Viability of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus* and *Lactobacillus casei* in fermented milk supplemented with isomalto-oligosaccharides derived from banana flour

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SUMMARY

Commercial yoghurts and yoghurt-type products do not always fulfill the minimum requirement of viable culture cell counts at the time of consumption, which is the most important factor in order for such products to exhibit therapeutic effects. This work aimed to investigate the survivability of *Streptococcus thermophilus*, combined with *Lactobacillus delbrueckii* ssp. *bulgaricus*, and *Lactobacillus acidophilus*, combined with *Lactobacillus casei*, in two fermented milk samples supplemented with isomalto-oligosaccharides (IMO) prepared from banana flour. The preferential fermentation of oligosaccharides by the combined starter cultures was also investigated. After 28 days of storage at 4 °C, the viable cell numbers of all bacterial strains in both samples were not changed ($p > 0.05$). Lactose, isomaltotriose, isomaltotetraose, and maltooligoheptaose and larger oligomers, were depleted by approximately 50%, 40%, 20% and 20%, respectively. The decrease of lactose and IMO in both fermented milk samples did not differ ($p > 0.05$). In this study, IMO could maintain the viable cell numbers of all bacteria used in the experiments. The order of oligosaccharide fermentation preference of the cultures was lactose > isomaltotriose > maltooligoheptaose and larger oligomers > isomaltotetraose.

Keywords

fermented milk; probiotic yoghurt; isomalto-oligosaccharides; lactic acid bacteria; prebiotics

Cultured or fermented milks are probably the oldest dairy products known to humans [1]. According to the Codex Standard [2], fermented milks are products derived by fermentation of milk by suitable organism(s), in which the milk's structure is altered by the reduction of pH. Among the fermented milk products range, yoghurt is one of the most well-known and developed product world-wide [3, 4]. Several factors, such as acidity, production of aroma compounds, textural characteristics, sensory attributes, nutritional value and therapeutic properties contribute to the creation of desirable products [5]. Strictly speaking, 'yoghurt' must contain two specific microorganisms belonging to the lactic acid bacteria (LAB) group, namely, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, as dominant organisms [2]. In many countries, such as France, Portugal and USA, this concept has been adopted as product identification. However, in the United Kingdom, the whole range of yoghurt-type fermented milk products are labelled as 'yoghurt' [6].

Since the 1980s, therapeutic yoghurts and other fermented milk products containing probiotic bacteria, in particular lactobacilli and bifidobacteria, have been increasingly studied and marketed [7]. The manufacturing process of therapeutic or probiotic yoghurts is similar to that of the conventional type; the main difference is the culture starters used in the process. There have been many studies reporting that some *Lactobacillus* spp., and in particular *Bifidobacterium* spp., contained in yoghurt can colonize the large intestine, reduce its pH and control the growth of undesirable microorganisms [3, 4, 8]. Therefore, bacterial cultures including *L. rhamnosus, L. reuterii, L. acidophilus,*

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L. plantarum, L. casei, B. bifidum and *B. longum* are widely used as inocula in therapeutic fermented milks in various combinations [7, 9]. However, to exhibit the desirable therapeutic effects, starter culture(s) must be present in the product in high viable counts at the time of consumption, which is at least 10^7 CFU × ml^{-1} of combined organisms and at least 10^6 CFU × ml^{-1} of other supplementary microorganisms [2]. Many researchers recommended ranges of minimum daily dose higher than 10^7–10^8 CFU × ml^{-1} [10, 11], or even as high as 10^8–10^9 CFU × ml^{-1} [12]. Despite the importance of viability of probiotics in the products, many surveys have reported poor viability of probiotics in some preparations of commercially available products, especially when the products were stored for a long period of time (28–35 days) or kept at a higher temperature (10–12 °C) [3, 11, 13, 14].

Some studies took interest in combining probiotics and prebiotics in order to solve the bacterial survivability problem, and reported various combinations of pro-prebiotics that could improve the viability of the cultures [15–18]. Prebiotic has been widely known since 1995 after GIBSON and ROBERFROID [19] gave its definition as 'a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one, or limited number of, bacteria in the colon that can improve the host health'. Since the 1990s, oligosaccharides with various structures and production technologies have been available on the market, especially in Japan [20, 21].

This study aimed to investigate the application of isomalto-oligosaccharides, which were prepared from banana flour, on survivability of probiotic cultures and their preferences of oligosaccharide fermentation in fermented milk.

MATERIALS AND METHODS

Materials

Chemicals used were of analytical grade and were obtained from BDH (Poole, United Kingdom). All chemicals used in high performance liquid chromatography (HPLC) were of HPLC grade obtained from Merck (Darmstadt, Germany). Diluents and culture media were obtained from Difco (Detroit, Michigan, USA). Selective media de Man Rogosa and Sharpe (MRS) and M17 broth were obtained from Oxoid (Cambridge, United Kingdom). Two α-amylase enzymes, Termamyl SC (E.C. 3.2.1.1) and Fungamyl 800L (E.C. 3.2.1.1; Novozymes, Bagsvaerd, Denmark), were kindly provided by East Asiatic (Bangkok, Thailand). The enzyme *trans*-glucosidase L (E.C. 3.2.1.20) was a gift from Amano Enzyme (Nagoya, Japan). Commercial isomalto-oligosaccahride (IMO) syrup was kindly provided by Corn Products International (Seoul, Korea). Skimmed-milk and whole-milk powder were kindly supplied by S&P Syndicate (Bangkok, Thailand) and Pepsi-Cola (Thai) Trading (Bangkok, Thailand), respectively.

Lyophilized bacterial cultures *Lactobacillus acidophilus* TISTR450, *Lactobacillus casei* TISTR1463, *Lactobacillus delbrueckii* ssp. *bulgaricus* TISTR451 and *Streptococcus thermophilus* TISTR458 were purchased from Thailand Institute of Science and Technological Research (TISTR; Bangkok, Thailand).

Preparation of isomalto-oligosaccharides

Isomalto-oligosaccharides (IMO) were prepared from banana flour. Banana flour slurry (250 g·kg^{-1}) was hydrolysed by applying Termamyl SC (0.15 ml, 95 °C, pH 5.5–6.0, 2 h) and Fungamyl 800L (0.3 ml, 50 °C, pH 5.5–6.0, 24 h), and subsequently used for IMO synthesis by applying *trans*-glucosidase l (0.3 ml, 50 °C, pH 5.5–6.0, 12 h). The synthesized mixture was subjected to baker's yeast fermentation (10 g·l^{-1}, 24 h). Ethanol produced during fermentation was removed by rotary evaporation [22]. Of total oligosaccharides with various degrees of polymerization (DP), the final IMO mixture was composed of 53% isomaltotriose/panose (IMO DP3), 21% isomaltotetraose (IMO DP4) and 26% maltooligoheptaose and larger oligomers (MO DP ≥ 5). The syrup was diluted to get a solution of 30 g·l^{-1} of total oligosaccharides and filtered through 0.2 mm membrane filters before applying into milk bases.

Starter culture preparation

Lyophilized bacterial cells *S. thermophilus* (ST), *Lb. acidophilus* (LA), *Lb. delbrueckii* ssp. *bulgaricus* (LB), and *Lb. casei* (LC) were revitalized as follows. ST was rehydrated by M17 broth and all lactobacilli were rehydrated by MRS broth. The bacterial suspensions were then transferred onto their respective agar media. The plates were incubated anaerobically at 37 °C for 72 h in anaerobic jars with gas-generating kits (Anaerobic system BR 38; Oxoid). Cultures were transferred three times successively for activation. A single colony of each strain was selected and inoculated into 10 ml aliquot of 100 g·l^{-1} sterile reconstituted skim milk (RSM) supplemented with 20 g·l^{-1} glucose and 10 g·l^{-1} yeast extract [23]. The cultures were anaerobically incubated at 37 °C for 24 h. The cultures were subsequently transferred into fresh RSM me-

dium at an inoculation level of 50 ml·l^{-1} and were anaerobically incubated at 37 °C for 24 h before being used as starters.

Fermented milk production

Two fermented milks were made using different combinations of starter cultures. The first combination was conventional yoghurt starters (ST and LB), and the second combination was two probiotic strains (LA and LC). Milk base (150 ml) was prepared by dissolving whole milk powder (110 g·l^{-1}) in the 30 g·l^{-1} IMO solution in 250 ml bottles. The milk base was then pasteurized by immersion in a water bath at 90 °C for 30 min [23]. After cooling down to approximately 35 °C, 40 ml·l^{-1} of starter cultures, which were composed of ST and LB, or LA and LC, in a ratio of 1:1, were inoculated into the milk bases. The mixes were gently stirred for 30 s before incubation at 42–44 °C for ST-LB fermented milk, and 37–40 °C for LA-LC fermented milk. When the pH of the milk reached 4.5, the fermentation was stopped by refrigeration at (4 ± 1) °C. The fermented milks were maintained at such temperature thereafter. Samples were taken at days 1, 7, 14, 21, and 28 to determine the viability of the cultures. The experiments were carried out in triplicate.

Enumeration of bacteria

Fermented milk samples (1 g) were taken at days 1, 7, 14, 21, and 28. Each sample was 10-fold serially diluted in 1.5 g·l^{-1} sterile peptone water. Cultures were enumerated by the pour plate technique using a presumptive medium and incubation conditions for each culture. ST colonies were enumerated on M17 agar and anaerobic incubation at 37 °C for 48 h [23]. LB and LC were enumerated on MRS agar and MRS-NaCl agar, respectively, with anaerobic incubation at 37 °C for 72 h. LA cells were grown on pH-modified agar with anaerobic incubation at 43 °C for 72 h [24]. Plates containing 25–250 colonies were counted and bacterial numbers calculated as colony forming units (CFU) per gram of product.

Oligosaccharide analysis by high performance liquid chromatography

Proteins were removed from the samples by means of a membrane filter (10 k molecular weight cut-off), and the saccharide composition of the samples was determined by high performance liquid chromatography (HPLC) as described elsewhere [25] with modifications as follows.

An HPLC unit equipped with an isocratic pump, a refractive index (RI) detector and an autosampler (1100 Series; Agilent, Santa Clara, California, USA) was used. Oligosaccharide profile was determined using Sugar-Pak 1 resin-based column (6.5 × 300 mm; Waters, Milford, Massachusetts, USA); injection volume was 10 μl. The column was heated to 70 °C and degassed HPLC water was used as a mobile phase at a flow rate of 0.5 ml·min^{-1}. Glucose, maltose, a commercial IMO, and a commercial MO were used as standards. Their ratios of peak areas to concentrations were used to convert peak areas of oligosaccharides found in the samples into concentrations.

Statistical analysis

Differences of oligosaccharide depletions in two fermented milk samples during storage were treated by *t*-test. Analysis of variance (ANOVA) with Duncan's new multiple range test ($\alpha = 0.05$) was used to compare the bacteria counts during storage time using SPSS for Windows version 11.01 software (IBM, Somers, New York, USA).

RESULTS AND DISCUSSION

Fermentation of IMO by probiotic strains

Two IMO-added set fermented milk samples were made using different combination of lactic acid producers (ST and LB; LA and LC). The ST-LB fermented milk was incubated at 42–44 °C, which was the optimum temperature of traditional yoghurt making [26]. The LA-LC fermented milk was incubated at 37–40 °C since *Lb. casei* has the optimum growth temperature of 37–40 °C and the upper limit of growth at 43–48 °C [24, 27]. Fig. 1 shows viable cell counts of all strains monitored for 28 days. It was found that in ST-LB fermented milk, the numbers of ST were comparable to those of LB (8.13 and 8.17 $\log_{10}$ CFU·g^{-1}, respectively). The viability of ST was maintained for 14 days before slightly decreasing afterwards (0.11 log cycle). The numbers of LB, on the other hand, were slightly increased during the first 21 days (0.09 log cycle) and then slightly decreased at the end of the storage time. In LA-LC fermented milk, the logarithms of viable cells counts of both strains were in the range of 7.9–8.0 throughout the storage time. The population changes of all cultures were not different over the observation period ($p > 0.05$).

The results showed that the synthesized IMO mixture could not increase the viable cell numbers of all starter cultures, but rather maintained them. Other studies on probiotic viability of other strains of lactobacilli (such as *Lb. reuteri*, *Lb. rhamnosus*, *Lb. acidophilus*) and bifidobacteria (*B. infantis*, *B. longum*, *B. pseudolongum*, *B. animalis*, *B. bifidum*) in fermented milk products supplemented

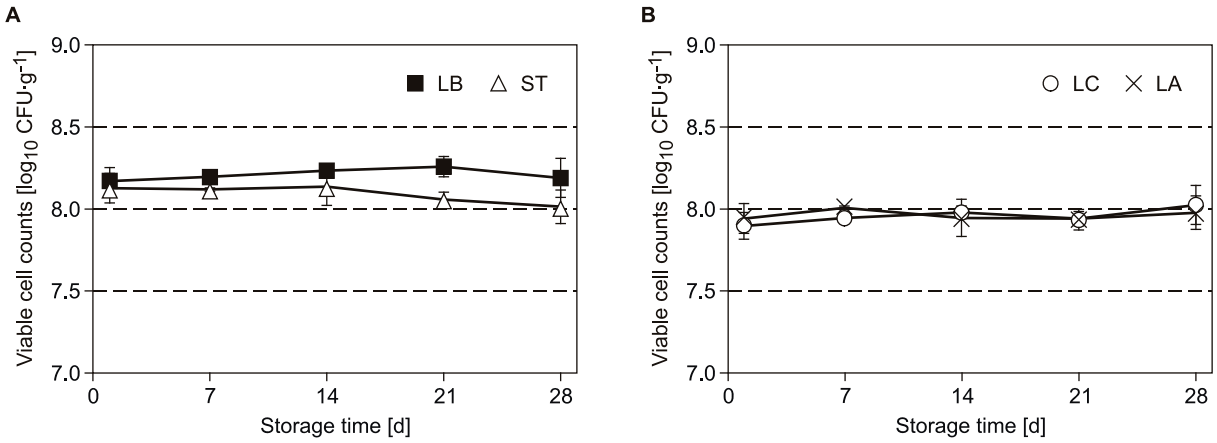

Fig. 1. The viability of starter cultures in fermented milk samples containing 30 g·l^{-1} IMO mixture during storage at 4 °C.

A – *Streptococcus thermophilus* (ST) and *Lactobacillus bulgaricus* (LB) fermented milk; B – *Lactobacillus acidophilus* (LA) and *Lactobacillus casei* (LC) fermented milk.
Values are means of triplicate analyses with standard deviation.

with various prebiotics (inulin, fructooligosaccharides, galactooligosaccharides, oligosaccharides of raffinose family) have been reported with a consensus to exhibit a higher retention of bacterial numbers in the respective medium compared to control [16–19]. The mechanism of maintaining viability of probiotics in dairy products in the presence of prebiotics is not thoroughly elucidated. It is probably due to higher viscosity of the medium after addition of prebiotics, and such conditions may have resulted in better protection of probiotic cells [18], or probably because the bacteria were supplied with plenty of carbon sources that they could metabolize over the storage time.

The changes of the concentrations of oligosaccharides available in the fermented milk samples are illustrated in Tab. 1 and Fig. 2. It was found that, in both samples, all oligosaccharide classes decreased over storage time, particularly lactose and isomaltotriose/panose, which decreased sig-

Tab. 1. Concentrations of oligosaccharides available in IMO-added fermented milk samples during storage at 40 °C.

Storage time [d]	Concentration [g·l^{-1}]				
	Lactose	Isomaltotriose/Panose (IMO DP3)	Isomaltotetraose (IMO DP4)	Maltooligosaccharides (MO DP ≥ 5)	Total IMO
ST-LB Fermented Milk					
1	34.5 ± 1.0^{a}	15.1 ± 0.9^{a}	6.4 ± 0.2^{a}	7.4 ± 0.2^{a}	21.4 ± 1.0^{a}
7	21.7 ± 1.0^{b}	13.5 ± 0.3^{b}	6.0 ± 0.2^{a}	6.5 ± 0.3^{b}	19.5 ± 0.3^{b}
14	18.9 ± 0.8^{c}	10.4 ± 0.3^{c}	6.0 ± 0.1^{a}	6.2 ± 0.1^{bc}	16.4 ± 0.3^{c}
21	17.8 ± 1.0^{cd}	9.1 ± 0.4^{d}	5.6 ± 0.1^{b}	5.9 ± 0.2^{cd}	14.7 ± 0.5^{d}
28	16.1 ± 0.9^{d}	8.8 ± 0.2^{d}	5.1 ± 0.3^{c}	5.6 ± 0.2^{d}	14.0 ± 0.4^{d}
LA-LC Fermented Milk					
1	34.6 ± 1.2^{a}	16.6 ± 0.4^{a}	6.5 ± 0.2^{a}	7.0 ± 0.2^{a}	23.1 ± 0.2^{a}
7	23.4 ± 0.9^{b}	14.4 ± 0.5^{b}	6.1 ± 0.1^{a}	5.8 ± 0.2^{b}	20.6 ± 0.6^{b}
14	22.8 ± 0.7^{b}	10.3 ± 0.4^{c}	6.2 ± 0.1^{a}	6.2 ± 0.3^{b}	16.6 ± 0.3^{c}
21	20.6 ± 0.6^{c}	9.7 ± 0.2^{c}	5.3 ± 0.3^{b}	5.9 ± 0.2^{b}	15.0 ± 0.5^{d}
28	17.4 ± 0.9^{d}	8.9 ± 0.4^{d}	5.3 ± 0.2^{b}	5.9 ± 0.1^{b}	14.3 ± 0.4^{d}

Values are means ± standard deviations of triplicate analyses. Within the same fermented milk sample, columns with different superscripts mean significant difference ($p < 0.05$).

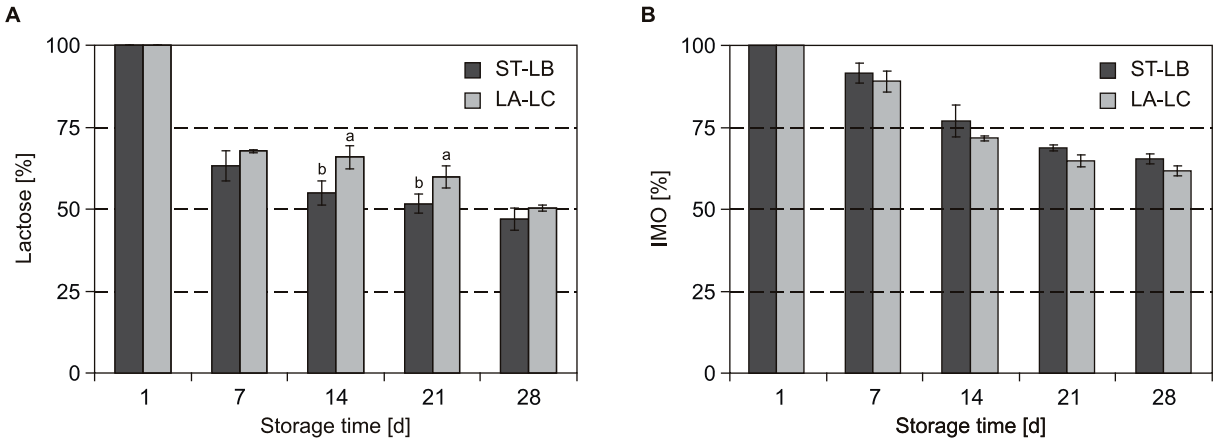

Fig. 2. Comparison of percentage changes of (A) lactose and (B) IMO in ST-LB and LA-LC fermented milk samples containing 30 g·l^{-1} IMO mixture during storage at 4 °C.

Values are means of triplicate analyses with standard deviation. Bars with the different superscript letters are significantly different ($p < 0.05$).

nificantly during 14–21 days. Isomaltotetraose and high DP maltooligosaccharides were also changed significantly but not to such high extent as those of lactose and isomaltrose/panose. The result presented in Fig. 2B shows that IMO concentration in LA-LC fermented milk was lower than that in ST-LB fermented milk. However, the differences were not statistically significant ($p > 0.05$). Lactose is a fermentable disaccharide for streptococci and lactobacilli. These bacteria initiate lactose fermentation by either hydrolysis catalysed by β-D-galactosidase, or hydrolysis of phosphorylated lactose by β-D-phosphogalactosidase [28]. Several species of lactobacilli including *Lb. bulgaricus* possess both enzymes [29].

Homofermentative conversion of lactose to lactic acid is the most important fermentative reaction in dairy processing including yoghurt production. The fate of lactose in fermentation of conventional yoghurt starters has been well documented [28]. Lactic acid formation resulting from the fermentation plays a very important role in yoghurt characteristics, it reduces the pH of the yoghurt mix, preserves the flavour and contributes to it, and modifies the texture of yoghurt by causing coagulation of casein micelles [1, 30]. It has been reported that lactic acid bacteria, especially lactobacilli and streptococci, express the ability to metabolize various carbon sources available in their environments [31–34]. The reduction of IMO in the fermented milk samples suggested that the cultures used in the experiment could metabolize them. This is in agreement with previous reports using a static batch culture fermentation and a three-stage continuous gut culture model using IMO as a sole carbon source to maintain lactic acid flora, and also to facilitate lactate, acetate, propionate and butyrate generation [35, 36]. As for carbon utilization preference in both samples (Fig. 3), lactose was the most preferred carbon source. Comparing IMO fermentation preference, it seems that isomaltotriose/panose was preferred to isomaltotetraose. This is probably due to the less complicated structure, so it was easier metabolized by the bacteria. The percentage changes of isomaltotetraose and maltooligoheptapse and larger oligomers were comparable in LA-LC fermented milk (Fig. 3B), but this was not the case in ST-LB fermented milk (Fig. 3A).

With regards to ST-LB fermented milk, SANDERS and co-workers [37] reported that all *S. thermophillus* and most *Lb. bulgaricus* strains have a high β-D-galactosidase activity, which could explain the higher depletion levels of lactose compared to those in LA-LC fermented milk (Fig. 2A). The interaction of *Lb. bulgaricus* and *S. thermophilus* has been described as mutual interaction [38], since in a mixed culture *S. thermophilus* produces formic acid that stimulates the growth of *Lb. bulgaricus*, while *Lb. bulgaricus* produces certain amino acids (glycine, histidine, valine, leucine, isoleucine) that stimulate the growth of *S. thermophilus*. The decrease of viable cell counts of *S. thermophilus* after 14 days, although not significantly different from those of *Lb. bulgaricus*, was in agreement with an earlier study which reported that the growth rate of *Lb. bulgaricus* was higher than that of *S. thermophilus* when the concentra-

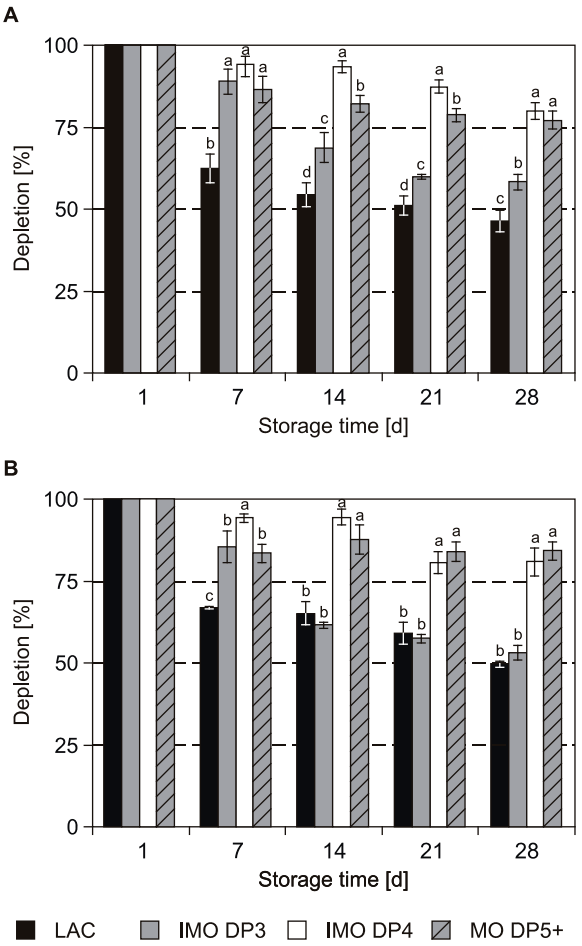

Fig. 3. Comparison of percentage changes of lactose (LAC), isomaltotriose/panose (IMO DP3), isomaltotetraose (IMO DP4) and maltooligoheptaose and larger oligomers (MO DP5+) in (A) ST-LB and (B) LA-LC fermented milk samples containing 30 g·l^{-1} IMO mixture during storage at 4 °C.

Values are means of triplicate analyses with standard deviation. Bars with the different superscript letters mean significantly different ($p < 0.05$).

tion of milk-solid-non-fat was between 100 g·l^{-1} and 150 g·l^{-1} [30]. Earlier studies on monitoring the population changes of *S. thermophilus* and *Lb. bulgaricus* in conventional set yoghurt after 28 days of storage showed inconsistency in 0.5–2.0 log-cycle range [3, 39]. However, inconsistencies between studies are often seen probably due to different bacterial strains, culture proportions in culture mix, chemical composition of the yoghurt mixes, final acidity of the products and oxygen contents [40]. Nevertheless, it can be seen from the results of this study that yoghurt supplemented with 30 g·l^{-1} IMO mixture could maintain the viable cell numbers of *S. thermophilus* and *Lb. bulgaricus* for at least 28 days, which is considered to be the shelf-life of the product.

With regard to LA-LC fermented milk, the fermentation of *Lb. acidophilus* results in the formation of acetaldehyde and lactic acid, which contribute to the characteristic flavour of 'bio' yoghurt [40]. Both *Lb. acidophilus* and *Lb. casei* are gram-positive, non-spore-forming rods, and require complex nutrients including saccharides, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives and vitamins for their growth [41]. As end products of lactose fermentation, apart from lactic acid as the main product, other volatile short-chain fatty acids (SCFA) such as formic, acetic and butyric acid could also be formed [27]. These SCFA are thought to be a desirable metabolite of colonic function since they lower the pH, which results in preventing the establishment of exogenous pathogenic or putrefactive microflora (colonization resistance) [42].

In this study, the results showed that the carbon sources available in the system could maintain the viable cell numbers of both *Lb. acidophilus* and *Lb. casei* (Fig. 1B). *Lb. casei* is tolerant to low temperature (10–15 °C) [27, 43] and therefore seems to be advantageous when incorporated in dairy products, which generally require storage at low temperature. Results on bacterial population changes show that the interaction between these two strains probably was of mutual type since their numbers were comparable throughout the storage time. However, further studies are needed to prove this statement, since the concentration of carbon sources in this experiment was high (almost 65 g·l^{-1} of total oligosaccharides, approximately 50% left after 28 days of storage), and therefore their behaviour in conditions of carbon source competition could not be observed. The results showed that, as seen in ST-LB combination, the population numbers of *Lb. acidophilus* and *Lb. casei* were satisfactorily preserved for at least 28 days in fermented milk supplemented with 30 g·l^{-1} IMO mixture. Other properties of the IMO-added yoghurt-type product, such as viscostity, syneresis, curd tension and organoleptic properties are yet to be studied, since the addition of various probiotics affects these characteristics in various ways [44].

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