# CHAPTER 3
# MATERIALS AND METHODS

## 3.1 Materials

Test strains: *Bifidobacterium longum*, *Bifidobacterium bifidum*, and *Bifidobacterium infantis* were obtained from the collection of Chr. Hansen A/S (Denmark) and were stored at –20°C and used within 12 months.

Yogurt cultures (YC-380, Yo-Flex®): Available from Chr. Hansen A/S (Denmark) in the freeze-dried culture form. The culture were stored at –18°C and used before the expiration date. The Yo-Flex® cultures are defined single strain of the lactic acid bacteria of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*.

Tapioca starch beads (TSB): Three leading brands of commercial TSB were used :

- Golden Chef® (Oriental Food Co., LTD., Bangkok, Thailand)
- Special Sacoo® (Lotus Co., LTD., Chiang Mai, Thailand)
- Thaiworld® (Thai world Import Export Co., LTD., Bangkok, Thailand)

Freeze-dried-gelatinized tapioca starch beads (FDTB) preparation: The TSB were heated in boiling water at 100°C for 15 min to completely gelatinize the beads. The beads were then cooled down immediately in deionized water at 25°C and kept at 4-5°C for 24 h, to allow the beads to completely swell. The swelled beads were either quick freezed at -176°C, 5-10 min (QF) by liquid nitrogen or slow freezed at -20°C, 24 h (SF) in frozen room (Shan-Yang *et al.*, 1999; Saxelin *et al.*, 1999). After freezing, the beads were kept in freeze-drying system at 25°C for 72 h to ensure the completed drying. Two different supporting materials were obtained; a) quick-freeze-dried-gelatinized tapioca starch beads (QF-FDTB) and b) slow-freeze-dried-gelatinized tapioca starch beads (SF-FDTB).

The samples were kept in sealed double plastic bags for further use. The moisture content and physical evaluations included bulk volume, diameter, microstructure, porosity, water-holding capacity, adsorption capacity, adsorption behavior, and adsorption capacity of bifidobacterial cells, namely *B. longum*, *B. bifidum*, and *B. infantis*, respectively.

### 3.2 Chemicals and media

Chemicals and media for culture and enumeration of bifidobacteria

- Difco™ Lactobacilli MRS broth was from BBL Microbiology System (Becton Dickinson, Rockville, MD, USA).
- Granulated agar was from Merck KGaA (Darmstadt, Germany).
- BBL™ GasPak™ disposable anaerobic indicator was from BBL Microbiology System (Becton Dickinson, Rockville, MD, USA). The aqueous solution composed percent aqueous solution of 18.0 fructose, 1.53 potassium phosphate, 0.35 sodium hydroxide, and 0.25 methylene blue solution, plus preservative on fiber carrier.
- Anaerocult®A was from Merck KGaA (Darmstadt, Germany). Each Anaerocult®A consisted of kieselguhr, iron powder, citric acid, and sodium carbonate. The chemical mixture inside the sachet contained free crystalline silica that required 35 mL water to activate the reaction. Anaerocult®A was used to produce an anaerobic condition in an anaerobic jar (contents 2.5 L) that was used for the cultivation of obligatory and facultative anaerobes.
- Peptone water was from Merck KGaA (Darmstadt, Germany).
- L-cysteine hydrochloride ($C_3H_7NO_2S$.HCl anhydrous) was from Sigma Aldrich (Louis, MO, USA).
- Gram strain kit BD® was from Becton, Dickinson and Company (Oakville, Ontario, Canada).

Coating materials

- Sodium caseinate was from Sigma-Aldrich (Louis, MO, USA).

- Palmitic acid (Hexadecanoic acid, $C_{16}H_{32}O_2$) was from Sigma-Aldrich (Louis, MO, USA).
- PANODAN® 150 K was from Danisco A/S (Copenhegen, Denmark). PANODAN® 150 K is a kosher approved blend of diacetyl tartaric acid ester of mono-diglyceride (DATEM) and mono-diglycerides
- White beeswax was from Fisher Scientific (Ottawa, Ontario, Canada).

Chemicals for simulated gastrointestinal fluids

- Potassium chloride (KCl) was from Merck KGaA (Darmstadt, Germany).
- Monobasic potassium phosphate ($KH_2PO_4$) was from Fisher Scientific (Loughborough, Leics, UK)
- Sodium dihydrogen orthophosphate ($Na_2H_2PO_4$) was from Sigma-Aldrich (Louis, MO., USA).
- Sodium hydroxide (NaOH) was from Merck KGaA (Darmstadt, Germany).
- Sodium chloride (NaCl) was from Merck KGaA (Darmstadt, Germany).

### 3.3 Equipments

- Anaerobic jar (Merck, Germany)
- Centrifugation (Model Hamle Z 200 A, Hamle Labtechniks, Germany)
- Helium displacement multivolume pycnometer 1330 (Micromeretics Instrument Corp., USA)
- Micrometer (Mitutoyo, Japan).
- Automatic volumetric sorption analyzer (Quantachrome, USA)
- Freeze dryer (FFD-42-WS Freeze-Dryer, The VirTis Co. Inc., GarDiner, NY., USA)
- Scanning electron microscopy (Model JSM-5910 LV, Jeol Ltd., Japan)
- Texture analyzer (Texture Exponent 32, Stable Micro Systems, England)
- Bags Zipper Seal® (2x3x0.002 mil.; Fisher Scientific, Ontario, Canada)
- 10-mL Graduated cylinder (10:0.2±0.15 mL, Witag, Germany)

- 4 decimal Mettler® analytical balance model AE163 (American Laboratory Trading LLC, USA)
- 3 decimal Precisa® balance model XT 320 M (Precisa Instrument AG, Switzerland)
- 1 decimal Petit® balance (Japan)
- Microprocessor pH meter (Hanna /instruments HI 9321, Portugal)
- 15 mL Polypropylene-disposable sterile centrifuge tubes with plug seal cap (Fisher Scientific, Ontario, Canada)
- 50 mL Blue Max® Polypropylene-disposable sterile centrifuge tubes (Becton Dickinson Labware, USA)
- 1 mL and 5 mL Stripette® disposable serological polystyrene pipette, individually wrapped and sterile (Corning, NY, USA)

**3.4 Media, simulated gastrointestinal fluids, and film solution preparation**

deMan-Rogosa-Sharpe (MRS) broth: MRS consisted of 10 g/L proteose peptone No.3, 10 g/L beef extract, 5 g/L yeast extract, 20 g/L dextrose, 1 g/L polysorbate 80, 2 g/L ammonium citrate, 5 g/L sodium acetate, 0.1 g/L magnesium sulphate, 0.05 g/L manganese sulphate, 2.0 g/L dipotassium phosphate. The media in powdered form is available from Difco™ and was used according to the manufacturers instruction which required 55 g powdered MRS media per litre of distilled water. Once the media was dissolved in the water, it was sterilized by autoclaving at 121°C for 15 min. The final pH was 6.5±0.2.

Modified MRS (mMRS) broth: to MRS broth prepared as above, 0.5% L-cysteine hydrochloride was added (on dry weight). The pH of the medium was adjusted to 7.0 before sterilization.

Modified MRS agar: mMRS broth and 15 g/L solidified agar were warmed up by microwave oven on medium power. The media was then adjusted to pH 7.0 and sterilized in an autoclave at 121°C for 15 min. The media was poured into petri-plates after cooling down to approximately 45°C.

Phosphate-buffered saline (PBS): Nine g/L NaCl, 0.2 g/L $KH_2PO_4$ (anhydrous), 2.9 g/L $Na_2HPO_4$ (anhydrous) and 2 g/L KCl were dissolved in distilled water. The pH was 7.2. The solution was autoclaved at 121°C for 15 min.

Peptone water saline: Consists of 1.0 g/L peptone water, 8.5 g/L NaCl in distilled water. After the solutes were dissolved, it was autoclaved at 121°C for 15 min.

Simulated gastric fluid without enzyme (USP, 2002): 2.0 g of NaCl was dissolved in 500 mL distilled water to which 7.0 mL of conc. HCl was added and then diluted to 1,000 mL with distilled water. The pH was about 1.2. The solution was autoclaved at 121°C for 15 min.

Simulated intestinal fluid without enzyme (pH 6.8 or 7.5) (USP, 2002): To 6.8 g of monobasic potassium phosphate in 250 mL of distilled water, 190 mL 0.2M NaOH solution, and 400 mL of distilled water were added. The pH of the solution was adjusted to 6.8±0.1 or 7.5±0.1 with 0.2N NaOH and then, diluted with distilled water to the total volume of 1,000 mL before sterilization at 121° C for 15 min.

Phosphate buffer (pH 6.0): The solution was prepared following the procedure of USP (2002) by adding 50 mL of 0.2 M monobasic potassium phosphate to 5.6 mL of 0.2 M sodium hydroxide and then adjusted the volume to 200 mL by distilled water before sterilization at 121°C for 15 min.

Phosphate buffer (pH 7.2): The solution was prepared following the procedure of USP (2002) by adding 50 mL of 0.2 M monobasic potassium phosphate to 34.7 mL of 0.2 M sodium hydroxide, and then adjusted the volume to 200 mL by distilled water before sterilization at 121°C for 15 min.

Film solution from sodium caseinate: The film solution was prepared according to Bustillos and Krochta (1993) with modification. 15% w/v of sodium caseinate was dispersed in sterilized water at 25-27°C. The solution was mixed well and warmed in a microwave oven on medium power. The film solution was kept at 4-5°C for 24 h before using.

## 3.5 Research designs and methods

### 3.5.1 Initial moisture content

The initial moisture content of the gelatinized TSB from three commercial brands (Golden Chef®, Special Sacoo®, and Thaiworld®) were measured before freezing and after drying by hot air oven at 130±3°C for 48 h or until the weight was constant (AOAC, 1998).

The moisture content of gelatinized TSB, QF- and SF-FDTB were statistical evaluated using completely randomized design with 3 replicates.

### 3.5.2 Physical properties of QF- and SF-FDTB

The physical properties of QF- and SF-FDTB from three commercial brands (Golden Chef®, Special Sacoo®, and Thaiworld®) included bulk volume, diameter, microstructure, porosity, specific surface area, water-holding capacity, adsorption capacity, adsorption behavior, and gel strength of the reformative beads. Analytical methods are as follows.

#### 3.5.2.1 Determination of bead diameter

The diameters of 100 QF- and SF-FDTB obtained from three commercial brands (Golden Chef®, Special Sacoo®, and Thaiworld®) were determined by micrometer and reported as average values in millimeter.

#### 3.5.2.2 Determination of the total bulk volume

The total bulk volume of 100 QF- and SF-FDTB from three commercial brands (Golden Chef®, Special Sacoo®, and Thaiworld®) were measured by 10-mL graduated cylinder. The average diameter and bulk volume were then calculated.

#### 3.5.2.3 Determination of porosity

The porosity (ε) of QF- and SF-FDTB from three commercial brands (Golden Chef®, Special Sacoo®, and Thaiworld®) were determined following the

procedure of Habib *et al.* (2002) with modification. The porosity was calculated using the following equation:

$$\varepsilon = 100\ (1 - \rho_a / \rho_t)$$

Where $\rho_t$ was the true density and $\rho_a$ was the granular density.

The true density ($\rho_t$) of the QF- and SF-FDTB from three commercial brands (Golden Chef®, Special Sacoo®, and Thaiworld®) was determined by helium displacement in Multivolume Pycnometer 1330. The true volume of the FDTB (an average of five runs) was calculated by determining the volume of helium displaced by the QF- or SF-FDTB during the test. The true density was then calculated by dividing the weight of the tested QF- or SF-FDTB by the average value of the true volume.

The granular density ($\rho_a$) of the QF- and SF-FDTB from three commercial brands (Golden Chef®, Special Sacoo®, and Thaiworld®) were determined using glycerol displacement in 25 mL-glass Pycnometer. The granular density was then calculated by dividing the weight of the tested QF- or SF-FDTB by the average value of granular volume.

### 3.5.2.4 Determination of specific surface area

The specific surface area of 100 QF- and SF-FDTB obtained from three commercial brands (Golden Chef®, Special Sacoo®, and Thaiworld®) were determined by Automatic volumetric sorption analyzer and reported the values in square meter per gram.



### 3.5.2.5 Determination of adsorption capacity

The adsorption capacity of 100-QF- and SF-FDTB from three commercial brands (Golden Chef®, Special Sacoo®, and Thaiworld®) were determined by measurement the weight of 100-rehydrated beads stored in PS at 4-5°C during the first minute. The adsorption capacity was then calculated by

dividing the weight of 100-rehydrated beads by the weight of 100-QF- or SF-FDTB.

**3.5.2.6 Determination of adsorption behavior**

The adsorption behavior of 100-QF- and SF-FDTB from three commercial brands (Golden Chef®, Special Sacoo®, and Thaiworld®) were determined by monitoring the weight of 100-rehydrated QF- and SF-FDTB stored in PS at 4-5°C for 48 h. The data were collected at 0, 1, 60, 120, 180, 240, 300, 360, 420, and 480 min, or until the weight was constant.

**3.5.2.7 Determination of water-holding capacity**

The water-holding capacity of 100-QF- and SF-FDTB from three commercial brands (Golden Chef®, Special Sacoo®, and Thaiworld®) were calculated by dividing the weight of completely rehydrated QF- or SF-FDTB by the dried weight of QF- or SF-FDTB.

**3.5.2.8 Determination of gel strength**

The gel strength of the rehydrated QF- and SF-FDTB were determined following the method of Kuo-Cheng and Jer-Yiing (1997) by placing the rehydrated QF- or SF-FDTB in a semi-sphere hole of a holder. Then the rehydrated QF- or SF-FDTB was compressed to deformation by a tested needle (probe type P/2), at a constant deformation rate of 2 mm/s, employing a Texture Analyzer. Mechanical strength measurement for gelatinized beads was expressed by a critical compressive stress when abrasion of gelatinized beads occurred.



**3.5.2.9 Determination of microstructure**

The microstructures of the SF-FDTB and QF-FDTB from three commercial brands (Golden Chef®, Special Sacoo®, and Thaiworld®) were determined by following the method of Habib *et al.* (2002) with modification. The beads were placed on aluminum mounts using double sided Scotch® tape

and stored overnight in tightly sealed glass desiccator. The cross-sections of QF- and SF-FDTB were then sputter coated with a gold-palladium mixture before examined by scanning electron microscopy.

#### 3.5.2.10 Statistical evaluation of physical properties of QF- and SF-FDTB

The physical properties of QF- and SF-FDTB from three commercial brands (Golden Chef®, Special Sacoo®, and Thaiworld®) were statistically evaluated using factorial experiment (2 x 3) with 3 replicates.

### 3.5.3 Culture conditions of *Bifidobacterium* spp.

The dried cultures of *B. longum, B. bifidum* or *B. infantis* were first propagated in mMRS containing 0.5% L-cysteine.HCl, incubated under anaerobiosis at 37°C for 24 h. The anaerobic condition was achieved by using the AnaeroCult® and Anaerobic Jar. The BBL™ GasPak™ disposable anaerobic indicator was used to indicate the anaerobic condition. Cells that were 24 h old were maintained on mMRS agar plates. The cultures were incubated anaerobically at 37°C for 24-48 h before further sub-culturing to mMRS broth. The cultures were incubated under the anaerobic conditions at 37°C for 24 h. The method followed the modified procedure of Hansen *et al.*(2002). The bifidobacterial cells were grown to the late exponential growth phase within 24 h (Hansen *et al.*, 2002). The bifidobacterial cells in mMRS broth were cooled to 4°C and harvested according to method of Hansen *et al.* (2002) by centrifugation at 3,500 x g for 1 min, washed once with PBS, and resuspended in 5 mL of PS.

### 3.5.4 Immobilization technique

One hundred beads of QF- or SF-FDTB from three commercial brands (Golden Chef®, Special Sacoo®, and Thaiworld®) were added to 5 mL of bifidobacterial suspension and kept in a 50-mL screw-cap sterilized plastic test tube. The tube was then gently mixed for 5 s and stored for 16-18 h at 4-5°C to

allow the bifidobacterial cells to be completely adsorbed into the beads. The mixture was gently mixed for 5 s after 1, 2, 3, 4, 5, and 6 h storage time to facilitate encapsulation of bacteria in QF- or SF-FDTB.

### 3.5.5 Cell enumeration

One mL of free cell was serially diluted in PS and 1 mL of diluted samples from the appropriated dilutions was determined by the pour plate method using mMRS agar. The viable cell count was determined after 72 h under anaerobic condition at 37°C. Enumeration of bifidobacteria encapsulated in gelatinized beads was determined following the procedure of Sun and Griffiths (2000) with modification. One mL of beads, measured by displacement of sterile water in a 10-mL graduated cylinder was washed once with sterile water, and then grounded completely with a porcelain mortar and pestle. Bifidobacterial cells released from the beads were suspended in PS and serially diluted. Viable cell counts were performed in duplicate and expressed in log CFU/mL of immobilized beads.

### 3.5.6 Effects of immobilization time and freezing methods of FDTB on the viability of immobilized *B. infantis*

*B. infantis* was cultured, harvested, suspended in 5 mL PS and immobilized in Special Sacoo® 100-QF- or SF-FDTB, respectively. The samples were taken out at the interval of 6, 18, and 24 h for enumeration. The results were reported in log CFU/mL of the immobilized bifidobacterial cells.

The effect of immobilization times on the viability of immobilized *B. infantis* in Special Sacoo®QF- and SF-FDTB were statistical evaluated using completely randomized design with 3 replicates.

### 3.5.7 Effects of commercial brands and freezing methods of FDTB on the immobilization of *Bifidobacterium* spp.

*B. longum, B. bifidum* or *B. infantis* was cultured, harvested, suspended in 5 mL PS and immobilized in 100-QF- or SF-FDTB from three commercial brands (Golden Chef®, Special Sacoo®, and Thaiworld®), respectively. The samples were kept at 4-5°C for 18 h before enumeration. The results were reported in log CFU/mL of the immobilized bifidobacterial cells. Immobilization of bifidobacterial cells in 100-QF- or SF-FDTB from three commercial brands were calculated as a percentage of free cells using the following equation:

$$\text{Immobilization (\%)} = \left(\frac{\text{Cell counts obtained from 100 immobilized beads}}{\text{Cell counts obtained from 5 mL of free cells}}\right) \times 100$$

The effects of freezing methods and commercial brands of FDTB on the immobilization of *Bifidobacterium* spp. were statistical evaluated using factorial experiment (2 x 3) with 5 replicates.

### 3.5.8 Effects of freezing methods of FDTB and bifidobacterial cell concentrations on the viability of immobilized *Bifidobacterium* spp., stored at 4-5°C for 16-18 h

The capacity of the QF- and SF-FDTB from selected commercial brands to load the bifidobacterial cells, were studied by suspended the cultures in 10, 20, 30, 40, and 50 mL of mMRS broth, harvested by centrifugation at 3,500 x g for 1 min, washed once with 5 mL PBS, and resuspended in 5 mL of PS. The concentrations of the cell collected from 10, 20, 30, 40, and 50 mL of mMRS broth were represented as 2x, 4x, 6x, 8x, and 10x, respectively. The concentrated cells were then immobilized into 100-QF- or SF-FDTB and kept at 4-5°C for 16-18 h before enumeration. The results were calculated and reported in log CFU/bead.

The effects of freezing methods of FDTB and bifidobacterial cell concentrations on the immobilization of *Bifidobacterium* spp. were statistical evaluated using factorial experiment (2 x 5) with 3 replicates.

### 3.5.9 Effect of freeze-drying on the viability of immobilized *Bifidobacterium* spp.

*B. longum, B. bifidum* or *B. infantis* was cultured, harvested, suspended in 5 mL PS and immobilized in 100-QF- or SF-FDTB from selected commercial brand, respectively. The immobilization proceeded at 4-5°C for 18 h to get fresh beads. Next, some of the fresh beads were frozen at –18°C for 24 h and dried out using freeze-drying at 25°C for 72 h. The viable counts of immobilized fresh beads and immobilized-dried beads were compared and reported in log CFU/mL. The microstructures of dried-immobilized beads were determined by SEM following the modified method of Habib *et al.* (2002).

The effects of freeze-drying method on the viability of immobilized *Bifidobacterium* spp. were statistical evaluated using completely randomized design with 3 replicates.

### 3.5.10 Effect of coating materials on the viability of dried-immobilized *Bifidobacterium* spp.

*B. longum, B. bifidum* or *B. infantis* was cultured, harvested, suspended in 5 mL PS and immobilized in 100-QF- or SF-FDTB from selected commercial brand, respectively. The samples were kept at 4-5°C for 18 h and –18°C for 24 h, respectively, before drying in freeze-drying system. The dried-immobilized beads were then coated with molten edible fat (palmitic acid, PANODAN®, and beeswax) at 50-53°C and followed by coating with film solution of 15% (w/v) sodium caseinate. Then film layer was dried by blower cold air for 3-4 min. The three types of edible fat: beeswax, PANODAN®150K, and palmitic acid were investigated for weight of coating materials and diameter of the

bilayer-coated-immobilized bead. The viable cell counts of the coated-immobilized bifidobacteria were compared with the non coated-immobilized bifidobacteria. The cell counts in log CFU/mL were reported. The microstructures of coated-immobilized beads were determined by SEM following the modified method of Habib *et al.* (2002).

The effects of coating materials on the viability of immobilized *Bifidobacterium* spp. were statistical evaluated using completely randomized design with 2 replicates and with 9 replicates for the weight of fat coating.

### 3.5.11 Survival of free cells, non-coated, and coated-dried-immobilized *Bifidobacterium* spp. in simulated gastrointestinal fluids without enzyme at 37°C for 310 min

One mL of free cells, non-coated, and coated-dried-immobilized *B. longum*, *B. bifidum* or *B. infantis*, were incubated in 9 mL of pre-warmed simulated gastrointestinal fluids at 37°C as described by the modified method of the United State of Pharmacopia (USP, 2002), Klein *et al.* (2002) and that of Sun and Griffiths (2000). The simulated gastrointestinal system composed of the series of simulated gastric fluid without pepsin (pH 1.2) for 120 min, phosphate buffer (pH 6.0) for 10 min, simulated intestinal fluid without pancreatin (pH 6.8) for 120 min, phosphate buffer (pH 7.2) for 30 min, and simulated intestinal fluid without pancreatin (pH 7.5) for 120 min, respectively. The temperature was controlled at 37°C. After incubation in simulated gastrointestinal fluids, the samples were enumerated and reported in log CFU/mL.

The change of the three-dimension structure of immobilized beads after storage in simulated gastrointestinal fluids without enzyme at 37°C for 310 min were determined. The beads were freeze-dried, sputter coated with a gold-palladium mixture before examined by SEM.

The survival of free cells, non-coated, and coated-dried-immobilized *bifidobacteria* in simulated gastrointestinal fluids without enzyme were statistical evaluated using completely randomized design with 3 replicates.

### 3.5.12 Effects of coating materials and storage time on the survival of non coated and coated-immobilized *Bifidobacterium* spp. in pasteurized yogurt stored at 4-5°C for 4 wk and in simulated gastrointestinal fluids without enzyme at 37°C for 310 min

Yogurt was manufactured following the modified method of Sun and Griffiths (2000) for the lab scale. The culture of freeze-dried yogurt was inoculated into the commercial pasteurized skim milk at concentration of 0.5% and incubated at 37°C until the pH reached 4.2-4.4. Nine mL of yogurt was then separated in a 15-mL polypropylene tube and pasteurized at 85-90°C for 30 min. One mL of immobilized beads was mixed with 9 mL of pasteurized yogurt and stored at 4-5°C for up to 4 wk. The viability of the bifidobacterial cells was determined at the weekly intervals by taking the entire contents from each container. The pH of the pasteurized yogurt was measured throughout the storage period. The cell counts (log CFU/mL) and pH values were reported.

The survival of non-coated and coated-immobilized *B. longum*, *B. bifidum*, *and B. infantis* in simulated gastrointestinal fluids without enzyme were determined. One mL of non-coated or coated-immobilized *B. longum*, *B. bifidum* or *B. infantis*, stored in yogurt at 4-5°C for 4 wk, were incubated in 9 mL pre-warmed simulated gastrointestinal fluids at 37°C following the modified methods of USP (2002), Klein *et al.* (2002), and that of Sun and Griffiths, (2000).

The changes of the three-dimension structure of immobilized beads during storage in pasteurized yogurt were investigated. The samples were taking out after storage at 4-5°C for 4 wk. The beads were freeze-dried, sputter coated with a gold-palladium mixture before examined by SEM.

The effects of coating materials and storage time on the survival of non-coated and coated-immobilized bifidobacteria in pasteurized yogurt were statistical evaluated using factorial experiment (3 x 5) with 3 replicates. The survival of non-coated and coated-dried-immobilized *bifidobacteria* in simulated gastrointestinal fluids without enzyme were statistical evaluated using completely randomized design with 3 replicates.

### 3.5.13 Effect of storage time on the survival of free cells and non-coated immobilized *Bifidobacterium* spp. in sterilized yogurt, stored at 4-5°C for 4 wk and in simulated gastrointestinal fluids without enzyme at 37°C for 310 min

Yogurt was manufactured following the method of Sun and Griffiths (2000) with modification of incubated temperature from 42°C to 37°C. The yogurt was sterilized at 121°C for 15 min and 9 mL of yogurt was then separated in the 15-mL sterilized-polypropylene tube. One mL of free cells or non-coated-immobilized beads was mixed with 9 mL of sterilized yogurt and stored at 4-5°C for up to 4 weeks. The viability of the bifidobacterial cells was determined at the weekly intervals by taking the entire contents from each container. Free cells (1 mL) were subjected to the same conditions as that of the immobilized cells. The cell counts in log CFU/mL were reported.

The survival of free cells and non-coated-immobilized *B. longum*, *B. bifidum*, *and B. infantis* in simulated gastrointestinal fluids without enzyme was determined. One mL of free cells or non-coated-immobilized *B. longum*, *B. bifidum* or *B. infantis*, stored in sterilized yogurt at 4-5°C for 4 wk, were incubated in 9 mL pre-warmed simulated gastrointestinal fluids at 37°C following the modified method of USP (2002), Klein *et al.* (2002), and that of Sun and Griffiths (2000).

The effect of storage time on the survival of free cells and non-coated immobilized bifidobacteria in sterilized yogurt were statistical evaluated using completely randomized design with 3 replicates.

### 3.5.14 Statistical evaluation

All results when possible were statistically analyzed by analysis of variance (SPSS ver. 8, 1998). If a significant main effect was detected, the means were separated by the Duncan's multiple range test (Cochran and Cox, 1957). The predetermined acceptable level of probability was 5% ($p \leq 0.5$) for all comparisons.