Viability during storage of two *Bifidobacterium bifidum* strains in set and stirred flavoured yoghurts containing whey protein concentrate

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Abstract

The viability of two strains of *Bifidobacterium bifidum* (NCDC 229/A and DSM 20456), during storage at refrigerated temperature, in flavoured set and stirred yoghurts prepared without or with whey protein concentrate (WPC), and the overall acceptability of these probiotic yoghurts for human consumption were assessed. Supplementation of either set or stirred probiotic yoghurts with 0.5 or 1.0% WPC followed by storage at 4°C improved the viability of *B. bifidum* strains. However, both the yoghurts supplemented with WPC, at 0.5% level, were acceptable up to four weeks of refrigerated storage.

Keywords: *Bifidobacterium bifidum*, Flavoured set yoghurt, Flavoured stirred yoghurt, Probiotic yoghurts, Viability, Whey protein concentrate.

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Introduction

*Bifidobacterium* spp. constitute the most important group of probiotic cultures frequently used as dietary adjuncts in functional dairy products such as bio-yoghurts¹. Some of the important health benefits attributed for *Bifidobacterium* spp. are control of undesirable microorganisms in intestinal tract, reduction in serum cholesterol levels to maintain a healthy cardiovascular system, prevention of colon cancer, improvement in lactose utilization, etc.². When used as dietary adjunct, one of the main requirements of probiotic microorganisms is the ability to maintain their viability and activity in the carrier food before consumption. It has been suggested that for any therapeutic effect, the minimum number of viable cells of *Bifidobacterium* spp. should be more than 10⁶ CFU/g in a fermented milk product³. However, several strains of probiotic microorganisms intrinsically lack the ability to survive harsh conditions and may not be suitable for use as dietary adjunct and many research workers have established the poor viability of *Bifidobacterium* spp. in yoghurts⁴⁻⁸. Poor survivality of *Bifidobacterium* spp. in fermented milk products could be due to acidic conditions caused by lactic acid bacteria during post-incubation (post-acidification), oxygen levels in the product, hydrogen peroxide produced by yoghurt cultures, etc.⁹,¹⁰. Manufacturing or storage processes that may cause cell death will also eliminate the benefits associated with *Bifidobacterium* spp. Survival of *Bifidobacterium* spp. can be improved by means of modification and control of manufacturing process and storage conditions and by better selection of probiotic bifidobacterial cultures. Over-acidification can be prevented by controlling pH, applying heat shock to yoghurt cultures before the addition of probiotic cultures, lowering storage temperature below 3–4°C, and improving the buffering capacity of yoghurt¹¹. We reported earlier that the addition of whey protein concentrate (WPC) at 0.5% level increased the viability of *B. bifidum* strains¹². The present investigation was aimed to study further the viability of two strains of *B. bifidum* (NCDC 229/A and DSM 20456) in flavoured set and stirred probiotic yoghurts supplemented with WPC during storage at 4°C, and to assess these yoghurts for their acceptability for human consumption.

Materials and Methods

Bacterial cultures

*Streptococcus salivarius* ssp. *thermophils* NCDC074 and *Lactobacillus delbrueckii* ssp. *bulgaricus* NCDC 009 were procured from the National Collection of Dairy Cultures (NCDC), National Dairy Research Institute (NDRI), Karnal, India, and used as the yoghurt cultures. Yoghurt cultures were maintained routinely in sterile skim
milk (11% TS) and in litmus milk. The cultures were subcultured at weekly intervals.

*Bifidobacterium bifidum* NCDC 229/A was procured from NCDC, Karnal, and *B. bifidum* DSM 20456 was obtained from Germany. The cultures of *B. bifidum* were routinely propagated in MRS-C (deMann Rogosa Sharpe broth with L-cystein hydrochloride) broth by following anaerobic incubation at 37°C for 48 h and subcultured at weekly intervals. Stab and slant cultures were maintained using NPNL (nalidixic acid - paramomycin sulphate - neomycin sulphate - lithium chloride) agar at refrigerated temperature.

**Preparation of yoghurts**

Standardized milk (4% milk fat and 12% SNF) was divided into three lots, and WPC (70% protein) obtained in dried form (Mahaan Proteins Limited, New Delhi) was used for supplementation by replacement of solids not-fat in yoghurt milk at 0, 0.5 and 1.0% levels. Sugar, at 8% on milk basis, was added to each lot of standardized yoghurt milk before pasteurization. Standardized milk was homogenized at 60°C using a two-stage homogenizer (FBF Italia, Italy) maintaining 2500 psi pressure at stage I and 500 psi at stage II, and was pasteurized at 95°C for 15 minutes. Mixed yoghurt culture propagated in sterile skim milk (11% TS) was added at 2% (v/v) to pasteurized and cooled milk at 43°C. Cultures of *B. bifidum* grown for 18 h in MRS-C broth were adjusted to 0.9 OD<sub>600nm</sub> using Spectronic-20 (Bausch & Lomb, USA) and centrifuged at 2,500 x g for 10 minutes at 4°C. The pellet was washed twice in normal saline and suspended in sterile skim milk. Based on pre-experimentation trials, a calculated volume of this suspension was added to pasteurized and cooled milk at 43°C to achieve a minimum viable population of *B. bifidum* at 10<sup>8</sup> CFU/g of the final product.

Probiotic set yoghurt (Fig. 1) was obtained by packing yoghurt milk in 100 ml polyethylene cups with lids and incubating at 40-41°C for 4 to 4.5 h till a final desired *pH* of 4.7 was obtained. Flavoured yoghurt culture was added aseptically (Figs 1 and 2). All types of yoghurts contained.
Research Paper

Fresh cow milk
↓
Preheating to 45 °C
↓
Separation of milk
↓
Skim milk was concentrated to 12% TS and standardized to 4% fat using fresh cream
↓
Heating to 65 °C
↓
Homogenization
↓
Addition of sugar @ 8%
↓
Pasteurization to 95 °C for 15 min
↓
Cooling to 43 °C
↓
Inoculation with yoghurt cultures @ 2.0% along with flavours

Stirred yoghurt
↓
Incubation at 40–41°C for 4 to 4.5 h (to get a pH of 4.7)
↓
Cooling to 4 °C
↓
Stirring
↓
Packing in 100 ml polyethylene cups
↓
Storage at 4°C

Stirred probiotic yoghurt
↓
Incubation at 40–41°C for 4 to 4.5 h (to get a pH of 4.7)
↓
Cooling to 4 °C
↓
Stirring with addition of probiotic culture cell suspension (to get a minimum of 10⁷ cfu/ml of the product)
↓
Packing in 100 ml polyethylene cups
↓
Storage at 4°C

Fig. 2: Protocol for flavoured plain and probiotic stirred yoghurt

in polyethylene cups were transferred to a refrigerator for storage at 4°C.

Analysis of yoghurt during storage period

The samples of probiotic set and stirred flavoured yoghurts stored at 4°C in 100 ml polyethylene cups were withdrawn at 0, 3, 7, 14, 21, 28 and 35 days of storage for determination of pH, counts of viable population of B. bifidum strains and for overall acceptability of the yoghurts for human consumption. The pH of the plain or probiotic yoghurt samples was determined at 25 °C by using bench top digital pH meter (Thermo Orion 420 A+, USA). The yoghurt samples were evaluated for overall acceptability using a 9-point hedonic scale by a panel of five experienced judges. NPNL medium (Teraguchi et al., 1978) was used for the enumeration of viable population of B. bifidum strains in probiotic yoghurts. Serially-diluted samples of probiotic yoghurts were pour-plated on NPNL agar and incubated at 37°C for 72 h in anaerobic chamber (Ruskin Technology, UK) by providing a gas mixture of 5, 10 and 85% of H₂, CO₂ and N₂, respectively.

Statistical analysis

All experiments were replicated four times, and all analyses and enumerations were done in duplicate. Statistical analysis for enumeration of B. bifidum strains and coliforms was conducted after log transformation. Data were subjected to analysis of variance (ANOVA) using SPSS software. The differences among mean values were tested for significance (P < 0.05) by Duncan’s new multiple range test.

Results and Discussion

Changes in pH of probiotic yoghurts

The initial pH for set and probiotic stirred yoghurts, without or with whey protein concentrate (WPC) supplementation (Fig. 3), ranged from 4.66 to 4.77 (Fig. 4). The pH of probiotic

Fig. 3: Set (a), and Stirred (b), banana flavoured yoghurts
set yoghurts prepared with *B. bifidum* NCDC 229/A or DSM 20456 and without WPC dropped from an initial value of ~ 4.68 (one day post-manufacture) to ~ 4.33 over a storage period of 21 days at 4°C, and finally reaching to 4.36 at the end of 35 days of storage. Supplementation of WPC at 0.5 and 1.0% in set probiotic yoghurt resulted in a lesser drop in pH from an initial ~ 4.72 to a final <4.44. The pH of probiotic set yoghurts was almost stable up to 3 days of storage and started declining on further storage. An overall of 0.35 units drop in pH was observed in control yoghurts by the end of 3 weeks of storage, but the drop in pH in probiotic set yoghurts supplemented with WPC at 0.5 and 1.0% levels was marginal with 0.25 units. The pH of probiotic set yoghurts was influenced by the storage period and differed significantly up to three weeks.

The trend in pH decline was consistent with probiotic stirred yoghurts, with the pH dropping from initial 4.66-4.77 to 4.35-4.39 at the end of 35 days of storage. However, there was no significant variation in the performance of two *B. bifidum* cultures in terms of change in pH in both set and stirred yoghurts, prepared with the addition of 0, 0.5 or 1.0% WPC. The final pH at the end of 5 weeks storage period of yoghurts varied between 4.36 and 4.39. The reduction in pH could be due to residual enzymes produced by starters during fermentation or due to the yoghurt cultures that were active even at refrigerated temperature producing small amounts of lactic acid by fermentation of lactose, while a smaller drop in pH of probiotic yoghurts supplemented with WPC may have been due to increased buffering action of proteins.

![Fig. 4: Changes in pH of probiotic set and stirred probiotic yoghurts.](image-url)
Viability of *B. bifidum* strains

The initial viable population of *B. bifidum* DSM 20456 and NCDC 229/A was 8.8-8.9 and 8.4-8.5 log$_{10}$ CFU/g, respectively, in flavoured set and stirred yoghurts, prepared without or with WPC supplementation (Table 1). Viable population of *B. bifidum* after fermentation of yoghurts containing no WPC was ≥ 8.4 log$_{10}$ CFU/g, but decreased gradually to < 5.0 and < 4.835 log$_{10}$ CFU/g, after 35 days of refrigerated storage of probiotic set and stirred yoghurts, respectively. There was ≥ 2 log reduction in viable population of *B. bifidum* DSM 20456 and NCDC 229/A in probiotic set and stirred yoghurts prepared without WPC at the end of 14 days of storage, and reduction was as high as 3 to 4 log at the end of 35 days storage at 4°C. The decline in viable counts of *B. bifidum* was significant within the periods of storage for both varieties of yoghurts. The trend in reduction of viable population of *B. bifidum* in yoghurts containing the strains DSM 20456 and NCDC 229/A was almost similar. Interestingly, none of the probiotic yoghurts without WPC, had viable counts of more than 6.0 log$_{10}$ CFU/g (reported minimum levels necessary for health benefits) after 21 days of storage, indicating that the yoghurts were best for consumption before 3 weeks of storage. The results of the present study are not in agreement with those of Roy *et al*¹⁴ who reported that the viable cells of bifidobacteria in yoghurts could not be maintained in more than 10⁶ CFU/g for more than a week during storage at 4°C, but are in close conformity with those of Martin¹⁵ who found that *B. bifidum* and *B. longum* were acid-tolerant and can survive in sufficiently higher numbers to remain viable in cultured dairy products even during prolonged storage.

Decline in viable counts of *B. bifidum* was less marked in probiotic yoghurts prepared with WPC at 0.5 and 1.0% supplementation. It was about ≥ 6.0 log$_{10}$ CFU/g even after 28 days of storage in probiotic set and stirred yoghurts. It is evident from the present results that the viability of *B. bifidum* can be improved by supplementation of yoghurts with WPC, as the viability of *B. bifidum* cells accounts for more than one log when compared with the yoghurts made without WPC. The improved viability in probiotic yoghurts containing WPC could be due to low acidity or amino nitrogen present in

### Table 1: Viable counts (Log$_{10}$ CFU/g) of *B. bifidum* strains in flavoured set and stirred yoghurts supplemented without or with WPC during refrigerated storage

<table>
<thead>
<tr>
<th>Yoghurt</th>
<th>WPC (%)</th>
<th>Storage in days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Set yoghurt with</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. bifidum</em> DSM 20456</td>
<td>0.0</td>
<td>8.84&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>8.86&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>8.94&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Set yoghurt with</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. bifidum</em> NCDC 229/A</td>
<td>0.0</td>
<td>8.45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>8.46&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>8.48&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stirred yoghurt with</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. bifidum</em> DSM 20456</td>
<td>0.0</td>
<td>8.88&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>8.93&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>8.94&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stirred yoghurt with</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. bifidum</em> NCDC 229/A</td>
<td>0.0</td>
<td>8.40&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>8.46&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>8.49&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means (n = 4) of a sampling in a column for each yoghurt having the same letter are not significantly (P ≤ 0.05) different from each other according to Duncan's new multiple range (DMR) test.
Table 2: Scores of overall acceptability of set plain, set flavoured and stirred flavoured yoghurts during storage at 4°C

<table>
<thead>
<tr>
<th>Storage period (days)</th>
<th>Set plain yoghurt</th>
<th>Set flavoured yoghurt</th>
<th>Stirred flavoured yoghurt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yoghurt culture (YC)</td>
<td>Yoghurt + strain DSM 20456</td>
<td>Yoghurt + strain NCDC 229-A</td>
</tr>
<tr>
<td>0</td>
<td>8.5a</td>
<td>8.3a</td>
<td>8.2a</td>
</tr>
<tr>
<td>3</td>
<td>8.4a</td>
<td>8.2a</td>
<td>8.1a</td>
</tr>
<tr>
<td>7</td>
<td>8.2a</td>
<td>8.1a</td>
<td>7.9a</td>
</tr>
<tr>
<td>14</td>
<td>8.1a</td>
<td>7.9a</td>
<td>7.8a</td>
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<td>21</td>
<td>7.8a</td>
<td>7.7a</td>
<td>7.5a</td>
</tr>
<tr>
<td>28</td>
<td>7.6a</td>
<td>6.8a</td>
<td>6.9b</td>
</tr>
<tr>
<td>35</td>
<td>7.1a</td>
<td>6.2a</td>
<td>5.9b</td>
</tr>
</tbody>
</table>

Means (n = 4) in a row for a sampling of a yoghurt having the same letter are not significantly (P ≤ 0.05) different from each other according to DMR test.

WPC. Developed acidity is one of the important factors that exert influence on the growth and viability of probiotic bacteria in any cultured dairy product. Lankaputhra et al. reported that the viability of bifidobacteria was greatly affected by any drop in pH below 4.3. However, Dave & Shah stated that the viability of Bifidobacterium spp. can be increased by 3 logs during 35 days of refrigerated storage by incorporating WPC, acid casein hydrolysate or tryptone, with the highest viability observed being in the yoghurt supplemented with WPC as these organisms lack proteolytic activity. These deviations observed in the viability of strains of B. bifidum could be due to strain variations. Dave & Shah also stated that the viability was affected by the bifidobacterial strain used in yoghurt manufacture. In a similar study, Martin & Chou reported that the viability of Bifidobacterium spp. varied greatly and was species and strain-dependent.

Acceptability of probiotic yoghurts

The initial score for overall acceptability for set plain probiotic yoghurt made without or with 0.5% WPC supplementation ranged between 8.2 and 8.3 on hedonic-scale, but the scores were 7.8-7.9 for set and stirred probiotic yoghurt supplemented with 1% WPC (Table 2). Irrespective of B. bifidum strain, there was no significant difference in scores for overall acceptability of probiotic set yoghurts up to one week of storage. On subsequent storage, the scores differed significantly between the storage periods. The scores for overall acceptability of set probiotic yoghurt were as low as 4.8-5.0 by the end of 35 days storage, and the scores for the corresponding storage period were 4.3-4.5 in case of stirred probiotic yoghurt supplemented with 1.0% WPC. However, the overall acceptability scores for the probiotic yoghurts prepared without and with WPC up to 0.5% were similar throughout the storage period.

Conclusion

It is evident that supplementation of either set or stirred probiotic yoghurts with WPC, even at 1.0% level, improved the viability of B. bifidum strains during refrigerated storage. The addition of WPC at 1.0%, but not 0.5%, level had an adverse effect on the scores of overall acceptability of the flavoured set or stirred probiotic yoghurts. In all, WPC at 0.5% supplementation could help maintain viability of B. bifidum strains without affecting the overall acceptability of probiotic yoghurts during storage at 4°C for a period of at least four weeks.
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References


