highest count was found in yoghurt of about 8.88 $\log_{10}$cfu/g while market curd sample had low count of 7.53 $\log_{10}$cfu/g. But when readymade M17 medium was used, lactococci colonies ranged from 7.95 to 8.95 $\log_{10}$cfu/g in fermented products in which more viable count of 8.95 was observed in one brand of market yogurt sample and low viable count of 7.95 in one market curd sample (Table 1). Among formulated and readymade medium used for lactococci (M17 agar) readymade media gave better recovery of viable cells of lactic acid bacteria, which might be due to use of pure forms of ingredients in the dehydrated media compared to formulated ones where each ingredient is weighed which was time consuming. Readymade media are easy to prepare as it was just reconstitution and warming and do not require any pH adjustment which was not true with formulated media. Hence in the further studies readymade M17 agar medium was used for lactic streptococcal viable counts. BRIGGS (Briggs, 1953), a medium for streptococci was developed. M17 agar medium has remained the most commonly used standard media, exhibiting consistent growth for lactic streptococci (Hayek *et al*., 2019).

Enumeration of lactic acid bacteria from fermented milk products using calcium propionate and sodium benzoate incorporated M17 readymade medium for the inhibition of aerobic spore former and yeast:

Market yogurt showed more acidity of 1.10 while domestic curd sample had lower acidity of 0.68 per cent, with range of 0.68 to 1.10 per cent lactic acid in fermented milk products (Table 2). The viable count of lactococci in market curd, yogurt and domestic curd samples on control M17 agar was 8.98, 8.70, 8.80 that showed reduced counts of 8.60, 8.38, 8.48 on M17 with 1.5+1.5 per cents of calcium propionate and sodium benzoate both, which might be attributed to inhibition of aerobic spore formers as well as yeasts. Reduction in counts was noticed in M17 agar after incorporation of calcium propionate and sodium benzoate at 1.5 per cent each helped in control of both aerobic spore formers and yeasts that appeared as surface colonies on both the media and thus could be successfully used in the laboratories for the microbiological analysis of fermented milk products to get actual counts of lactococci. Taye *et al.* (2021) enumerated the load of *Lactococcu*s spp. as 1.12×10^7, 8.01×10^7 and 2.75×10^9 CFU/ml from raw cow's milk, cheese and yogurt, respectively, using M17 agar.

Effect of addition of bromocresol purple (BCP) to yeast glucose agar differentiation of colonies of lactic acid bacteria:

Bromcresol purple is a pH indicator that differentiated the colonies of lactic based cocci and bacilli present in fermented milk products. With this background, 0.005, 0.05 and 0.5 per cent of BCP was added to yeast glucose agar and poured to plated market and domestic curd samples. Viable counts of surface, yellow and purple colonies on yeast glucose agar containing BCP of 0.005, 0.05 and 0.5 per cents did not show much difference, BCP added at 0.005 per cent faded the colour of colonies on 2nd day and became difficult to take the counts while 0.05 per cent the colour of the colonies remained the same throughout the incubation period. BCP with 0.5 per cent led to dark colouration and again the colonies were difficult to count. Significant difference (P=0.05) was not noticed among the per cent of BCP used in yeast glucose agar with respect to type of colonies obtained on yeast glucose agar. Yeast glucose agar with 0.05 per cent bromocrsol purple was used to obtain viable counts of lactic acid bacteria in further studies (Table 3). On par with the present study, Matalon and Sandine (1986), selected 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, a redox indicator to produce small, red *Streptococcus thermophilus* colonies and larger, white *Lactobacillus bulgaricus* colonies. Lee and Lee (2008) compared PCA-BCP, mMRS-BPB as BPB changed colour of colonies within a range from pH 3 to 5, it was useful for detection of the pH change produced during fermentation of LAB. mMRS-BPB showed advantages in enumeration of LAB due to incubation time than PCA-BCP, supported growth of all LAB and it allowed differentiation of each LAB in a mixed culture.

Optimization of medium for the enumeration of lactic streptococci from curd samples using yeast glucose agar with BCP, calcium propionate and sodium benzoate:

Addition of calcium propionate at 0.2, 0.4, 0.6 and 0.8 per cents to yeast glucose agar with 0.05 per cent BCP when used for lactic counts in market and domestic curd samples, the surface colonies means aerobic spore forming bacterial colonies reduced to 0.00 while counts of purple colonies indicated lactococci were more than 8.50 $\log_{10}$cfu/g. Sodium benzoate when added at 0.3, 0.6, 0.9 and 1.2 per cents to yeast glucose agar with 0.05 per cent BCP same trend was noticed with respect to surface colonies that drastically came to nil where as purple colonies (lactococci) increased in their numbers. Concentration of calcium propionate and sodium benzoate at 0.2 and 0.3 percent incorporated in yeast glucose agar did not show significant difference (P=.05) in the counts of lactic acid bacteria present in fermented milk samples. But as the concentration of calcium propionate and sodium benzoate increased like 0.4, 0.6, 0.8 and 0.6, 0.9, 1.2, respectively, affected the counts of lactic acid bacteria on yeast glucose agar (0.05 per cent BCP) with significant differences in surfaces and purple colonies. Overall reduction of surface colonies and increase in lactic streptococcal colonies were observed when 0.8 per cent calcium propionate that inhibited spore forming bacteria and 1.2

per cent sodium benzoate inhibited yeast incorporated in yeast glucose agar containing 0.05 per cent BCP (Table 4). The purple colonies on the selective agar when inoculated to sterile skim milk took 8 hours to curdle the milk with 0.65 % lactic acid indicating the presence of lactic streptococci. Guley *et al.* (2022) modified FSDA (mFSDA) with increased selectivity of nalidix acid (inhibit gram negative bacteria), ascorbic acid and yeast extract stimulated the growth of lactic streptococci and the pH indicator bromocresol purple enabled the chromogenic discrimination between LAB with different acid production capability.

Table 1: Enumeration of lactic streptococci from commercial and domestic fermented dairy products using Formulated media (FM) and Readymade (RM).

Sample name	Code	Titratable Acidity	Lactococci in curd/ Str. Thermophilus in yogurt	
			M17	
			FM	RM
		%Lactic acid	Viable count (log_{10}cfu/g)	
Commercial fermented dairy products samples				
Curd	MC1	0.76 [a]	7.59 [a]	8.00 [a]
	MC2	0.80[a]	7.56 [a]	7.95 [a]
	MC3	0.81[a]	7.53 [a]	8.00 [a]
Yogurt	MY1	1.02[a]	8.88 [a]	8.95 [a]
	MY2	1.00 [a]	8.80 [a]	8.91 [a]
Domestic samples				
Curd	HC1	0.68[a]	8.68 [a]	8.88 [a]
	HC2	0.72[a]	8.65 [a]	8.90 [a]
	HC3	0.83[a]	8.50 [a]	8.78 [a]
CD (P=.05)		**2.53**	**2.61**	**2.59**

Note: CD – Critical difference; For lactococci M17 medium (formulated –components weighed & readymade media) was used with incubation at 30°C/48h in candle jar; All the values are average of three trials; Same superscripts in the column indicate non-significance while different superscripts indicate significance difference

Table 2: Enumeration of lactic streptococci from curd sample using calcium propionate and sodium benzoate incorporated M17 readymade media.

Name of the sample	Sample code	Titratable Acidity (per cent LA)	Control M17	Addition of calcium propionate + sodium benzoate %			
				0.50+0.50	0.75+0.75	1.0+1.0	1.5+1.5
				M17			
			Viable count (log_{10}cfu/g)				
Market curd	MC1	0.72[a]	8.98[a]	8.90[a]	8.86[a]	8.80[a]	8.60[a]
Market yogurt	MY1	1.10[a]	8.70[a]	8.62[a]	8.58[a]	8.46[a]	8.38[a]
Domestic curd	HC1	0.68[a]	8.80[a]	8.68[a]	8.60[a]	8.51[a]	8.48[a]
CD (P=.05)		**1.21**	**1.23**	**1.23**	**1.23**	**1.23**	**1.23**

Note: CD – Critical difference; Readymade media for lactococci M17 agar with incubation at 30 °C /48 h & for lactobacilli MRS agar in candle jar at 37 °C /48 h was used; All the values are average of three trials; Same superscripts in the column indicate non-significance while different superscripts indicate significance

Table 3: Optimization of addition of bromocresol purple to yeast glucose agar for the enumeration of lactic colonies in curd sample.

Name of the sample	Yeast glucose agar with Bromocresol purple %					
	0.005		0.05		0.5	
	Type of colony					
	S	P	S	P	S	P
	Viable count (log_{10}cfu/g)					
Market curd (MC1)	7.88[a]	8.60[a]	7.70[a]	8.56[a]	7.50[a]	8.58[a]
Domestic curd (HC1)	7.69[a]	8.80[a]	7.60[a]	8.78[a]	7.40[a]	8.72[a]
CD (P=.05)	**0.30**	**0.44**	**0.51**	**0.32**	**0.47**	**0.38**

Note: CD- Critical difference; S – surface colony; Y – Yellow colony; P – Purple colony; Incubation of plate s done at 30 °C for 48 h. in candle jar; All the values are average of three trials; Same superscripts in the column indicate non-significance while different superscripts indicate significance difference.

Table 4: Enumeration of LAB from curd sample using Yeast Glucose Agar (YGA) with Bromocresol purple (BCP), calcium propionate and sodium benzoate.

Sample name	Medium used for plating lactic streptococci									
	YGA with 0.05 % Bromocresol purple	Colony Type	YGA + 0.05 % BCP							
			Calcium propionate (%)				Sodium benzoate (%)			
			0.2	0.4	0.6	0.8	0.3	0.6	0.9	1.2
			log_{10}cfu/g							
Market curd (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]
		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]
Domestic curd (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]
		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: CD – Critical difference; Incubation of the plates at 30 °C for 48 h. in candle jar; All the values are average of three trials; Same superscripts in the column indicate non-significance while different superscripts indicate significance difference.

CONCLUSIONS

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. The calcium propionate and sodium benzoate along with pH indicator bromocrseol purple helped in proper expression of viable lactic streptococci inhibiting the common contaminants aerobic spore formers and yeast in fermented milks with differentiating purple colonies.

FUTURE SCOPE

The components that separate the lactic streptococcal flora in mixed cultured fermented milk products need to be identified for better differentiation of lactococci and their species and biovar.

Conflict of Interest. None.

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How to cite this article: Gopal S., Yadav S., Ramachandra B. and Prabha R. (2023). Enumeration of Lactic streptococci from Fermented Milk Products using Differential Medium. *Biological Forum – An International Journal, 15*(4): 632-636.