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BIOTECHNOLOGICAL INTERVENTION USING PROBIOTIC MICROBES TO SUSTAIN THE SHELF LIFE QUALITY OF MILK PRODUCTS

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ABSTRACT

Dairy products are highly perishable at room temperature. To enhance their storage at ambient temperatures, biotechnological intervention is warranted. For this operation microbes can be used as tools. Probiotic microbes isolated from fermented milk product like curd can be used to extend the shelf life of milk products by combining it with other commercial strains. Prolonged storage of milk products like curd in ambient temperature promotes spoilage by enhancing the growth of microbes and converts it in to a highly acidic product with unpleasant odour and rejection. To overcome these issues and to enhance the shelf life period at ambient temperature starter microbial inoculums can be applied. In the present study. *Bacillus brevis* (MMI strain) a bacterial isolate from curd and yoghurt was combined with a commercial probiotic starter strain *Lactobacillus acidophilus* in different ratios and this bacterial combination was used as starter culture to ferment raw and pasteurized milk in the curd formation. *L. acidophilus* and *B. brevis* mixture in the ratio 1:1(v/v) was found to yield a good quality curd with a short curdling period without any whey formation. The acidity of the curd was 0.45% to 0.64% and P^H ranged between 6.95 - 6.61 during the storage time of 0 – 144 hours. The microbial count ranged between 10 - 342 10^8CFU/ml, during 0 - 144 hours. The shelf life, taste and sensory qualities of curd developed from raw and pasteurized milk remained in good quality for five days while applying *L. acidophilus* and *B. brevis* combinations as starter cultures. The present report will ensure the commercial availability of the curd for an enhanced shelf life period.

KEYWORDS: *B .brevis, L. acidophilus*, pasteurized milk, curd, dairy products.

INTRODUCTION

Enhancing the stability and shelf life of food using microflora is an innovative approach in the preservation of foods and beverages. The bio- preservative microbes prevent premature spoilage by extending the shelf life and safety of food products (Lianou et al., 2016) Food products get spoiled due to the initial contamination of the product during production, harvesting, post harvesting, processing and distribution. Further endogenous factors like P^H, water activity and substrate and extrinsic influence from temperature relative humidity and atmosphere are also counted for food spoilage (Batt 2016).

Microbial spoilage of milk and dairy products is an area of great concern. To decontaminate milk and, probiotic microbes mostly lactic acid bacteria (LAB) serves good. They not only create unfavorable environment for most spoilaging microbes but also exerts probiotic role (Griffth and Ro-beiro, 2012). Lactic acid bacteria produces bacteriocin which helps to control pathogens in milk yogurt and cheeses (Silva et al., 2018). The LAB comprises Gram – positive, non-sporulating, anaerobic or facultative aerobic bacilli and cocci primarily of the genera Lactobacillus, Leuconostoc, Pediococcus and Streptococcus.

Bacteriocin of LAB origin tolerates P^H variations, resistant to high temperature active against food spoiling pathogens, sensitive to digestive proteases and do not affect gut microbiota (Ahmad et al, 2017). The peptides or proteins in bacteriocin promote a good bio-preservative activity (Singh 2018).

Milk is supposed to be a vehicle for many human pathogenic bacteria (Claeys et al, 2013). Pasteurisation is of course useful to reduce background spoilage microbiota, it can't yield a total sterile product (Galvez et al, 2014). The curd prepared from both raw and sterilized milk if kept at ambient temperature without refrigeration it gets spoiled and its taste, flavor and acidity altered rendering it to a unpalatable food. This can be overcome by using beneficial microbes as starter cultures. Kiran et al., (2012) reported that the use of *Brevibacillus brevis* as starter culture sustained the quality of the curd for 8 days even in ambient conditions and it is proved that the curd formation period and its quality retention is influenced by the starter cultures. Lactic acid bacteria are used as

starter culture in dairy fermentations because they produce lactic acid, which adds texture, aroma, flavor and anti spoilage effect. In addition promote probiotic action in the gut (Hati et al., 2013). LAB produces different types of bacteriocin, of which nisin have been identified for wider applications in food industry (Singh 2018).

The effect of bacteriocin produced by the bacteria, *Enterococcus faecium*, *Pediococcus acidilactici*, and *Lacto bacillus planatarum* were found effective in inhibiting the milk products spoiling *Listeria innocua* (Patrovsky et al 2016). To enhance the shelf life period of curd at ambient conditions ,to sustain its palatable quality and incorporating beneficial probionts in the present study a microbial isolate from curd mixed with well proved *L.acidophilus* bacteria was tried to produce a high quality commercial food grade curd.

MATERIALS AND METHODOLOGY

MILK SAMPLE

The pasteurized milk (500ml) retail pack was collected from Aavin Brand of Tamilnadu. The raw milk was collected directly from the cow sheds found in the nearby village regions of Periyapalayam, Thiruvallur district, A North Tamilnadu region.

CURD AND YOGURT

The commercially available curd samples of two different brands namely Heritage of 20ml pack and Milky mist of 50ml cup and a fruit yogurt sample of Mother Dairy of 50g cup were collected for the isolation of the bacterial strain.

DETERMINATION OF P^H

The P^H of the pasteurized milk and the raw milk was determined using the P^H digital meter manufactured by the Electronics India Laboratory Instruments.

DETERMINATION OF ACIDITY (%)

The acidity was determined using the titration method of (Kiran et al., 2009). The samples were tested for the presence of LAB using the MRS agar plating method. The samples were prepared in different (10^{-2}, 10^{-3} and 10^{-4}) dilutions and plated in the Man Rogosa Sharpe medium (MRS) agar. After 48h of incubation at 35-42^{0}C the colonies were counted. The commercially purchased curd and yogurt samples were diluted (10^{-2} to 10^{-6}) using sterile distilled water. The prepared dilutions were plated over the solidified MRS agar and left for 24-48 hours of incubation at 35-42^{0}C. The isolated colonies were morphologically analysed and identified. As per Kiran et al., (2012) work the pasteurized milk sample of 15ml and raw milk sample of 15ml were inoculated with the three different colonies.

Five morphologically district colonies were isolated for further testing. Using the five isolates, MM1, MM2, H1, Y1 and Y2 as starter inoculums to ferment milk to form curd, experiments were carried out. Using the starter culture, the time taken for curd formation, whey formation, whey off period and setting of curd were analysed. The culture that performed well with lowest time taken for curdling was again purified. The purified culture MMI was further identified using biochemical testing. It was found to be *Brevibacillus brevis*. To combine *B.brevis* with other probiotic culture, the commercial culture of *Lactobacillus acidophilus* was procured from IMTECH, Chandigarh, India. The cultures were maintained on skim medium. Sensory analysis of the curd was examined with experts in dairy products. The flavor, color, cutting quality, sourness, taste and overall acceptance were evaluated using a 10-point hedonic scale (McWilliams, 2001). The mean intensity scores of the entire attributes to rate the quality were if 10 - highly acceptable and if 2 - rejected.

PREPARATION OF THE MOTHER CULTURE AND IDENTIFICATION OF THE DESIRED RATIO

The pasteurized and raw milk of 20ml were sterilized for 20 minutes using autoclave. The isolated strain which produced desired result (MMI) and the *Lactobacillus acidophilus* were mixed in a ratio of 1:1, 2:1 and 1:2(v/v) and inoculated to the milk samples for incubation at 37^{0}C. Based on the curd quality such as the cutting quality, whey off period, whey formation and curdling time, the desired ratio for the inoculums was chosen. To 100ml of the pasteurized and raw milk sample the chosen desired mother culture was added. The inoculated milk samples were incubated at 37^{0}C for the curdling of the milk. The milk sample were analysed for P^H, acidity % and microbial load for every 30 minutes after the inoculation of the microbes.

STARTER CULTURE INNOCULATION

Before the experiments, 20ml of both the raw and pasteurized milk were sterilized in an autoclave for 20 minutes. To the sterilized milk *B. brevis* and *L. acidophilus* were added in different ratios 1:1, 2:1 and 1:2. A loopful of freshly prepared culture in different ratios were inoculated into the sterilized milk sample and incubated at temperature of 37^{0}C till curd was produced.

CURD QUALITY ANALYSIS

The acidity, microbial load and P^H of the curdling sample were periodically analysed with a time internal of 0, 24, 48, 72, 96, 120 and 144 hours. Acidity was measured using the technique of. AOAC (2005).

In the present study Lactic acid bacteria were isolated from curd and yogurt of the different isolates, the strains that were found to induce short curdling duration were further isolated and identified. The identified microbe, *Brevibacillus brevis* was used along with commercial strain *L.acidophilus* for milk products protecting starters with different dose combinations. The effect of these two bacteriocin producing LAB on the curd formation time and curd quality were tested in raw and pasteurized milk. The new combination of *L.acidophilus* and *B.brevis* is a

kind of new approach in using LAB as starter to prevent microbial spoilage of dairy products. The bacteriocins to be isolated from these strains can be further used in food industry.

Bacteriocins are ribosomally synthesized extracellular antimicrobial low molecular mass proteins or peptides offering bacteriostatic or bactericidal action on closely related bacteria – Gram positive bacteria by interfering with the molecular mechanism of cytoplasmic membrane of bacteria. Bacteriocin produced by the LAB are of different categories.

Small, heat stable, peptide – Lantibioticc (<5KDa) eg. Nisin, Lactin
Larger (<10KDa) heat stable pediocin peptides.
Two peptide bacteriocin (Lactococcin).
Large (>30KDa) peptides sensitive to heat (Enterocin, Helveticin)
All these products from LAB provide the bio preservative action from their starter culture (Kesankas et al., 2006 and Cleveland et al., 2001).

RESULTS AND DISCUSSION

In order to find out a new LAB bio-preservative for milk products particularly to use it as starter culture to obtain quality curd at a short duration, the present study was planned. For the present study the raw material used were pasteurized milk and non-pasteurized milk. The biochemical characteristics of the raw milk are given in Table 1 and 2. In the pasteurized milk the LAB count was 10 x 10^8 CFU/ml but in non-pasteurized milk it was 26x10^8CFU/ml. The pasteurized milk was found to contain 6.4% fat, 9.2% SNF, P^H of 6.93, 0.82% of acidity, LAB microbial count of 10 (*108CFU/ml), aerobic plate count of 1 (*108CFU/ml), 6.2g of fat, 3.5g of protein, 4.8g of carbohydrate, 445mg of minerals and energy value of 92 KCal for 100 ml of milk(Table 1). The raw milk was found to contain 14.5% fat, P^H of 6.85, 0.93% of acidity, LAB microbial count of 26 (*10^8CFU/ml), aerobic plate count of 4 (*10^8CFU/ml), 3.5g of fat, 3.4g of protein and 4.8g of carbohydrate for 100ml of milk. (Table2). To obtain a desirable LAB that can be used as good starter inoculums, preliminary screening was done. From the commercially available yogurt and curd five bacterial isolates are isolated and selected (MM1, MM2, H1, Y1 and Y2). The selected isolates were cultured again to get pure colonies and used as inoculums in the curdling process of pasteurized milk. In this curdling process, the time taken for curdling time, whey formation, whey off time and setting time were noted (Table 3) of the different cultures used MM1 and MM2 showed less time for the formation of curd, 3h and 4.5h respectively. In MM2 isolates used curd, whey formation was absent and setting of curd was little less than MM1 used curd. So, the culture MM1 was selected as desired LAB and it was identified to be *Brevibacillus brevis* strain (Fig. 1a – 1e).

The isolated *B. brevis* was tested for its efficacy to prepare quality curd. Like *B. brevis* the commercially available LAB, *Lactobacillus acidophilus* was also used as starter culture to prepare curd in pasteurized milk. Further both *B brevis* and *L. acidophilus* were mixed in a ratio 1:1, 1:2 and 2:1 and inoculated as starter culture to prepare the curd. The curdling time and curd quality were recorded and it was found that the curd prepared out of *L .acidophilus and B brevis* in a ratio 1:1 was having less curdling time and superior curd quality (Fig. 3, Table4-6). Further the three cultures, *B. brevis*, *L.acidophilus* and their mixing in the ratio 1:1 were tested and their efficacy was compared. The acidity percentage, P^H, microbial count and curd quality were recorded every 24h till the curd formation is complete. For the curd quality assessment flavor, color, cutting quality, sourness and taste were recorded by an expert panel using a 10 point quality scale assessment. The score for the flavor of the curd was between 7.0-7.3. during 24 hrs to 144 hrs. It was 7.1 for *Brevibacillus brevis* fermented product, 7.0 for *L. acdiphillus* and 7.2 for mixed culture at 144 hrs. At 192 hrs except *L.acidophilus* treated curd the other two treated curds showed poor score for flavor. The scoring for the colour of the mixed culture fermented product was 8.5 at 144 hrs but it was 8.1 and 8.0 for *L.acidophillus and B.brevis* used curd at 144 hrs respectively. Even at 192 hrs there was no marked change in colour of the curd. The cutting quality of curd was comparatively higher for mixed culture treatment (7.5) than the other two treatments at 144 hrs. The cutting edge quality did not show much variation even at 192 hrs.

The score for the sourness of the curd was 6.4 at 144 hrs and 6.2 at 192 hrs. For the other two treatment curds the sourness did not change much even at 192 hrs. The score for taste was high for mixed culture treated curd samples than the other two treatments. It was 7.3 at 144 hrs and 7.0 at 192 hrs. The sensory score for the curd prepared using the mixed culture of microbes was higher than the other two. The elevation of the flavor in mixed culture treated groups could be due to the proteolytic release of small peptides and amino acids (Liu et al., 2010). Also lipolysis was reported to influence the flavor formation(Casaburi et al., 2008). The use of mixed culture protected the curd from early flavor deterioration. The score for the colour of the curd was good for the preparation made using mixed culture even after 192hrs. It is reported that the curd get colour change due to microbial contamination(Seo et al., 2007) In the present study the incorporated microbial strains inhibited the growth of the contaminants and prevented the change in colour till 192 hrs in ambient conditions. The sensory score for the cutting quality of the curd was also high for mixed culture treated samples(7.5 at 144 hrs and 7.4 at 192 hrs.). Kiran et al (2012) reported that the use of probiotic microbes enhanced cutting quality of the curd or the firmness of the curd. The sourness of the prepared curd using the mixed culture remained good till 192 hrs and the score was 6.4 at 144 hrs and 6.2 at 192 hrs.

Acidity increase is associated with sourness (Kiran et al., 2012). In the present study the incorporation of mixed culture inhibited acid formation and prevented sourness well till 192 hrs. Acid production are reported to affect the sensorial characteristics by inducing sour taste and gas formations (El Zubeir et al. 2007). The acidity values for standard curd need to be in the range of 0.70% to 0.90%. (Johanson and Alford 1987). In the present study the acidity and pH values were in the range 0.42% to 0.54% (pH --6.87-6.60) for non- pasteurized milk turned curd and 0.45% to 0.64% (pH -6.95-6.61) for pasteurized milk turned curd from day 1 to day 5. The acidity, P^H and microbial count varied in relation to the age of the curd and type of milk source. In the pasteurized milk the initial microbial load was $10x10^8$ CFU/ml on day1 and $392x10^8$ CFU/ml at 120 hrs but decreased to $342x10^8$ CFU/ml at 144 hrs. In non pasteurized milk it was 27 $x10^8$ CFU/ml on day1 and $363x10^8$ CFU/ml at 72hrs and reduced to 342 $x10^8$ CFU/ml at 96 hrs. From the study it is clear that the curd prepared using the combination of *B.brevis* and *L.acidophilus* in a ratio 1:1 was found highly efficient in fermenting the milk to form curd.

The decrease in the microbial count, acidity and P^H after 120h in raw milk curd and 72h in pasteurized milk curd may be due to the reduction of nutrient source and accumulation of toxic metabolites as reported (Kiran et al., 2012).

If acidity increases the sensorial characters are affected due to sour taste and gas formations. As per Indian Standard (acidity percentage 0.6 – 0.8) the acidity in the curd formed after the inoculums is 0.67% at 120hrs for non- pasteurized milk and 0.58% for pasteurized milk at 72h. The lower value of acidity less than 0.6% as per Indian standard may be due to less lactose fermentation as reported earlier (Kiran, et al., 2012).

The sensory analysis of the curd that was obtained after fermentation with *L acidophilus, B brevis* and its mixture in 1:1 ratio showed that the curd fermented with the mixed culture of *B brevis and L acidophilus* showed a high consumer standard when compared to the other starter cultures (Fig. 3 Table5&6).

CONCLUSION

The Lactic acid bacteria belong to the genus Lactococcus, Streptococcus, Lactobacillus, Pediococcus, Leuconostoc, Enterococcus and Propioni bacterium play an important role in food fermentations and are used as starter culture in dairy products. In addition to accelerate the fermentation process, some of them release bio preservative antimicrobials and organic acids to inhibit the growth of spoiling microbes. The organic acids like lactic acid and propionic acid interfere with the functions of cytoplasmic membrane of bacteria and these acids interfere with the molecular mechanism of yeasts and moulds and inhibit their growth. The antimicrobials produced by the LAB, *Lactobacillus reuteri* (Reuterin) interferes with ribonucleotide reductase in spoilage microbes (Kesenkas et al., 2006). The sensory analysis of the curd that was obtained after fermentation with *L acidophilus, B brevis* and its mixture in 1:1 ratio showed that the curd fermented with the mixed culture of *B brevis and L acidophilus* showed a high consumer standard when compared to the other starter cultures.

Table 1: Nutritional Profile of pasteurized milk (500ml).

	Description	**Standardized Full Cream milk**
	Pack colour	Red
	Quantity	500ml
	Fat (%)	6.4
	SNF (%)	9.2
	P^H	6.93
	Acidity (%)	0.82
	LAB microbial count	10 (*10^8CFU/ml)
	Aerobic plate count	1(*10^8CFU/ml)
	Fat (g) (per 100ml)	6.2
	Protein (g) (per 100ml)	3.5
	Carbohydrate (g) (per 100ml)	4.8
	Minerals(mg) (per 100ml)	745
	Energy value (Kcal) (per 100ml)	92

Table 2: Nutritional Characteristics of raw milk.

	DESCRIPTION	**Raw milk**
	P^H	6.85
	Acidity (%)	0.93
	Fat (%)	14.5
	Fat (g) (per 100ml)	3.5
	Carbohydrate(g)(per 100ml)	4.8g
	Protein (g) (per 100ml)	3.4g
	LAB microbial count	26 (*10^8CFU/ml)
	Aerobic plate count	4 (*10^8CFU/ml)

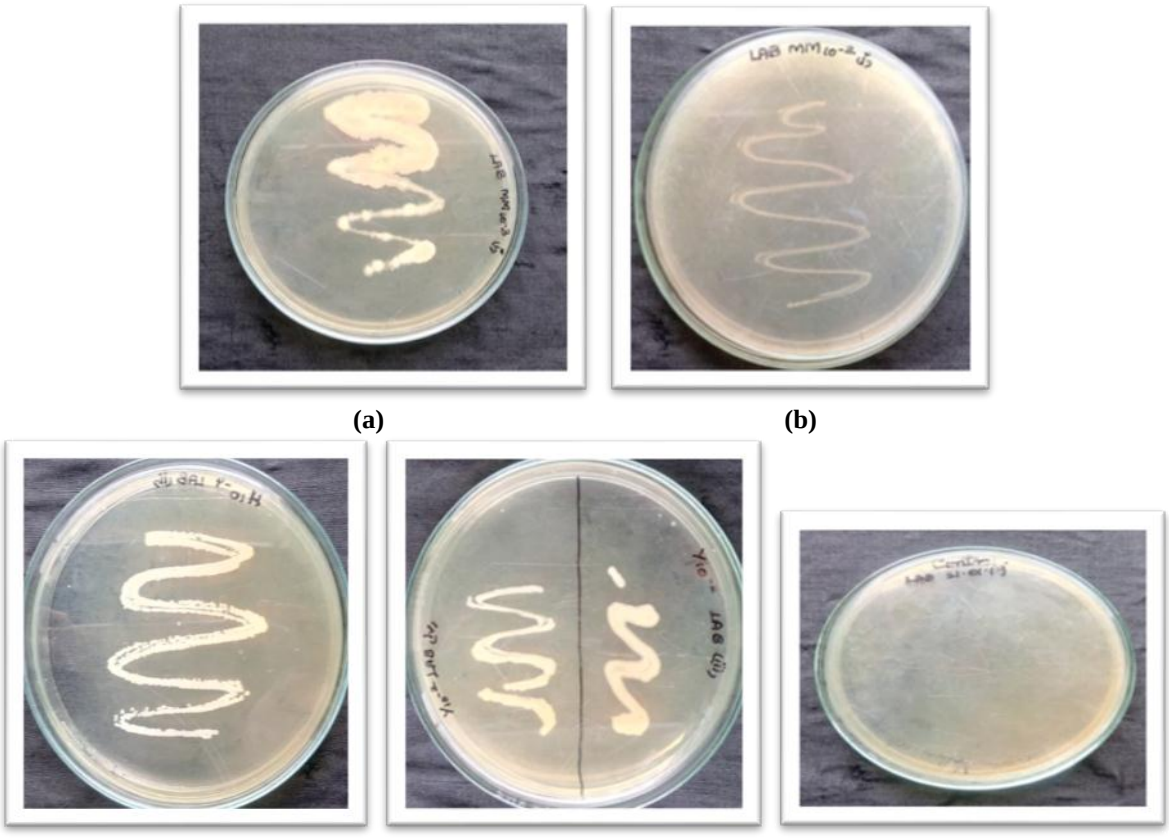
Table 3: Characteristics of the Curd Formed By Inoculation by Isolates MM1, MM2, H1, Y1 AND Y2.

Parameters	MM1	MM2	H1	Y1	Y2
Curdling Time	3 Hours	4.5 Hours	8 Hours	6 Hours	5.5 Hours
Whey Formation	Nil	Positive	Positive	Nil	Positive
Whey off period	4 Days	2 Days	10 Hours	20 Hours	1 Day
Setting of Curd	Good	Better	Poor	Good	Poor

Table 4: Sensory score of the characteristics of the curd formed by the inoculation of *B brevis* (BB) *and L acidophilus* (LA) and its combination of 1:1 ratio (MIX)(v/v).

Time of Fermentation (hours)	Flavour			Colour			Cutting Quality			Soureness			Taste		
	LA	BB	MIX	LA	BB	MIX	LA	BB	MIX	LA	BB	MIX	LA	BB	MIX
0	7	7.1	7.5	8	8.1	8.6	7	7.3	7.6	6.0	6.1	6.4	6.3	6.7	7.4
20	7	7.1	7.4	8	8.0	8.8	68	7.2	7.6	6.0	6.1	6.5	6.2	6.7	7.3
40	7	7.0	7.4	8	8.0	8.4	7.0	7.1	7.5	5.7	6.0	6.4	6.4	6.6	7.6
72	7	7.1	7.4	8	8.1	8.5	6.9	7.2	7.6	5.4	6.0	6.1	6.5	6.8	7.5
96	7	7.0	7.4	8.1	8.0	8.3	6.6	7.4	7.3	5.3	6.0	6.1	6.4	6.5	7.4
120	7.1	7.0	7.3	8	8.0	8.4	6.3	7.0	7.6	5.4	5.9	6.4	6.1	6.6	7.4
144	7.0	7.1	7.3	8.1	8.0	8.5	6.8	7.3	7.5	5.2	5.9	6.4	6.1	6.6	7.3
168	7	-	7.2	8.0	8.0	8.2	6.4	7.1	7.4	5.0	5.6	6.3	6.0	6.3	7.0
192	7	-	-	8.0	8.0	8.2	6.4	7.1	7.4	5.2	5.6	6.2	6.0	6.3	7.0
			-												

Fig 1 (a): MRS media plated with MM1 isolate
Fig 1 (b): MRS Media plated with MM2 isolat
Fig 1 (c): MRS media plated with H1 isolate
Fig 1 (d): MRS media plated with Y1 and Y2 isolate
Fig 1 (e): MRS media as Control



(a) **(b)** **(c)** **(d)** **(e)**

Fig 1 (a)

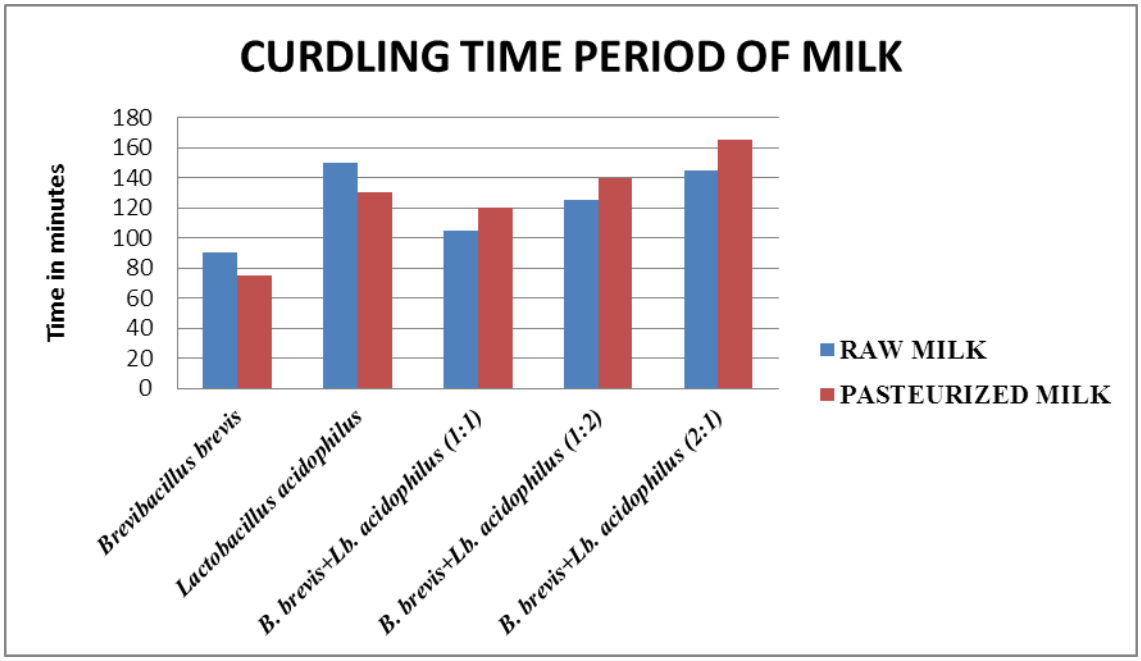


FIG 2: Curdling time period of raw and pasteurized milk in minutes for various starter culture.

Table 5: Quality of curd from the raw milk (Acidity %, pH and Microbial count).

AGE OF CURD	ACIDITY (%)	pH	MICROBIAL COUNT IN MRS ($* 10^8 CFU/ml$)
0	0.42	6.87	27
24	0.54	6.73	325
48	0.57	6.70	347
72	0.58	6.67	363
96	0.54	6.60	354

Table 6: Quality of curd prepared from the Pasteurised milk (Acidity %, pH and Microbial count).

AGE OF CURD	ACIDITY (%)	pH	MICROBIAL COUNT IN MRS ($* 10^8 CFU/ml$)
0	0.45	6.95	10
24	0.60	6.79	273
48	0.61	6.70	289
72	0.62	6.65	310
96	0.65	6.63	352
120	0.67	6.61	392
144	0.64	6.61	342

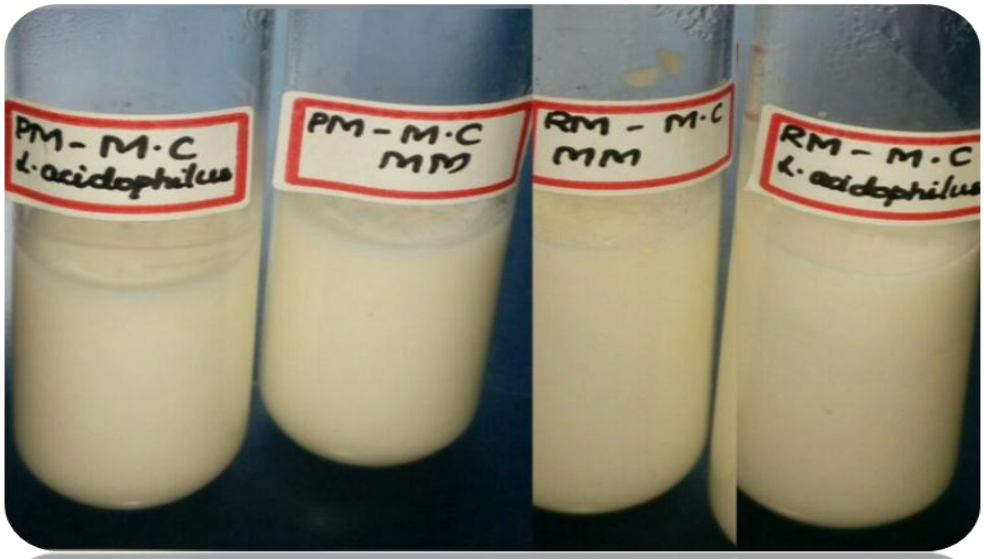


FIG 3: Mother culture prepared by inoculation of *B. brevis* and L *acidophilus* in Raw and Pasteurized milk.

FIG 4: Pasteurized and Raw milk samples inoculated with the *B.brevis, L. acidophilus* and 1:1 ratio of the *B brevis* and *L acidophilus.*

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