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Effect of *Asparagus falcatus* and *Taraxacum javanicum* Inulins on Growth of *L. acidophilus* La-5 and *B. animalis* subsp lactis Bb-12, Co-cultured in Skim Milk

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***ABSTRACT*:** *A study was conducted to evaluate the effect of inulin from Asparagus falcatus and Taraxacum javanicum on growth of Lactobacillus acidophilus (La-5) and Bifidobacterium animalis (Bb-12) in skim milk. Commercially available chicory inulin and chicory fructooligosaccharides (FOS) were used as positive controls. Skim milk (13% (w/v)) samples supplemented with different concentrations (0.5, 1, 2, 3 and 5%) of AF or TJ inulin, 5% Chicory inulin and 5% FOS, inoculated with mix culture of La-5 and Bb-12 (1:1), were incubated at 37 ^{o}C for 24 hours. The skim milk samples prepared the same way, but without adding inulin were used as negative controls. Optical density and pH of the milk samples were checked during 0, 6, 12 and 24 hours of incubation. Selective enumeration of La-5 and Bb-12, at 6 hours of incubation, was done using MRS-sorbitol agar and Beerens agar, respectively. The AF and TJ inulin showed enhanced bifidobacterial growth in skim milk, similar to chicory inulin. The growth promotion effect of AF and TJ inulin on bifidobacteria was maximal at 3% inulin concentration. None of the inulin types or FOS significantly influenced the La-5 growth in skim milk. The pH values of fermented milk samples were significantly ($P<0.05$) lower in inulin or FOS supplemented skim milks compared with the control. AF and TJ inulin showed clear bifidogenic properties in skim milk, indicating their potential use as alternative inulin sources for the Food Industry.*

Keywords: *bifidobacteria, inulin, lactobacilli, prebiotics, symbiotic*

1. INTRODUCTION

Consumers across the world are becoming more interested in consuming functional foods. Among such functional foods, probiotics, prebiotics and synbiotics are becoming promising groups of foods, which specifically target the gut health.

Inulin is one of the most extensively studied prebiotic. Chemically, inulin is a linear polymer of fructose, joined by β 2-1 fructosyl fructose linkages and typical terminal glucose (Apolinario *et al.,* 2014). At present commercially available inulin is extracted and purified from two plants namely; Chicory and Jerusalem artichoke, which are not grown in Sri Lanka.

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Recent study by Mudannayake *et al.* (2015a) and Mudannayake *et al.* (2015b) revealed that inulin derived from roots of *Asparagus falcatus* (AF) (*Hathawariya*) and *Taraxacum javanicum* (TJ) (Dandelion), has potential to be used as inulin sources in food industry.

The main aim of incorporating inulin in foods is to selectively stimulate the growth and activity of the beneficial microorganisms reside in the colon, thus generating the beneficial health effects in the host (Kolida and Gibson, 2011). However, in manufacturing synbiotic milk products, additional advantages of inulin can be obtained through enhancement of probiotic counts in the milk medium (Desai *et al.*, 2004).

Numerous studies have reported that the growth of different *Lactobacillus* and *Bifidobacterium* strains in the skim milk medium was enhanced in the presence of inulin or fructooligosachcharides (Shin *et al.*, 2000; Bruno *et al.*, 2002; Aryana and McGrew, 2007; Aryana *et al.*, 2007; Allgeyer *et al.*, 2010; Oliveira *et al.*, 2011; Oliveira *et al.*, 2012). However, all these studies, used inulin or fructooligosaccharides derived from Chicory plant. There is limited research on use of inulin derived from alternative plants as a source of prebiotics in fermented milks.

It has been documented that each prebiotic is not compatible with every probiotic strain. The ability of probiotics to ferment prebiotic carbohydrate is both strain and substrate specific (Kaplan and Hutkins, 2000; Schrezenmeir and Vrese, 2001; Huebner *et al.*, 2007). Moreover, the ability of fermentation of inulin by probiotic bacteria depends on various characteristics of inulin, including degree of polymerization, composition of monomer units, glycosidic linkage, degree of branching, level of purity and solubility (Biedrzycka and Bielecka, 2004; Al-Sheraji *et al.*, 2013).

Therefore, prior to formulating a synbiotic product using a new prebiotic source, it is important to investigate whether prebiotic carbohydrate and probiotic organism have a positive interaction (Holzapfel and Schillinger, 2002; Oliveira *et al.*, 2012).

This study aimed to investigate whether newly developed inulin from AF and TJ plants can enhance the growth of *L. acidophilus* (La-5) and *B. animalis* (Bb-12) co-cultured in skim milk in comparison with commercial inulin, and to identify the optimum concentrations of those inulins for the growth of probiotics.

2. METHODOLOGY

The lab experiments were carried out in the Dairy Science laboratory of the Department of Animal Science, Faculty of Agriculture, University of Peradeniya, Sri Lanka and Food Science Laboratory, Bio Sciences Section, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Victoria 3010, Australia.

2.1 Materials

The single strain probiotic cultures; *Bifidobacterium animalis* subsp. *lactis* Bb-12 and *Lactobacillus acidophilus* La-5 (Chr Hansen®) were kindly provided by J L Morison Son & Jones Plc, Kelaniya, Sri Lanka. Chicory inulin (Beneo Orafti®-GR) and Chicory fructooligosaccharides (FOS) (Beneo Orafti® -P95) were kindly provided by DPO International, Colombo, Sri Lanka. Instant skim milk powder was purchased from Fonterra

brands, Sri Lanka. All the bacteriological media (Oxoid®), media ingredients, anaerobic atmosphere generation sachets (AnaeroGen, Oxoid, UK) and anaerobic indicator strips (BR55, Oxoid, UK) were purchased from Hemsons International (Pte) Ltd, Colombo, Sri Lanka.

2.2 Methods

2.2.1 Inulin extraction and powder preparation

Asparagus falcatus (AF) and *Taraxacum javanicum* (TJ) inulin powders were prepared from fresh tubers of *A. falcatus* and *T. javanicum* plants as described by Mudannayake *et al.* (2015a).

2.2.2 Selective media for enumeration of probiotic bacteria

Selective enumeration of *Lactobacillus acidophilus* (La-5) and *Bifidobacterium animalis* (Bb-12) were carried out using MRS-Sorbitol agar (Shah, 2000) and Beerens agar (Campos *et al.*, 2012), respectively. MRS-Sorbitol agar was prepared as described by Aryana *et al.* (2007) with slight modifications, using (g/L) of; peptone (10.0), lab-lemco powder (8.0), yeast extract (4.0), D-Sorbitol (20.0), tween-80 (1.0), di pottassium hydrogen phosphate (2.0), sodium acetate $3H_2O$ (5.0), Triammonium citrate (2.0), Magnesium sulphate $7H_2O$ (0.2), Manganeese sulphate $4H_2O$ (0.05), agar (10.0) (pH adjusted to 6.2). Beerens agar medium, was prepared by using 47 g/L brain heart infusion agar, 3 g/L Glucose, 5 g/L L-cysteine HCl, 5 g/L agar and 5 mL/L propionic acid in 1L deionized water, and pH was adjusted to 5.6 with 1 M NaOH (Beerens, 1990; Campos *et al.*, 2012).

2.2.3 Pre-culture preparation

Each single strain probiotic culture (La-5 and Bb-12) pack (50 U) was fully dissolved in 1 L of sterile, 10% (w/v) reconstituted skim milk. After complete dissolving, 20 mL aliquots of suspended pre-cultures were dispensed into sterile screw capped MaCarthy bottles, stored at -20 $^{\circ}$C and used within 1 month. Prior to the inoculation, pre-culture bottles were kept in a water bath at 37 $^{\circ}$C for 30 minutes for activation. Bacterial counts in these pre-culture bottles ranged from 7.6 to 8.0 log cfu/mL.

2.2.4 Milk preparation and fermentation

As Shin *et al.* (2000) demonstrated that the maximum growth of lactic acid bacteria in milk was evident in the presence of 5% inulin or FOS. This study followed those guidelines and used 5% (w/v) inulin as the maximum concentration.

Milk preparations were done as described by Oliveira *et al.* (2012) with slight modifications. Skim milk powder was dissolved in distilled water (1.5 L) to make a solution of 13% (w/v) reconstituted skim milk. Prepared reconstituted skim milk samples were divided into 100 mL aliquots and blended with different levels (0.5%, 1%, 2%, 3% and 5%) of Asparagus or Taraxacum inulins separately. Skim milk samples prepared with 5% Chicory inulin (Beneo Orafti®GR) and 5% Chicory FOS (Beneo Orafti®P95) were used as positive controls while skim milk samples prepared with no added inulin were used as negative controls. Milk samples with added inulin, in 100 mL sterilized Schott bottles with screw caps were heat treated at 90°C for 10 min in a water bath. Each pasteurized milk bottle was then cooled to

40±2°C and aseptically inoculated with equal volumes (1 mL) of each single strain pre-activated cultures (La-5 and Bb-12). Consequently, each 100 mL milk sample contained a mixed culture of 1 mL La-5 and 1 mL Bb-12. Each inoculated milk sample was then aseptically dispensed into labelled, sterile McCarthy bottles in 20 mL aliquots, tightly closed and incubated aerobically at 37 °C for 24 hours.

2.2.5 Optical density measurements

Fermented milk samples (500 µL) were collected at 0, 6, 12 and 24 hours during incubation and cell density was measured using a UV-visible spectrophotometer as described by Bruno *et al.* (2002), with some modifications. Briefly, 500 µL of fermented milk samples were transferred into 20 mL culture tubes and diluted with 10 mL of 0.2% (w/v) ice cold EDTA, which was previously adjusted to pH 12.5 with 0.5% (w/v) NaOH. Then samples were thoroughly vortexed and turbidity was measured at 640 nm using a UV-visible spectrophotometer. Each measurement was done in triplicate. Un-inoculated pasteurized milk samples with respective added inulins, diluted with 0.2% (w/v) EDTA (pH 12.5) were used as the blanks (Bruno *et al.*, 2002).

2.2.6 pH Measurement of fermented milk samples

The pH values of fermented milk samples were measured at 0, 6, 12 and 24 hours of incubation using a pH meter (Eutech 6, Eutech instruments, Singapore). Triplicate pH measurements were done for each sample.

2.2.7 Bacteria enumeration in fermented milk samples

Spread plate technique was used to assess the bacterial counts in fermented milk samples. The initial experiments on growth of mix culture (Bb-12 and La-5) in skim milk revealed that maximum growth can be achieved at 6 hours of incubation (section 3.1). Therefore, bacterial counts were enumerated only after 6 hours of incubation.

Duplicate fermented milk samples (1 mL each) were obtained at 6 hrs of incubation and serially diluted in sterile buffered peptone water added with 0. 5 g/L L-cysteine HCl. Triplicate aliquots (100 µL) from each three dilutions (10^{-5}, 10^{-6}, and 10^{-7}), were plated on MRS-sorbitol agar and Beerens agar for selective enumeration of *L. acidophilus* and *Bifidobacterium* species, respectively. Inoculated plates were then incubated anaerobically in 2.5 L anaerobic jars (AnaeroGen™, Oxoid, UK) at 37 °C for 48 hrs. Anaerobic atmosphere generation sachets and anaerobic indicator strips were used during incubation to ensure anaerobic conditions.

2.2.8 Statistical analysis

Experiments were designed as random, full factorial split-plot in time design exploring the influence of inulin type and concentration as the main effects. The milk preparation for analysis of bacterial counts, optical density and pH was done two times, with quadruplicated measurements of each parameter at each time. Statistical analysis was conducted using Minitab 16 statistical software, at the University of Melbourne. One-way analysis of variance was conducted to see the effect of inulin concentration on bacterial growth. Post hoc Tukey's tests were conducted at 95% confidence level to separate means.

3. RESULTS AND DISCUSSION

3.1 Impact of inulin on growth of mix culture *B. animalis* (Bb-12) and *L. acidophilus* (La-5) in milk

Overall OD results showed that mixed cultures (Bb-12 and La-5) achieved the maximum bio mass growth during the first 6 hrs of fermentation (Fig. 1). Further, incubation beyond 6 hrs revealed insignificant ($P>0.05$) increase in bacterial growth. The results reveal that the bacterial growth was improved significantly ($P<0.05$) in the presence of all types of inulins compared to that of control.

The optical density values in milk samples supplemented with AF inulin (Fig. 1. A) Showed the greatest bacterial growth in the presence of 3% AF, 5% Chi inulin and 5% FOS. Similarly, the highest bacterial growth was observed in the presence of 3% TJ, 5% Chi inulin and 5% FOS (Fig 1.B).

Bacterial growth in the presence of smaller inulin concentration (1-2%) was smaller than the growth at 3% (Fig 1). Non-inulin supplemented milks (controls) revealed lower growth than those containing inulins (either AF, TJ or Chi), in all concentrations.

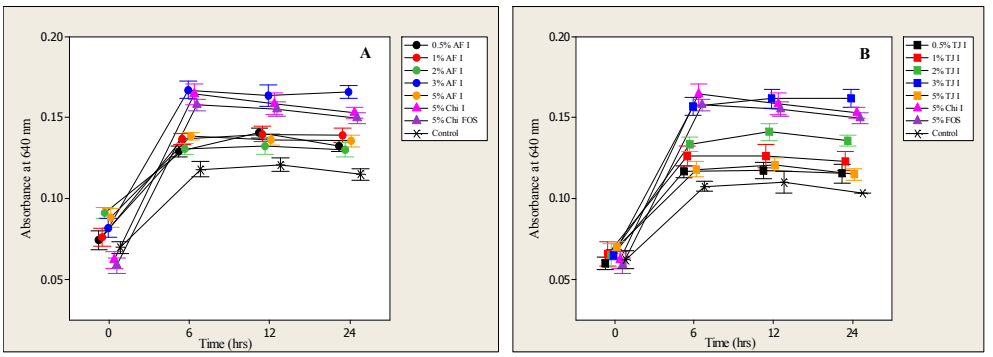

Fig 1. Growth of mix cultures of Bb-12 and La-5 in skim milk containing different types of inulins at various concentrations, during incubation at 37 °C for 24 hrs. (A): different AF inulin concentrations (B): different TJ inulin concentrations. AF I- Asparagus inulin, TJ I- Taraxacum inulin, Chi I- commercial Chicory inulin, FOS commercial Chicory fructooligoscaccharide, Control: no added inulin. n=4.

3.2 Changes in milk pH in the presence of inulin and mixed bacterial culture

Changes of pH in skim milk inoculated with mix cultures of Bb-12 and La-5, in the presence of different inulins, and during 24 hours incubation are presented in Figure 2. The pH of all milk samples declined dramatically at a higher rate during the first six hours of incubation. The pH values dropped sharply from 6 to about 4.5 during the first 6 hours of incubation, and continued to decrease until 12 hours of incubations. The pH values were 3.83± 0.04 and 3.73± 0.12, after 12 hours and in the presence of 3% AF and 3% TJ inulins, respectively. However, the decline in pH during the last 12 hours of incubation was insignificant. The pH values in milk samples containing 3% of AF and TJ inulins after 24 hours were 3.80±0.04

and 3.67±0.11, respectively. These results were in agreement with the OD data, which showed significant increase in bacterial growth during the first 6 hours of incubation (Fig 2). It is clear that growth of La-5 and Bb-12 during incubation caused hydrolysis of lactose into organic acids such as lactic acid, and consequently reduced the pH of the medium. The pattern of pH reduction was same with all samples. However, milk samples with no added inulin showed significantly ($P<0.05$) higher pH compared with all inulin or FOS supplemented samples. This indirectly indicates the increased growth of probiotic bacteria in inulin supplemented samples compared to control.

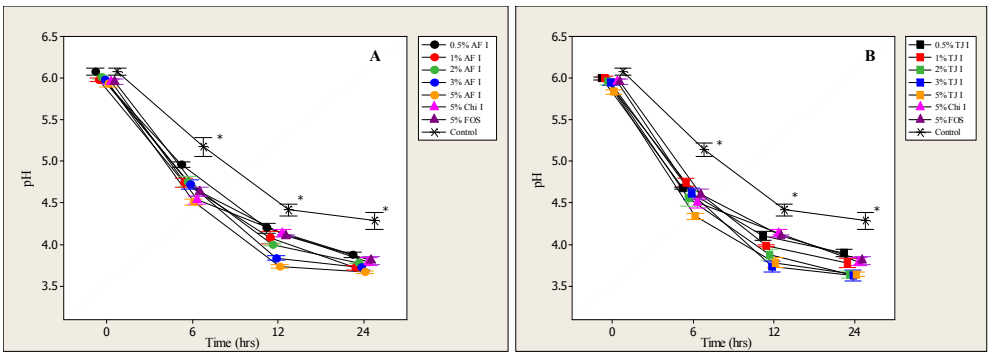

Fig 2. Changes in pH of skim milk, containing different inulin concentrations during incubation at 37 °C for 24 hrs. (A): different AF inulin concentrations (B): different TJ inulin concentrations. AF I- Asparagus inulin, TJ I- Taraxacum inulin, Chi I- commercial Chicory inulin, FOS- commercial Chicory fructooligoscaccharide, Control: no added inulin. n=4.

Fig 1 showed that OD readings did not increase after the first 6 hours of incubation, while pH values (Fig 2) continued to decrease up to 12 hours of incubation. However, these observations clearly indicated a negative correlation between changes in OD and pH measurements (Figs 1 and 2) during fermentation. Similar patterns of OD and pH changes were reported by Li *et al.* (2015) who studied the growth of probiotic strains during incubation of yoghurt in the presence of Jerusalem artichoke inulin.

Such decline in pH values and increase in the growth of Bb-12 and La-5 bacteria in the presence of AF and TJ inulins support the view that these newly developed inulins could be good alternatives to the commercial Chicory inulin.

3.3 Effect of inulin concentration on bacterial counts

The counts of Bb-12 and La-5 (log cfu/mL) co-cultured in skim milk, containing different inulin concentrations, and incubated at 37 ^{o}C for 6 hours are given in Table 1. The effect of AF or TJ inulins at various concentrations (0.5, 1, 2, 3, and 5%) was compared with that of 5% commercial Chicory inulin and 5% Chicory fructooligosaccharide (FOS). It should be noted here that 5% (w/v) concentration was used as the maximum added level of inulin in this study following the recommendation of Shin *et al.* (2000). These authors reported that maximum growth and activity of bifidobacteria were observed when 5% inulin or FOS was added to skim milk.

3.3.1 Effect of added inulin concentration on *Bifidobacterium* counts

Results revealed that increasing AF or TJ inulin concentration from 0.5 to 5% had significantly ($P<0.05$) improved the growth of bifidobacteria (Bb-12) co-cultured in skim milk. The viable count of Bb-12 in the presence 0.5% of AF inulin (8.22±0.13 log cfu/mL), was not significantly ($P>0.05$) different from the control (8.15±0.12 log cfu/mL) (Table 1). However, 1, 2, 3 and 5% AF inulin containing milk samples showed significantly greater Bb-12 counts compared to the control. However, it should be noted that increasing the added AF inulin concentration from 3 to 5%, reduced the Bb-12 counts in milk by less than 0.5 log cfu/mL. Apparently Bb-12 gained their optimum growth at 3% AF inulin concentration. The same results revealed that Bb-12 counts in the presence of AF inulin at 2 and 3% were comparable to the Bb-12 counts in the presence of 5% Chi inulin or FOS.

Table 1. Total counts (log cfu/mL) of Bb-12 and La-5 in skim milk containing different inulin concentrations after incubation at 37 °C for 6 hrs.

Type of inulin	Concentration (%)	Bb-12 (log cfu/mL)	La-5 (log cfu/mL)
AF I	0.5	8.22±0.13 $^{z\,C}$	8.53±0.22 $^{x\,A}$
	1	8.59±0.13 $^{z\,B}$	8.57±0.25 $^{x\,A}$
	2	8.83±0.31 $^{y\,A}$	8.79±0.11 $^{x\,A}$
	3	9.06±0.21 $^{x\,A}$	8.74±0.10 $^{x\,A}$
	5	8.53±0.27 $^{z\,B}$	8.70±0.12 $^{x\,A}$
TJ I	0.5	8.39±0.16 $^{z\,B}$	8.53±0.22 $^{x\,A}$
	1	8.61±0.14 $^{z\,B}$	8.79±0.22 $^{x\,A}$
	2	8.89±0.04 $^{y\,A}$	8.89±0.14 $^{x\,A}$
	3	9.23±0.30 $^{x\,A}$	8.89±0.17 $^{x\,A}$
	5	8.36±0.30 $^{z\,B}$	8.74±0.13 $^{x\,A}$
Chi I	5	8.97±0.17 ^{A}	8.61±0.21 ^{A}
FOS	5	8.49±0.13 ^{B}	8.63±0.17 ^{A}
No I (Control)	-	8.15±0.12 ^{C}	8.68±0.24 ^{A}

Means with different superscripts (x, y, z), along the column, and within each inulin type are significantly different ($P<0.05$). Means with different superscripts (A, B, C) along the entire column are significantly different ($P<0.05$). AF I- Asparagus inulin, TJ I-Taraxacum inulin, Chi I- commercial chicory inulin, FOS- commercial chicory fructo oligoscaccharide, No I: no added inulin.

Similar growth patterns of Bb-12 were observed in the presence of TJ inulin at all added concentrations (Table 1). Taraxacum (TJ) inulin in milk at 0.5, 1, 2, 3 and 5% significantly ($P<0.05$) increased the Bb-12 counts, compared to the control. For example, Bb-12 counts in skim milk containing 1, 2, 3 and 5% TJ inulin were 8.61±0.14, 8.89±0.04 and 9.23±0.30 and 8.36± 0.30 log cfu/mL, respectively compared with 8.15±0.12 log cfu/mL in the control. Similar to the effect of AF inulin that was discussed before, the same results indicated that adding TJ inulin at concentration more than 3% did not cause any further increase in Bb-12 counts. The maximal counts of Bb-12 were observed at 2% or 3% TJ inulin added to the milk samples. Furthermore, it was noted that, Bb-12 counts in skim milk containing 2% or 3% TJ inulin were comparable to the counts in samples with 5% chicory inulin.

Bifidobacterium growth in the presence of inulin was dose dependent. For example, statistically significant increase in bifidobacteria (Bb-12) count was observed with increasing concentration of AF and TJ inulin from 0.5 to 5%. These results were consistent with Shin *et al.* (2000), who reported the dose dependent nature of bifidobacteria in the presence of inulin in skim milk.

These results of growth enhancement of bifidobacteria in the presence of inulin in skim milk were in agreement with those reported by several other authors including Oliveira *et al.* (2012), Oliveira *et al.* (2011), Ozer *et al.* (2005) and Bruno *et al.* (2002).

Data in table 1 also revealed that counts of bifidobacteria in skim milk containing 5% FOS were significantly ($P<0.05$) smaller than counts in the presence of 5% Chicory inulin. These results were in disagreement with those reported by Shin *et al.* (2000) and Paseephol and Sherkat (2009), who showed that FOS caused larger growth stimulation to bifidobacteria than inulin. However, Ito *et al.* (2011), showed that longer chain length inulin preferentially supported the growth of bifidobacteria over short chain length FOS.

3.3.2 Effect of added inulin concentration on *Lactobacillus* counts

Data in table 1 revealed that, added inulin (AF, TJ, Chi inulin) or FOS had no significant effect ($P>0.05$) on the growth of *L. acidophilus* (La-5) in skim milk. Unlike the effect of Bb-12 that was discussed in section 3.3.1, increasing AF or TJ inulin concentrations from 0.5 to 5% showed no significant effect ($P>0.05$) on growth of La-5 in skim milk.

Several other studies also reported that adding inulin has no effect on the growth of *Lactobacillus* species in skim milk (Ozer *et al.*, 2005; Paseephol and Sherkat, 2009; Allgeyer *et al.*, 2010; Oliveira *et al.*, 2011). Similarly, Adebola *et al.* (2014), who studied the effect of inulin on the growth of five different strains of *Lactobacillus* reported that none of the tested *Lactobacillus* strains could utilize inulin. Consequently, the results in this current study are consistent with observations reported by those authors. However, conflicting results had been also reported by Aryana *et al.* (2007) and Sadek *et al.* (2006) who stated that *Lactobacillus* growth was promoted by adding inulin. Such contradictory conclusions by different investigators may be due to the differences of strains used in these studies. Similarly, Kaplan and Hutkins (2000) and Huebner *et al.* (2007) reported that use of inulin or FOS by probiotics depends on the species as well as the strains of tested bacteria.

4. CONCLUSION

Optical density measurements as an indicator of microbial growth were very representative and showed that maximum growth of mix probiotic culture (Bb-12 and La-5) could be achieved after 6 hours of incubation. Although OD measurement method to measure bacterial growth have the drawback of accounting both viable and dead cells, these results give an clear idea of the growth pattern of tested mix probiotic strains.

Experimentally prepared AF and TJ inulin showed bifidogenic properties in skim milk. However, none of the inulins (AF, TJ and Chi) nor FOS showed valuable effect on *Lactobacillus* (La-5) growth in skim milk. Based on results of this study, 3% inulin (AF and TJ) was the optimum concentration for *Bifidobacterium* growth.

The results showed that both AF and TJ inulins could be good alternatives for commercial chicory inulin in terms of effective promotion of *Bifidobacterium* (Bb-12) growth in skim milk. Thus both TJ and AF inulins could be considered as potential prebiotic sources for the food industry.

Nevertheless, further studies on viability and activity of probiotic cultures during cold storage, physicochemical properties and sensory properties in the presence of new inulin in milk will be essential in order to fully estimate the potential usage of AF and TJ inulins as substitutes to commercial chicory inulin in probiotic milk products.

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