LACTIC ACID FERMENTATION OF SYNBIOTIC CREAM: EFFECTS ON PHYSICOCHEMICAL CHARACTERISTICS AND FORMATION OF L (+), AND D (–)-LACTIC ACID ISOMERS

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(Received 4th May 2018; accepted 27th Jun 2018)

Abstract. In this study, the effect of Probiotic bacteria (*Lactobacillus acidophilus* and *Lactobacillus casei*) and inulin had been evaluated on some of the chemical properties, viability of probiotics and the production of L- and D-lactic acid isomers in synbiotic cream during fermentation. UHT cream supplemented with 0, 1.5 and 3% (w/w) inulin was fermented for 4 h at 37 °C. Two strains showed viable cell numbers above 9 log cfu/g after 4 h fermentation at 37 °C. HPLC evaluation showed that both of strains produced L-isomer. *Lactobacillus acidophilus* achieved L-lactic acid concentration close to 1.922 ± 0.11 g L^{-1} and *Lactobacillus casei* 2.148 ± 0.03 g L^{-1} over storage. Using *Lactobacillus acidophilus*, the amount of D-isomer is significantly increased (0.47 g L^{-1}). The probiotic strains produced different amounts of metabolic products according to temperature and fermentation time illustrating the importance of controlling these parameters.
Keywords: *HPLC, lactic acid isomers, cream, inulin, Lactobacillus acidophilus, Lactobacillus casei*

Introduction

According to the rules of the International Union of Applied Chemistry (IUPAC), Lactic acid is known as 2-hydroxypropanoic acid. Because of the partial separation of this acid in water and aqueous media, it is classified as a weak organic hydroxylated acid of low molecular weight and PKa = 3.86 at 25 °C (Liu and Zhang, 2011). Lactic acid was first isolated from the milk by Scheele, a Swedish chemist in 1780. Louis Pasteur then examined the fermentation process and, using a variety of lactic bacteria, used them to prepare fermented foods from milk, vegetables, cereals, and meat, and showed that it protects foods from microbial degradation (Ameen and Caruso, 2017). For the first time, Lactic acid was commercially produced in 1881 by Charles Avery in the United States (Schubert et al., 2011). Lactic acid is widely used in food, pharmaceutical, and chemical industries, and is provided by the lactate dehydrogenase from pyruvate regeneration and plays a significant role in biotechnology (Gaspar et al., 2013). The use of lactic acid and its main derivatives are mainly related to food, pharmaceutical, and chemical industries. In the food industry, lactic acid is used to produce cheese, yogurt, sour cucumber, essential oils, lemon juice, extracts of juices and other food products, as well as microbial contamination of canned foods and as a natural supplement to the taste of foods (Ebersole et al., 2018). It may also be used in

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1606_74477466

the production of other organic acids, acrylic acid, acetaldehyde, and ethanol. The most recent use of lactic acid is the preparation of biodegradable plastics, including poly carboxylic acids, which are used in clinical applications and the manufacture of medical instruments such as suture, bonded tools, slow-release agents, and insecticides. Lactic acid with an asymmetric carbon atom has two forms of (+) L, and (-) D isomer (Witkin et al., 2013). L (+) Lactic Acid is a biological isomer that is naturally present in the human body. Because of the biological significance of this form of lactic acid, its production has always been targeted by researchers (Schubert et al., 2011). Since the human body lacks the enzyme D- lactate dehydrogenase, the high consumption of lactic acid D (-) isomer and its accumulation in the body can be a health hazard. For this reason, the presence of lactic acid D (-) isomer in the food and pharmaceutical industry is not appropriate, and the WHO limits its use. Nowadays L-(+) isomer is widely used in the pharmaceutical industry, intermediate medications, disintegrating polymers, and the manufacture of medical equipment (Witkin et al., 2013). This acid (with D and L isomers and a mixture of racemic isomers) is used to create acidic conditions in a variety of foods, such as dairy products. In addition to the sensory properties formed by the addition of lactic acid, inhibition against harmful and pathogenic microorganisms is among the most important reasons for its attempts to apply (Lassprilla et al., 2012). Since the production of pure optical isomers is not possible with the chemical method, the use of fermentation methods in its production has increased today. As biological production of lactic acid accounts for more than half of global production (Rasal et al., 2010). Probiotics are living microorganisms that have important characteristics such as Viability, metabolic activity, and positive effects on the health of consumers (Ohland and Mac Naughton, 2010). For this purpose, the number of live probiotics should be at least about 10^6- 10^7 colonies per gram of product (Joint, 2002).

Lactic acid is almost the only metabolite produced by lactic acid bacteria and about 90% of the total lactic acid in the world by fermentation with lactic acid bacteria (Abdel- Rahman et al., 2013). Lactic acid bacteria are among the most-producing lactic acid microorganisms. These bacteria produce lactic acid as the major product of sugar fermentation via lactate dehydrogenase activity (Martinez et al., 2013). Significant new expansions have been made in the research of lactic acid bacteria in the areas of multidrug resistance, bacteriocins, osmoregulation, autolysins, and bacteriophages. These have opened new potential applications for these microorganisms in various industries (Witkin etal., 2013). Fermentation increase shelf life and enhanced safety of foods by the use of natural or controlled microbiota and antimicrobial compounds is an approach to the problem of food preservation that has gained increasing attention in recent years. Consequently, individual lactic acid bacteria (LAB) have demonstrated antimicrobial properties which derive from the production of one or more antimicrobial active metabolites such as organic acids (lactic and acetic), hydrogen peroxide, and antimicrobial peptides (bacteriocins) (Rasal et al., 2010). On the other hand, microbially produced lactic acid is usually a mixture of the L (+) - and D (–)-forms. The common producing lactic acid microorganisms are probiotic bacteria belong to the genus *Lactobacillus* and *Bifidobacterium. Lactobacillus casei* is a gram-positive bacterium-free of spores (Jespersen et al., 2015). It has antioxidant properties, high resistance to fermented milk products and antimicrobial activity (Nag and Das, 2013). *Lactobacillus acidophilus* is a kind of gram-positive bacterium, fermenting sugars into lactic acid, and grows at rather low pH values (below pH 5.0) and has an optimum growth temperature of around 37 °C (Yadav et al., 2007). *Lb. acidophilus* occurs commonly in the human

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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and animal gastrointestinal tract and mouth (Bedani et al., 2013). To enhance the survival and viability of probiotics in food and the effects of nutrition and health more often in foods containing probiotics, combinations of prebiotic used in this way, It is well known to a synbiotic (Jespersen et al., 2015). Inulin is one of the most important dietary fiber and prebiotic used in foods. This combination of fructan is indigestible that enhances calcium absorption and thereby improve bone mineral density, reduce cholesterol levels in blood serum (Dewulf et al., 2013), increasing the viability of probiotics And stimulate their growth and activity (Muzzarelli et al., 2012). One of the dairy products that has the potential to preserve and transport probiotics is cream. The cream is part of the milk that is relatively rich in milk fat content and has been isolated by milk creaming and converted into the fat-free emulsion in milk (Jespersen et al., 2015). By considering the importance of the use of probiotics in dairy products and the decisive role of prebiotics in stimulating the growth and activity of probiotic and aspects such as increasing the quality and acceptability of the product to the consumer and enhance the nutritional value of food, in this study, the effect of Probiotic bacteria (*Lactobacillus acidophilus* and *Lactobacillus casei*) and inulin had been evaluated on some of the chemical properties and viability of probiotics in sterile cream. In addition the production of L- and D-lactic acid isomers were evaluated during fermentation of cream by *Lactobacillus* spp (*Lactobacillus acidophilus* and *Lactobacillus casei*). The influence of lacto-fermentation on some of the chemical properties and viability of probiotics of fermented cream samples are also investigated.

Materials and methods

Commercial UHT cream samples containing 30% fat for this study was obtained from Pegah dairy plant Tehran, Iran. Inulin was used (Sigma -Aldrich, USA). Commercial single probiotic strains lyophilized culture of *Lactobacillus acidophilus* (La-5) and *Lactobacillus Casei* (*L*- 431) both were supplied from Chr. Hansen Horsholm, Denmark. Man Rogosa Sharpe (MRS) agar and Ringer Salt Pills were all prepared from Merck Company, Germany.

Preparation of probiotic strains

Each lyophilized Probiotic strain which was prepared using 1 g of lyophilized culture was inoculated into 100 ml of MRS broth medium and was incubated at 37 ° C for 6 to 8 hours to make probiotic culture. Finally, the resulting biomass was separated through a refrigerator centrifuge in 3000 × *g* for 10 min at 4 °C, washed with the sterile solution of 0.1% peptone water in two stages and stored at 4 °C for use in artificial inoculation. The cells were placed in a suitable volume of sterile distilled water, resulting in a concentration equivalent to 10^8 cfu/g (Dong et al., 2012).

Production of fermented synbiotic cream

10% from prepared probiotic bacteria is inoculated to the UHT cream containing 0, 1.5 and 3% levels. Containers of inoculated creams are transferred to an incubator at 37 °C containing 5% of CO_2 and are incubated for 4 hours; then, After the fermentation, the cream samples were cooled down to 4 °C and stored at 4 °C during 30 days. A sweet cream was chosen as the control sample. Then, changes in viability, pH, acidity,

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and lactic acid enantiomers were evaluated after 1, 15, and 30 days of storage at 4 °C (Ravi et al., 2017).

Microbiological analysis

The viability of probiotic bacteria in fermented cream samples was assessed on the 1nd, 15nd and 30nd days of storage by plate count method, after 48h of anaerobic incubation at 37 °C. *Lb. acidophilus* and *Lb. case*i were counted on MRS agar modified with the addition of 0.15% (w/v) of bile (MRS-Bile). The cell concentrations were expressed as the logarithm of colony-forming units per gram of product (log cfu /g) (Ravi et al., 2017).

pH and acidification measurements of cream

pH changes in cream samples during storage were measured the pH changes during fermentation were monitored every 1 h by a digital pH meter (pH meter, 730, WTW, Germany). The pH meter was calibrated using standard buffer solutions (Merck) at pH 4.0 and 7.0 (Beheshtipour et al., 2012).

Acidity of samples was determined according to the general titration method and based on lactic acid percentage. 10 mL of sample was titrated against 0.1 N NaOH in presence of phenolphthalein (James, 2013).

HPLC characterization

Reagents and apparatus

L-(+) Lithium lactate was reagent grade and supplied from Merck (Darmstadt, Germany). Lithium D-lactate was provided from Sigma (Steinheim, Germany) and was used without any further purification. These compounds were used for preparing stock and standard solutions. DL-Malic acid was reagent grade used as internal standard and supplied from Merck (Darmstadt, Germany). All solvents used in chromatography analyses were HPLC grade and provided by Merck. The lactic acid stock solutions (1000 μg L^{-1}) were prepared weekly and stored at 4◦C. Intermediate standard solution of 10 μg L^{-1} was prepared weekly by dilution of stock solutions with water. Working standard solutions of different concentrations were prepared daily by diluting the intermediate standard solution with mobile phase solution.

Determination of L, D-lactic acid

The chromatographic analysis was carried out in a Dionex high-performance liquid chromatography (Munchen, Germany, and Sunnyvale, Ca, USA) equipped with a Dionex P680 pump and Dionex reodyne injection valve. The separations were performed with a Chiracell, OD-RH, 150 × 4.6 mm, 5 μm analytical chiral column with the mobile phase acetonitrile as solvent A and 0.2 M phosphate buffer (the pH was adjusted to 2.0 with H3PO4, solvent B) with 40:60 v/v ratio of A: B in an isocratic elution program. The flow rate and injection volume were set at 0.7 ml min^{-1} and 25 μl, respectively. The column temperature was adjusted to 30 ºC. Ultraviolet detection of lactic acids was performed at 215 nm with a Dionex UVD 170U UV-Vis detection system. HPLC data were acquired and processed using a PC and Chromeleon Chromatogram Manager software (Version 6.6 Dionex). The pH of solutions was

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adjusted using a model 630 digital Metrohm pH meter (Herisau, Switzerland) equipped with a combined glass calomel electrode (Codari et al., 2012).

Statistical analysis

All experiments were replicated three times and for analysis of quantitative data in this research, the analysis of variance (ANOVA) of a factorial experiment is used in a completely random design (CRD), was designed with the product type and the storage time as the main factors. Results were expressed as the mean values ± standard deviations. Besides, for a comparison between averages, Duncan's multiple range test (DMRT) is used at the confidence interval of 95% ($p < 0.05$) significance levels. Software Microsoft Excel 2010 is used for organization of data and drawing of diagrams. SPSS version 20 (SPSS, IBM, Chicago, IL, USA) is also applied for parametric statistical analysis.

Results and discussion

The viability of probiotic bacteria in fermented cream

Survival of probiotics after they were added to the cream and fermentation was evaluated, and the results indicated that the cell concentrations of two probiotic bacteria in cream reached above 10^9 cfu /g by the end of the fermentation and the number of bacteria decreased by 1.1 log cycles during storage.

Figure 1 shows the changes of probiotic bacteria viability in cream samples during the 30 day storage at 4 °C. According to the results of this research, two strains of probiotic bacteria showed a slight similar decline in viability within the 15 days period and by 30 days. However, *Lb. acidophilus* had greater viable numbers than *Lb. casei.* One of the reasons is the high resistance *Lb. acidophilus* to acid condition and its ability to intake nutrients (Gebara et al., 2013). The variability the number of probiotics in cream samples resulted in a reduction of 0.6 log cycle for *Lb. acidophilu*s, and 1 log cycle for *Lb. casei*. Storage time had a significant effect on viable cell counts in cream samples ($P < 0.05$) of probiotic bacteria in cream samples during the 30-days storage at 4 °C. After 15 days of refrigerated storage, the concentration of bacteria increased and the cream had the highest viability of *Lb. acidophilus* was recognized at 4 °C *(Fig. 1),* because according to, when free amino acids are low, lactic acid bacteria that are linked to the proteolytic system By providing adequate hydrolysis of proteins. This action causes a sudden increase in microorganisms, resulting in a sudden increase in bacterial growth, which increases the concentration of acids and decreases pH (Papadimitrious et al., 2016). Synbiotic cream samples (coincident with inulin, probiotics), due to the inulin inducing effect, the metabolic activity of the probiotics was significantly increased and, as a result, the acidification capacity of this Strains will increase (Bedani et al., 2013). These conditions usually lead to a reduction in the number of probiotic bacteria during the storage. The number of bacteria in the absence of inulin (0%) showed a significant difference with these numbers at 1.5 and 3% ($p < 0.05$). Studies have shown that inulin can stimulate the growth of probiotic bacteria (Wilson and Whelan, 2017). In a study, the effect of adding inulin to probiotic soybean yogurt was studied. The increase in the number of probiotic bacteria in the final product (as compared to control) was the result of this study. On the other hand, probiotic bacteria were able to grow and increase more in vitro with inulin, and ultimately, they were

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more viable (Krasaekkoopt and Watcharapoka, 2014). As a result, during the fermentation process, more of these microorganisms were multiplied and continued to function during cream storage.

Probiotics survival in-vivo and in-vitro systems are the most critical factor for the application of these microorganisms. Survival of probiotic bacteria in probiotic fermented milk depends on factors such as the species, the culture conditions, the chemical composition of the fermentation medium (e.g., carbohydrate), the final acidity, the milk solids, the nutrients present, the dissolved oxygen (to Specific for *Bifidobacterium*), inoculation levels and temperature, fermentation time and storage temperature (Ohland and Macnaughton, 2010). There was a > 10^8 CFU/g of probiotic bacteria still present after thirty days of storage (*Fig. 1*). Thus fermented cream can act as a suitable matrix for probiotic bacteria. In a related work, the cell viability of *Bifidobacterium lactis* in butter was evaluated during long-term storage using plate count and fluorescence techniques. They reported that butter could serve as an additional source of probiotic delivery and give to the diversity of the probiotic market (Olszewska et al., 2012).

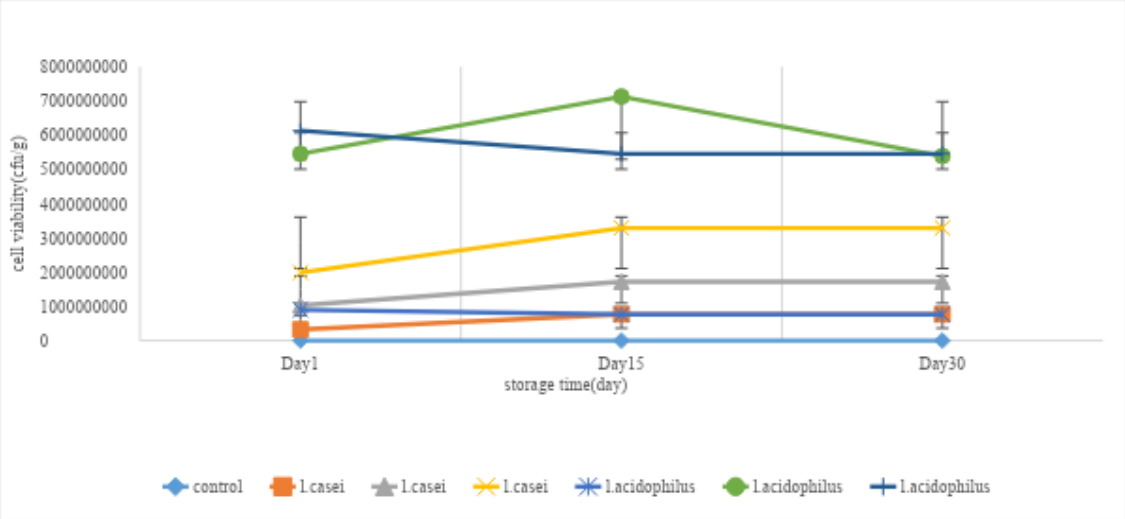

Figure 1. *Survival of Lactobacillus casei and Lactobacillus acidophilus (log cfu/g) incorporated into cream during 30 days of storage at 4 °C*

Measurement of the acidification profile during fermentation

The variations in pH values and titrable acidity of probiotic cream samples during fermentation are given in *Figures 2* and *3*. There were significant differences in pH values among the samples depending on the probiotic bacteria used ($P < 0.05$). The initial pH of the cream was 6.70 at 0 h and decreased gradually throughout the fermentation period to 6.14 and 6.10 for samples *Lb. casei*, and *Lb. acidophilus*, respectively. During the fermentation process, the pH of sample concerning the presence of probiotics (*Lb. Casei* and *Lb. acidophilus*) gradually was decreased due to acidifying the probiotics. This result is undoubtedly due to the metabolic activities of bacteria and the production of lactic acid in the product. This result is confirmed by numerous studies (Tachedjian et al., 2017). Inulin has a remarkable solubility than other prebiotics and fibers, and it has a very high solubility in milk and fermentation products

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(Vandeputte et al., 2017). Therefore, in the presence of this combination of bacteria, the bacteria are better and, with the increase in its amount, the number of bacteria is reduced. Of course, the acidic conditions also have a negative effect on the survival of probiotics, and another reason is to reduce pH.

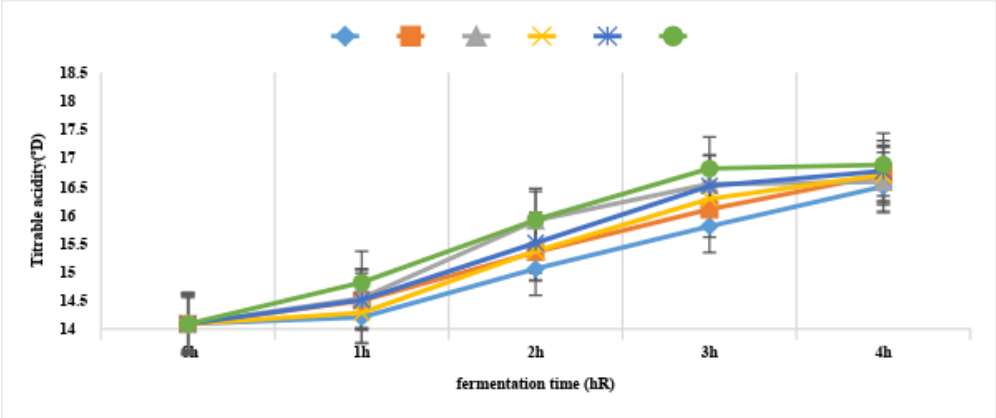

Figure 2. *Variation trend in titrable acidity during fermentation of synbiotic cream samples*

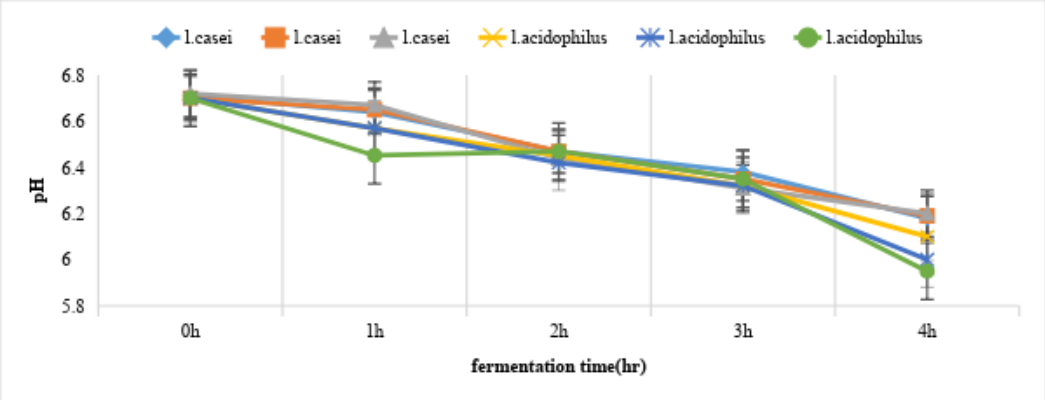

Figure 3. *Variation trend in pH value during fermentation of synbiotic cream samples*

Lactic acid production is monitored via pH and titrable acidity measurements, allowing accurate determination of the completion time of fermentation, and improving the final product's sensorial and textural quality. The variations in pH values and titrable acidity of probiotic cream samples during fermentation are given in *Figures 2* and *3*. There were significant differences in pH and titrable acidity values among the samples depending on the probiotic bacteria used ($P < 0.05$). The acidification profile was obtained for each type of cream samples and the distinct experimental conditions. *Figure 3* showed that the pH decreased for the *Lb. casei* samples is slower when compared to the *Lb. acidophilus* samples. The acidification profile exhibited three distinct and noticeable phases. Initially, a slight decrease in pH and increase titrable acidity was shown, followed by the second period where the pH values decreased faster and a steep curve inclination was detected. Finally, the third acidification phase was characterized by a tendency to stabilization, with little variation in the pH and acidity levels. This behavior was previously reported by researchers. They are justified by

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several chemical and biochemical reactions that take place during the lactic acidification process (Olivera et al., 2016).

pH changes during the storage of synbiotic cream

pH is one of the essential factors in fermentation, production and maintenance of fermented food (Zare et al., 2012). pH of the food matrix, is a significant factor that defines the stability of probiotics during storage (Delgado et al., 2017). The fermentation process had a significant effect on the pH of the cream samples so that during the fermentation, the pH of the cream samples decreased significantly. The pH changes of probiotic cream samples were found to decrease from 6.70 to 5.70 over 30 days of cold storage (*Table 1*). The addition of probiotic bacteria to cream formulation resulted in a significant decrease in pH, and the effect of *Lb. acidophilus* on this reduction was significantly higher than *Lb. casei,* because of *Lb. acidophilus* showed to be more acid-tolerant than *Lb. casei*. ($p < 0.05$) Moreover, *Lb. casei* was shown to be far slower for *Lb. acidophilus*. It is known that *Lb. casei* as compared with *Lb. acidophilus*, are less capable of acid production and reduction of pH levels (*Table 1*). The pH of the cream containing probiotic bacteria changed from 6.70 to the final pH was 5.7 during storage (*Table 1*). The cream containing the probiotic bacteria had an initial pH of 6.14 after fermentation and changed to a final pH of 5.7 at the end of the storage period (*Table 1*).

According to the findings of (Ekinci et al., 2008), final pH values of cream samples fortified with probiotic bacteria and plant oils were between 5.02 and 4.28 to obtain the desired viability of a fermented dairy product, maintaining the pH above 4.0 during the storage is recommended. In this study, the role of fermentation process along with the presence of different levels of inulin on pH reduction in fermentative and inulin-containing samples is debatable. Several studies have shown that inulin increases the stimulation and growth of probiotic bacteria and increases the metabolic activity of probiotics (Amirdivani and Baba, 2011). Therefore, the pH values will decrease with the passage of time, which will be reduced to the prebiotic effect of inulin (Bedani et al., 2013). This combination stimulates the growth and activity of probiotic bacteria in fermentation products, increases the production of lactic acid, increases acidity and subsequently decreases pH. The use of inulin with *bifidobacterium* increases the production of short-chain fatty acids (SCFA) and decreases pH (Pătruică and Mot, 2012).

Table 1. *pH changes of synbiotic cream samples during storage*

Probiotics	Sample type	Days		
		1	15	30
Lactobacillus casei	Blank (without bacteria)	$6.70 \pm .01^{A,a}$	$6.70 \pm .01^{A,a}$	$6.70 \pm .03^{A,a}$
	0% inulin	$6.14 \pm .01^{A,b}$	$6 .00 \pm .01^{B,d}$	$5.90 \pm .04^{C,f}$
	1.5% inulin	$6.10 \pm .01^{A,c}$	$6.00 \pm .02^{B,d}$	$5.90 \pm .01^{C,f}$
	3% inulin	$6.10 \pm .02^{A,c}$	$6.00 \pm .02^{B,d}$	$5.87 \pm .04^{C,g}$
Lactobacillus acidophilus	0% inulin	$6.10 \pm .02^{A,c}$	$6.00 \pm .02^{B,d}$	$5.81 \pm .01^{Ch}$
	1.5% inulin	$6.10 \pm .05^{A,c}$	$6.00 \pm .02^{B,d}$	$5.80 \pm .01^{C,h}$
	3% inulin	$6.00 \pm .01^{A,d}$	$5.93 \pm .02^{B,e}$	$5.70 \pm .05^{C,i}$

a, b: Different superscript lowercase letters denote significant differences ($P < 0.05$) between different three probiotic bacteria, A, B: Different superscripts capital letters denote significant differences ($P < 0.05$) between different storage time. Note. Means with different letter in a column are significantly different ($p < 0 .05$).

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Titrable acidity changes during the storage of synbiotic cream

The most important thing that occurs during fermentation in fermented dairy products is the production of lactic acid (PKa = 3.86), along with other compounds, thereby increasing the acidity of the final product. According to the *Figure 4,* titrable acidity of cream samples increased during storage, and significant differences were Samples with high titrable acidity ($P < 0.05$).

The titrable acidity was strictly linked to the pH of a sample. The fermentation of probiotic bacteria in cream resulted in slightly higher titrable acidity values compared with the control. During storage, the titrable acidity increased somewhat in all probiotic cream samples. The higher level of titrable acidity in cream samples containing *Lb. acidophilus* might be attributed to the relatively higher cell counts, which leads to higher lactic acid production. Previous studies have shown that lactic acid production is affected by the quantity of probiotics present (Olivera et al., 2009). The nature of the saccharolytic fermentation of probiotics requires that prebiotics, including inulin. Therefore, the hydrolysis of their chains requires sufficient time, and there will be enough time to storage cream samples. As a result, the inulin stimulation process and the growth and activity of *Lb. acidophilus* and *Lb. casei* will be well done over time (Al-sheraji et al., 2013). The type and percentage of inoculation of starter culture, the initial composition (solid matter and enrichment) and the incubation temperature are three important factors in bacterial growth and acidification of inoculum milk in fermentation (Settachaimongkon et al., 2014).

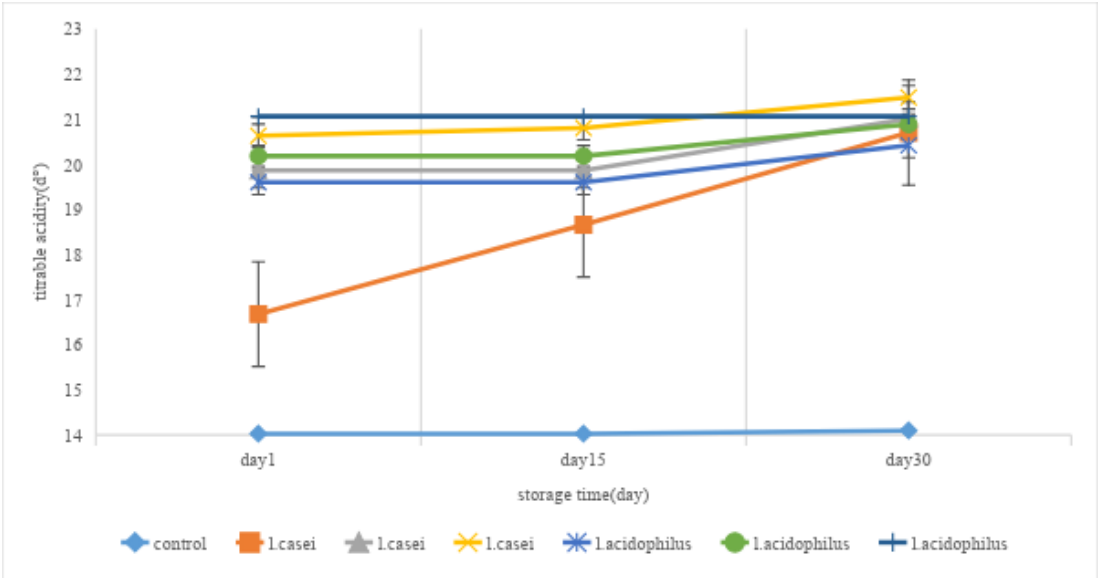

Figure 4. *Titrable acidity changes of synbiotic cream samples during storage*

The changes of optical isomer of (L-and-D) lactic acid during lacto-fermentation of synbiotic cream

Lactic acid is produced as a significant source of fermentation by the enzyme lactate dehydrogenase in probiotics, which is an essential chemical compound in the food and pharmaceutical industry (Martinez et al., 2013). Numerous organisms produce lactic acid by Fermentation, but most industrially significant strains are from *Lactobacillus*

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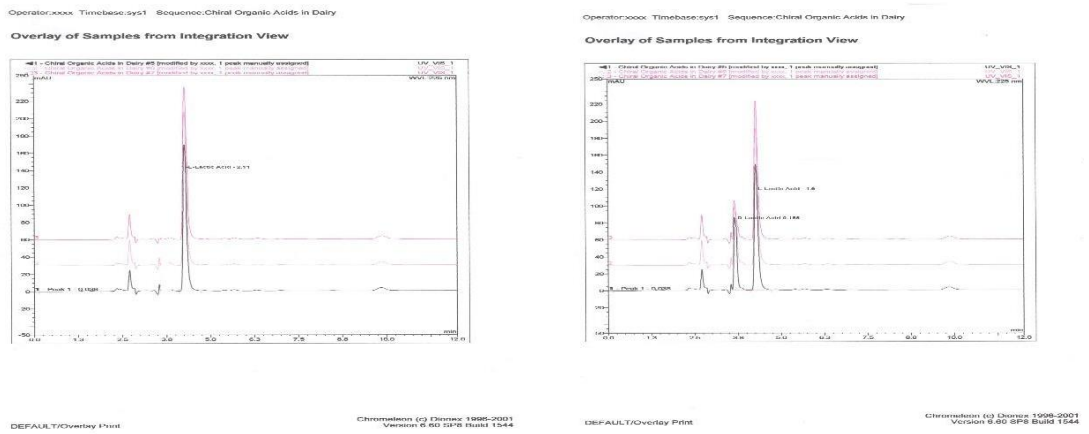
genus (Holzapfel and Wood, 2012). Lactic acid (with D and L isomers and a mixture of racemic isomers) is used to create acidic conditions in a variety of foods, such as dairy products (Martinez et al., 2013). Lactic acid optical isomers were examined by HPLC. The results of optical isomers of lactic acid produced by two strains are shown in *Figure 5*. Results showed that all analyzed sample containing *Lb. acidophilus* produced the mixture of L- and D-lactic acid (*Fig. 5*). In most of the samples, the amount of lactic acid isomer was gradually increased during the 30-day maintenance period. By increasing the shelf life of cream samples from the fifteenth to the thirteenth day, the production of lactic acid isomer was increased and there was a significant difference between these two days ($p < 0.05$), and on the final day, the highest amount of isomeric D -Lactic acid was produced (*Fig. 6*) although there was no significant difference between the mean values of the isomers on the fifteenth day and the 30th day. Using *Lb. acidophilus* as a probiotic strain, the amount of D-lactic acid isomer is significantly increased; by contrast, *Lb. casei* did not have the ability to produce this kind of isomer in this research. There was a significant difference between two fermentation treatments with *Lb. acidophilus* and *Lb. casei* ($p < 0.05$). The difference between the treatments on the amount of L-lactic acid isomer was statistically significant ($p < 0.05$). The addition of probiotic bacteria to cream formulation resulted in a significant increase in the amount of this isomer and the effect of *Lb. casei* was significantly higher than *Lb. acidophilus* ($p < 0.05$) (*Fig. 6*). The highest levels of both forms were determined in *Lb. casei* and *Lb. acidophilus* samples (2.148 ± 0.03) and (1.922 ± 0.11) g L^{-1}, respectively.



a **b**

Figure 5. *Separation of D- and L-lactic acid on a chiral column. (a) Chromatogram of the enantiomers of lactic acid and the internal standard of sample containing lactobacillus casei.(b) Chromatogram of the enantiomers of lactic acid and the internal standard of sample containing lactobacillus acidophilus*

The main products of the metabolism are lactose, L or D-lactate or the racemic mixture of both of them. *Lb. acidophilus* has the ability to produce a mixture of racemic (DL). Therefore, this probiotic strain produces amounts of D-and L lactic acid in the optimum conditions *(Fig. 7)*. The probiotic bacteria obtain their energy through the metabolism of sugars during the fermentation process and produce lactic acid as the main and final product (Martinez et al., 2013). The metabolism of lactose to lactate (lactic acid) is essential for the production of fermented milk products such as cheese

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and cream. Based on the type of initiator, lactose is metabolized by phosphoketalose due to glycolytic pathways (Holzapfel and Wood, 2012).

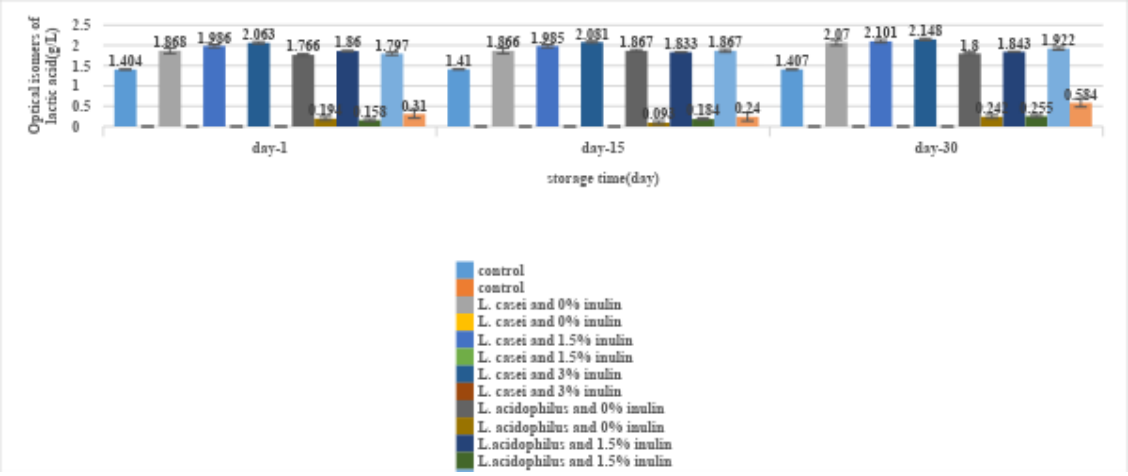

Figure 6. *Variation trend in optical isomers of lactic acid during storage of synbiotic cream samples*

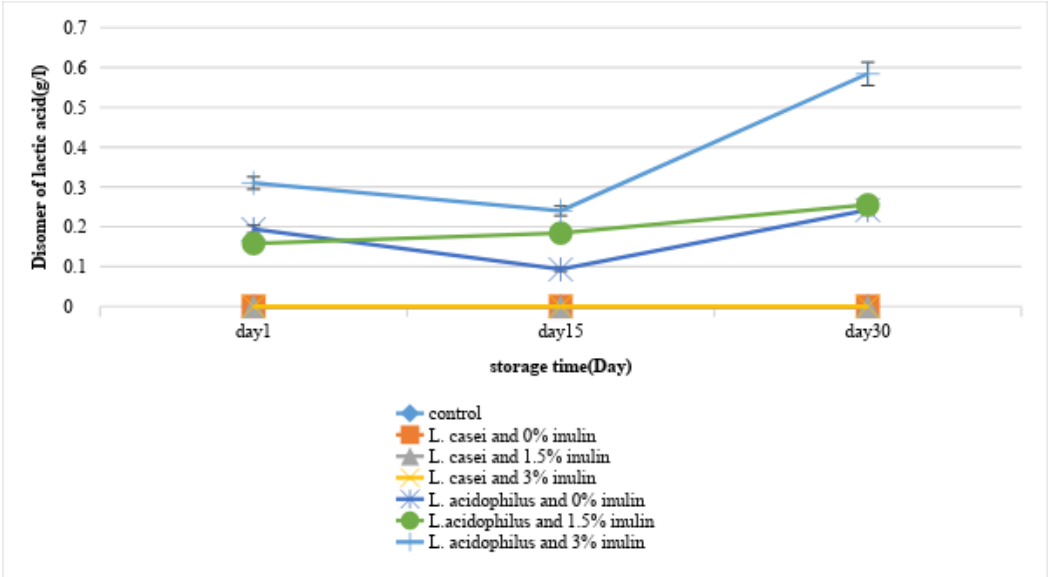

Figure 7. *Variation trend in D-isomer value during storage of synbiotic cream samples*

L (+)-lactic acid production by *Lb. casei* occurs in during fermentation and storage time. By comparing two strains, *Lb. casei* 431 produced high concentration of L (+)-lactic acid (*Fig. 7*). This confirmed experimental work of Martinez et al. (2013). They compared several strains of *Lb. casei* and reported that lactic acid produced by *Lb. casei* subsp *casei* were 97.6% optically L (+) form. In this research, high purity of L (+) -lactic acid was produced by C3 sample (containing *Lb casei* and 3%inulin) and *Lb. acidophilus* was produced both optical isomers of lactic acid (*Fig. 8*).

Lb. acidophilus strains were produced combination of both optical isomers of lactic acid as shown in *Figure 8*. On the last day, the lowest and the highest levels of lactic

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acid D-isomer were, in the control sample (0 g/l) and the sample containing *Lb. acidophilus* with 3% inulin (0.584 g/l), respectively (*Fig. 8*). The ability of *Lb. acidophilus* strain has been demonstrated to produce a mixture of racemic or DL lactic acid isomers (mainly L-isomer) (Thamacharoensuk, 2015). In the study, the amount of lactic acid isomer is produced by *Lb. acidophilus* (L. NCFM) is 33.8%, *Lb. acidophilus* (ATCC4356) is equal to 21.5% and *Lb. acidophilus* (ATCC393) was reported to be 6.2% (Molinaro, 2013). In fermentation process, carbohydrates are metabolized to lactic acid and other compounds, depending on the microorganisms. Lactic acid Fermentation is one of the characteristics of lactic acid bacteria that belong to the *lactobacillaceae*. The Fermentative bacteria initially catalyzed the sugars through the pathway of glycolysis to pyruvate, and subsequently, pyruvate produced by the NADH coenzyme produced by the reaction of glyceraldehyde phosphate dehydrogenase to the glycolysis pathway was restored to lactate. This reaction provides a natural cycle for the use and generation of NAD +, which provides relative oxidation of carbohydrates for the release of energy in the form of ATP. Recovery of pyruvate and lactate is done by the dehydrogenase. The enzyme is in the form of Stereospecific in nature and hence leads to the production of lactate, which is either (-) D or (+) L, and this is dependent on the type of microbial strain. There are also certain strains that synthesize both lactate (Niu and Guo, 2014). As reported by (Molinaro, 2013) and (Olivera, 2016), *Lactobacillus amylophilus, Lb. bavaricus, Lb. casei, Lb. maltaromicus and Lb. salivarius* predominantly yield the L-isomer. Strains such as *Lb. delbrueckii, Lb. jensenii* or *Lb. acidophilus* yield the D-lactic acid or mixtures of both forms. Lactic acid bacteria such as *Lb. pentosus, Lb. brevis* and *L. lactis* can ferment glucose to lactic acid homolactic fermentation. lactobacilli strains, *Lb. casei* subsp *casei* produced high concentration of L(+)-lactic acid with 98%purity.

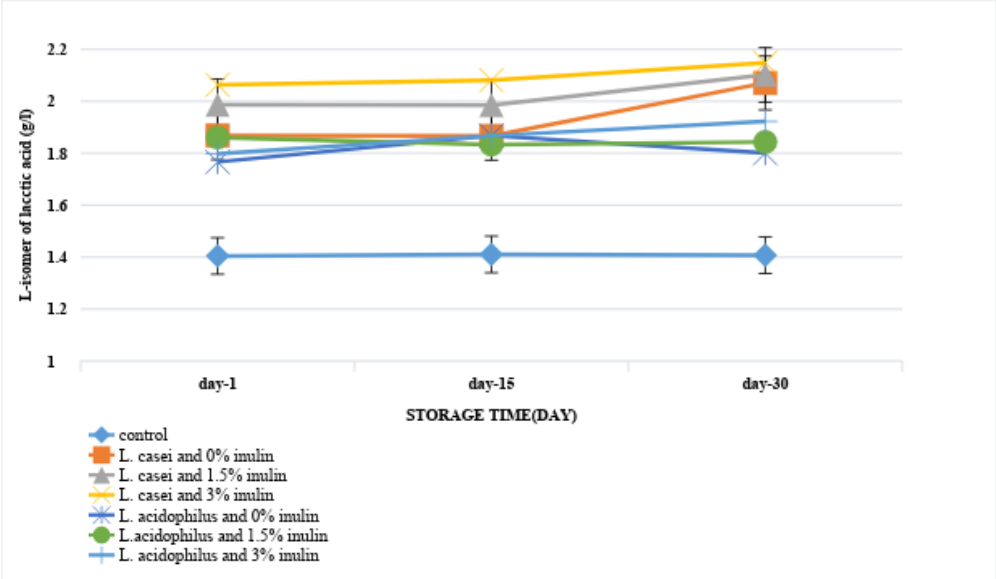

Figure 8. *Variation trend in L-isomer value during storage of synbiotic cream samples*

The use of a variety of fermentative microorganisms and their strains can affect the production of various types of lactic acid isomers. In particular, in the production of certain isomers of lactic acid, they use certain microorganisms. Cream fermentation has

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increased the amount of isomer L, which can be attributed to the production of this isomer by fermentative strains. The results in this regard contradict the results of (Okano et al., 2010) reports. In their studies, the amounts of lactic acid produced in fermented samples were less relevant than fermentation samples, which were linked to the decline in growth or loss of species producing this isomer.

Fermentation conditions such as temperature, pH, culture medium, dissolved oxygen content, and neutralizing agent type greatly affect the growth of lactobacillus bacteria and thus the production of lactic acid (Ercan and Demirci, 2015). The phosphorylation reactions carried out during fermentation play a major role in the production of ATP from these microorganisms. In fermented lactic acid bacteria, fermentation of hexoses is carried out by enzymes of the glycolysis (Embden–Meyerhof–Parnas) (Shiby and Mishra, 2013). One of the major enzymes in this pathway is aldolase, which routes the sugars into the metabolic pathway through the decomposition of fructose 1, 6 diphosphate into phosphate. The Embden–Meyerhof–Parnas produces both mole hexoses, 2 moles of pyruvate and 2 mole of ATP (Khalid, 2011). The resulting pyruvate is restored by the enzyme lactate dehydrogenase to l or di-lactate. More than 90% of the substrate is converted into lactic acid in the metabolism of bacteria-like fermentation (Adsul et al., 2011). Martinez (2013) showed that a number of *lactobacillus* strains, by increasing the concentration of dissolved oxygen in the culture medium, the heterogeneous fermentation mechanism replaces the hemo fermentation mechanism, which significantly increases the production of lactic acid D-isomer.

The creation of a suitable substrate for the production of more lactic acid by fermentation has yielded some of the expected results. Inulin can play a role in the production of lactic acid. Inulin, added to supplemented foods, indirectly increases their beneficial and beneficial effects, as these compounds and related compounds (oligophyric enzymes) are indigestible to humans and are used to intestinal bacteria (Schaafsma and Slavin, 2015). In fermented foods, these compounds also increase the process of fermentation and production of lactic acid by providing energy for probiotic bacteria (Gaspar et al., 2013).

Between the amounts of lactic acid isomer is produced by fermentation with *Lb. acidophilus* containing different levels of inulin (0, 1.5 and 3%) compared to *Lb. casei*, there was a significant difference ($p < 0.05$). The highest production rate for fermentation with *Lb. acidophilus* and 3% inulin content (*Fig. 9*). In samples containing *Lb. casei*, with increasing inulin level, the amount of lactic acid isomer increased significantly and the difference between the three treatments was significant ($p < 0.05$), while in samples containing *Lb. acidophilus* was irregular and the highest mean was observed in the treatment containing 1.5% inulin, which was significantly different with other treatments containing this strain ($p < 0.05$). In general, the treatment contains *Lb. casei* and 3% inulin, had the highest average production of this isomer (*Fig. 9*). The results obtained in this study are in line with the findings of the study, because with the presence of inulin and an increase in its level, as a rule, the amounts of D -and L-lactic acid isomers increased. In the case of in vivo prebiotic effect on probiotics in this study, the following sections were presented. There are reports that *Lb. casei* and *Lb. Plantarum* in a medium containing sugar, such as sucrose, has a higher lactic acid content than strain *Lb. brevis* produce.

Microorganisms that can produce lactic acid isomers include Lactobacillus delbrucki subsp. Bulgaricus, Lactobacillus rhamnosus, Lactococcus lactis, Lactobacillus plantarum and Lactobacillus amilophilus (Gaspar et al., 2013).

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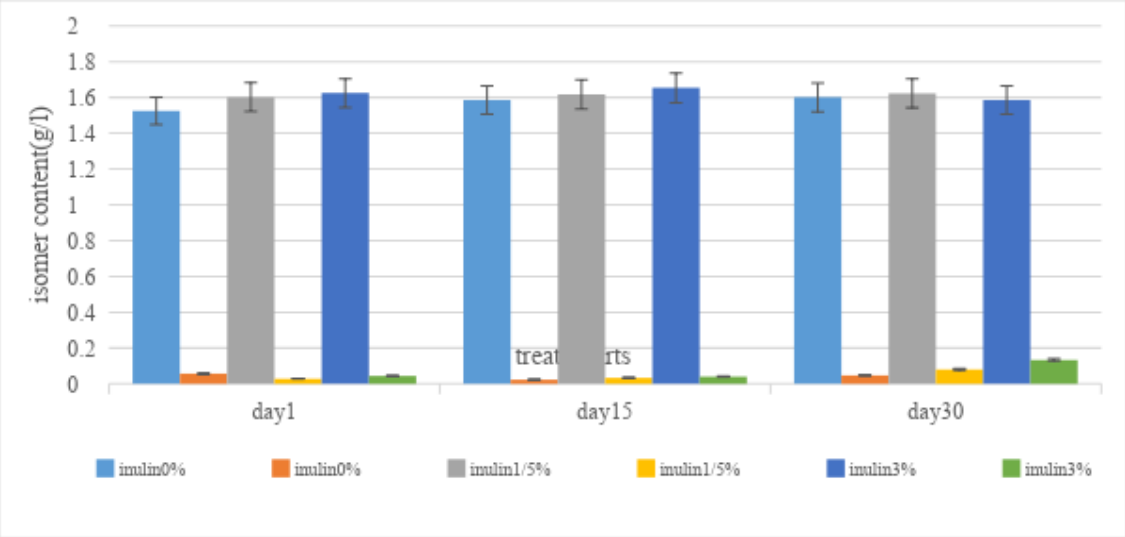

Figure 9. *The influence of inulin concentration on D-isomer and L-isomer formation during storage*

Therefore, determining the reason for the difference between the isomers produced in the fermentation of the cream requires more experiments, such as determining the species, which can be attributed to the production of this isomer more. Cream fermentation has increased the amount of isomer in fermentation samples by fermentative strains. The type of microorganism, the type of fermentation, the nutritional composition used, temperature and pH are among the factors that influence the type and amount of each isomer production (Kondo and Miura, 2010).

Conclusion

We worked to evaluate the viability of two probiotic strains in cream. In general, this study represented that the viability of *Lb. casei* and *Lb. acidophilus* can be significantly enhanced in cream. ($p > 0.05$) After 30 days of storage at 4 °C, the number of bacteria was within the international standards. Moreover, pH and titrable acidity evaluation of the cream suggested that addition of the probiotics used in this research had a considerable effect on the pH and acidity of the product. The D- and L- lactic acid in a cream determined with the HPLC-method described. All examined samples containing *Lb. acidophilus* produced the mixture of L- and D-lactic acid, the latter isomer being at a lower level. Because of the potential toxicity of D-lactic acid, cream samples prepared using culture of *Lb. casei* in all cases were found safe than *Lb. acidophilus* ($p > 0.05$). Fermentation of cream with selected lactic acid bacteria such as *Lb. casei* resulted in a greater L-lactic acid bioavailability accompanied by an increase in inulin content. According to our results, *Lb. acidophilus* and *Lb. casei* may be useful for preservation of cream, which could be recommended as a way of obtaining more functional value products.

Acknowledgements. This research was supported by Reference Food and Drug Control Laboratories, Tehran, Iran. Hereby, I wish to express my appreciation to Food Science Research Center, Islamic Azad University, Qazvin, Iran and Kimia Novin Azma Particular Laboratory (Techno Azma for their contribution in this study.

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APPENDIX

Basic field data

day	treat	fermentation	probiotic	inulin	incaps	rep	D- ISOMER(g/l)	L -ISOMER(g/l)	acidity(g/l)	pH	acidity	survival
1	check	0	0	0	0	1	0	1,41	1,41	6,7	14,1	0
1	check	0	0	0	0	2	0	1,4	1,4	6,7	14	0
1	check	0	0	0	0	3	0	1,4	1,4	6,7	14	0
1	treatmented	0	c	0	f	1	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	c	0	f	2	0	1,4	1,4	6,7	14	180000000
1	treatmented	0	c	0	f	3	0	1,4	1,4	6,7	14	180000000
1	treatmented	0	c	0	i	1	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	c	0	i	2	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	c	0	i	3	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	c	1,5	f	1	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	c	1,5	f	2	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	c	1,5	f	3	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	c	1,5	i	1	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	c	1,5	i	2	0	1,4	1,4	6,7	14	180000000
1	treatmented	0	c	1,5	i	3	0	1,4	1,4	6,7	14	180000000
1	treatmented	0	c	3	f	1	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	c	3	f	2	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	c	3	f	3	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	c	3	i	1	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	c	3	i	2	0	1,4	1,4	6,7	14	180000000
1	treatmented	0	c	3	i	3	0	1,4	1,4	6,7	14	180000000
1	treatmented	0	a	0	f	1	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	a	0	f	2	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	a	0	f	3	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	a	0	i	1	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	a	0	i	2	0	1,4	1,4	6,7	14	180000000
1	treatmented	0	a	0	i	3	0	1,4	1,4	6,7	14	180000000
1	treatmented	0	a	1,5	f	1	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	a	1,5	f	2	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	a	1,5	f	3	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	a	1,5	i	1	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	a	1,5	i	2	0	1,4	1,4	6,7	14	180000000
1	treatmented	0	a	1,5	i	3	0	1,4	1,4	6,7	14	180000000
1	treatmented	0	a	3	f	1	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	a	3	f	2	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	a	3	f	3	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	a	3	i	1	0	1,41	1,41	6,7	14,1	180000000
1	treatmented	0	a	3	i	2	0	1,4	1,4	6,7	14	180000000
1	treatmented	0	a	3	i	3	0	1,4	1,4	6,7	14	180000000
1	treatmented	1	c	0	f	1	0	1,874	1,874	6,14	18,74	7800000000
1	treatmented	1	c	0	f	2	0	1,864	1,864	6,14	18,64	1180000000
1	treatmented	1	c	0	f	3	0	1,867	1,867	6,14	18,67	1300000000
1	treatmented	1	c	0	i	1	0	1,67	1,67	6,4	16,7	270000000
1	treatmented	1	c	0	i	2	0	1,6	1,6	6,4	16	470000000
1	treatmented	1	c	0	i	3	0	1,647	1,647	6,4	16,47	230000000
1	treatmented	1	c	1,5	f	1	0	1,933	1,933	6,1	19,33	17000000000
1	treatmented	1	c	1,5	f	2	0	1,993	1,993	6,1	19,93	1300000000
1	treatmented	1	c	1,5	f	3	0	2,031	2,031	6,1	20,31	7800000000
1	treatmented	1	c	1,5	i	1	0	1,67	1,67	6,3	16,7	170000000
1	treatmented	1	c	1,5	i	2	0	1,6	1,6	6,3	16	2700000000
1	treatmented	1	c	1,5	i	3	0	1,647	1,647	6,3	16,47	230000000
1	treatmented	1	c	3	f	1	0	2,03	2,03	6,1	20,3	31000000000
1	treatmented	1	c	3	f	2	0	2,08	2,08	6,1	20,8	37800000000
1	treatmented	1	c	3	f	3	0	2,08	2,08	6,1	20,8	8300000000
1	treatmented	1	c	3	i	1	0	1,77	1,77	6,3	17,7	990000000
1	treatmented	1	c	3	i	2	0	1,7	1,7	6,3	17	4090000000
1	treatmented	1	c	3	i	3	0	1,747	1,747	6,3	17,47	890000000
1	treatmented	1	a	0	f	1	0,199	1,791	1,99	6,1	19,9	11100000000
1	treatmented	1	a	0	f	2	0,284	1,606	1,89	6,1	18,9	7340000000
1	treatmented	1	a	0	f	3	0,1	1,9	2	6,1	20	8700000000
1	treatmented	1	a	0	i	1	0,35	1,42	1,77	6,2	17,7	1700000000
1	treatmented	1	a	0	i	2	0,2	1,5	1,7	6,2	17	770000000
1	treatmented	1	a	0	i	3	0,407	1,34	1,747	6,2	17,47	230000000
1	treatmented	1	a	1,5	f	1	0,2	1,8	2	6,1	20	8700000000
1	treatmented	1	a	1,5	f	2	0,24	1,78	2,02	6,1	20,2	8800000000
1	treatmented	1	a	1,5	f	3	0,033	2	2,033	6,1	20,33	18000000000
1	treatmented	1	a	1,5	i	1	0	1,87	1,87	6,2	18,7	7800000000
1	treatmented	1	a	1,5	i	2	0,03	1,67	1,7	6,2	17	7800090000
1	treatmented	1	a	1,5	i	3	0,177	1,57	1,747	6,2	17,47	780000000
1	treatmented	1	a	3	f	1	0,437	1,64	2,077	6	20,77	91000000000
1	treatmented	1	a	3	f	2	0,415	1,75	2,165	6	21,65	3780000000

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1606_74477466

1	treatmented	1	a	3	f	3	0,077	2	2,077	6	20,77	8300000000
1	treatmented	1	a	3	i	1	0,07	1,8	1,87	6,2	18,7	9800000000
1	treatmented	1	a	3	i	2	0	1,8	1,8	6,2	18	780000000
1	treatmented	1	a	3	i	3	0,047	1,7	1,747	6,2	17,47	7800000000
15	check	0	0	0	0	1	0	1,41	1,41	6,7	14,1	0
15	check	0	0	0	0	2	0	1,41	1,41	6,7	14	0
15	check	0	0	0	0	3	0	1,41	1,41	6,7	14	0
15	treatmented	0	c	0	f	1	0	1,45	1,45	6,6	14,1	280000000
15	treatmented	0	c	0	f	2	0	1,45	1,45	6,6	14	4680000000
15	treatmented	0	c	0	f	3	0	1,45	1,45	6,6	14	430000000
15	treatmented	0	c	0	i	1	0	1,461	1,461	6,7	14,2	180000000
15	treatmented	0	c	0	i	2	0	1,41	1,41	6,7	14,1	110000000
15	treatmented	0	c	0	i	3	0	1,415	1,415	6,7	14,1	130000000
15	treatmented	0	c	1,5	f	1	0	1,415	1,415	6,6	14,1	3800000000
15	treatmented	0	c	1,5	f	2	0	1,415	1,415	6,6	14,1	780000000
15	treatmented	0	c	1,5	f	3	0	1,415	1,415	6,6	14,1	530000000
15	treatmented	0	c	1,5	i	1	0	1,41	1,41	6,7	14,1	180000000
15	treatmented	0	c	1,5	i	2	0	1,4	1,4	6,7	14	1100000000
15	treatmented	0	c	1,5	i	3	0	1,4	1,4	6,7	14	130000000
15	treatmented	0	c	3	f	1	0	1,416	1,416	6,6	14,1	2800000000
15	treatmented	0	c	3	f	2	0	1,416	1,416	6,6	14,1	8800000000
15	treatmented	0	c	3	f	3	0	1,416	1,416	6,6	14,1	460000000
15	treatmented	0	c	3	i	1	0	1,41	1,41	6,7	14,1	180000000
15	treatmented	0	c	3	i	2	0	1,4	1,4	6,7	14	110000000
15	treatmented	0	c	3	i	3	0	1,47	1,47	6,7	14	130000000
15	treatmented	0	a	0	f	1	0,07	1,4	1,47	6,6	14,1	471000000
15	treatmented	0	a	0	f	2	0,08	1,39	1,47	6,6	14,1	5310000000
15	treatmented	0	a	0	f	3	-0,01	1,42	1,41	6,6	14,1	430000000
15	treatmented	0	a	0	i	1	0	1,41	1,41	6,7	14,1	180000000
15	treatmented	0	a	0	i	2	-0,01	1,41	1,4	6,7	14	180000000
15	treatmented	0	a	0	i	3	-0,01	1,41	1,4	6,7	14	180000000
15	treatmented	0	a	1,5	f	1	0,051	1,41	1,461	6,6	14,1	1800000000
15	treatmented	0	a	1,5	f	2	0,051	1,41	1,461	6,6	14,1	38000009000
15	treatmented	0	a	1,5	f	3	0,051	1,41	1,461	6,6	14,1	6130000000
15	treatmented	0	a	1,5	i	1	0	1,41	1,41	6,7	14,1	980000000
15	treatmented	0	a	1,5	i	2	-0,01	1,41	1,4	6,7	14	1800000000
15	treatmented	0	a	1,5	i	3	-0,01	1,41	1,4	6,7	14	1800000000
15	treatmented	0	a	3	f	1	0,06	1,41	1,47	6,5	14,1	2300000000
15	treatmented	0	a	3	f	2	0,06	1,41	1,47	6,5	14,1	8180000000
15	treatmented	0	a	3	f	3	0,06	1,41	1,47	6,5	14,1	2100000000
15	treatmented	0	a	3	i	1	-0,01	1,42	1,41	6,7	14,1	790000000
15	treatmented	0	a	3	i	2	0,03	1,42	1,45	6,6	14	190000000
15	treatmented	0	a	3	i	3	0	1,42	1,42	6,6	14	8900000000
15	treatmented	1	c	0	f	1	0	1,874	1,874	6	18,74	78000000000
15	treatmented	1	c	0	f	2	0	1,864	1,864	6	18,64	1180000000
15	treatmented	1	c	0	f	3	0	1,86	1,86	6	18,6	1300000000
15	treatmented	1	c	0	i	1	0	1,77	1,77	6,3	17,7	1700000000
15	treatmented	1	c	0	i	2	0	1,7	1,7	6,3	17	370000000
15	treatmented	1	c	0	i	3	0	1,747	1,747	6,3	17,47	230000000
15	treatmented	1	c	1,5	f	1	0	1,933	1,933	6	19,33	97000000000
15	treatmented	1	c	1,5	f	2	0	1,993	1,993	6	19,93	1300000000
15	treatmented	1	c	1,5	f	3	0	2,031	2,031	6	20,31	7800000000
15	treatmented	1	c	1,5	i	1	0	1,77	1,77	6,3	17,7	170000000
15	treatmented	1	c	1,5	i	2	0	1,7	1,7	6,3	17	2700000000
15	treatmented	1	c	1,5	i	3	0	1,747	1,747	6,3	17,47	2300000000
15	treatmented	1	c	3	f	1	0	2,08	2,08	6	20,8	91000000000
15	treatmented	1	c	3	f	2	0	2,08	2,08	6	20,8	3780000000
15	treatmented	1	c	3	f	3	0	2,08	2,08	6	20,8	8300000000
15	treatmented	1	c	3	i	1	0	1,874	1,874	6,2	18,74	4900000000
15	treatmented	1	c	3	i	2	0	1,864	1,864	6,2	18,64	4090000000
15	treatmented	1	c	3	i	3	0	1,86	1,86	6,2	18,6	890000000
15	treatmented	1	a	0	f	1	0,09	1,9	1,99	6	19,9	11100000000
15	treatmented	1	a	0	f	2	0,19	1,7	1,89	6	18,9	13400000000
15	treatmented	1	a	0	f	3	0	2	2	6	20	8700000000
15	treatmented	1	a	0	i	1	0,07	1,8	1,87	6,2	18,7	1700000000
15	treatmented	1	a	0	i	2	0,1	1,6	1,7	6,2	17	370000000
15	treatmented	1	a	0	i	3	0,047	1,7	1,747	6,2	17,47	230000000
15	treatmented	1	a	1,5	f	1	0	2	2	6	20	87000000000
15	treatmented	1	a	1,5	f	2	0,32	1,7	2,02	6	20,2	88000000000
15	treatmented	1	a	1,5	f	3	0,233	1,8	2,033	6	20,33	18000000000
15	treatmented	1	a	1,5	i	1	0,07	1,8	1,87	6,2	18,7	7800000000
15	treatmented	1	a	1,5	i	2	0	1,7	1,7	6,2	17	5800090000
15	treatmented	1	a	1,5	i	3	0,047	1,7	1,747	6,2	17,47	7800000000
15	treatmented	1	a	3	f	1	0,377	1,7	2,077	6	20,77	9100000000
15	treatmented	1	a	3	f	2	0,265	1,9	2,165	5,9	21,65	37800000000
15	treatmented	1	a	3	f	3	0,077	2	2,077	5,9	20,77	83000000000
15	treatmented	1	a	3	i	1	0,17	1,7	1,87	6,2	18,7	7800000000
15	treatmented	1	a	3	i	2	0	1,8	1,8	6,2	18	780000000

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15	treatmented	1	a	3	i	3	0,047	1,7	1,747	6,2	17,47	7800000000
30	check	0	0	0	0	1	0	1,41	1,41	6,7	14,1	0
30	check	0	0	0	0	2	0	1,41	1,41	6,7	14,1	0
30	check	0	0	0	0	3	0	1,4	1,4	6,7	14,1	0
30	treatmented	0	c	0	f	1	0	1,451	1,451	6,5	14,11	5800000000
30	treatmented	0	c	0	f	2	0	1,451	1,451	6,5	14,11	610000000
30	treatmented	0	c	0	f	3	0	1,451	1,451	6,5	14,11	600000000
30	treatmented	0	c	0	i	1	0	1,431	1,431	6,6	14,11	180000000
30	treatmented	0	c	0	i	2	0	1,431	1,431	6,6	14,11	110000000
30	treatmented	0	c	0	i	3	0	1,431	1,431	6,6	14,11	130000000
30	treatmented	0	c	1,5	f	1	0	1,41	1,41	6,5	14,11	600000000
30	treatmented	0	c	1,5	f	2	0	1,471	1,471	6,5	14,11	670000000
30	treatmented	0	c	1,5	f	3	0	1,471	1,471	6,5	14,11	680000000
30	treatmented	0	c	1,5	i	1	0	1,481	1,481	6,6	14,11	180000000
30	treatmented	0	c	1,5	i	2	0	1,42	1,42	6,6	14,11	110000000
30	treatmented	0	c	1,5	i	3	0	1,43	1,43	6,6	14,11	130000000
30	treatmented	0	c	3	f	1	0	1,471	1,471	6,5	14,11	7800000000
30	treatmented	0	c	3	f	2	0	1,491	1,491	6,5	14,11	8800000000
30	treatmented	0	c	3	f	3	0	1,51	1,51	6,5	14,11	980000000
30	treatmented	0	c	3	i	1	0	1,41	1,41	6,6	14,12	290000000
30	treatmented	0	c	3	i	2	0	1,437	1,437	6,6	14,12	290000000
30	treatmented	0	c	3	i	3	0	1,428	1,428	6,6	14,12	390000000
30	treatmented	0	a	0	f	1	0,04	1,37	1,41	6,4	14,11	390000000
30	treatmented	0	a	0	f	2	0,04	1,37	1,41	6,4	14,11	450000000
30	treatmented	0	a	0	f	3	0,04	1,37	1,41	6,4	14,11	480000000
30	treatmented	0	a	0	i	1	0,06	1,35	1,41	6,6	14,15	190000000
30	treatmented	0	a	0	i	2	0,1	1,3	1,4	6,6	14,15	190000000
30	treatmented	0	a	0	i	3	0,05	1,35	1,4	6,6	14,15	290000000
30	treatmented	0	a	1,5	f	1	0,11	1,4	1,51	6,4	14,11	2300000000
30	treatmented	0	a	1,5	f	2	0,06	1,4	1,46	6,4	14,11	8180000000
30	treatmented	0	a	1,5	f	3	0,091	1,4	1,491	6,4	14,11	2100000000
30	treatmented	0	a	1,5	i	1	0,131	1,3	1,431	6,6	14,15	790000000
30	treatmented	0	a	1,5	i	2	0,05	1,4	1,45	6,6	14,15	1900000000
30	treatmented	0	a	1,5	i	3	0	1,4	1,4	6,6	14,17	890000000
30	treatmented	0	a	3	f	1	0,091	1,4	1,491	6,4	14,11	2300000000
30	treatmented	0	a	3	f	2	0,11	1,4	1,51	6,4	14,11	8180000000
30	treatmented	0	a	3	f	3	0,09	1,4	1,49	6,4	14,11	2100000000
30	treatmented	0	a	3	i	1	0,081	1,4	1,481	6,6	14,16	790000000
30	treatmented	0	a	3	i	2	0,1	1,36	1,46	6,6	14,17	1900000000
30	treatmented	0	a	3	i	3	0,05	1,4	1,45	6,6	14,15	890000000
30	treatmented	1	c	0	f	1	0	2,07	2,07	5,9	20,7	7800000000
30	treatmented	1	c	0	f	2	0	2,07	2,07	5,9	20,7	118000000
30	treatmented	1	c	0	f	3	0	2,07	2,07	5,9	20,7	1300000000
30	treatmented	1	c	0	i	1	0	1,87	1,87	6,1	18,7	1700000000
30	treatmented	1	c	0	i	2	0	1,8	1,8	6,1	18	370000000
30	treatmented	1	c	0	i	3	0	1,747	1,747	6,1	17,47	230000000
30	treatmented	1	c	1,5	f	1	0	2,1	2,1	5,9	21	9700000000
30	treatmented	1	c	1,5	f	2	0	2,1	2,1	5,9	21	130000000
30	treatmented	1	c	1,5	f	3	0	2,1	2,1	5,9	21	7800000000
30	treatmented	1	c	1,5	i	1	0	1,87	1,87	6	18,7	170000000
30	treatmented	1	c	1,5	i	2	0	1,8	1,8	6,1	18	2700000000
30	treatmented	1	c	1,5	i	3	0	1,747	1,747	6,1	17,47	2300000000
30	treatmented	1	c	3	f	1	0	2,14	2,14	5,9	21,4	9100000000
30	treatmented	1	c	3	f	2	0	2,158	2,158	5,9	21,58	3780000000
30	treatmented	1	c	3	f	3	0	2,145	2,145	5,8	21,45	8300000000
30	treatmented	1	c	3	i	1	0	1,87	1,87	6	18,7	4900000000
30	treatmented	1	c	3	i	2	0	1,8	1,8	6	18	4090000000
30	treatmented	1	c	3	i	3	0	1,747	1,747	6	17,47	890000000
30	treatmented	1	a	0	f	1	0,242	1,8	2,042	5,8	20,42	1110000000
30	treatmented	1	a	0	f	2	0,242	1,8	2,042	5,8	20,42	1340000000
30	treatmented	1	a	0	f	3	0,242	1,8	2,042	5,8	20,42	870000000
30	treatmented	1	a	0	i	1	0,17	1,7	1,87	6,2	18,7	1700000000
30	treatmented	1	a	0	i	2	0	1,8	1,8	6,2	18	370000000
30	treatmented	1	a	0	i	3	0,047	1,7	1,747	6,2	17,47	230000000
30	treatmented	1	a	1,5	f	1	0,355	1,8	2,155	5,8	21,55	8700000000
30	treatmented	1	a	1,5	f	2	0,155	1,9	2,055	5,8	20,55	8800000000
30	treatmented	1	a	1,5	f	3	0,255	1,8	2,055	5,8	20,55	1800000000
30	treatmented	1	a	1,5	i	1	0,27	1,53	1,8	6,1	18	7800000000
30	treatmented	1	a	1,5	i	2	0,18	1,62	1,8	6,1	18	580009000
30	treatmented	1	a	1,5	i	3	0,27	1,53	1,8	6,1	18	7800000000
30	treatmented	1	a	3	f	1	0,517	1,56	2,077	5,7	20,77	9100000000
30	treatmented	1	a	3	f	2	0,6495	1,5155	2,165	5,7	21,65	378000000
30	treatmented	1	a	3	f	3	0,587	1,49	2,077	5,7	20,77	8300000000
30	treatmented	1	a	3	i	1	0,27	1,53	1,8	6	18	7800000000
30	treatmented	1	a	3	i	2	0,27	1,53	1,8	6	18	7800000000
30	treatmented	1	a	3	i	3	0,3654	1,4616	1,827	6	18,27	780000000

This manuscript also has an electronic appendix.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1606_74477466