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## ORIGINAL ARTICLE

# Encapsulation For Short Term Preservation of Potentially Probiotic *Pediococcus pentosaceus*Dm101 to Supplement Pasteurized Donor Human Milk

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**ABSTRACT**
*This study aimed to appraise the upshot of alginate encapsulation on the storage stability of a potentially probiotic lactic acid bacterium Pediococcus pentosaceusDM101 (NCBI accession number: MK774704) isolated from breast milk. The isolate exhibited an autoaggregation value of 67.9±0.74% and a cell surface hydrophobicity value of 60.7±0.67%. The galactose utilizing potential of the isolate makes it useful as secondary starters. Encapsulation was achieved by extrusion using 2% alginate, 0.1 M $CaCl_2$ and a gelling time of 20 minutes. The alginate beads so obtained were characterized in terms of swelling ratio and dissolution behavior. The studies revealed that alkaline and neutral pH favored the alginate matrix to release the bioactive components. On 60$^{th}$ day of storage log reduction of viable cells in encapsulated form at 4 °C and -18 °C was 1.10 and 3.30 respectively. For the same time period, corresponding values were 9.17 and 9.30 for non-encapsulated cells. These findings highlight the defensive role of alginate matrix and thereby a preservation strategy for conserving breast milk microflora.*
***Keywords***: *Breast milk, Encapsulation, Pediococcus pentosaceus*

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## INTRODUCTION

Breast milk with its unique maternal signature is the most complete food that is tailor made to meet the requirements of infants. Breast milk is the gold standard for infant nutrition. Beyond its nutritional benefits, breast feeding reduces the respiratory and gastrointestinal infections in early life stages. Microorganisms have emerged as the most important bioactive components of human milk (10). A large multicentre study by confirmed breast feeding as the most significant factor that influence microbiome structure in early life of infants (21). Recognizing the exhaustive literature documenting breast feeding for optimal infant health, even proposed human milk as nature's archetypal probiotic food (17). Science based evidences advocate the use of probiotic lactic acid bacteria in the management of life style diseases especially in adults. In neonates also, gut bacteria are considered as the earliest and the most important stimulus for the development of Gut associated lymphoid tissue (GALT) and promotion of antiallergic processes. In circumstances where mother's milk is unavailable for neonates pasteurized donor human milk (PDHM) is the first option. This has resulted in the resurgence of human milk banks that usually supply frozen pasteurized breast milk (6). Breast milk banking resort to pasteurization and so its microbiome remains substantially different from natural milk. Neonates fed with pasteurized donor human milk are deprived of the rich beneficial microflora of breast milk predisposing them to untoward health effects in later life. Preservation of breast milk flora for restoring and modulating gut microbiome of preterm infants open up new avenues for bacteriotherapy and probiotics (16). Microbiological customization of pasteurized donor human milk is an exciting approach for seeding the infant gut with

good microbes. Isolation identification and preservation of breast milk microbes will be the first step in designing human milk bacterial consortia tailored for the target population Considering the importance of microorganisms for the various biotechnological applications, there is a need for preserving microorganisms in a way appropriate to its intended use. A simple short term preservation technique facilitating easy storage and retrieval will be helpful in maintaining a library of breast milk microflora that can be used to fortify pasteurized donor human milk. Conventionally this is accomplished by using low temperatures which reduce cellular metabolic activity. Among the different delivery systems, microencapsulation is widely used in pharmaceutical and food industries as an immobilization technique for providing functionality at the target site in the right manner. More recently, microencapsulation has also been explored as a novel method of microbial preservation for probiotic applications. Microencapsulation of organisms can be achieved by incorporating them into an encapsulation matrix that could act as a shield protecting the cells from the destructive and degrading factors in the environment. Apart from facilitating controlled release of bioactive components, encapsulation assists in sustaining the viability of cells during storage. Considering the profound long term health benefits imparted by breast milk microflora, this study aimed to isolate, identify and characterize a lactic acid bacterium from breast milk and also to evaluate the efficacy of using an alginate matrix for preservation and targeted delivery of these organisms.

## MATERIAL AND METHODS

### Isolation of lactic acid bacteria from human breast milk

Aseptically collected breast milk sample, after pre-enrichment in Nutrient broth (Hi-Media) (37 °C/18 h) was appropriately diluted and pour plated in De Man, Rogosa, and Sharpe (MRS) agar (Hi-Media). The plates were incubated at 37 $^0$C for 48 h to get discrete colonies. The typical spindle-shaped colonies were selected and streaked on MRS agar for further purification. The purified isolate was maintained in MRS agar slant at 4 $^o$C and also in 70% glycerol at -18 °C for long term storage.

### Identification of lactic acid bacteria

Preliminary characterization was done by assessing morphological and biochemical characteristics as detailed in Bergey's Manual of systematic Bacteriology for identification of Lactic acid bacteria (22). Freshly activated cultures were used for doing all the tests. Molecular-level confirmation of the isolate was done by 16S rRNA sequencing using the primers 16S-R-S-F Forward 5'CAGGCCTAACACATGCAAGTC3' and 16S-R-S-R Reverse 5' GGGCGGWGTGTACAAGGC 3. The gene sequences obtained was searched with the NCBI BLAST program for their closest relatives/reference strains having a homology of over or equal to 99%. The phylogenetic tree was constructed using nucleotide sequences of interesting isolates by neighbor joining method. The evolutionary distances were compared using the Maximum Likelihood method and the evolutionary analysis was conducted in MEGA X.

### Evaluation of adhesion potential

Adhesion potential was evaluated qualitatively by determining autoaggregation and cell surface hydrophobicity (CSH). Autoaggregation assay was performed as per (12). The cell pellet from freshly activated culture (37 °C/18 h) was suspended in phosphate buffered saline (PBS) to give a final optical density of 0.60±0.02 at 600 nm. 0.1 mL of the suspension was mixed with 3.9 mL of phosphate buffered saline and the absorbance (A1) was measured at 600 nm. The sample was kept undisturbed at 37 $^o$C to allow sedimentation. Optical density (OD) of the sample (upper portion) (A2) was again determined at sixth hour. The autoaggregation was calculated and expressed as percentage.

Autoaggregation (%) = [(A1 - A2)/ (A1) x 100]

Cell-surface hydrophobicity was determined using apolar solvent xylene (5). The cell pellet from 18 h old freshly activated cultures was suspended in phosphate buffered saline to give an optical density of 0.25±.05 at 600 nm. Equal volume of xylene was added to this cell suspension, vortexed thoroughly and the optical density was measured. The mixture was kept undisturbed for six hour to allow separation of phases. The optical density of upper phase was determined after carefully pipetting it out. Difference in optical density immediately after vortexing and after keeping undisturbed for six hours was expressed as percentage.

$$\text{Cell surface hydrophobicity}(\%) = \frac{\text{Initial optical density} - \text{Final optical density} * 100}{\text{Initial optical density}}$$

### Encapsulation of bacteria

Freshly activated cells of the isolate in its late log phase were used for encapsulation (13). The cells were harvested by refrigerated centrifugation at 12,000 g /10 min / 4 $^0$C (Velocity 18R Refrigerated, Dyna

MICO, UK). After washing, the cell pellet was suspended in minimum volume of normal saline. This suspension was added to ten times volume of 2% sodium alginate (INS No: INS 401, CAS No: 9005383-Food Grade acc. to BIS specification) and vortexed thoroughly to ensure uniform and homogenous distribution of the cells in the alginate matrix. 0.1 M calcium chloride was used as the gelling solution. The alginate-cell mixture was carefully added drop by drop into the gelling solution (29 ℃) using a sterile dropper. During preparation, care was taken to maintain a constant distance between dropper tip and gelling solution surface (collecting distance) to facilitate formation of uniform sized spherical beads. The beads were allowed to remain in the same solution for 20 minutes to attain firmness. The beads/encapsulates containing probiotic cells were collected, washed with 0.1% peptone water and stored in sterile containers at 4 °C till further use.

**Dissolution behavior of alginate beads**

The dissolution behavior of the beads at different pH was evaluated by exposing them to sterile phosphate buffered saline of pH 2.0, 7.0 and 8.0. To facilitate better visualization, beads for doing this study was prepared by incorporating 0.1% crystal violet at a level of 2% in sodium alginate mix. Physical changes happening to the beads were observed for a period of 8 h at 4 h intervals. The time required for complete dissolution of beads was recorded (4)

**Swelling ratio of alginate beads**

The uptake of water is decisive of the dissolution behaviour of beads. This was evaluated in terms of swelling ratio. 0.5 g beads were accurately weighed and placed in 100 mL of Phosphate buffered saline (pH 7.0). After 4 h beads were taken out, dried carefully and again weighed. The water uptake was calculated as the ratio of the increase in weight of beads after swelling to the initial dry weight (Menon and Sajeeth, 2013)

$$\text{Swelling ratio} = \frac{\text{Swollen weight - initial weight}}{\text{Initial weight}}$$

**Scanning electron microscopy of alginate beads**

To study the microstructures of the probiotic beads scanning electron microscopy was carried out (3). The probiotic beads were carefully washed in distilled water and dehydrated by putting in ethanol series: 15%, 30%, 50%, and 70% for 10 min each; 80% and 90% for 15 min each; and 100% for 1 h. The beads were cross sectioned and vacuum dried. The samples were fixed on aluminium stubs coated with double-faced metallic tape, sputter coated with gold and observed using Tescan Vega 3 (Czech Republic) Scanning electron microscope operated at 10.0 kV at different magnifications.

**Storage study of encapsulates**

Encapsulates containing the isolate were aseptically transferred to sterilized vials in such a way that each vial contained the beads prepared from one milliliter broth culture. The cell pellets from one milliliter broth culture suspended in one milliliter normal saline served as control. The vials containing encapsulated cells as well as non-encapsulated cells were stored at both 4 $^0$C and -18 $^0$C. To evaluate the storage stability, enumeration was done at 15 days interval for a period of 60 days and compared with the count on 0$^{th}$ day. The procedure was repeated and results were derived from average of six replications (Mandal et al., 2012).

**Statistical analysis**

Independent sample t-test was followed for storage studies with levels of significance of $p \leq 0.01$. Data analyses were carried out using the SPSS 24.0 software (Statistical Product and Service Solution). The results are presented as mean ± standard error (SE) of six replications.

## RESULTS AND DISCUSSION

**Isolation and characterization of the isolate**

Microscopic examination of gram stained smears of breast milk isolate revealed the presence of Gram positive cocci as tetrads and diplococci. Results of preliminary identification agreed with the features of Lactic acid bacteria provided in Bergey's manual of systematic bacteriology (22). The isolate was confirmed as *Pediococcus pentosaceus* with 16S rRNA sequencing. The strain denominated as *Pediococcus pentosaceus* strain DM101 was deposited in NCBI using BankIt with the accession number MK774704. BLASTing of the isolate was done against the sequences deposited in NCBI. The isolate exhibited 99.75% similarity to *P. pentosaceus* 2397 and *P. pentosaceus* OZF. Though isolated from milk, *Pediococcus pentosaceus* strain DM101 obtained in this study was incapable of fermenting milk sugar, lactose but fermented galactose, maltose, melezitose and ribose without gas production. Lactose fermenting strains of *P. pentosaceus* has been reported from goat milk (1). When non galactose fermenting lactic acid

bacteria are used as starters for preparing fermented milk products, galactose gets accumulated in products. Consumption of such products can lead to galactosaemia in individuals who are deficient in enzyme that converts galactose to glucose (2). If left untreated, galactosaemia might lead to jaundice, kidney problems, cataracts, mental retardation and possibly death (9). Galactose fermenting potential of this breast milk isolate enables its use as a secondary starter for preventing galactose accumulation in fermented products. Galactose fermenting strains of *P. pentosaceus* has been reported from goat milk (1). The phylogenetic tree of this isolate constructed with other closely related *P. pentosaceus* isolates available in gene bank is presented in (Fig 1). Accordingly, the isolate *P. pentosaceus* DM101 is closer to *P. pentosaceus* OZF, a human milk isolate from Turkey deposited in May 2010 and *P. pentosaceus* 2397 an isolate from China deposited in June 2020.

**Adhesion of the isolate**

Adherence to intestinal mucosa is essential for probiotics to impart health benefits. Auto aggregation and cell surface hydrophobicity are suggestive of cell surface properties that determine adhesion potential. An autoaggregation value of 67.9±0.74% was exhibited by the isolate obtained in this study(Table 1). According to (24), strains with an autoaggregation value of more than 40% are considered as good, while those with a value less than 10% are considered as poor. The cell surface hydrophobicity values are indicative of potential interactions that probiotic cells can have with intestinal mucosa. With the apolar solvent xylene, the cell surface hydrophobicity value of 60.7±0.67% was observed for the isolate. This confirms a hydrophobic cell surface for the breast milk isolate (Table 1). Age and surface chemistry of cells along with growth media components influence the cell surface hydrophobicity (17). (20) opined that a cell surface hydrophobicity value above 50% indicates high hydrophobicity. (23) found a positive correlation between cell surface hydrophobicity and adhesion potential.

**Table 1. Adhesion potential of *P. pentosaceus* DM101 and characteristics of alginate beads**

| **Adhesion potential** | | **Alginate bead characteristics** | | |
|---|---|---|---|---|
| Autoaggregation (%) | Cell surface hydrophobicity (%) | Weight(g) | Diameter(mm) | Swelling ratio |
| 67.9±0.74 | 60.7±0.67 | 0.03±0.003 | 3.75±0.25 | 0.368±0.006 |

Figures are mean± standard error of six replications (n=6)

**Table 2. Viable count of non-encapsulated and encapsulated *P. pentosaceus* DM101 at 4 °C and -18 °C**

| **Temperature** | **4 °C** | | | **-18 °C** | | |
|---|---|---|---|---|---|---|
| Days | Non-encapsulated cells(log CFU/ml) | Encapsulated cells(log CFU/beads from 1ml) | t-value | Non-encapsulated cells (log CFU/ml) | Encapsulated cells (log CFU/ beads from 1 ml) | t-value |
| 0 | 10.70 ± 0.153 | 7.10 ± 0.20 | 65.08** | 10.70 ± 0.153 | 7.10 ± 0.20 | 408.58** |
| 15 | 3.50 ± 0.058 | 6.19 ± 0.091 | 404.00** | 2.20 ± 0.058 | 6.03 ± 0.088 | 829.86** |
| 30 | 3.30 ± 0.173 | 6.32 ± 0.037 | 272.26** | 1.90 ± 0.058 | 5.63 ± 0.088 | 789.83** |
| 45 | 2.32 ± 0.079 | 6.23 ± 0.12 | 416.13** | 1.33 ± 0.033 | 3.92 ± 0.101 | 781.06** |
| 60 | 1.53 ± 0.085 | 6.00 ± 0.208 | 474.15** | 1.3 ± 0.058 | 3.80 ± 0.115 | 751.01** |

**significant at one per cent level (p≤0.01).

Figures are mean ± standard error of six replications (n=6)

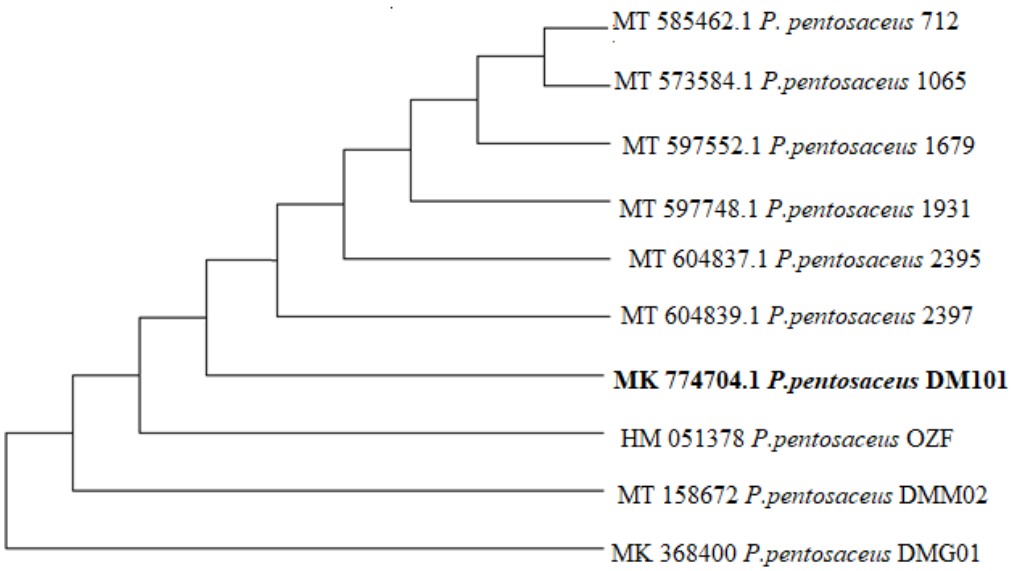
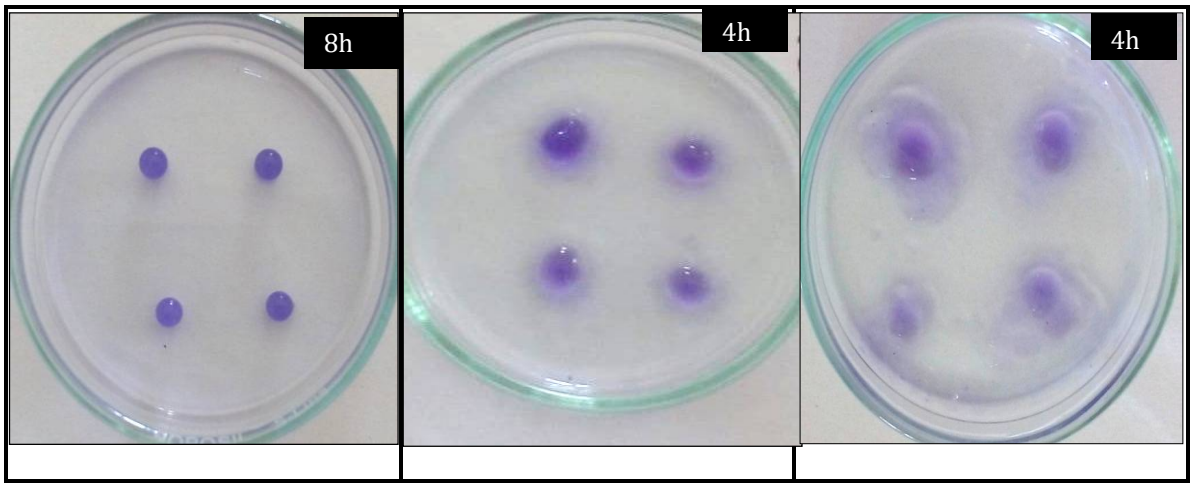


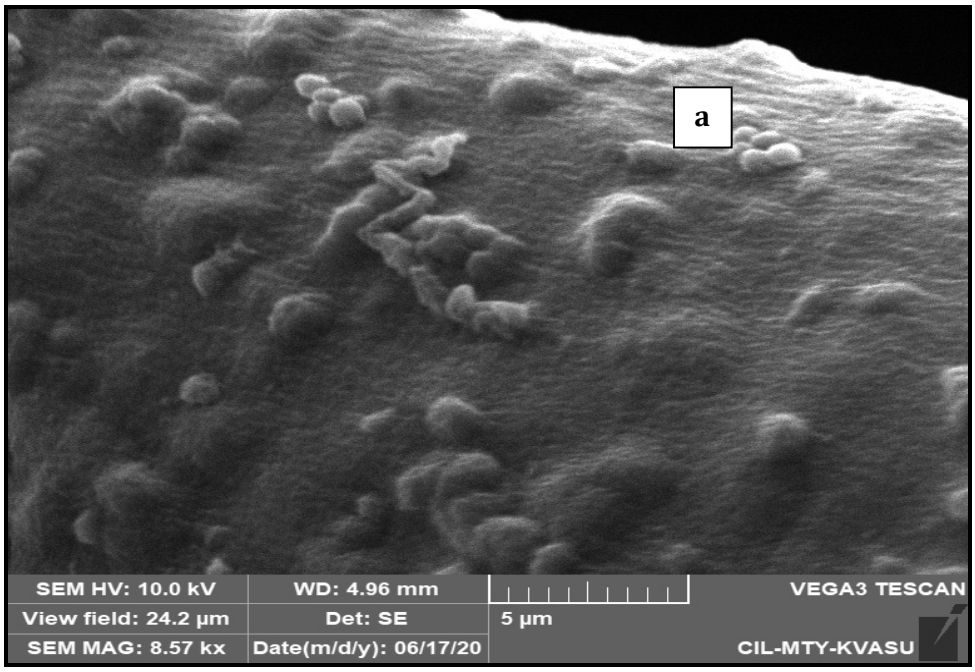

**Fig.1. Phylogenetic tree of *P. pentosaceus* DM101**




**pH 2.0** **pH 7.0** **pH 8.0**

**Fig.2. The dissolution behaviour of alginate beads at different pH**




a - *P. pentosaceus* DM101 in alginate matrix.

**Fig.3. Scanning electron micrograph of sodium alginate bead with *P. pentosaceus* DM101**

**Evaluation of probiotic beads**

The beads prepared in this work had an average diameter and weight of 3.75±0.25 mm and 0.03±0.003 g respectively (Table 1). (11) reported almost similar values for beads prepared by extrusion dropping. Active components in the matrix are generally released when the integrity of outer surface get compromised due to the pH changes in the suspending medium (4). Hence the dissolution behaviour of beads at pH 2.0, 7.0 and 8.0 were evaluated **(**Fig 2**).** The beads exhibited no change and remained intact even after 8 h at pH 2.0. But at pH 7.0 and 8.0, bead size increased gradually with time. According to (6), at low pH, alginate shrinks and the encapsulated ingredients remain confined to the beads. In the acidic pH of stomach, the hydrated sodium alginate becomes insoluble alginic acid skin and at higher pH of the intestine, this gets converted into a soluble layer. This pH dependent behaviour of alginate can be exploited to customize release profiles. At pH 7.0 the complete dissolution of the beads was observed within 5 h whereas at pH 8.0 dissolution was complete within 4 h itself. The dissolution behavior observed in this study indicated the preference of an alkaline pH for the release of active components. The water imbibing potential of the bead is also critical in deciding the dissolution behavior. Hence swelling ratio of the beads was also determined. Swelling ratio of beads is suggestive of the releasing behavior of encapsulated components. When the bead is having a loose texture, penetration of dissolution medium is fast and swelling ratio is high. Swelling is observed when the inert pore present in the polymeric chain gets filled by water (11). The swelling ratio of 0.368±0.006 (Table 1) observed in 4 h at neutral pH 7.0 is in line with the dissolution behavior shown by the beads at the same pH wherein bursting of the beads occurred by 5 h (11). The observations made in this study underscore the need for an alkaline pH for the retrieval of cells for later use. The scanning electron micrograph reveals the randomly distributed *P. pentosaceus* DM101 in an uneven alginate matrix **(**Fig 3**).**

**Storage study**

There are ample scientific evidences stating that any deviation from the evolutionary conserved process of breast feeding can alter the maturation of neonatal immune system. Breast feeding as the most significant factor that influence microbiome structure in early life of infants. The incidence of premature birth is on an increase and in most instances pasteurized donor milk is the only option for feeding. Such babies will be deprived of the breast milk flora that has the potential to positively condition the early gut microbiota. Strategies to ensure the availability of human milk microbiota early in life will significantly improve associated health benefits. Realizing the crucial role of breast milk microflora in imparting long term health benefits, this study attempted to evaluate the feasibility of using an alginate matrix for preserving the potentially probiotic *P. pentosaceus* DM101 isolated from breast milk (19). Comparison of viable counts of non-encapsulated and encapsulated cells (Table 2) demonstrated the potential of alginate

encapsulation to sustain the viability of probiotic cells for an extended period both under refrigeration and freezing. The significant difference in the viable count of encapsulated and non-encapsulated cells at zero day implies the need for improvising the conventional method followed for encapsulation. Even though, the zero-day count of encapsulated cells was significantly lower than non-encapsulated cells, as the storage period progressed, the encapsulated cells retained their viability in a better manner than their non-encapsulated counterparts. After 60 days of storage, when the free cells showed a 9.2 log reduction, encapsulated cells exhibited only 1.1 log reduction at 4 °C. For the same time period, viable count in beads stored at -18 °C showed a 3.3 log reduction and non-encapsulated cells showed 9.3 log reduction. This finding undoubtedly confirms the protective role of alginate matrix. The protective influence of encapsulation has been reported by [13]. From the observations it can be inferred that refrigerated storage is remarkably more beneficial than frozen storage to preserve alginate beads. The signs of shrinkage evident from 15$^{th}$ day onwards progressed steadily for beads stored in freezer. However, in refrigerated storage similar changes could not be appreciated even after 60 days. It can be concluded that freezer environment posed a greater threat to the integrity of alginate gel predisposing the embedded cells to cryoinjury and subsequent loss of vitality. Not even a single instance of contamination occurred for cells preserved in alginate beads during the entire study is noteworthy. Moreover, no extra steps for resuscitation were needed for retrieval of cells from the alginate encapsulates. The results of this study upshots alginate encapsulation as an effective and safeguarding tool for preserving the pioneer bacteria of breast milk.

## CONCLUSION

Preserving breast milk flora in alginate matrix can be a safe alternative for supplementing breast milk microflora in neonatal care units. This simple technique has ample scope in situations that cannot afford sophisticated inputs, especially in rural areas where low birth weight babies are higher. A well concerted study in this direction opens up promising opportunities for industrial exploitation of this technique in pediatric health care segment. Traditional approaches, if refined can ensure improved encapsulation efficiency.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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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