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Stimulating the Viability of *Bifidobacterium spp.* in Synbiotic Fermented Milk by Co-culturing with *Lactobacillus paracasei* 441 and Inulin

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Abstract*—The aim of this study is to examine the ability of three Bifidobacterium (bifidum, breve, and infantis) and Lactobacillus paracasei 441 strains to survive at different pH values, and different bile salts concentrations for 3h, also to determine the stimulatory effect of L. paracasei 441 strain in combination with inulin on the viability of Bifidobacterium strains in fermented milk during 15 days at 4°C. Each tested strain was cultured with and without L. paracasei 441 in fermented milk, then each treatment was split into three groups [0,1.0, and 3.0 inulin (w/v %)] and incubated at 37°C for 3h. Our results showed that the bacterial populations of Bifidobacterium strains co-culturing with L. paracasei 441, and 1% Inulin) were significantly (P > 0.05) higher than in the control samples [without (L. paracasei 441 and inulin). Our results reveal that the presents of (L. paracasei 441, and inulin) was stimulated the viability of Bifidobacterium strains during storage condition.*

***Keywords*—*Bifidobacterium, Inulin, Low pH and Low bile salts, Stimulating, and Synbiotic fermented milk*.**

I. INTRODUCTION

Probiotics are defined as living microorganisms, which can be consumed separately or with foods, which beneficially influence the health of the host by improving the composition of intestinal microflora (A. A. Ayad, 2016; Ranadheera, Baines, & Adams, 2010). Members of the genus *Bifidobacterium* and *Lactobacillus* are commonly used as probiotic microorganisms in probiotic foods (Granato, Branco, Cruz, Faria, & Shah, 2010). It has been suggested that food containing probiotic bacteria should contain at least 6-7 Log CFU/mL live microorganisms, at the time of consumption to achieve the desired health benefits. However, different factors have shown to affect the viability of probiotic bacteria in dairy products, including acidity level, pH, presence of other microorganisms, oxygen content, concentrations of lactic and acetic acids, storage time, and temperature (Barakat, Ibrahim, Tawfik, El-Kholy, & El-Rab, 2011; Florence, de Oliveira, Delile, & Béal, 2016). The main metabolic activity for most of the bacterial species is the degradation of different carbohydrates and related compounds to obtain mainly energy and carbon molecules. Most of the bacterial species including LAB express various metabolic activities that are necessary for their survival and growth. During growth, LAB break down sugars primarily to lactic acid through the fermentation process (König & Fröhlich, 2017).

Bifidobacterium are considered as a major part of the intestinal microbiota of humans and animals and play a significant role of improving the host gut health. The potential health benefits associated with *Bifidobacterium* include inhibition of bacterial pathogens, enhancing of the immune response, reduction of serum cholesterol levels and colon cancer risks, and improvement of lactose tolerance and vitamin synthesis.

Due to the probiotic health-promoting characteristics, most strains are related to different species and they have been used as supplement culture in dairy products, especially fermented milks (Champagne, Ross, Saarela, Hansen, & Charalampopoulos, 2011). Therefore, some of lactic acid bacteria and other probiotic strains used in

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combination could have a strong impact on the viability of each other in the fermented products (Granato, Branco, Cruz, et al., 2010). Mixed-strain culture fermentation is an effective approach to obtain the desired product characteristics and to reduce fermentation time in most food fermentation processes.

Prebiotics are non-digestible food components which beneficially affect the human health, because they selectively stimulate the activity of bacterial populations that are desirable in the gastrointestinal tract (Granato, Branco, Nazzaro, Cruz, & Faria, 2010). Since its introduction, the concept of prebiotics has drawn more attention from scientific researches as well as industrial interest (Mehanna, Tawfik, Salem, Effat, & Gad El-Rab, 2013). Therefore, many food components including inulin (de Souza Oliveira, Perego, de Oliveira, & Converti, 2012), oligosaccharides, and polysaccharides foods (including dietary fiber), claimed to have prebiotic properties.

Inulin proved to exert a protective effect towards *Bifidobacteria*, which improved this bacterial strain survival and activity during storage period. However, not all dietary carbohydrates are prebiotics, specific criteria have been established to classify all ingredients as following: (1) ingredients should be resistant to gastric acidity, and gastrointestinal absorption; (2) are able to be fermented by the intestinal microflora; (3) have the ability to stimulate the growth and/or activity of intestinal bacteria associated with being in a good health (A. A. Ayad et al., 2016).

A synbiotic is defined as a mixture of probiotics and prebiotics that has beneficial impact on the host by enhancing the survival of live microbial dietary supplements in the gastrointestinal tract, by enriching the growth and/or activating the metabolism of one or a limited number of health-promoting bacteria (Roberfroid et al., 2010). The aim of this study is to examine the ability of three *Bifidobacterium* (*bifidum, breve and infantis*) and *Lactobacillus paracasei* 441 strains to survive at different pH values, and different bile salts concentrations for 3h, also to determine the stimulatory effect of *L. paracasei* 441 strain in combination with inulin on the viability of *Bifidobacterium* strains in fermented milk during 15 days at 4°C.

II. MATERIALS AND METHODS

2.1 Bacterial culture activation and preparation

Starter cultures (*Lactobacillus delbrueckii subsp. bulgaricus* (Lb-12), and *Streptococcus thermophilus* (St) were obtained from the strain collection at CEPHT (NCAT-CEPHT, Kannapolis, USA), and *Bifidobacterium* (*bifidum, breve and infantis*) were obtained from Chr. Hansen's Lab., Denmark. While, *Lactobacillus paracasei* 441 (Lp-441) strain was obtained from Dairy Microbiology Lab, National Research Center, Egypt. All cultures (100 µL) were activated into 10 mL of fresh *Lactobacilli* MRS (deMan Rogosa and Sharp) broth (Neogen, Lansing, MI) containing 1% L-Cysteine. HCL (w/v) then incubated at 37°C for 24 h. The cultures were streaked on MRS, and MRS-LP agar, and then incubated at 37°C for 48 h.

2.2 Assay of acid resistance

Four different batches of fresh MRS broth (100 mL) were prepared. 100 µL of proper dilution (~3.1 Log CFU/mL) of each strain was sub-cultured into each batch, and adjusted to different pH values (3, 2.5, 2, and 1.5) with HCl (1M). The viable bacterial counts (CFU/mL) were determined on MRS agar hourly for 3h.

2.3 Assay of bile resistance

Activated cultures (100 µL) were transferred into different batches of MRS broth (10 mL) containing different concentrations [0.5 and 1.0% (w/v)] of (oxgall) bile salts (Difco, Detroit, Michigan, USA). The viable bacterial counts (CFU/mL) were determined on MRS agar hourly for 3 h incubation at 37°C under anaerobic conditions.

2.4 Synbiotic fermented milk (SFM) production

The stirred yogurt was prepared according to (A. A. F. Ayad, 2011) with slightly modification. Skim milk powder (Thermo Scientific, Oxoid) was dissolved into commercial pasteurized milk (3% fat) obtained from local store at ratio 1:4 (w/v %). The mixture was dispensed into sterile (1L) bottles. All batches were pasteurized at 85°C for 30 min, and then cooled down at 40°C.

Each batch was inoculated with active starter culture (*S. thermophilus* and *L. bulgaricus*) (~6 Log CFU/mL) for the production of yogurt base. Then, each activated *B.* (*bifidum, breve and infantis*) strains were individually sub-inoculated into three different batches of yogurt to form SFM as followed: T1: SFM only (as a Control), T2: Contains 3% inulin, and T3: Contains 1% inulin and L. paracasei 441. All the inoculated SFM batches were incubated at 42°C for 3-4 h. All yogurt batches were then stirred and stored at 4°C. The viable cell counts were enumerated at 0, 5, 10, and 15 days.

2.5 Determination of acidity

The titratable acidity was determined using the AOAC method (Association of Official Analytical Chemists) by titrating with 0.1 N NaOH using a pH meter and calculated as percentage of acid (AOAC International, 1995).

2.6 Inoculation procedure

A single colony of each strain was activated in 10 mL MRS broth and incubated at 37°C for 16 -18 h. The overnight-grown cultures (1mL) were serially diluted in 9 mL of phosphate buffer saline (PBS, pH 6.8).

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2.7 Determination of pH

10 mL from each sample was withdrawn after incubation to measure the pH. A pH meter (Orion Star, A211, Thermo Scientific, USA) was calibrated with pH standard buffers 4.0 and 7.0. After calibration, sample pH measurements were taken and recorded. The electrode was rinsed with DDW between different sample pH measurements.

2.8 Bacterial enumeration

Bacterial populations for all stains were determined by plating onto MRS agar. Samples were serially diluted in 9 mL of 0.1% peptone water and 100 µL from the appropriated dilution tube was then surface plated in triplicate. Plates of MRS agar were incubated aerobically, and anaerobically at 37°C for 48 h. Plates from dilutions having 25-250 colonies were counted and converted to Log CFU/mL.

2.9 Assessing viscosity of synbiotic fermented milk (SFM)

The viscosity of SFM was measured by using a cylinder viscometer (Brookfield LVDV-II+PRO, with Rheocalc™ v3.0 software for automated instrument control and data acquisition. All apparent viscosity measurements were performed in triplicates recorded in Pascal seconds (Pa.s).

2.10 Statistical analysis

Each experiment was independently replicated 3 times. The mean and the standard deviation values of each treatment were calculated. The experiment was done in a randomized design. Analysis of variance was performed to determine significant effects at significance level of $P < 0.05$ using Origin Lab corporation software (Northampton, MA).

III. RESULTS AND DISCUSSION

3.1 Survival of probiotics (*Bifidobacterium* and *Lactobacillus*) in bile salt

In order to provide a beneficial effect in gut health, probiotic cultures should be able to survive through the gastrointestinal tract and be tolerant to the bile salts concentrations in the small intestine (Patel, Singhania, Pandey, & Chincholkar, 2010). The survival of *B.* (*bifidum, breve*, and *infantis*) and *L. paracasei* 441 strains at different concentrations (0.5 and 1.0%) of bile salts were studied. The results presented in (Fig.1[a-d]) showed that the viability of all tested strains was decreased when exposed to bile salt 1% (w/v). Initial bacterial populations (Log CFU/mL) were determined using the bacterial enumeration method. The bacterial population of *B. infantis*, and *L. paracasei* 441 strains reached 7.35±0.1, and 7.23±0.1 Log CFU/mL after 3h incubation, respectively form an initial population of 8.50±0.1 Log CFU/mL on an average. However, the *B. breve* and *B. bifidum* strains were more tolerant to the bile salt 1% (w/v). Therefore, the population counts of B. breve and *B. bifidum* strains reached 7.50±0.3 and 8.16±0.2 Log CFU/mL after 3 h incubation, respectively from initial population of approximately 8.60±0.1 Log CFU/mL.

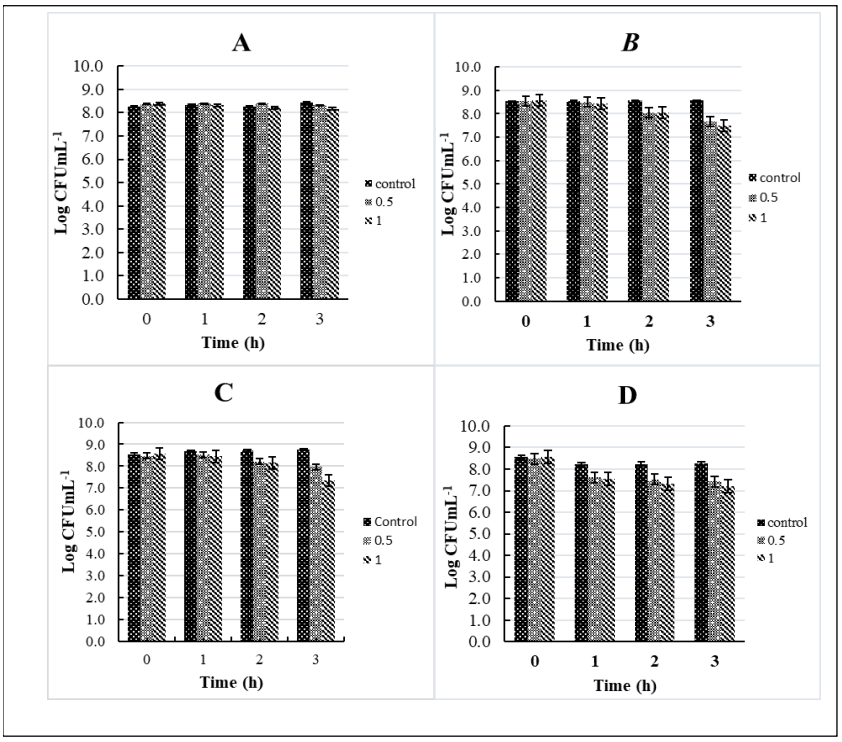

Fig. 1:Survival of B. ((a) bifidum, (b) breve, (c) infantis) and (d) L. paracasei 441 during incubation at 37 °C for 3 hours in MRS with different concentrations of bile salt (0.0, 0.5 and 1.0%).

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3.2 Survival of probiotics (*Bifidobacterium* and *Lactobacillus*) at different pH Values

The survival of *B.* (*bifidum, breve, and infantis*) and *L. paracasei* 441 strains at different pH values (3.0, 2.5, 2.0 and 1.5) for 180 min was assessed. Initial bacterial populations (Log CFU/mL) were determined using the bacterial enumeration method. The data in (Fig. 2 [a-d]) shows that the total viable counts of all strains were slightly decreased on incubation at pH 3, 2.5 and 2.0 for 3 h. However, when the pH adjusted to 1.5, all strains showed varying levels of acid tolerance. Therefore, the bacterial population of *B. breve*, and *B. infantis* reached 4.85±0.1, and 4.95±0.1 Log CFU/mL. While, the bacterial population of *B. bifidum* and *L. paracasei* 441 retained about 5.67±0.1, and 5.05±0.1 Log CFU/mL from initial population 8.54±0.1 Log CFU/mL on an average.

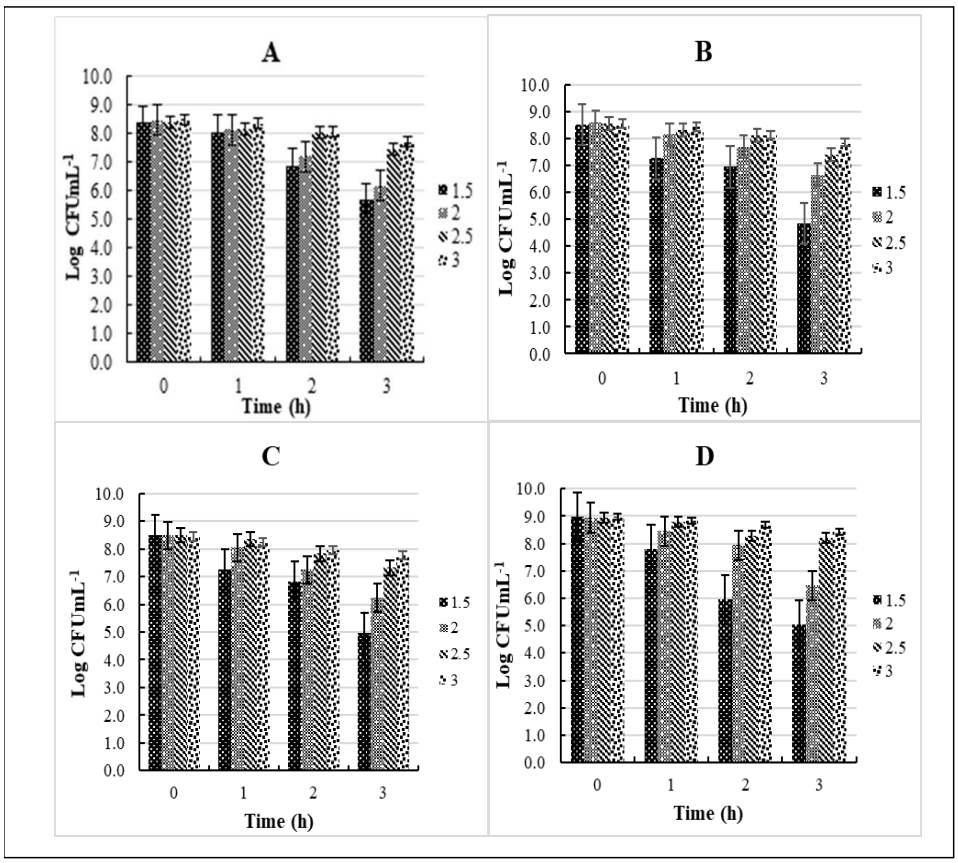

Fig. 2: Survival of B. ((a) bifidum, (b) breve, (c) infantis) and (d) *L. paracasei 441 during incubation at 37 °C for 3 hours in MRS with different values of pH (1.5, 2.0, 2.5 and 3.0).*

3.2 Change of Titratable Acidity (TA) and pH in SFM

The results presented in (Table 1 [a-c]) shows the effect of changing the TA and pH values on the viability of *B.* (*bifidum, breve, and infantis*) strains in three different treatments during storage for 15 days at 4°C. The initial TA, and pH values of synbiotic fermented milk (SFM) were 0.74, 4.75 on an average. When *B. bifidum* grown in a batch of T1 the TA, and pH values reached 0.89, and 4.52 after 15 days storage on an average, respectively. However, the TA, and pH values reached 0.91, and 4.50 when *B. bifidum* grown in T2. Therefore, the TA, and pH values reached 0.95, and 4.44, respectively in T3. Equivalent results were observed when *B. breve*, and *B. infantis* strains grown under the same conditions. Based on our observation, the acidity values in *B.* (*bifidum, breve, and infantis*) strains for (T3) are higher than those in treatment (T2) indicating that the *L. paracasei* 441 are active and more tolerant to acidity. Nevertheless, the three treatments were studied showed increasing values of acidity and decreasing values of pH during storage for 15 days due to the growing of starter culture and acid production during fermentation, particularly for the product containing both the probiotic (*L. paracasei* 441) and the prebiotic ingredients (1% inulin). Our results are consistence with other findings that increase the acidity value during fermentation and storage period increased the curd stability due to increase the protein water-binding ability (SERT, Akin, & Dertli, 2011).

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Table.1: Change of pH and titratable acidity (TA) of synbiotic fermented milk inoculated with three B. ((a) bifidum, (b) breve, (c) infantis) during incubation at 4°C for 15 days.

A

Storage Time (days)	T1		T2		T3	
	pH	TA	pH	TA	pH	TA
Control	4.78±0.0^{B}	0.74±0.0^{d}	4.73±0.0^{A}	0.73±0.0^{de}	4.72±0.0^{A}	0.75±0.0^{e}
5	4.71±0.0^{C}	0.8±0.0^{c}	4.69±0.0^{B}	0.79±0.0^{c}	4.69±0.0^{C}	0.85±0.0^{c}
10	4.68±0.0^{DE}	0.84±0.0^{c}	4.61±0.0^{C}	0.8±0.06^{b}	4.58±0.0^{D}	0.89±0.0^{b}
15	4.51±0.0^{F}	0.89±0.0^{b}	4.45±0.0^{E}	0.91±0.0^{a}	4.44±0.0^{G}	0.95±0.0^{a}

B

Storage Time (days)	T1		T2		T3	
	pH	TA	pH	TA	pH	TA
Control	4.78±0.0^{A}	0.79±0.0^{g}	4.76±0.0^{A}	0.72±0.0^{h}	4.69±0.0^{C}	0.74±0.0^{h}
5	4.72±0.0^{B}	0.81±0.0^{fg}	4.71±0.0^{BC}	0.78±0.0^{g}	4.65±0.0^{D}	0.85±0.0^{de}
10	4.62±0.0^{E}	0.83±0.0^{ef}	5.62±0.0^{E}	0.85±0.0^{d}	4.55±0.0^{G}	0.88±0.0^{c}
15	4.59±0.0^{F}	0.90±0.0^{bc}	4.54±0.0^{G}	0.92±0.0^{ab}	4.44±0.0^{H}	0.93±0.0^{a}

C

Storage Time (days)	T1		T2		T3	
	pH	TA	pH	TA	pH	TA
Control	4.80±0.0^{A}	0.78±0.0^{gh}	4.77±0.0^{B}	0.76±0.0^{h}	4.78±0.0^{B}	0.75±0.0^{g}
5	4.72±0.0^{BC}	0.82±0.0^{f}	4.74±0.0^{C}	0.82±0.0^{f}	4.73±0.0^{C}	0.84±0.0^{de}
10	4.66±0.0^{C}	0.84±0.0^{e}	4.71±0.0^{D}	0.87±0.0^{d}	4.67±0.0^{D}	0.88±0.0^{c}
15	4.62±0.0^{F}	0.88±0.0^{c}	4.60±0.0^{E}	0.91±0.0^{b}	4.60±0.0^{E}	0.93±0.0^{a}

*Mean values in the same rows with different superscript are significantly different at $P < 0.05$, values are the means ± S.D (n=3)

3.3 Viscosity of synbiotic fermented milk

The viscosity of the synbiotic fermented milk (SFM) batches on 0 day and at every 5 days intervals during storage conditions was measured. The data presented in (Fig. 3 [a-c]) shows the apparent viscosities of all batches of SFM during 15 days of storage at 4°C. When *B. bifidum* was grown in a batch of T2 the viscosities values were higher than the viscosities values of the T1 after 15 days of storage period at 4°C. However, the apparent viscosities of the T3 were significantly ($P < 0.05$) higher than the apparent viscosities of T1 after 15 days of storage period at 4°C. Thus, the obtained results when B. breve, and *B. infantis* strains grown under the same conditions were quite similar.

A reduced fat content in fermented milk products could cause a lack in viscosity and structure, which will affect the appearance, texture, and mouthfeel of the yogurt (Krzeminski, Großhable, & Hinrichs, 2011). Interestingly, our results consisted with other findings that supplementing synbiotic fermented milk with inulin could substantially increase the viscosity due to the interaction between inulin and milk protein which increase the product total solid (Meyer, Bayarri, Tárrega, & Costell, 2011). Similarly, other observations revealed that some strains of *Lactococci* have the potential to produce viscosity-generating exopolysaccharides (Goddik, 2012). Therefore, the presents of *L. paracasei* 441 strain in T3 have a considerable influence on raising the viscosity of the SFM, due to the ability of *L. paracasei* 441 strain to produce polysaccharides (data not shown) which helps to hold the water content and increase the total solid of the SFM.

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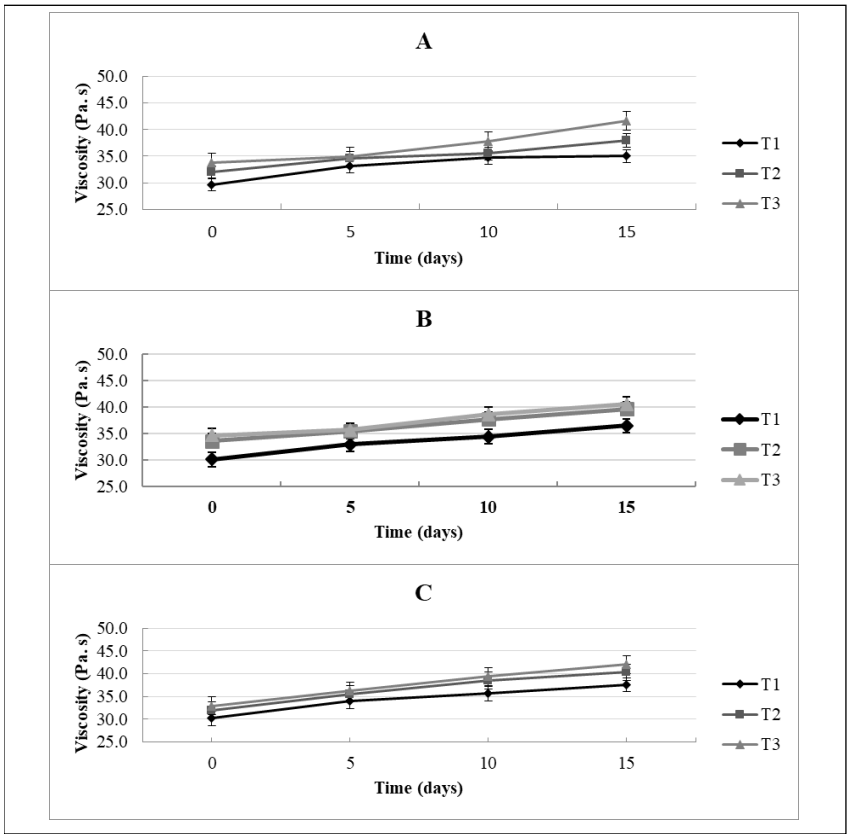

Fig. 3: The synbiotic fermented milk variation of viscosity at different treatments (T1, Control), (T2, supplemented with 3% inulin), and (T3,inoculated with L. paracasei 441 and 1% inulin) using B. ((a) bifidum, (b) breve, (c) infantis) with during storage for15 days at 4°C.

3.4 Viability of selected Bifidobacterium strains in SFM

All fresh yogurt batches (T1, T2, and T3) contained similar populations of starter culture (S. thermophilus and L. bulgaricus) after 1 and 15 days of storage, and always above 9 Log CFU/mL (data not shown). The survival of three Bifidobacterium spp. strains grown in three (T1, T2, and T3) was studied. To maintain the health benefit, the recommended concentration of the probiotic bacteria is at least 6 Log CFU/mL of any product during their shelf life (Champagne et al., 2011).

The data in (Fig. 4 [a-c]) presents the viability of three *B.* (*bifidum, breve,* and *infantis*) strains during storage period for 15 days at 4°C. The bacterial population of *B. bifidum* strain growing in T1, T2, and T3 reached 7.45, 8.13, and 8.67 Log CFU/mL on an average, respectively from the initial population 8.00 Log CFU/mL. The similar growth pattern was observed on the other two strains of *B.* (*breve,* and *infantis*) under the same conditions as the *B. bifidum* strain.

Our results have shown that the viable count of *B.* (*bifidum, breve,* and *infantis*) strains were consistently stable in the synbiotic fermented milk during 15 days of storage. Thus, the viable population of *B. bifidum* strain obtained in SFM could be referring to the presents of *L. paracasei* 441. Furthermore, our results agreed with other findings that mixing cultures *B. longum* and *L. fermentum* was the best proportion to improve the growth of each strain and increased the survival of both cultures in fermented soy milk during 28 days of storage (Rivera-Espinoza & Gallardo-Navarro, 2010).

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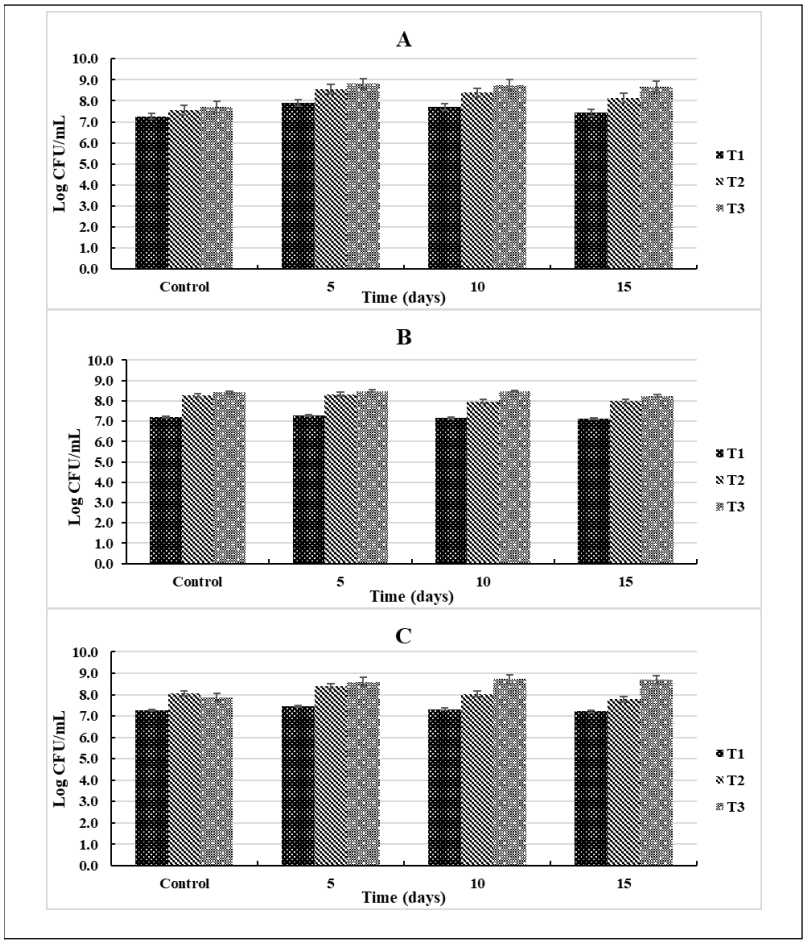

Fig.4:Viability of Bifidobacterium ((a) bifidum, (b) breve, (c) infantis) in synbiotic fermented milk for 15 days storage period at 4°C.

IV. CONCLUSION

Our results revealed that the three *Bifidobacterium* (*bifidum, breve,* and *infantis*), and *L. paracasei* 441 strains showed varying levels of acid, and bile salt tolerance. Therefore, the *B. bifidum* and *L. paracasei* 441 strains were more acid (pH 1.5), and bile salt (%1) tolerant than *B. breve*, and *B. infantis* strains. Our findings indicated that the viability of three *B.* (*bifidum, breve,* and *infantis*) strains were consistently stable in the synbiotic fermented milk during refrigerated storage for 15 days. In this regard, we have found that the L. paracasei 441 strain and inulin 1% had a potential to stimulate the growth of the *B.* (*bifidum, breve,* and *infantis*) strains. This study can affirm that the incorporate between *L. paracasei* 441 strain and inulin could be used as a reference to restore the balance of gut microbiota and related malfunction of human gastrointestinal tract.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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