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Formation of oligosaccharides in skim milk fermented with mixed dahi cultures, *Lactococcus lactis* ssp *diacetylactis* and probiotic strains of lactobacilli

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Milk fermented with mixed dahi cultures NCDC167, *Lactococcus lactis* ssp *diacetylactis* NCDC60 and two probiotic strains; *Lactobacillus acidophilus* NCDC14 and *Lb. casei* NCDC19 were evaluated after fermentation (14 h) and during 8 d storage at 7 °C. The β-galactosidase activity was found to increase after fermentation leading to the hydrolysis of lactose and production of glucose, galactose and oligosaccharides; that subsequently decreased during storage. The viable counts of lactococci and lactobacilli decreased during storage yet remained $>10^6$ cfu/ml after storage. The results of present study indicate that all the selected cultures have ability to produce oligosaccharides (prebiotics) due to transgalactosidal and lactose hydrolysis activities of β-galactosidase. The cultures developed an active synbiotic formula by maintaining sufficient probiotic viable counts to exert health benefits to the consumers.

Keywords: Lactococcus, Lactobacilli, Probiotics, Prebiotics, Synbiotics, Oligosaccharides.

Abbreviations: NCDC, National collection of dairy culture; TA, Titratable acidity.

Lactic acid bacteria (LAB) provide health benefits for hosts such as elimination of lactose intolerance by β-galactosidase activity (Saltzman et al. 1999; Lin, 2003), and production of oligosaccharides (Aronson, 1952; Hung & Lee, 2002) that may function as prebiotics. The presence of probiotics in a product along with prebiotics i.e. synbiotics improves the survival of probiotic bacteria during storage and passage through the harsh conditions of gastrointestinal tract, ultimately helping in increasing implantation of probiotic bacteria into the gut (Ziemer & Gibson, 1998). The development of a synbiotic product using external sources is cost and labour intensive. However, if a bacterial strain has ability to produce oligosaccharides (prebiotics) from an internal source (i.e. lactose) along with probiotic attributes, this can overcome the problem by serving a dual purpose. There are few reports on the production of galacto-oligosaccharides by LAB during milk fermentation (Greenberg & Mahoney, 1983; Zarate & Lopez-Leiva, 1990). Garman et al. (1996) reported that partially purified β-galactosidase from *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lb. casei*, *Lactococcus lactis* ssp *lactis*, *Streptococcus thermophilus*, *Pseudomonas pentosaceus* and *Bifidobacterium bifidum* produced oligosaccharides due to transgalactosidation activity of β-galactosidase. *Lb. reuteri* also has ability to synthesize oligosaccharides during fermentation of milk (Tzortzis et al. 2004).

As dietary adjunct, probiotic cultures must remain viable and active during storage until consumption (Gilliland & Rich, 1990). However, the incorporated probiotic bacteria could not survive well in yogurt and other commercial preparations during retail storage (Hull et al. 1984; Shah et al. 1995; Dave & Shah, 1997). Hence, there is an urgent need to search for new LAB before developing bioactive synbiotic products with extended shelf life and added health benefits. Mixed dahi cultures and *Lc. lactis* ssp biovar *diacetylactis* are the commonly used starters for dahi, a traditional Indian fermented milk product, which is well known for its nutritional and therapeutical values (Abbas & Jafri, 1992; Sinha & Sinha, 2000). *Lb. acidophilus* and *Lb. casei* used in present study are well characterized probiotic strains with good acid and bile tolerance, high surface hydrophobicity, adherence to epithelial cells, cholesterol removal, ACE inhibitory activity and antioxidant properties (unpublished data). The present study evaluated dahi starters and probiotics cultures for the production of oligosaccharides, β-galactosidase activity and viability in buffalo skim milk after fermentation and during storage.

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Table 1. Changes in counts of total lactococci and lactobacilli (log_{10} cfu/ml) during storage of milk fermented with selected bacterial cultures

Values are mean and standard deviation of triplicate analysis

	Storage time (d)					
	0	1	3	5	7	8
Total Lactococci						
Mixed dahi cultures	$8{\cdot}81 \pm 0{\cdot}14^{a}$	$8{\cdot}73 \pm 0{\cdot}13^{aA}$	$8{\cdot}67 \pm 0{\cdot}17^{bA}$	$8{\cdot}12 \pm 0{\cdot}16^{cA}$	$7{\cdot}51 \pm 0{\cdot}13^{dA}$	$7{\cdot}28 \pm 0{\cdot}05^{eA}$
Lc. lactis	$8{\cdot}85 \pm 0{\cdot}07^{a}$	$8{\cdot}18 \pm 0{\cdot}17^{bB}$	$7{\cdot}95 \pm 0{\cdot}04^{cB}$	$7{\cdot}80 \pm 0{\cdot}04^{dB}$	$6{\cdot}96 \pm 0{\cdot}07^{eB}$	$6{\cdot}22 \pm 0{\cdot}13^{fB}$
Total Lactobacilli						
Lb. acidophilus	$8{\cdot}97 \pm 0.18^{a}$	$8{\cdot}81 \pm 0{\cdot}12^{bA}$	$8{\cdot}72 \pm 0{\cdot}19^{bA}$	$8{\cdot}23 \pm 0{\cdot}16^{cC}$	$7{\cdot}6\ 3 \pm 0{\cdot}09^{dC}$	$7{\cdot}45 \pm 0{\cdot}04^{eC}$
Lb. casei	$8{\cdot}93 \pm 0{\cdot}09^{a}$	$8{\cdot}43 \pm 0{\cdot}15^{bC}$	$8{\cdot}46 \pm 0{\cdot}007^{bC}$	$7{\cdot}90 \pm 0{\cdot}02^{cD}$	$7{\cdot}45 \pm 0{\cdot}16^{dA}$	$7{\cdot}08 \pm 0{\cdot}11^{eD}$

^{a,b,c,d,e,f} Values with common superscripts within a row were not significantly different at $P<0{\cdot}05$
^{A,B,C,D} Valeues with common superscripts within a column were not significantly different at $P<0{\cdot}05$

Materials and methods

Bacterial strains

Mixed dahi culture, *Lc. lactis* ssp *diacetylactis, Lb. acidophilus* and *Lb. casei* were obtained from National Collection of Dairy Cultures (NCDC) of the institute. The lactobacilli cultures were activated by inoculating into MRS broth (DeMan et al. 1960) whereas mixed dahi culture and *Lc. lactis* were activated on M17 media (Terzaghi & Sandine, 1975), and incubated for 18–20 h at 37 °C and 30 °C, respectively before being stored under refrigeration (7 °C) and sub-cultured routinely. Cultures were revived by two subsequent inoculations (10 g/l) into 10 ml sterilized skim milk by incubating 18–20 h at the respective temperatures.

Preparation of samples

Skim milk from buffalo was adjusted to 90 g/l by adding lactose externally (Sisco Research Laboratories Pvt. Ltd, Mumbai, India), an optimum concentration for high production of oligosaccharides in milk during fermentation (unpublished data). Fermented milk samples were obtained by inoculating respective bacterial culture ($\sim 10^7$ cfu/ml) in 100 ml reconstituted sterilized skim milk followed by incubation at their respective temperatures for 14 h. After fermentation samples were stored at 7 °C up to 8 d. Samples were withdrawn at 0, 1, 3, 5, 7 and 8 d for analysis of pH, titratable acidity (TA), viable cell counts, β-galactosidase activity and sugars as described below.

Physico-chemical composition

Protein, fat, total solids and ash contents were determined by the protocols of AOAC (1995). The pH was determined at 18–20 °C using digital pH meter equipped with a combination of spear tip glass body electrode and thermometer. TA was measured according to the procedure described in Part I of IS: 1479 (ISI, 1960).

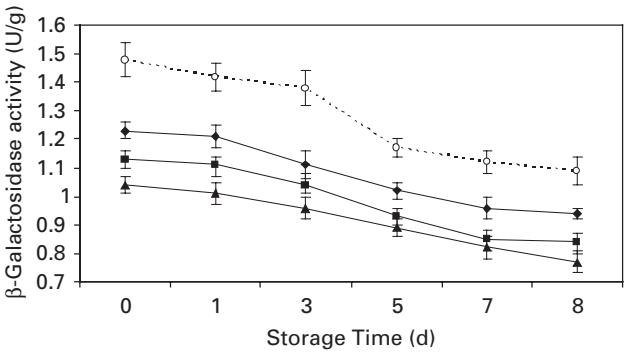

Fig. 1. Changes in β-galactosidase activity (U/ml) during storage of milk fermented with mixed dahi cultures (◆), *Lc. lactis* (▲), *Lb. acidophilus* (○) and *Lb. casei* (■).

Enumeration of viable micro-organisms

One ml fermented milk sample was transferred aseptically to 9 ml sterile peptone water (1g/l), vortexed and serially diluted. Total lactobacilli and lactococci counts were made of appropriately diluted samples on MRS agar (DeMan et al. 1960) and M17 (Terzaghi & Sandine, 1975), respectively.

Assaying β-galactosidase activity

β-Galactosidase activity in fermented milk was estimated as per Lin et al. (1989) with slight modifications. The milk sample was diluted in 0·1 M-sodium phosphate buffer (SPB; pH 7·0). One ml diluted sample was added to 4 ml SPB and 0·2 ml chloroform in a centrifuge tube. The mixture was thoroughly mixed by vortexing and centrifuged at 29 000 ***g*** for 15 min at 4 °C. After removing the supernatant, pellet was suspended in 1 ml 10 g triton X-100/l and 2 ml SPB, subsequently vortexed and incubated at 37 °C for 15 min. Then, 1 ml 15 mM-o-nitrophenol-β-D-galactopyranoside solution prepared in SBP was added, vortexed for 10 s and incubated again for 10 min at 37 °C. After incubation, the reaction was

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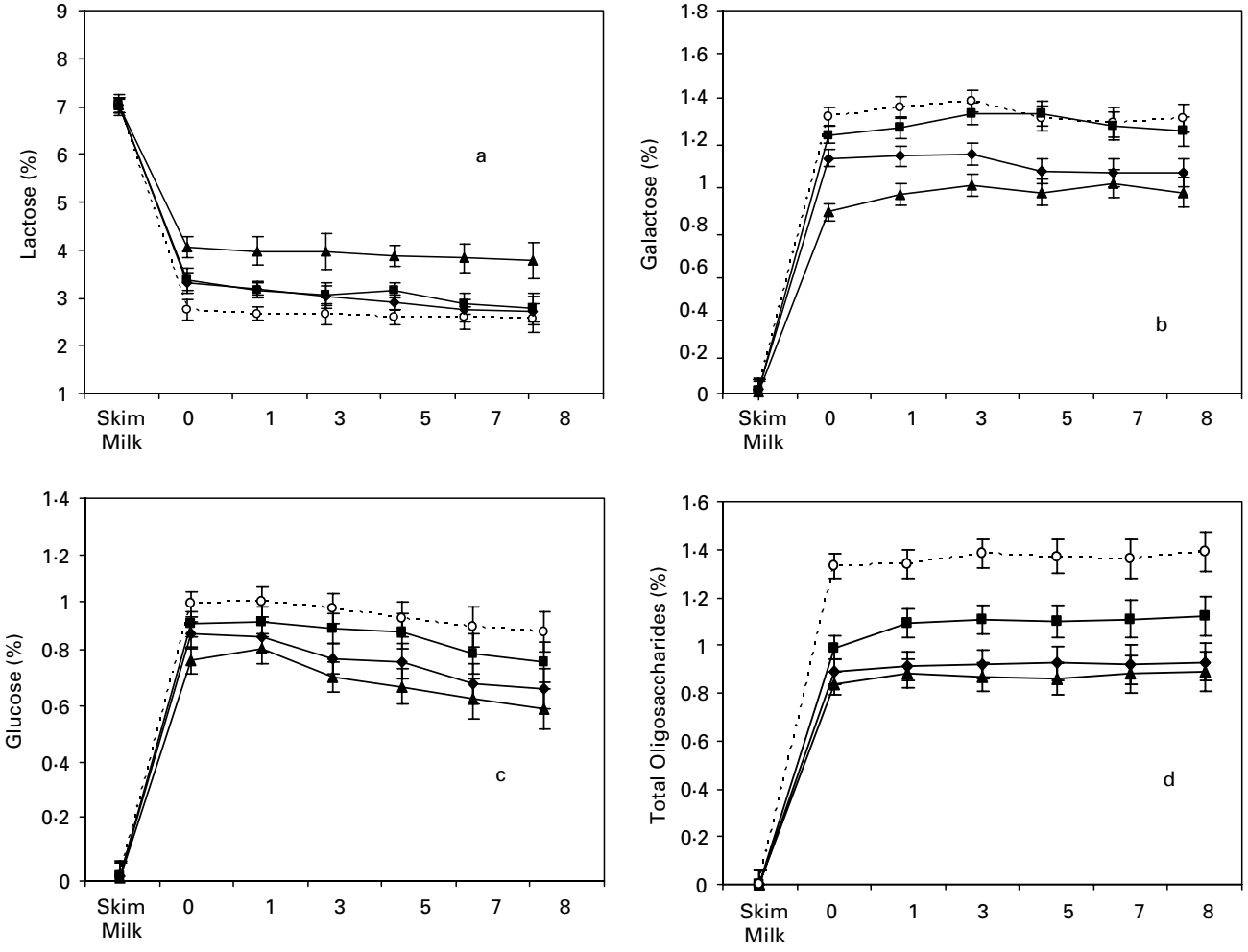

Fig. 2. Hydrolysis of (a) lactose, (b) evolution galactose and (c) glucose and (d) total oligosaccharide. Concentrations (%) during storage of milk fermented with mixed dahi cultures (◆), *Lc. lactis* (▲), *Lb. acidophilus* (○) and *Lb. casei* (■).

stopped by adding 1 ml 0·5 M-sodium carbonate and centrifuged at 25 000 ***g*** for 15 min at 4 °C to remove cell debris. The release of o-nitrophenol was determined at 420 nm and units of activity were expressed as micromoles o-nitrophenol released per min (Hughes & Hoover, 1995; Lamoureux et al. 2002).

Determination of oligosaccharides

Sugars in fermented milk samples were measured using HPLC system (Waters Chromatography Division, Milford, Massachusetts, 01757, USA). The separation of sugars was carried out using ion exchange/size exclusion based Sugar Pak I column (300 mm × 6·5 mm) maintained at 90 °C with highly purified HPLC grade water mobile phase (maintained at 60 °C) at the flow rate of 0·5 ml/min. The peaks were determined by differential refractive index detector maintained at 35 °C. The carbohydrate standards (stachyose, raffinose, lactose, glucose and galactose) were obtained from Sigma Chemicals Co., St. Louis, MO, USA for identification of peaks. Waters Millenium[32] Chromatography Manager Software (version 3.20, 2000; Waters Corporation) was used to process and calculate peak areas. The stachyose and raffinose were used to quantify the presence of oligosaccharides. The method for the preparation of samples for HPLC analysis of sugars was followed as described by Indyk et al. (1996). For this, fermented milk (2·5 ml) was dissolved in 15 ml warm HPLC grade water. Carrez reagent 1 and 2 (prepared by separately dissolving 3·6 gm potassium hexocyanoferate (II) and 7·2 gm zinc acetate in 100 ml HPLC grade water, respectively) with 0·25 ml were added sequentially, mixed and allowed to stand for 20 min. The extract was made up to final volume of 25 ml with water and filtered through Whatman grade 540 filter paper. The aliquot was passed through a 0·22 µm syringe-driven disposable filter unit. The clear filtrate was diluted (1 : 5) from which, 20 µl was injected for analysis.

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Table 2. The production (g/100 ml) and stability of oligosaccharides during fermentation and storage of fermented milks

Values are mean and standard deviation of triplicate analysis

Storage time (d)	Oligosaccharide(s)	Mixed dahi cultures	*Lc. lactis*	*Lb. casei*	*Lb. acidophilus*
0	Trisaccharides	$0{\cdot}39 \pm 0{\cdot}09^{aA}$	$0{\cdot}33 \pm 0{\cdot}01^{bA}$	$0{\cdot}40 \pm 0{\cdot}01^{aA}$	$0{\cdot}53 \pm 0{\cdot}02^{cA}$
	Tetrasaccharides	$0{\cdot}28 \pm 0{\cdot}10^{aA}$	$0{\cdot}29 \pm 0{\cdot}02^{aA}$	$0{\cdot}33 \pm 0{\cdot}02^{bA}$	$0{\cdot}48 \pm 0{\cdot}01^{cA}$
	Pentasaccharides	$0{\cdot}23 \pm 0{\cdot}09^{aA}$	$0{\cdot}22 \pm 0{\cdot}01^{aA}$	$0{\cdot}31 \pm 0{\cdot}05^{bA}$	$0{\cdot}41 \pm 0{\cdot}02^{cA}$
1	Trisaccharides	$0{\cdot}44 \pm 0{\cdot}02^{aB}$	$0{\cdot}34 \pm 0{\cdot}05^{bA}$	$0{\cdot}43 \pm 0{\cdot}03^{aA}$	$0{\cdot}55 \pm 0{\cdot}02^{cA}$
	Tetrasaccharides	$0{\cdot}26 \pm 0{\cdot}08^{aA}$	$0{\cdot}30 \pm 0{\cdot}10^{aA}$	$0{\cdot}34 \pm 0{\cdot}02^{aA}$	$0{\cdot}44 \pm 0{\cdot}08^{bA}$
	Pentasaccharides	$0{\cdot}21 \pm 0{\cdot}02^{aA}$	$0{\cdot}20 \pm 0{\cdot}04^{aA}$	$0{\cdot}29 \pm 0{\cdot}03^{bA}$	$0{\cdot}42 \pm 0{\cdot}09^{cA}$
3	Trisaccharides	$0{\cdot}43 \pm 0{\cdot}09^{aB}$	$0{\cdot}31 \pm 0{\cdot}12^{bA}$	$0{\cdot}44 \pm 0{\cdot}09^{aA}$	$0{\cdot}52 \pm 0{\cdot}13^{cA}$
	Tetrasaccharides	$0{\cdot}27 \pm 0{\cdot}13^{aA}$	$0{\cdot}28 \pm 0{\cdot}12^{aA}$	$0{\cdot}31 \pm 0{\cdot}06^{aA}$	$0{\cdot}46 \pm 0{\cdot}06^{bA}$
	Pentasaccharides	$0{\cdot}22 \pm 0{\cdot}08^{aA}$	$0{\cdot}23 \pm 0{\cdot}02^{aA}$	$0{\cdot}32 \pm 0{\cdot}02^{bB}$	$0{\cdot}39 \pm 0{\cdot}05^{cA}$
5	Trisaccharides	$0{\cdot}44 \pm 0{\cdot}09^{aB}$	$0{\cdot}35 \pm 0{\cdot}09^{bA}$	$0{\cdot}44 \pm 0{\cdot}08^{aA}$	$0{\cdot}49 \pm 0{\cdot}09^{cA}$
	Tetrasaccharides	$0{\cdot}28 \pm 0{\cdot}03^{aA}$	$0{\cdot}27 \pm 0{\cdot}02^{aA}$	$0{\cdot}35 \pm 0{\cdot}01^{bA}$	$0{\cdot}46 \pm 0{\cdot}08^{cA}$
	Pentasaccharides	$0{\cdot}20 \pm 0{\cdot}09^{aA}$	$0{\cdot}21 \pm 0{\cdot}03^{aA}$	$0{\cdot}30 \pm 0{\cdot}10^{bA}$	$0{\cdot}45 \pm 0{\cdot}13^{cA}$
7	Trisaccharides	$0{\cdot}42 \pm 0{\cdot}07^{aB}$	$0{\cdot}34 \pm 0{\cdot}03^{bA}$	$0{\cdot}47 \pm 0{\cdot}03^{cB}$	$0{\cdot}53 \pm 0{\cdot}01^{dA}$
	Tetrasaccharides	$0{\cdot}29 \pm 0{\cdot}03^{aA}$	$0{\cdot}25 \pm 0{\cdot}02^{bB}$	$0{\cdot}38 \pm 0{\cdot}04^{cB}$	$0{\cdot}44 \pm 0{\cdot}11^{dA}$
	Pentasaccharides	$0{\cdot}23 \pm 0{\cdot}04^{aA}$	$0{\cdot}19 \pm 0{\cdot}01^{bB}$	$0{\cdot}32 \pm 0{\cdot}03^{cA}$	$0{\cdot}39 \pm 0{\cdot}12^{dA}$
8	Trisaccharides	$0{\cdot}44 \pm 0{\cdot}09^{aB}$	$0{\cdot}32 \pm 0{\cdot}02^{bA}$	$0{\cdot}48 \pm 0{\cdot}01^{cB}$	$0{\cdot}55 \pm 0{\cdot}08^{dA}$
	Tetrasaccharides	$0{\cdot}30 \pm 0{\cdot}04^{aA}$	$0{\cdot}28 \pm 0{\cdot}03^{aA}$	$0{\cdot}35 \pm 0{\cdot}02^{cA}$	$0{\cdot}43 \pm 0{\cdot}04^{dA}$
	Pentasaccharides	$0{\cdot}19 \pm 0{\cdot}02^{aA}$	$0{\cdot}24 \pm 0{\cdot}05^{bA}$	$0{\cdot}37 \pm 0{\cdot}06^{cB}$	$0{\cdot}42 \pm 0{\cdot}03^{dA}$

[a,b,c,d] Values with common superscripts within a row were not significantly different at $P<0{\cdot}05$
[A,B] Values with common superscripts within a column were not significantly different at $P<0{\cdot}05$

Statistical analyses

The multi-factor analysis of variance with interaction was performed to determine the effects of time, temperature and strains on different parameters in triplicate with *Bonferroni* test by using SPSS (version 10.1, SPSS Inc. Chicago, IL). The values were expressed as means and standard deviation (SD). The P values $<0{\cdot}05$ were considered statistically significant.

Results and discussion

Physico-chemical composition

The gross chemical composition was approximately 45·5 g protein, 4·1 g fat, 114·1 g total solids and 9·5 g ash content/l in milk fermented with four different bacterial strains. The values were within typical ranges for fermented milks prepared from buffalo milk (Aneja et al. 2002) and means were not significantly different for all the experimental samples ($P<0{\cdot}05$).

The pH decreased (from ~6·67 to ~4·87) and TA increased (from ~0·13 to ~0·91) significantly during fermentation and again during storage (pH: from ~4·87 to ~4·56; TA: from 0·91 to ~1·21) in samples tested. However, the pH and TA were lowest and highest, respectively in milk fermented with *Lb. acidophilus* followed by mix dahi culture, *Lb. casei* and *Lc. lactis*. The elevation of TA indicates that the lactic acid content increased continuously due to metabolic activities of dahi and probiotic microorganisms during fermentation as well as storage. Moreover, metabolites i.e. organic acids, volatile flavour components and free fatty acids produced in fermented milks (unpublished data), might have decreased pH and increased TA.

Survival of lactobacilli and lactococci in fermented milk

Bacterial cultures used in this study had almost the same initial populations and, as duration of storage increased, the total numbers of lactobacilli and lactococci in fermented milks significantly decreased, but were maintained $>10^6$ cfu/ml till the end of d 8 to exert health benefits (Table 1). The viable lactobacilli counts in milk fermented with *Lb. acidophilus* did not decline rapidly during first 3 d, as observed in *Lb. casei* containing fermented milk samples, though, the numbers of total lactococci in milk fermented with mixed dahi cultures and *Lc. lactis* declined similarly to that of lactobacilli. The highest viable counts of lactobacilli and lactococci were in milk fermented with *Lb. acidophilus* and mixed dahi cultures, respectively after 8 d. The lactococci in milk fermented with *Lc. lactis* declined to a greater extent compared with mixed dahi cultures. Similarly lactobacilli in milk fermented with *Lb. casei* declined at a faster rate compared with *Lb. acidophilus*. Similar trends were reported by Gilliland & Rich (1990).

β-Galactosidase activity

The initial and final β-galactosidase activities were highest in fermented milk containing *Lb. acidophilus*. This might

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be due to this milk having the highest viable counts, as total β-galactosidase activity depends on cell growth and total viable cells (Lin et al. 1989). However, it decreased significantly during storage (Fig. 1). Gilliland & Lara (1988) also reported that storage of three strains of *Lb. acidophilus* in peptonized milk nutrient for 7 d at 5 °C, exhibited significant decline β-galactosidase activity with increased storage time.

Production of oligosaccharides in fermented milk

Figure 2 indicates that mixed dahi culture, *Lc. lactis, Lb. acidophilus* and *Lb. casei* produced glucose, galactose and galactooligosaccharides during fermentation and storage by hydrolysing lactose. This activity was highest in milk fermented with *Lb. acidophilus* compared with other bacterial cultures (Fig. 2B and C). These results are in agreement with the study of Kim & Gilliland (1983), who observed that the hydrolysis of lactose was highest in milk fermented with *Lb. acidophilus*, which was used as a dietary adjunct for elimination of lactose intolerance in humans. The formation of all types of oligosaccharides by β-galactosidase of *Lb. delbrueckii* subsp. *bulgaricus* in lactose solutions has also been reported by Vesiljevic & Jelen (2003). However, they reported that only *Lc. lactis* produced tetrasaccharides at detectable levels in skim milk. Smart (1991) studied β-galactosidase from *Str. thermophilus* to produce transferase products during hydrolysis of lactose and reported up to three trisaccharides by GLC analysis. β-Galactosidase of *Aspergillus oryzae* (a widely studied organism) has been reported to produce all types of oligosaccharides including trisaccharides by different workers (Toba et al. 1985; Prenosil et al. 1987).

Figure 2D and Table 2 show that the tested bacterial strains could also produce oligosaccharides during fermentation of skim milk. The amount of oligosaccharide production was different among the strains/species of LAB. The oligosaccharide production was highest in milk fermented with *Lb. acidophilus*, which might be due to the high β-galactosidase activity and viable counts. The oligosaccharides of different fermented milks were significantly higher after fermentation than in the original reconstituted skim milk. Slight increase in oligosaccharide contents was also observed during storage but it was not statistically significant. After storage period, the levels of oligosaccharides in fermented milk varied from 24 to 34%. The results indicate that oligosaccharides were not hydrolysed during storage. Our results are in agreement with Lamoureux et al. (2002), who reported that oligosaccharides synthesised during preparation of yoghurt did not undergo degradation during storage. In contrast, Mozaffar et al. (1985) observed that oligosaccharides synthesised by lactose hydrolysis, were subsequently hydrolysed to glucose and galactose after a short time of storage. In present study, tri-saccharides were the major component among the oligosaccharides produced, being greater than tetra and penta-sachharides (Table 2). The results of present study suggest that milk fermented with *Lb. acidophilus* could be a better choice for the production of the useful bio-ingredients *viz.* galacto-oligosaccharides compared with other bacterial cultures. The study needs further work on isolation, purification, characterization and biological effects of oligosaccharides produced in these fermented milk samples.

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