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Incorporation of microencapsulated *Lactobacillus rhamnosus* into infant-foods inhibit proliferation of toxicogenic *Bacillus cereus* strains

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ABSTRACT

Considering the hygienic quality of infant-food is of great important, especially it is the primary source of kids nutrition. *Bacillus cereus* has been recognized as a serious food-borne pathogen, causing outbreaks due to the produced enterotoxins including cytotoxin K (CytK), non-hemolytic enterotoxin (Nhe) and hemolysin BL (Hbl). In this study, two bacterial isolates, carrying the virulence encoding genes *cytK, nheC,* and *hblA,* were obtained from infant-foods. Using 16S rDNA sequence analysis, they were identified as *Bacillus cereus* MH031700 and MH031701. Then, they were tested for the antagonistic effect of some lactic acid bacterial strains. Results showed that cell free supernatant from *Lactobacillus rhamnosus* B-445 exhibited the highest antagonistic activity. In addition, this strain have reduced the growth of the toxicogenic *Bacillus* spp in mixed cultures. In further experiments, proliferation of *B. cereus* strains was evaluated in presence/absence of microencapsulated or spray-dried *L. rhamnosus* B-445 in formulated infant-food mixtures. Microencapsulated cells exhibited higher effeciency for growth inhibition than spray dried cells. Incorporating microencapsulated or spray dried *L. rhamnosus* B-445 not only inhibited the proliferation of *B. cereus* strains that existed in infant-foods but also it reduced their count from ~ Log 3.0 to ~ Log 2.0 after 2 and 3 h at room temperature (25 °C), respectively. Similar results were achieved at low temperature (7.0 ± 2.0 °C) but after 4 and 8 h, respectively. Our results showed the potential efficiency of *L. rhamnosus* B-445 in suppressing toxicogenic *B. cereus* strains by its supplementation into infant-foods.

1. Introduction

Infant-foods contain high values of proteins, minerals, fats and vitamins that are considered as the primary source of kid's nutrition before they able to digest other types of food. At the same time, babies have weak immunity and any infection in their foods causes illness. Therefore, the hygienic quality and controlling of baby foods is of great importance. Foodborne diseases cause health problem include a wide spectrum of illnesses by bacteria, virus, parasite, or chemical contamination of food (Thurm and Gericke, 1994).

Some spore forming microorganisms such as *Bacillus* spp. can survive after high temperature treatments used in the infant-food manufacture (Sadek et al., 2018). Di Pinto et al. (2013) isolated *B. cereus, B. licheniformis, B. subtilis*, and *B. mycoides* from infant milk formula in Italy. Whereas, El-Gamal et al. (2013), found that, 25% of infant milk samples were positive for spore-forming microbes as *B. cereus*, *B. licheniformis* and *B. subtilis* were isolated from dairy products samples including infant formula and full fat milk powder. *Bacillus cereus* was reported as the most common bacterium in several pasteurized food products (Samapundo et al., 2011), milk powder, liquid milk, mixed food products, and infant-food formula (Arshak et al., 2007; Carlin et al., 2010). This bacterium can cause two different types of food poisoning, the diarrhoeal type caused by complex enterotoxins and the emetic type produced by growing cells in the food (Stenfors Arnesen et al., 2008). In addition, it can cause many infections including fulminant bacteremia, brain abscesses, meningitis, endophthalmitis, pneumonia and gas gangrene-like cutaneous infections (Bottone, 2010).

Lactic acid bacteria (LAB) are a group of Gram-positive, cocci or rods, non-spore forming microorganisms that produce lactic acid as the major end product from sugars fermentation. They are generally recognized as safe (GRAS) facilitating their use *in situ* and *ex situ* in food preservation (Abdel-Rahman et al., 2013). They include an important

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group of probiotic strains such as *Lactobacillus* and *Bifidobacterium* species. LAB are thriving in fermented or fortified dairy products (Feng et al., 2015). They can also produce low-molecular-mass compounds such as diacetyl (2,3-butanedione) and antimicrobial peptides (bacteriocins). These characteristics make them important agents to antagonize and control the growth of unwanted spoilage/pathogenic microbes in foods (Olaoye and Onilude, 2011; Cortés-Zavaleta et al., 2104; Arena et al., 2016; Elshahawy et al., 2018). Moreover, LAB have additional benefits in different clinical ailments such as allergic pathologies diarrhea, necrotizing enterocolitis, inflammatory bowel disease, type 2 diabetes, and viral infections (Presti et al., 2015).

Recently, there is an increased consumers' demand for non-dairy and drink based LAB products, as well as its use as food supplements in different forms [tablets, capsules, and freeze–dried preparations] (Shah, 2001) to be used as bio-preservative against various food borne pathogens (Goyal and Kannan, 2018). The bacteriocins produced by LAB are extremely important for preventing the growth of spoilage and pathogenic microbes (Castro et al., 2017; Hu et al., 2017).

This study aimed to identify potent toxin producing bacteria in infant-food and to investigate the antagonistic activity of some LAB strains against these isolates. This study also aimed to evaluate the effect of the most promising LAB strain as possible supplement (micro-encapsulated or spray dried form) on pathogenic microbes in formulated infant-food preparations.

2. Material and methods

2.1. Lactic acid bacteria strains

Leuconostoc mesenteroides B-118 was provided by Chr. Hansen's Lab, Denmark. *L. cremoris* was obtained from the Dairy Department, National Research Centre, Egypt. *L. rhamnosus* B-445 (ATCC 10863) was provided by the Northern Regional Research Laboratory, Illinois, USA. *L. dulbrueckii* subsp. *bulgaricus* Lb-12 DRI-VAC provided by the Northern Regional Research Laboratory, Illinois, USA.

Leuconostoc mesenteroides B-118, *L. dulbrueckii* subsp. *bulgaricus* Lb-12 DRI-VAC and *L. rhamnosus* B-445 were previously proved to be probiotics (Mabrouk, 2009; Ibrahim, 2012) by their characteristics (tolerance to acid, antibiotic susceptibility, survivability in different concentrations of bile salt, and their antimicrobial activities against specific pathogens as indicated by FAO/WHO (2002)).

2.2. Pathogenic strains

Two toxigenic bacteria were isolated from infant-foods and preliminary identified as *Bacillus cereus* using API 50 CHB as described by Sadek et al. (2018).

2.3. Growth and preservation of LAB strains

Before use in main experiments, each LAB strain used in this study was sub-cultured twice in MRS medium (Oxoid) and then maintained in 11% (*w/v*) sterile reconstituted skim milk powder at 37 °C for 24 h, except for *L. mesenteroides* that was incubated at 30 °C for 18 h.

2.4. Molecular identification of the most potent toxicogenic isolates

Genomic DNA of the two most potent toxicogenic bacterial isolates (coded: MOS8 and MOS9) were extracted using enzymatic lyses [lysozyme (20 mg/ml) and Proteinase K (1 mg/ml)] and then purified using isopropanol buffer as described by Eida et al. (2018). Amplification of the 16S rRNA gene was conducted by Polymerase Chain Reaction (PCR) using DNA as template, forward primer (5′ d AGAGTTTGATCCTGGCT CAG 3′) and the reverse primer (5′ d TACGGTTACCTTGTTACGACTT 3′). The reaction mixture (50 µl) contained 1 × PCR buffer (NEB, England), 1 *p*mol of 2 mM $MgSO_4$, 1 nmol of dNTPs, 1 unit Taq DNA polymerase (NEB, England), 0.25 *p*mol of forward and reverse primers, and 5 µl template DNA. PCR products were purified by QIAquick Gel Extraction Kit (QIAGEN, USA) and 16S rRNA fragments were confirmed on agarose gel before sequencing. The obtained sequences were submitted to GenBank. The obtained sequences were compared with 16S rRNA sequence data available in public databases in GenBank using the BLAST program. The analysis was conducted with MEGA 6 using neighbor-joining method with bootstrap value (1000 replicates).

2.5. Activities of LAB' cell free supernatants against B. cereus strains

2.5.1. Preparation of LAB' cell free supernatant

Cell-free supernatant broth was obtained by centrifuging the overnight culture (grown at 37 °C for 24 h in MRS broth media) of the tested LAB strain at 4000 *rpm* for 15 min at 4 °C. The supernatant was then passed through 0.22 µm Millex-GV membranes (Millipore) and the pH was adjusted to 6.0 to exclude organic acids effect.

2.5.2. Antibacterial activity

The activity of the resulting metabolites were tested against *B. cereus* MH031700 and *B. cereus* MH031701 using agar well diffusion assay as described by Darwesh et al. (2018). Metabolite samples were tested in triplicate, and the plates were incubated for 24 h at 37 °C. The ability to inhibit the growth of *B. cereus* was determined by measuring the diameter (in millimeter) of the clear inhibition zones formed around the wells.

2.5.3. Antagonistic activities of various LAB cells against Bacillus spp

B. cereus MH031700 and MH031701 strains were transferred into tryptone soya broth (Oxoid), and each tested LAB strain was cultured in MRS broth medium and incubated for 24 h at 37 °C. The cultures were separately diluted in saline solutions to an appropriate inoculums size (10^5 CFU/ml for *B. cereus* and 10^7 CFU/ml for LAB) and then inoculated together (mixed culture) into 100 ml tryptone soya broth. Each of *L. rhamnosus* B-445 and *L. dulbrueckii* subsp. *bulgaricus* Lb-12 DRI-VAC strain was inoculated with *B. cereus* MH031700 and MH031701 with two controls for pathogens. The count of *B. cereus* strain MH031700 and strain MH031701 in the mixed cultures was estimated after incubation at different time intervals (0, 6, 24, 48 h).

2.6. Biocontrol of B. cereus in formulated infant-foods

Manufacturing of infant-foods: Ingredients used for the formulation of infant-foods were summarized in Table (1). Infant-foods were formulated in lab. as described by Soliman et al. (1996). The composition and percentage of ingredients used are given in Table (2). Banana, apple pineapple, carrot, pea and potato were washed with tape water to remove dirt's, adhering latex and other foreign matters, as well as to reduce the initial contamination with microbes. Banana was hand peeled and the seeds of pineapple and apple were carefully removed.

Banana, pineapple, pea and potato were cut into small parts, while, carrot was peeled using stainless steel peeler. The prepared vegetables and fruits were washed and blanched for appropriate time (5 min), then dried at 60 °C for 12 h in an electric oven drier. The dried materials were milled in an electrical miller, then sieved through a silk sieve (60

Table 1
Ingredients of materials used in the manufacture of infant-foods used in this study.

Ingredients	Description
Banana, apple, pineapple, carrot, pea and potato	Purchased from farmers at Cairo city area, Egypt
Rice powder	Purchased from markets in Cairo, Egypt
Skim milk powder (USA)	

Table 2
Ingredients percentage (%) used in the preparation of blends for fruits and milk-based and vegetables and milk-based infant-food.

Ingredients	Percentage (%)	Fruits and milk-based infant-food	Vegetables and milk-based infant-food
Banana	20		–
Apple	20		–
Pineapple	20		–
Carrot	20	–	
Pea	20	–	
Potato	20	–	
Rice powder	28		
Skim milk powder	10		
Probiotic	2		

–, means not included in the prepared food.

mesh) and grounded to a particle size of 500–600 µm. All prepared materials were bottled in glass jars individually and stored at refrigerator until using in preparation of infant-food formula.

All batches were performed at National Research Centre (NRC), Cairo, Egypt. The first batch (infant-food with fruit and milk-based) was divided into three equal portions and inoculated with *L. rhamnosus* B-445 and mixed strains of *Bacillus cereus*. Every portion was divided to three parts according to infant-foods mixer (water, milk and orange juice) as following:

Treatment 1: Inoculation with 2% microencapsulated *L. rhamnosus* and *B. cereus*.
Treatment 2: Inoculation with 2% spray dried *L. rhamnosus* and *B. cereus*.
Treatment 3: Inoculation with mixed strain of *B. cereus* only as control.

All of these treatments were inoculated separately with 10^3 CFU/ml of *B. cereus* mixed strain. pH values of water, milk and orange juice that used for prepartion of infant-foods [before and after adding to infant-foods] were represented in Table (3).

The second batch was divided into the same equal portions as mentioned previously for the first batch, but used infant-foods with vegetables and milk-based. Also, every portion from-infant foods with vegetables and milk-based was divided to three parts and mixed with water, milk or orange juice.

All the infant-foods treatments were filled into sterilized glass jars and covered with aluminum foil. Half of these containers from each treatment were stored at 25.0 °C and the other half stored at 7.0 ± 2.0 °C. All samples including the contaminated controls and the treated contaminated infant-foods (fruits and milk-based, vegetables and milk-based) were subjected for bacteriological analysis to investigate the behavior of the tested pathogens and LAB at zero time, 0.5, 1, 2, 4, 6, 8, 12, 24 h of storage at refrigerator and after 0, 0.5, 1, 2, 3 h of storage at room temperature. Each bacterium was recovered on its selective agar media.

2.6.1. Bacteriological analysis

All infant-foods samples (ca. 25 g) were homogenized for 1 min in 225 ml of sterile peptone water, from which ten-fold serial dilutions were prepared. *B. cereus* counts were carried out by spreading 0.1 ml of the appropriate dilution onto PEMBA medium (Oxoid). The incubation temperature was 37 °C for 18–24 h (Diop et al., 2007). While *L. rhamnosus* B-445 and *L. dulbrueckii* subsp. *bulgaricus* Lb-12 DRI-VAC counts were carried out by De Man, Rogosa and Sharpe agar MRS (Oxoid), the plates were incubated anaerobically at 30 °C for 3 days (De Man et al., 1960).

Table 3
pH values of water, milk and orange juice that used for preparation of infant-foods for children consumption.

Dissolver	Control	Infant-food with fruit and milk-based	Infant-food with vegetables and milk-based
Water	7.43	5.48	6.55
Milk	6.8	5.31	6.35
Orange juice	3.33	4.12	4.36

2.6.2. Microencapsulation of L. rhamnosus B-445 strain

Elliker broth medium [Oxide] (500 ml) was inoculated with 5% (*v/v*) fresh active culture of *L. rhamnosus* B-445 and incubated at 37 °C for 18 h. Cells were aseptically harvested by centrifugation (8400 × *g*, 20 min, 4 °C). The pellets were re-suspended in buffer to a final concentration of 4 g/L dry cell weight (DCW) and then mixed with an equal volume of 4% (*w/v*) sodium alginate, yielding a final cell concentration of 2 g/L DCW (Ivanovska et al., 2014). This mixture was added drop wisely into a sterile solution of NaCl (0.2 M) and $CaCl_2$ (0.05 M). The beads were stirred for at least 45 min to ensure complete gelling. One gram of encapsulated bead was liquefied in 0.1 M sodium citrate (pH 6), decimally diluted in 0.1% (*w/v*) peptone water and incubated at 30 °C for 3 days, then viable cell number were determined.

2.6.3. Spray drying of L. rhamnosus B-445 strain

The culture of *L. rhamnosus* B-445 was propagated in 11% (*w/v*) sterilized skim -milk using 2% inoculum from the bulk starter and incubated for 24 h at 30 °C. Sample was spray-dried in apparatus (BUCHI Mini Spray Dryer B-290, Switzerland). Spray-drier conditions were: outlet air temperature 60 °C, inlet air temperature 120 °C, aspirator 90%, speed 25–30 rpm, and noozle cleaner 2. Powder was collected in a single cyclone separator.

The culture was blend in a mixer for 2 min and filtered by sterile cheese cloth. pH of the culture was adjusted to 6.8 using 10% ammonium solution, and then 2% dextrin was added. After drying, the solids were collected and stored in small sealed polyethylene bags and examined for viable cell count (Silva et al., 2002). One gram of spray dried bead or 1 ml of free cell were liquefied in 0.1 M sodium citrate (pH 6), decimally diluted in 0.1% (*w/v*) peptone water and incubated at 30 °C for 3 days, then viable cell number were determined by viable count plate method.

3. Results and discussion

3.1. Molecular identification of the most potent toxicogenic isolates

Two bacterial isolates (coded, MOS8 and MOS9) obtained from infant-food were selected as the most potent toxigenic strains as reported by Sadek et al. (2018) owing to the presence of 3 enterotoxigenic virulence genes (*cytK, nheC, and hblA*). These isolates were previously characterized using API 50 CHB as *Bacillus cereus* strains. 16S rRNA gene sequences of these isolates were investigated. The genomic DNA of tested strains were isolated and purified using isopropyl method. The 16S rRNA gene was amplified by PCR. The size of PCR product was approximately 1000 bp. The obtained sequences were compared with those available in GenBank using BLAST program NCBI web page as shown in Fig. 1. The phylogenetic tree showed that these strains are very close to the type strains of *Bacillus cereus* deposited in culture collection centre of National Centre for Biotechnology Information. Similarity percentages of these isolates were accounted for 96 and 97% with strain *Bacillus cereus* for isolates MOS8 and MOS9, respectively. The sequences of these strains were submitted to GenBank and given accession number of MH031700 and MH031701 for isolates MOS8 and MOS9, respectively.

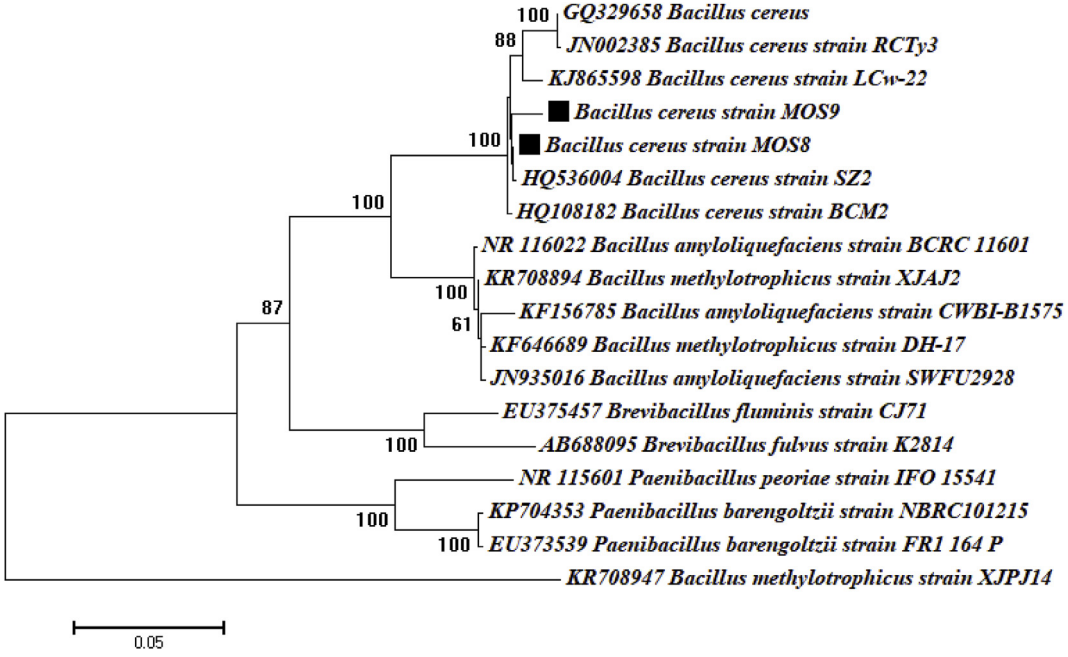

Fig. 1. Phylogenetic analysis of 16S rRNA sequences of the bacterial isolates with the sequences from NCBI. MOS9 and MOS8 refer to 16S rRNA sequences retrieved from bacterial isolates. The analysis was conducted with MEGA 6 using neighbor-joining method with bootstrap value (1000 replicates).

3.2. Activities of some LAB' cell free superanatans against B. cereus strains

LAB is well known to produce a variety of important metabolic by-products having antibacterial activity such as bacteriocins and bacteriocin-like substances of biomedical advantages (Choi and Chang, 2015). The antibacterial activity of different LAB strains against *B. cereus* MH031700 and *B. cereus* MH031701 strains were investigated. Results were expressed by the detected clear zone of inhibition as shown in Table (4). The results revealed that, *L. rhamnosus* B-445 and *L. dulbrueckii* subsp. *bulgaricus* Lb-12 DRI-VAC had variable antibacterial activity whereas their supernatants inhibited the growth of *B. cereus* MH031700 and MH031701. *L. rhamnosus* exhibited the highest inhibition zone of 18.0, 21.0 mm followed by *L. dulbrueckii* subsp. *bulgaricus* Lb-12 DRI-VAC that exhibited inhibition zones of 12.0 and 20.7 mm against *B. cereus* MH031700 and *B. cereus* MH031701, respectively (Fig. 2). In contrast, *Leuconostoc mesenteroides* B-118, *L. cremoris* and *Enterococcus mundtii* did not exhibit inhibition effect against *B. cereus* strains.

Several reports indicated that LAB had antimicrobial activities against *Bacillus* strains and various pathogens. *L. rhamnosus* LC705, LBA, LGG and LR1524 showed an inhibition activity against *B. cereus* with diameters of 17.0, 20.0, 17.0, and 19.0 mm, respectively (Tharmaraj and Shah, 2009; Khalil et al., 2017). *L. delbrueckii* subsp. *bulgaricus* was reported as potential natural bio-preservative in food products due to its effectiveness against *B. cereus* with inhibition zone of

Table 4
Antibacterial activity of some lactic acid bacteria against *B. cereus* strain MH031700 and strain MH031701.

Lactic acid bacteria	Diameter of inhibition zone (mm) against:	
	B. cereus MH031700	*B. cereus* MH031701
Leuconostoc mesenteroides B-118	0.00	0.00
Lactobacillus cremoris	0.00	0.00
Lactobacillus rhamnosus (B-445)	18.0	21.0
Enterococcus mundtii	0.00	0.00
Lactobacillus dulbrueckii subsp. *bulgaricus* Lb-12 DRI-VAC	12.0	20.7

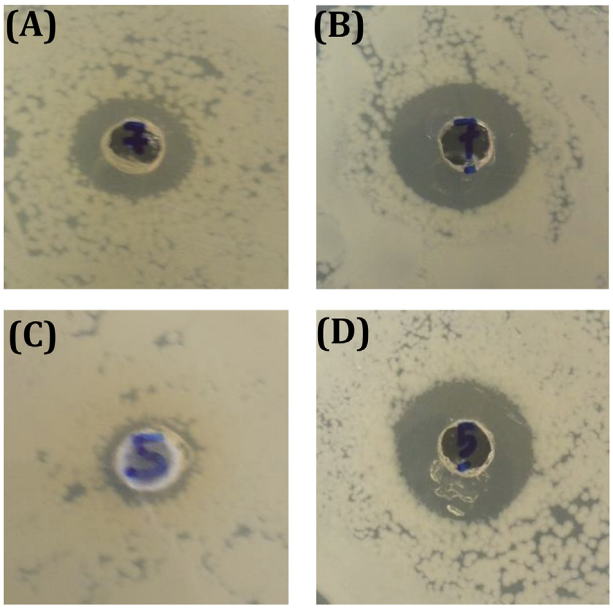

Fig. 2. The inhibition effect of *L. rhamnosus* against (A) *B. cereus* MH031700 and (B) *B. cereus* MH031701; and the inhibition effect of *L. dulbrueckii* subsp. *bulgaricus* Lb-12 DRI-VAC against (C) *B. cereus* MH031700 and (D) *B. cereus* MH031701.

12.0 ± 0.8 mm (Hasanin et al., 2018). Mohammed and Ijah (2013) reported that *L. bulgaricus, L. lactis* and *L. acidophilus* had antibacterial activities against *Staphylococcus, Salmonella, Bacillus, Shigella,* and *Pseudomonas* species due to bacteriocin production. Recently, Adel and Sari (2017) reported that *L. rhamnosus* Y1 showed great inhibition effect against *B. cereus* with diameter 60.0 mm. On the other hand, Okereke et al. (2012) found that the cell-free extract of *Leuconostoc mesenteroides* had no antimicrobial effects against *B. cereus*.

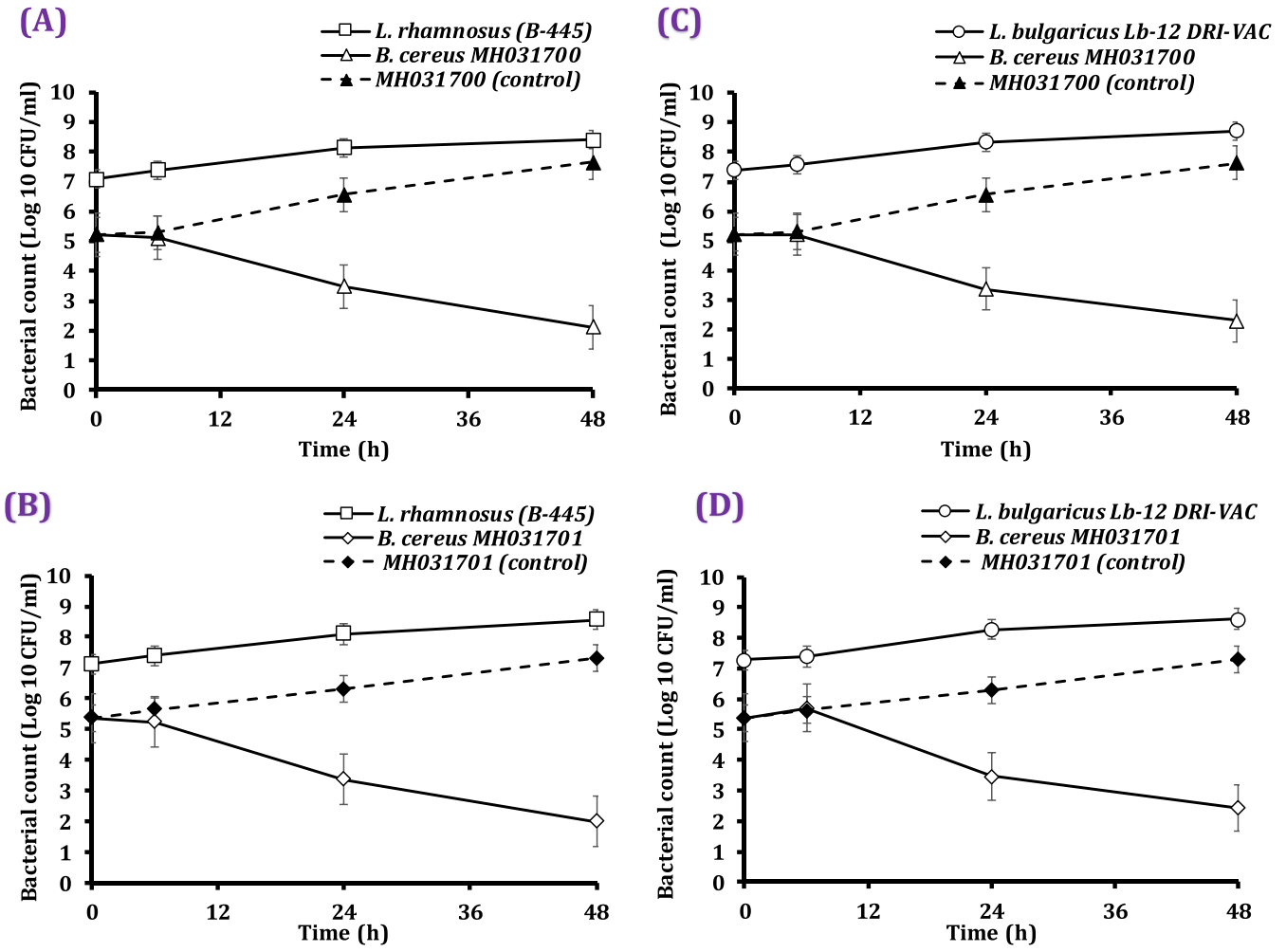

Fig. 3. Antagonism activity of *L. rhamnosus* B-445 (A&B) and *L. dulbrueckii* subsp. *bulgaricus* Lb-12 DRI-VAC (C&D) against *B. cereus* MH031700 and MH031701 in mixed cultures, respectively.

3.3. Antagonistic activity of LAB against B. cereus in mixed cultures

To determine the effect of the most potent LAB cells (*L. rhamnosus* B-445 and *L. dulbrueckii* subsp. *bulgaricus* Lb-12 DRI-VAC), They allowed to grow in mixed culture with *B. cereus* MH031700 and MH031701 (Fig. 3). Results indicated that, both *B. cereus* strains proliferated well in tryptone soya broth with increased cell counts by 2.46 and 1.96 Log cycle, respectively in control without LAB after 48 h as compared to initial cell count. However, in mixed culture with *L. rhamnosus* or *L. dulbrueckii* subsp. *bulgaricus*, a pronounced reduction in viability of *B. cereus* were observed. *B. cereus* MH031700 showed rapid decline in mixed culture with *L. rhamnosus* and *L. dulbrueckii* subsp. *bulgaricus* as its concentration numbers decreased by 3.06 and 2.90 Log cycle after 48 h of incubation, respectively. Also, *L. rhamnosus* exhibited high inhibitory effect against *B. cereus* MH031701 resulting in decreasing the counts from 5.30 to 3.30 Log_{10} CFU/ml after 24 h of inoculation with reduction percent of 37.7% reaching 2.00 Log_{10} CFU/ml with reduction percent of 62.2% after 48 h of inoculation. While, *L. dulbrueckii* subsp. *bulgaricus* inhibited the growth of *B. cereus* MH031701 leading to reduce count by 1.87 and 2.88 Log cycle, respectively after 24 and 48 h of incubation. These results showed that *L. rhamnosus* and *L. dulbrueckii* subsp. *bulgaricus* strains could effectively restrict the growth and survival of *B. cereus* MH031700 and MH031701.

The inhibition of *B. cereus* could be attributed to the antimicrobial metabolites [bacteriocin and similar inhibitory substances] produced by *Lactobacillus* strain as previously reported. Such substances showed potential application in food bio-preservaton (Heredia-Castro et al., 2015; Rossland et al., 2005). *L. rhamnosus*, and *L. bulgaricus* Y34 (Mohammed et al., 2013) showed antibacterial activity against *B. cereus*. Abdel-Shafi et al. (2014) reported that *L. bulgaricus* Z55 produce bacteriocin that inhibited many food-borne pathogens.

3.4. Biocontrol of B. cereus in formulated infant-foods

Recently, there has been considerable concern in identifying the potential beneficial roles of probiotics for improving human health and restricting the growth of unwanted microorganisms. The present experiments were implemented to evaluate the effect of *L. rhamnosus* B-445 (in microencapsulated or spray dried form) against *B. cereus* MH031700 and MH031701 as mixed strains in formulated manufactured infant-foods [vegetable- or fruit and milk-based food]. The control samples of infant-food were prepared with inoculation of *B. cereus* without the probiotic supplementation (*L. rhamnosus* B-445).

Enumeration of encapsulated beads and spray dried culture of *L. rhamnosus* showed that the number of viable cell concentrations were 10.48 and 8.45 Log_{10} CFU/g, respectively. Mixed culture of *L. rhamnosus* B-445 and *B. cereus* were allowed to grow in infant-foods stored at 25 °C for 3 h and at 7 °C for 24 h (Figs. 4 and 5). Generally, *B. cereus* showed a rapid decline in its cultural numbers in infant-foods supplemented with *L. rhamnosus* B-445 strain. Data in Fig. 4 (A-C) showed that

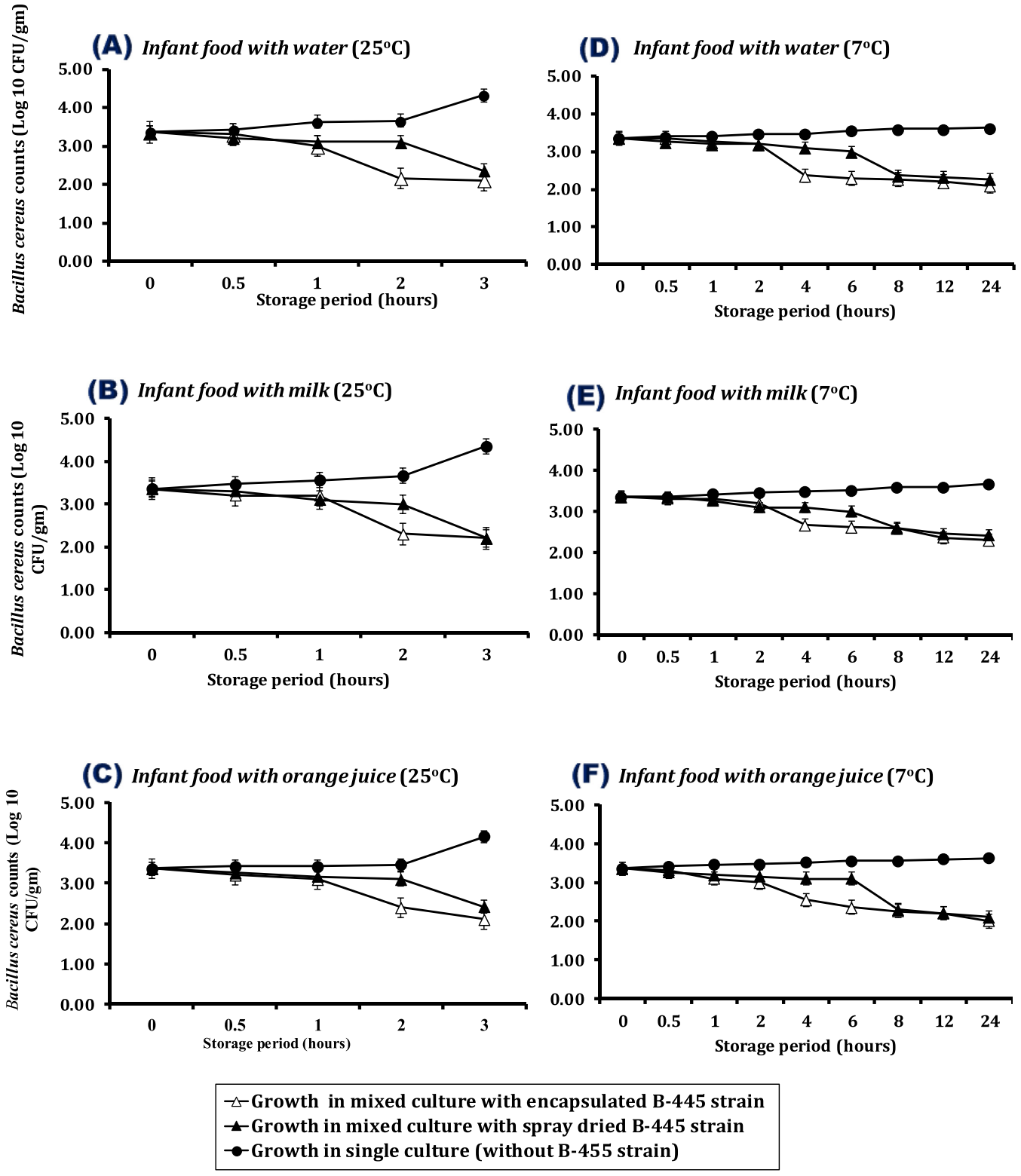

Fig. 4. Behavior of *B. cereus* during storage of infant-food [fruit and milk-based] after mixing with water, milk, and orange juice supplemented with *L. rhamnosus* (B-445) and incubated at 25 °C for 3 h (A, B & C, respectively) and at 7.0 ± 2.0 °C for 24 h (D, E & F, respectively).

B. cereus rapidly proliferated in control infant-food [fruit and milk-based] without *L. rhamnosus* B-445 supplementation and reached to growth concentration at 4.32, 4.36, and 4.16 Log_{10} for food mixed with water, milk and orange juice, respectively at 25 after 3 h. These counts are much higher than counts obtained at 7 °C for 24 h (Fig. 4D–F).

On the other hand, infant-food [fruit and milk-based] supplemented with microencapsulated *L. rhamnosus* B-445 and incubated at 25 °C exhibited great reduction in *B. cereus* counts by 1.20, 1.06, and 0.96 Log cycle after 2 h and by 1.26, 1.16, and 1.26 Log cycle after 3 h of incubation for food mixed with water, milk and orange juice, respectively as compared to the initial *B. cereus* count. While the count was decreased by 0.26, 0.36, and 0.26 Log cycle after 2 h and by 1.0, 1.16, and

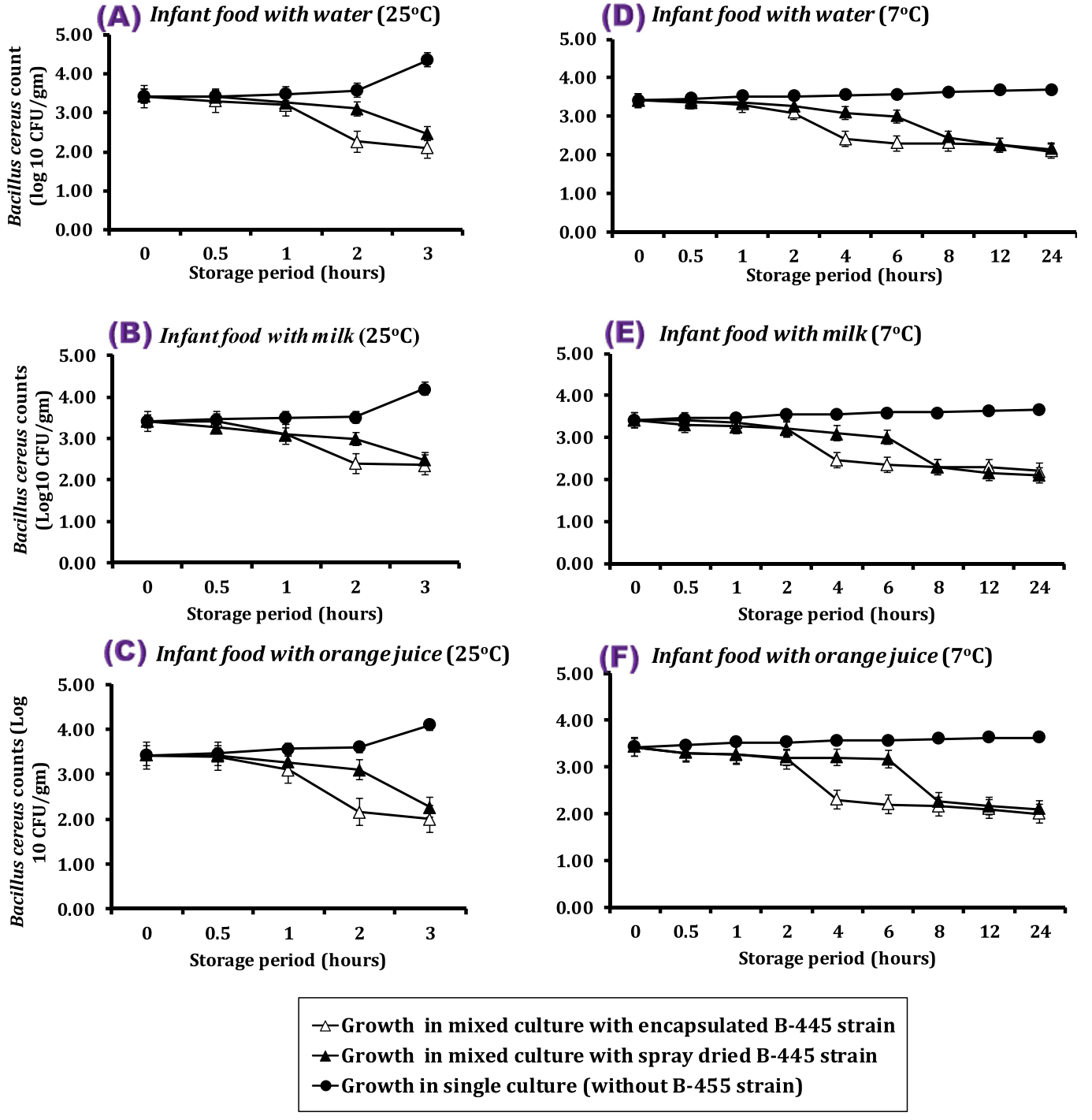

Fig. 5. Behavior of *B. cereus* during storage of infant-food [vegetables and milk-based] after mixing with water, milk, and orange juice supplemented with *L. rhamnosus* (B-445) and incubated at 25 °C for 3 h (A, B & C, respectively) and at 7.0 ± 2.0 °C for 24 h (D, E & F, respectively).

0.94 Log cycle, after 3 h of incubation at 25 °C for infant-food mixed in water, milk and orange juice, respectively when supplemented with spray dried *L. rhamnosus* B-445. It is worthy to compare such results with the increased growth of *B. cereus* count by 0.96, 1.0, and 0.80 Log cycle, respectively after 3 h of incubation in control treatments without *L. rhamnosus* inoculation. A decrease in *B. cereus* count was obtained when infant-food [fruit and milk-based] supplemented with encapsulated *L. rhamnosus was* incubated at 7 °C. It showed count reduction by 1.0, 0.69, and 0.81 Log cycle for infant-food mixed in water, milk and orange juice, respectively after 4 h while it decreased by only 0.26 Log cycle in food supplemented with spray dried *L. rhamnosus* for all treatments. Longer time is required (8 h) to achieve a decrease by 1.0, 0.77 and 1.06 Log cycle using spray dried *L. rhamnosus* (Fig. 4). Therefore, the reduction in counts of *B. cereus* with encapsulated *L. rhamnosus* (B-445) was higher than obtained with the same treatments using spray dried *L. rhamnosus* B-445.

Similarly, supplementation of infant-food [vegetables and milk-based] with *L. rhamnosus* B-445 had suppressed the growth and survival of *B. cereus* and showed higher inhibition effect with encapsulated cells than spray dried cells. At room temperature (25 °C) *B. cereus* counts reached to Log 2.26, 2.40 and 2.16 after 2 h and to Log 2.10, 2.36, and 2.0 after 3 h of incubation for infant-foods mixed in water, milk, or

orange juice, respectively as compared to initial count of Log 3.42. While, supplementation of infant-food [vegetables and milk-based] with spray dried *L. rhamnosus* had reduced the viability and survival of *B. cereus* counts that reached to Log 3.10, 3.0, and 3.10 after 2 h and to Log 2.46, 2.49 and 2.26 after 3 h of incubation at 25 °C for infant-food mixed in water, milk or orange juice, respectively (Fig. 5A–C). Furthermore, supplementation of infant-food [vegetables and milk-based] with encapsulated *L. rhamnosus* at 7 °C have resulted in decreased *B. cereus* counts by 1.0, 0.96 and 1.12 Log cycle after 4 h of incubation for infant-food mixed in water, milk or orange juice, respectively. However, it takes longer time (8 h) to achieve such results when food was supplemented with spray dried *L. rhamnosus* cells (Fig. 5D–F).

It is obvious that *B. cereus* can survive in control infant-food reaching maximum counts of > Log 4.0 after 3 h at 25 °C (Figs. 4 and 5). Such counts are very close to the estimated count to be sufficient for toxin production at high level (Van Netten et al., 1990). Granum et al. (1997) indicated that any food containing more than 10^3 of *B. cereus* per gram cannot be recognized to be safely consumed. The exact infective dose of *Bacillus cereus* is varied because of the differences in enterotoxin concentrations produced by different strains (Granum et al., 1997). Also, Mattila-Sandholm et al. (2002) reported that, fruit juice could serve as a good medium for cultivating probiotics. These results demostrated the importance of incorporation of microencapulated *L. rhamnosus* in infant-food as it showed potential and rapid effect for supressing food pathogens from further growth and proliferation.

It was previously reported that, the cell-free extract of *L. rhamnosus* culture inhibited the growth of *B. cereus* (Sadek et al., 2017; Emam et al., 2018; Goyal and Kannan, 2018). Rejiniemon et al. (2015) and Jagadeesh (2015) reported that *L. brevis, L. bulgaricus, L. lactis, L. fermentum, L. acidophilus, L. pentosus, L. rhamnosus* and *L. plantarum* are commonly probiotics used with various biological applications as biopreservative. Marie et al. (2011) indicated that bacteriocin produced by *L. rhamnosus* 1 K [isolated from traditionally fermented milk] had antibacterial effect against food spoilage and pathogenic Gram-positive and Gram-negative bacteria including not only *Bacillus* but also *Staphylococcus aureus* and *Salmonella typhi.*

4. Conclusion

The changes in total microorganism's count of infant-foods occur between preparation and consumption. Therefore, it is important to give the mothers and hospital employees training to ensure that prepared infant-food is consumed as quickly as possible under suitable conditions in order not to reach the biohazards cell concentrations. This study also clarify the importance of application of HACCP system in infant-foods factories or any of its components. Incorporation of *L. rhamnosus* B-445 as a probiotic bacteria as microencapsulation in infant-foods would greatly suppress or arrest the growth of toxigenic *B. cereus* and improve the hygienic quality and safety of the infant-foods. Besides, mixing of infant-foods in orange juice and keeping food at low temperature (7.0 °C) are more effective for biocontrol of pathogen proliferation than mixing it in water or milk and keeping at room temperature.

Conflict of interest statement

Authors declare that there are no conflicts of interest.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.01.051.

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