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Research Paper

Fermentation Kinetics and Sensory Attributes of Milk Fermented by Probiotic Bacteria

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Abstract

Two indigenous human originated potentially probiotic cultures *Lb. helveticus* MTCC 5463 (LH) and *Lb. rhamnosus* 231 (LR231) were used to ferment milk and products were examined for their fermentation characteristics and sensory attributes. *Lb. rhamnosus* GG (LGG) and commercial dairy starter YC-381 (YC) were used as control. Decline in pH and corresponding increase titratable acidity of milk fermented by probiotic strains were similar to YC. Amongst the probiotic strains, LH was found to produce highest acid which was comparable to dairy starters. Viability of all probiotic organism did not differ significantly and remained above 8 log cfu/g in freshly fermented product. LH produced significantly higher lactic acid whereas the concentrations of the acetic acid did not vary among the probiotic strains and dairy. Highest viscosity of fermented product obtained with LGG, however, whey separation was not found to be affected. Milk fermented by probiotic organism are found to be comparable, in their sensorial attributes, to commercial dairy starters. Hence, the indigenous probiotic strains could be utilize as dairy starter to produce fermented milk.

Keywords: Probiotic stain, concentration, fermented food.

The demands by the consumers are driving food manufacturers towards new products, which can help to maximize the physiological functioning on the one hand and reduce the risk of diseases on the other, ultimately led to the concept of optimal nutrition. To this concept, functional food is one of ruler to improve nutrition. Diplock *et al.,* (1999) defined the functional food as "A food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way

that is relevant to either improved stage of health and well-being and/or reduction of risk of disease. In this context, probiotics have received considerable attention of dairy and food industry. Probiotics can be defined as "living microbial feed supplements added to the diets which have beneficial effects on the host" (FAO/ WHO, 2002).

Role of LAB in fermented food is well known since ancient times. Of various LAB genera commonly used in probiotic preparations, *Lactobacillus spp.* isone of the most widely studied (Collins *et al.,* 1999). Health promoting effects both in vivo and in vitro of potentially probiotic lactobacilli are recently reviewed by many researchers (Gupta and Garg, 2009; Kleerebezem and Vaughan, 2009; Kolida and Gibson, 2010). Despite of their desired health properties, probiotics should meet several basic requirements for the development of marketable probiotic products including their survival and activity in the product, and stability during storage of the product. To realize the health benefits, the probiotics in fermented milk must be viable and available at a concentration of at least 10^6cfu per gram of the product (Ramchandran and Shah, 2010). Moreover, probiotics should not adversely affect the taste or aroma of the product nor acidification during the shelf life of the product (Tamime and Robinson, 2007).

Flavour compounds, primarily, lactic acid, acetic acid, butyric acid, carbonyl compounds (acetaldehyde or diacetyl) produced by lactobacilli couldcontribute to aroma of fermented milks and hence their quantitativedetermination of is not only important for monitoring bacterialgrowth and activity, but also due to their significance as natural preservatives and for sensory characteristicsof the product (Izco *et al.,* 2002; Fernandez-Garcia & McGregor, 1994). Similarly, ability of some lactobacilli strains to produce exopolysaccharides (EPS) during fermentation and gel formation contributes to the improvement in texture and viscosity in fermented milk product (Hassan *et al.,* 1995). Since many probiotic bacteria are sensitive to stresses such as oxygen, heat and acid exposure, they perform poorly in many food environments, particularly in fermented foods, which may ultimately lead to a short shelf-life of fermented milk products (Stanton *et al.,* 2005). Nevertheless, other factors like pre-heat treatments, standardization of milk base and post-fermentation handling could lead significant effects on physico-chemical properties and sensory characteristics of final product (Tamime and Robinson, 2007).

To date, most of the work has been focused on evaluation of acidification activity and survival ability of starter cultures and probiotic strains (Dave and Shah, 1997; Ramchandran and Shah, 2010). In addition, studies have also been carried out on influence of starter strains on physico-chemical properties and sensory attributes of fermented milk (Fernandez-Garcia & McGregor, 1994; Hassan *et al.,* 1995; Tamime and Robinson, 2007). This work has been carried out with an objective of studying the acidification activity and survivability of two indigenous

human originated potentially probiotic strains *Lb. helveticus* MTCC 5463 and *Lb. rhamnosus*231 in milk medium as well as their influence on physico-chemical properties and sensory profile of the final product. *Lb. rhmnosus* GG was used as control probiotic strain and commercial yoghurt starter containing *S. thermophilus* and *Lb. bulgaricus* was used to produce control yoghurt.

Materials and Methods

Bacterial strain and their activation

Pure strains of *Lb. helveticus* MTCC 5463 (LH) was acquired from the Culture Collection center of Department of Dairy Microbiology, Anand Agricultural University (Anand, India) and *Lb. rhamnosus* 231 (LR231) was obtained from Department of Microbiology, Saurashtra University (Rajkot, India). *Lb. rhamnosus* GG (LGG) was obtained from Belgian Co-ordinated Collection of Microorganisms (Ghent, Belgium). All these pure strains were activated from their frozen forms (stored in 40 g 100/mL glycerol at - 20°C) by giving one transfer in MRS broth. Thereafter, the cultures were transferred once each to sterile reconstituted skim milk (RSM; 12 g 100/mL). For each transfer, the rate of inoculation was 1 g/100 ml and the temperature of incubation was 37°C. Commercial starter cultures YC-381 (YC) (*S.thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) was obtained from Chr. Hansen A/S (Horsholm, Denmark) in freeze dried form and stored as per the recommendation of the manufacturer.

Preparation of products and sampling

Fermented milk was prepared using cow milk containing 3% fat (Skånemejerier, Sweden) that was standardized to 12 g/100ml total solids with skim milk powder. The milk was preheated temperature of 80°C and the heated mix was held at this temperature for 30 min followed by cooling to 45°C before inoculation. Active cultures of *S. thermophilus* MTCC 5460, *Lb. helveticus* MTCC 5463, *Lb. rhamnosus*231, *Lb. rhamnosus* GG were added at the rate of 2% (w/w) whereas Commercial dairy starters were added as per the recommendations of the manufacturer. The inoculated mix was then mixed thoroughly and dispensed in 50 mL polystyrene cups with lids and incubated at 42°C until either for 24 hours or the pH dropped to 4.5±0.1. The fermentation was stopped by transferring the cups immediately to refrigerator maintained at 4±1°C. The product making experiment was quadruplicate. Samples of inoculated mixes (0 h) were removed prior toincubation for enumeration of the viable counts, measuring pH,determining lactic acid content and acetic acid. Samples of freshly fermented milk were removed from the refrigeratedstorage at 18 h post-manufacture. This was referred to as day 1sample. All samples were analysed for changes in pH, lactic acidcontent,

and acetic acid content, viability of starter cultures and probiotics as well as forviscosity, whey separation and sensorial characteristics.

Determination of pH and titratable acidity

Samples were removed at 0, 4, 8 12 and 24h and analysed for pH and titratable acidity. The pH values of the samples during fermentation were monitored using a pH meter (Mettler Toledo, USA). The titratable acidity (TA) was determined after mixing a sample with 10 ml of hot (65±1°C) deionised water and titrated with 0.1 M NaOH using 1% (w/v) phenolphthalein (BDH Prolabo, Belgium) as an indicator. The results were expressed as per cent lactic acid. (Dave and Shah, 1997).

Viability of starter bacteria

Viability of starter bacteria was monitored only for probiotic grown in the fermented milk at 42°C at 0, 4, 8, 12 and 24 h as well as at 1d. Serial dilutions were carried out using sterile PBS. For the individually fermented blend, thelactobacilli colony counts were determined by enumeration on MRS agar (de Mann *et al*., 1960). The counts were expressed as $\log_{10}$ colony forming units (CFU) per gram of fermented milk.

Determination of organic acids

Determination of organic acids was carried out using high performance liquid chromatography (HPLC) according to Ramchandran and Shah (2010) with slight modification. Briefly, 3 mL yoghurt samples were mixed with 50 µL of 15.5 M nitric and 1.0 mL of 0.01M sulfuric acids. The resulting mixture was centrifuged at 14,000 x g for 30 min (Eppendorf 5415, USA) for removal of proteins. The supernatant was filtered through a 0.20 µm membrane filter (Schleicher &Schuell GmbH, Dassel, Germany) into an HPLC vial. The separation of organic acids was achieved using a HPLC (Waters, MA, USA) fitted with an Aminex HPX - 87H, 300 x 7.8 mm ion exchange column (Biorad Life Science Group, Hercules, USA) and a guard column maintained at 45°C. The mobile phase was 5mM H_2S0_4 with a flow rate of 0.6 mL/min. The column was equipped with a refrective index detector (RID-6A; Shimadzu, Kyoto, Japan). Quantification of acetic and lactic acids was performed from the standard curves obtained using solutions of pre-determined concentrations.

Determination of viscosity and whey separation

The viscosity of the fermented milk samples were measured at 25°C using Brookfield viscometer (model DV-II, Brookfield Engineering Laboratories, MA, USA) with a constant shear rate using spindle No. 2 during 60s. The spontaneous

whey separation in freshly fermented samples was determined using the siphon method by Ramchandran *et al.,* (2008) with slight modification. The whey was removed. Thereafter, the samples were weighed and phase separation calculated by dividing the weight of whey siphoned with the initial weight of thesample. The results were expressed as percentage spontaneous whey separation.

Sensory Evaluation

Six panellists were engaged for their liking and preference evaluation, which were performed on different occasions. Liking scales were defined using 9-point for mouthfeel, acidity, flavour, appearance, texture and overall acceptability as described by Stone and Sidel (2004). Coded samples of freshly fermented product were given to the panellist. The results of sensory evaluations were reported as mean value with standard deviation.

Statistical analysis

Data were processed using One-way ANOVA in Minitab software package version 14.0 (Minitab Inc, State College, PA, USA) with a least significant difference of 95%. Results represent the mean value and the standard deviation. Values with a $P<0.05$ were considered statistically significant. Correlation analysis was employed, where appropriate using Microsoft Excel Statpro software. All measurements were performed in duplicate except the sensory evaluation.

Results and Discussion

The changes in pH and titratable acidity of fermented milk prepared by potentially probiotic cultures and commercial dairy starters are illustrated in Figure 1 and 2 respectively. The initial pH of milk (6.68-6.7 at 0 h) decreased to 3.54-4.06 for probiotic cultures and commercial dairy starters at 24 h of continuous fermentation. The decline in pH was significantly higher in LH and LR 231 ($P < 0.05$) as compared to LGG and YC. After the initial drop, a gradual decrease in the pH was observed throughout the incubation period and the trend was identical for all the starter cultures, similar to that observed for titratable acidity (Figure 1). The initial TA of milk (0.12-0.13%) increased to 0.92-1.18% in fermented milk made from cultures LH, LR231, LGG and YC at 24 h incubation. The increase in titratable acidity was minimal for culture LGG and as compared with culture LH, LR 231 and YC. Since there was significant difference ($P<0.05$) in decline of pH and corresponding increase in titratable acidity, it is likely that these changes would have affected viability of probiotic organisms during fermentation. The results of the present study are in agreement with the results reported by Dave and Shah (1997), Ramchandran and Shah (2010).

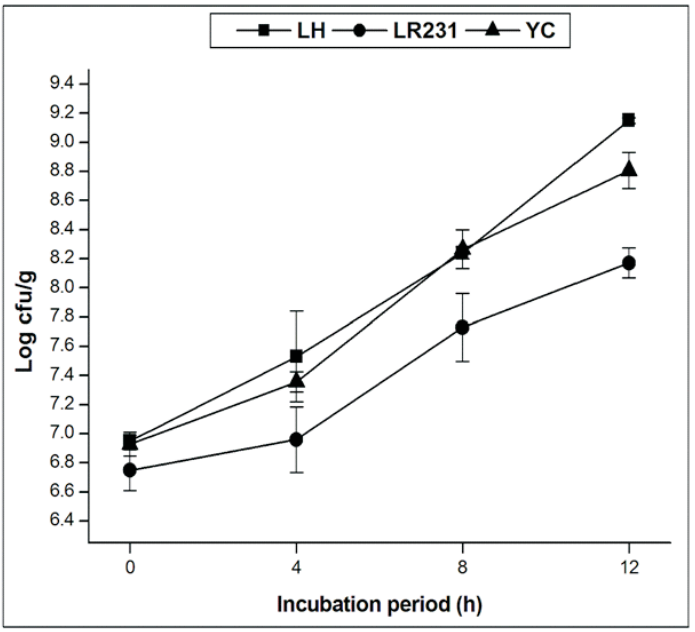

Fig. 1. Changes in pH of cow milk during fermentation at 42°C by potentially probiotic organisms and commercial dairy starter

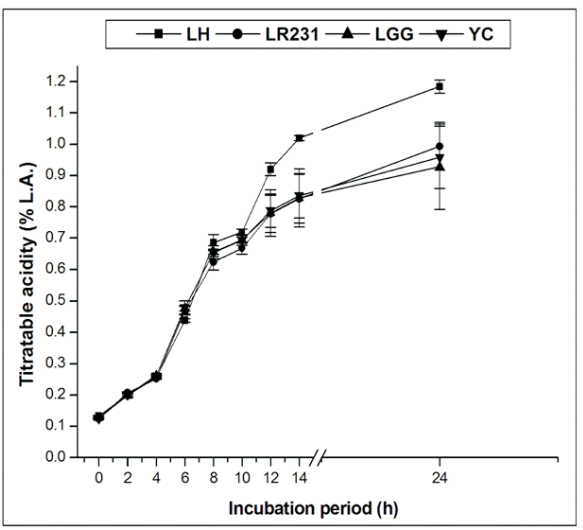

Fig. 2. Changes in titratable acidity (%L.A.) of cow milk during fermentation at 42°C by potentially probiotic bacteria

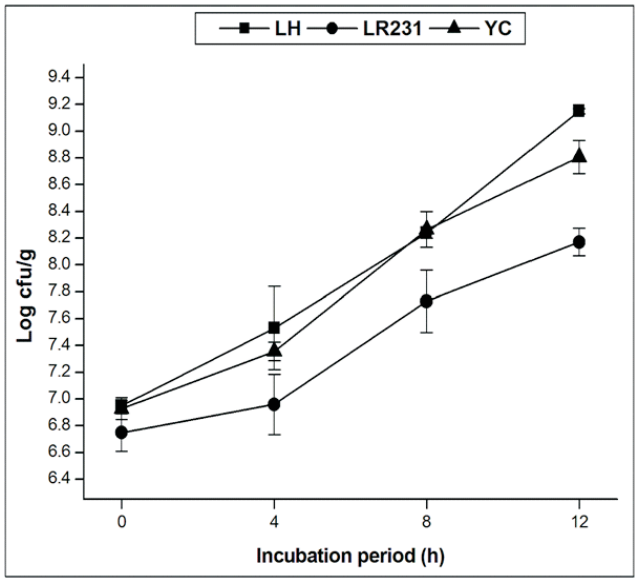

Fig. 3. Changes in total viable counts of potentially probiotic organisms during fermentation of cow milk at 42°C

The changes and survival of probiotic organism during the fermentation are given in Figure 3. The initial TVC of *Lb. helveticus* MTCC 5463, *Lb. rhamnosus* 231 and *Lb. rhamnosus* GG in milk were in the range of 6.7-6.9 log cfu/g at 0 h. Significant increase ($P<0.05$) in TVC of all probiotic cultures were observed throughout the incubation period of 12 h. Lb. helveticus MTCC 5463 and Lb. rhmanosus GG did not significantly differ ($P>0.05$) in their growth and survival in milk medium (Δ log cfu = 1.9-2.2) while Lb. rhmnosus 231 had lowest viability (Δ log cfu =1.42) amongst all studied probiotic cultures. The increase in survival of all probiotic organisms during the fermentation of milk in the present experiment might be affected by pH drop and accumulation of organic acids produce by same probiotic organism. Similar findings were reported by other researchers (Kailasapathy *et al.,* 2007; Vinderola *et al.,* 2002). The evaluation of suitable probiotic starter to fermenta milk is a crucial step in the development of new probiotic products. In thispresent work we show the capacity of three human originated probiotic organism to ferment the milk and provide similar fermentation characteristics as commercial dairy starters. Consequently, the milk was fermented with all four starters until pH 4.5 and examined post 18 h manufacture (1 d) for its fermentation characteristics and sensory properties.

The concentration of lactic acid and acetic acid in milk fermented by probiotic cultures and dairy starters at 1 d are given in Table 1. Yoghurt starter exhibited

Table 1. Fermentation characteristics of potentially probiotic culture and commercial dairy starters

Starter culture	Lactic acid (mg/ml)	Acetic acid (mg/ml)	TVC (log cfu/g)	Viscosity (pa.s)	Whey separation (%)
LH	13.63 ± 0.73a	5.27 ± 0.40a	9.33 ± 0.12a	21.91 ± 0.44a	1.79 ± 0.13a
LR231	12.9 ± 50.91b	5.10 ± 0.64a	8.22 ± 0.1a	24.10 ± 0.50a	1.73 ± 0.14a
LGG	11.74 ± 0.88c	4.60 ± 0.58a	8.88 ± 0.15a	31.4 ± 0.37b	1.84 ± 0.15a
YC	16.11 ± 0.81d	5.52 ± 0.68a	ND	22.68 ± 0.44a	1.54 ± 0.17a

Data represents Mean ± standard deviation

ND= Not determined

[abcd]Means with different superscript letters were significantly different ($P<0.05$) for particular starter organism

Table 2. Sensory attributes of fermented milk prepared from potentially probiotic culture and commercial dairy starters

Starter culture	Acidity	Flavor	Appearance	Texture	Overall acceptability
LH	7.50 ± 2.07a	6.33 ±1.21a	7.00 ± 0.89a	6.83 ± 1.47a	6.83 ±1.47a
LR231	6.33 ± 1.97b	7.17 ±1.33a	7.33 ± 1.03a	7.17 ±0.41b	7.00 ±1.55a
LGG	5.67 ± 2.58c	6.17 ±1.72a	7.50 ± 1.22a	6.67 ± 1.21a	5.67 ± 1.97a
YC	6.50 ± 2.26b	7.17 ±1.72a	7.17 ± 1.47a	7.67 ± 1.51b	7.50 ±1.38a

Data represents Mean ± Standard deviation

The intensity scales were set using 9-point hedonic scale.

Values represent mean (n=36) ± standard deviations.

[abcd]Means with different superscript letters were significantly different ($P<0.05$) for particular starter organism

significantly higher ($P<0.05$) lactic acid production as compared to probiotic starters. This could be attributed to positive associative growth of commercial starters which consisted of *S. thermophilus* and *Lb. bulgaricus*. Among the probiotic cultures, highest lactic acid was produced by LH whereas LGG was poor lactic acid producers. The concentration of acetic acid on the other hand remained more or less consistent. There was no significant difference were observed in survival of starter bacteria at 1 d (Table 1). Viscosity value showed that LGG exhibited moderately high viscous product as compared to product prepared by LH and LR231 (Table 1). This may be attributed to ability of some lactobacilli strains to produce exopolysaccharides (EPS) during fermentation and gel formation that contributes to the improvement in texture and viscosity in fermented milk product

(Hassan et al., 1995). Nevertheless, other factors like heat induced changes in milk base and post-fermentation handling could lead significant effects viscosity of final product (Tamime and Robinson, 2007). The spontaneous whey separation of all the products are given in Table 1. The value of percent whey separation varied non-significantly ($P>0.05$) from 1.5 to 2.0. Fermentation of milk by probiotics did not affect the whey separation. Sensory evaluation of the milk fermented by potentially probiotic organism and dairy starters was carried out at 1 d. Nine expert panellist judged the product for their sensorial quality which offers quality control criterion for final product by using 9 point hedonic scale. The results of the sensory evaluation are shown in Table 2. LH exhibited significantly highest preference for acidity whereas acidity of LR231 and YC were found to be comparable. There was no significant difference were observed amongst probiotics and dairy starters with respect to flavour, appearance and overall acceptability. However, dairy received moderately high score for texture ($P<0.05$) which comparable to LR 231. Overall, the milk fermented by probiotic organism are found to be comparable, in their sensorial attributes, to commercial dairy starters.

Conclusion

Utilizing food as a vehicle to deliver probiotics has been an interestto food scientists for decades due to their purported health-associatedbenefits to customers. In the present study, comparative evaluation of the indigenous two potentially probiotic strain LH and LR 231 with most widely studied probiotic strain LGG was carried out. All these probiotic strains were also compared with commercial dairy starter in order to evaluate their suitability for product manufacturing. Viable counts, pH reduction, production of organic acid and viscosity were found to be comparable amongst the studied cultures. Likewise, sensorial characteristics were also found to be similar to commercial dairy starters. Hence, it could be concluded that probiotic cultures could be used as starter to produce fermented dairy product that not only impart the benefits of metabolites produced by probiotics but also a cost effective solution for functional food industry.

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