

## Use of *Bifidobacterium bifidum* in the manufacture of bifidus milk and its antibacterial activity

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**Summary** — A method to prepare bifidus milk on a commercial basis was standardised by addition of 10% inoculum of *Bifidobacterium bifidum* NDRI to 9% reconstituted skim milk (0.5% fat, 8.7% non-fat solids) heated at 95 °C for 30 min and incubated at 37 °C for 18 h. The product was able to meet all the criteria regarding technological and dietetic properties required in a quality product. The effect of various factors influencing the antibacterial activity of bifidus milk against 4 test organisms viz *Escherichia coli*, *Shigella dysenteriae*, *Staphylococcus aureus* and *Bacillus cereus* was determined. There was a significant variation ( $P < 0.05$ ) in the antibacterial activity of bifidus milk made from various types of milk (reconstituted skim milk, cow milk, buffalo milk, reconstituted infant food) and with various types of heat treatment. Reconstituted skim milk was recommended for preparation of bifidus milk. Heating of milk to sterilization temperature or at 95 °C for 30 min had a maximum effect on antibacterial activity. There was no significant variation ( $P > 0.05$ ) in the antibacterial activity at different levels of inoculum (5.10 and 15%) or due to varying concentrations of sugar. Incubation at 37 °C showed highest inhibitory activity. Bifidus milk had a minimum storage life of 3 weeks under refrigeration, retaining a sufficiently good taste and the requisite amount of microbial population ( $10^8$  cfu/g) to be potentially beneficial.

### ***Bifidobacterium* / bifidus milk / antibacterial activity**

**Résumé** — Fabrication et activité antibactérienne de lait au bifidus préparé en utilisant *Bifidobacterium bifidum*. Un lait au bifidus a été préparé industriellement par addition de 10% d'inoculum de *Bifidobacterium bifidum* NDRI à 9% de lait écrémé reconstitué (0,5% de matière grasse, 8,7% de matière sèche non grasse) chauffé à 95 °C/30 min, et incubé à 37 °C pendant 18 h. Le produit présentait toutes les propriétés technologiques et diététiques requises pour un produit de qualité. L'effet de plusieurs facteurs influençant l'activité antibactérienne du lait au bifidus contre 4 types de micro-organismes, à savoir *Escherichia coli*, *Shigella dysenteriae*, *Staphylococcus aureus* et *Bacillus cereus*, a été déterminé. Une variation significative ( $P < 0,05$ ) de l'activité antibactérienne du lait au bifidus était observée entre les différents types de lait (lait écrémé reconstitué, lait de vache, lait de bufflesse, lait infantile reconstitué) et entre les traitements thermiques. Le lait écrémé reconstitué était recommandé pour la préparation de lait au bifidus. Le chauffage du lait à des températures de stérilisation ou à 95 °C/30 min avait l'effet maximal sur l'activité antibactérienne. Par contre, les niveaux d'inoculation (5, 10 et 15%) et la teneur en sucre n'avaient pas d'influence significative ( $P > 0,05$ ). L'activité inhibitrice maximale était observée avec une incubation à 37 °C. Après 3 semaines de conservation au réfrigérateur, le goût et le niveau de population microbienne ( $10^8$  UFC/g) du lait au bifidus étaient considérés comme satisfaisant aux critères requis pour l'alimentation infantile.

### **Bifidobactérium / lait au bifidus / activité antibactérienne**

## INTRODUCTION

Bifodogenic microflora have been studied in various countries with a view to utilizing them in the manufacture of various fermented milk products, fresh cheese and dried milks for human nutrition. Regular consumption of these products can have a number of advantages as they contain physiological body-related bacteria (Sandine *et al*, 1972), have a generally larger content of L(+) lactic acid (Klupsch, 1983), correct various types of gastrointestinal disorders and maintain a favourable balance among the indigenous intestinal microflora (Schaedler *et al*, 1965).

The technology of bifidus culture in the milk processing industry and the usability of bifidus culture in the manufacture of fermented milks was first described by Schuler-Malyoth *et al* (1968). Successive efforts in optimizing qualities of bifidus-based fermented milks have also been reported (Kosikowa, 1978; Marshall *et al*, 1982; Collins and Hall, 1984; Goh *et al*, 1986), but the product obtained varied from one strain of starter culture to another. In addition there is little information on the anti-bacterial properties of these fermented milks.

The present communication includes a report on the preparation of bifidus milk and assessment of its antibacterial activity as well as its acceptability to consumers.

## MATERIALS AND METHODS

### Source and maintenance of cultures

*Bifidobacterium bifidum* NDRI and test cultures of pathogenic organisms, viz *Bacillus cereus*, *E coli*, *Shigella dysenteriae* and *Staphylococcus aureus* were obtained from the National Collec-

tion of Dairy Organisms, National Dairy Research Institute, Karnal, India.

*B bifidum* was maintained in sterile skim milk medium supplemented with 1% dextrose and 0.1% yeast extract. The pathogenic test cultures were maintained on nutrient agar slants (Oxoid) and were activated by 3 successive transfers at 24-h intervals in nutrient broth.

### Preparation of bifidus milk

The method of Nahaisi and Robinson (1985) was adopted with minor modifications to prepare a drink for direct consumption through bottle feeding for infants (fig 1).

The effect of some of the factors such as: i), type of milk, viz reconstituted skim milk, cow milk, buffalo milk and reconstituted infant milk food; ii), heat treatment, viz 121 °C for 15 min, 85 °C for 30 min, 95 °C for 30 min and steaming for 30 min; iii), size of inoculum, viz 2, 5, 10, 15 and 20%; iv) incubation temperature, viz 32, 37, 40 and 45 °C; v), concentration of sucrose, viz 0, 6, 8, and 12%; and vi) storage at refrigeration temperature (5–8 °C) for 3, 10, 17, 24 and 30 days – on the antibacterial activity of bifidus milk prepared by *Bifidobacterium bifidum* NDRI (National Dairy Research Institute) were also examined.

### Analysis

Bifidus milk products were analysed for titratable acidity (ISI, 1960), volatile acidity (Hempeniens and Liska, 1968), lactic acid (Barker and Summerson, 1941), proteolytic activity (Hull, 1947) and combined diacetyl and acetoin (King, 1948). The antibacterial activity of the product was estimated by the modified cup agar assay technique (BSI, 1968). Culture filtrates (or cell-free extracts) were collected by centrifugation at 3 000 rpm for 15–20 min. These were passed through a Seitz filter separately. Wells of 5.0 mm diameter were made on solidified nutrient agar (seeded with the pathogenic test organisms) in each plate, using a sterile hollow borer. With the help of a sterile serological pipette, 0.05 ml of the cell-free extract was transferred to

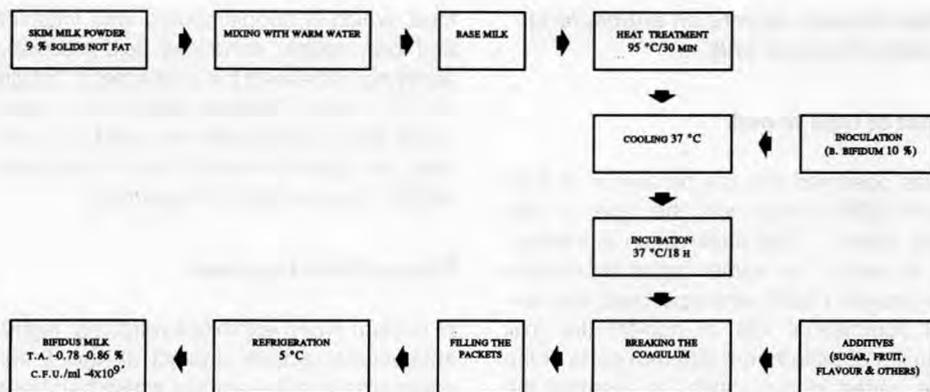


Fig 1. Schematic diagram for the manufacture of bifidus milk.

Diagramme schématique de fabrication de lait fermenté au bifidus.

different wells. The plates were incubated without inverting at 37 °C for 18–24 h and the diameters of zones of inhibition formed around the wells were measured. Results were statistically analysed for analysis of variance according to the method of Snedecor and Cochran (1967).

Samples of standardized bifidus milk products, viz bifidus sour milk and bifidus sweetened milk, were subjected to sensory evaluation by a panel of 7 judges using the 9-point hedonic scale (Amerine *et al*, 1967) and analysed statistically by 2-way classification.

## RESULTS AND DISCUSSION

### Characteristics of the product

On the basis of the results of different trials, the procedure shown in figure 1 was adopted for the preparation of bifidus milk using single culture of *Bifidobacterium bifidum* NDRI. The final product had a titratable acidity of 0.78–0.86%, 1.9–2.2 ml of volatile acidity, proteolytic activity of 30 mg% liberated tyrosine, a mild acidic flavour and a viable count of  $4 \times 10^9$  cfu/ml. The product also showed desirable anti-

bacterial activity against the 4 test organisms, namely *Escherichia coli*, *Shigella dysenteriae*, *Staphylococcus aureus* and *Bacillus cereus*. The most important criterion defining the usefulness of bifidus milk for intestinal therapy is the ability of *B bifidum* to be successfully implanted in the intestine. Several workers have reported that for successful seeding in the intestine, the viable population must be in the range of  $10^8$ – $10^9$  cells/ml of the product (Tanaka *et al*, 1982; Kim, 1988). The viable cell population obtained in the product satisfied this condition. The percent inoculum of *B bifidum* utilized by many workers ranged between 2–5%, and the normal time taken for setting was 24 h (Brown and Townsley, 1970; Marshall *et al*, 1982). Fonden and Holgersson (1985) characterized *Bifidobacterium* species as slow cultivators in milk and recommended a larger inoculum (5–20%) of starter culture in the final manufacture of the cultured milk. Similar experiences were observed in the present study and 10% inoculum was found to meet all the criteria regarding technological and dietetic properties required in the product.

### Effect of some factors on antibacterial activity of bifidus milk

#### Effect of type of milk

It was observed that the behaviour of *B bifidum* NDRI varied with the type of milk used (table I). This observation is interesting in view of an earlier report by Greene and Jezeski (1957) who observed that several commercial lots of non-fat dry milk samples obtained from different parts of the USA varied in their ability to promote the growth and activity of starter cultures. The acid production in all the types of milk was within the range of 0.81–0.87% lactic acid and there was a significant variation in the antibacterial activity according to the types of milk used ( $P < 0.05$ ). Reconstituted skim milk (0.5% fat and 8.7% non-fat solids) and cow milk (3.5% fat and 8.5% non-fat solids) had a similar effect on the antibacterial activity of *B bifidum*. Reconstituted infant milk

food, which is supplemented with vitamins and iron, neither enhanced acid-producing ability nor increased the antibacterial activity of the culture. Similar observations were made when buffalo milk was used as a medium for growth except that it produced slightly higher acidity in the product.

#### Effect of heat treatment

*B bifidum* NDRI exhibited relatively higher antibacterial activity against all the 4 test organisms in milk samples which had been sterilized at 15 psi for 15 min (table II). Milk samples heated at 95 °C for 30 min produced similar results. As regards production of acid, the highest amount was produced in milk heated at 95 °C for 30 min. A similar trend was noticed in the case of sterilized milk. These results are consistent with similar observations made by other workers, although there appears to be some divergence of opinion in this respect.

**Table I.** Effect of various types of milk on the antibacterial activity of bifidus sour milk \*.  
Effet de différents types de lait sur l'activité antibactérienne du lait fermenté au bifidus \*.

| Type of milk              | Fat (%) | SNF (%) | Acidity (% LA) | Diameter of zone of inhibition (in mm) * |                      |                       |                 |
|---------------------------|---------|---------|----------------|--|----------------------|-----------------------|-----------------|
|                           |         |         |                | E coli                                   | Shigella dysenteriae | Staphylococcus aureus | Bacillus cereus |
| Reconstituted skim milk   | 0.5     | 8.7     | 0.86           | 11                                       | 11                   | 8.5                   | 10.5            |
| Cow milk                  | 3.5     | 8.5     | 0.81           | 11.5                                     | 8.5                  | 8.0                   | 9.5             |
| Buffalo milk              | 5.0     | 9.0     | 0.87           | 7.0                                      | 8.0                  | 8.0                   | 5.0             |
| Reconstituted infant food | 3.3     | 8.7     | 0.83           | 8.0                                      | 8.5                  | 6.0                   | 9.0             |

\* Inoculated with 10% starter and incubated at 37 °C for 48 h; \* included diameter of well (5 mm); NFS: non-fat solids. Amount of supernatant added to well: 0.05 ml.

\* Inoculé avec 10% de levain et incubé à 37 °C pendant 48 h. \*: incluant le diamètre du puits (5 mm); SNF : matière sèche non grasse; (0,05 ml de surnageant ajouté dans le puits).

**Table II.** Effect of heat treatment of milk on the antibacterial activity of bifidus sour milk\*.  
*Effet du traitement thermique du lait sur l'activité antibactérienne du lait fermenté au bifidus\**.

| Type of heat treatment                 | Acidity (% LA) | Total cell count (cfu/ml) | Diameter of zone of inhibition (in mm) * |                      |                       |                 |
|--|----------------|---------------------------|--|----------------------|-----------------------|-----------------|
|  |                |                           | E coli                                   | Shigella dysenteriae | Staphylococcus aureus | Bacillus cereus |
| Sterilization (121 °C, 15 psi/ 15 min) | 0.87           | 14 x 10 <sup>8</sup>      | 11.5                                     | 11.5                 | 8.5                   | 11.0            |
| Steaming (30 min)                      | 0.84           | 1 x 10 <sup>8</sup>       | 8.5                                      | 11.0                 | 9.0                   | 10.0            |
| 95 °C for 30 min                       | 0.91           | 10 x 10 <sup>8</sup>      | 11.0                                     | 11.0                 | 8.5                   | 10.5            |
| 85 °C for 30 min                       | 0.85           | 11 x 10 <sup>8</sup>      | 8.5                                      | 8.5                  | 8.0                   | 9.0             |

\* Skim milk (0.5% fat, 8.7% non fat solids) inoculated with 10% starter and incubated at 37 °C for 48 h; \* included diameter of well (5 mm). Amount of supernatant added to well: 0.05 ml.

\* Lait écrémé (0,5% matière grasse, 8,7% matière sèche non grasse) inoculé avec 10% de levain et incubé à 37 °C pendant 48 h. \* incluant le diamètre du puits (5 mm) (0,05 ml de surnageant ajouté dans le puits).

Foster (1952) and Babel (1967) considered that higher heat treatments at 120 °C for 15 min and 85 °C for 30 min enhanced acid production, whereas Goh *et al* (1986) observed a decrease in the acid production of *B bifidum* ATCC11863 when the milk medium was heated at 95 °C for 30 min, 121 °C for 1 min or 85 °C for 15 min than when it was heated at 85 °C for 30 min. The total colony count of *B bifidum* was the highest in milk samples which were sterilized, giving a viable count of 14 x 10<sup>8</sup> cfu/ml. This increase in acid production and viable count can be attributed to the increased amounts of usable nitrogen provided due to partial hydrolysis of casein, as suggested by Foster (1952). On statistical analysis, a significant variation in the antibacterial activity of the *B bifidum* culture in milk was observed with the various types of heat treatment ( $P < 0.05$ ).

Heating of milk to sterilization temperatures and heating at 95 °C for 30 min had a similar effect on the antibacterial activity of *B bifidum* in milk. On the basis of these results, heating of milk at 95 °C for 30 min was employed as it could easily be adopted by dairy plants and under household conditions for production of bifidus milk.

#### Effect of the levels of inoculum

There was no significant variation in the antibacterial activity of different levels of inoculum of *B bifidum* culture ( $P > 0.05$ ), but there was a significant relationship of percent inoculum and the acidity produced which was directly related to the total viable count. The antibacterial activity against the 4 test organisms was almost the same at 5, 10 and 15% levels of inocu-

lum. Similar observations for 1, 2 and 3% inoculum of *L acidophilus* have been reported by Gandhi (1978).

### Effect of the incubation temperature

The effect of different incubation temperatures on the antibacterial activity of *B bifidum*-NDRI in milk as presented in table III showed poor acid production and reduced antibacterial activity at 32 °C. There was no zone of inhibition when tested against *E coli* and *Staphylococcus aureus*. As the temperature was raised from 32 to 37 °C the acid production and antibacterial activity were increased but at 40 °C antibacterial activity was not exhibited against *Shigella dysenteriae*, *Staphylococcus aureus* and *E coli*. The optimum temperature for the growth of *B bifidum* was 37 °C and thus the maximum production of antibacterial substances was expected at this temperature. Similar results have been reported by Shahani *et al* (1976) for *L acidophilus*.

### Effect of addition of sugar

Since bifidus sour milk tastes too bitter for infants, sweetened bifidus milk was prepared using different concentrations of sugar. It was observed that as the level of sugar addition increased there was no significant variation in the antibacterial activity ( $P > 0.05$ ). However, it was observed that the product with 12% level of sucrose was excellent in taste and had acidity within the desirable limit.

### Effect of storage at refrigeration temperature

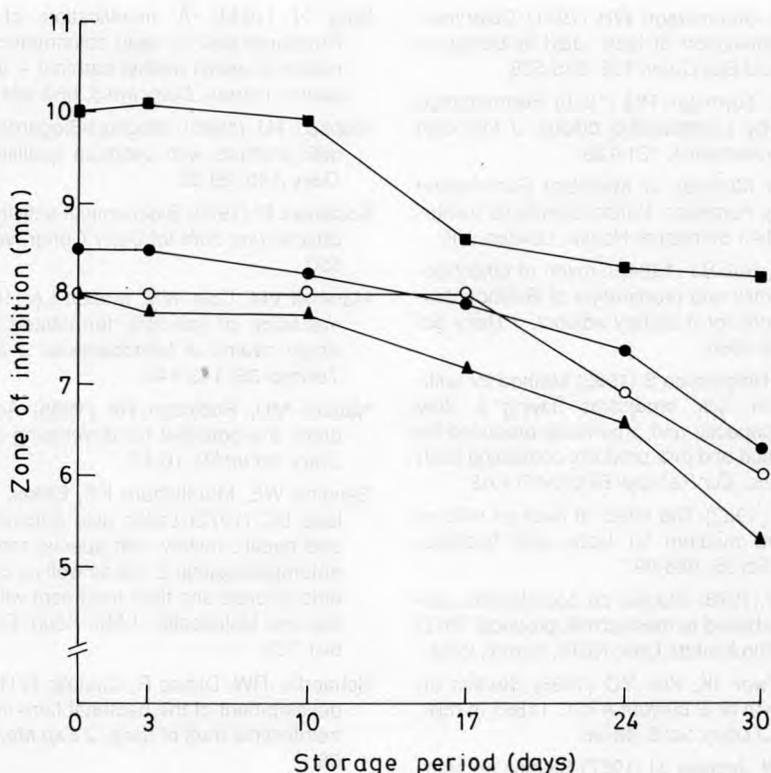
Refrigerated storage (5–8 °C) of bifidus sour milk for 30 days indicated that as the storage time increased beyond 3 days there was a decrease in the antibacterial activity against the 4 test organisms, with a sharp decline by the end of second week (fig 2). The total viable count was also found to decrease from  $4.1 \times 10^9$  cfu/ml to

**Table III.** Effect of incubation temperature on the antibacterial activity of bifidus sour milk \*.  
*Effet de la température d'incubation sur l'activité antibactérienne du lait fermenté du bifidus\**.

| Incubation temperature (°C) | Acidity (% LA) | Diameter zone of inhibition (in mm) * |                      |                       |                 |
|-----------------------------|----------------|---------------------------------------|----------------------|-----------------------|-----------------|
|                             |                | E coli                                | Shigella dysenteriae | Staphylococcus aureus | Bacillus cereus |
| 32                          | 0.56           | —                                     | 7.5                  | —                     | 8.0             |
| 37                          | 0.86           | 8.5                                   | 10.0                 | 8.0                   | 8.0             |
| 40                          | 0.71           | 8.0                                   | —                    | —                     | 10.0            |
| 45                          | 0.65           | —                                     | —                    | —                     | 6.5             |

\* Skim milk inoculated with 10% starter and incubated for 48 h; \* included diameter of well (5 mm); —: no inhibition observed. Amount of supernatant added: 0.05 ml.

\* Lait écrémé inoculé avec 10% de levain et incubé pendant 48 h; \* incluant le diamètre du puits (5 mm); — : pas d'inhibition observée; (0,05 ml de surnageant ajouté dans le puits).



**Fig 2.** Effect of storage at refrigeration temperature on antibacterial activity of bifidus sour milk. ●—● *E coli*; ■—■ *Shigella dysenteriae*; ▲—▲ *Staphylococcus aureus*; ○—○ *Bacillus cereus*.

*Effet de la conservation au réfrigérateur sur l'activité antibactérienne du lait fermenté au bifidus.*

$2.1 \times 10^6$  cfu/ml after 30 days of storage. The product did not have any off-flavour after 30 days of storage, but it tasted very sour. Products stored up to 17 days were good in taste and also had the requisite amount of total viable count ( $10^8$  cfu/g) to be utilized for implantation in the intestine by direct consumption of bifidus milk.

## CONCLUSION

Sensory evaluation studies revealed that sweetened bifidus milk was preferred over

normal bifidus milk. Thus direct consumption of sweetened bifidus milk can be recommended for infants through bottle feeding with the aim of providing viable cells suitable for intestinal implantation.

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