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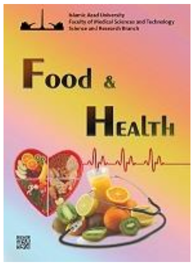

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The survival of probiotic *Bifidobacterium bifidum* and *Lactobacillus acidophilus* in synbiotic yogurt enriched with *Agave tequilana* aqueous extract

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A B S T R A C T

In this study the effect of incorporation of aqueous extract of *Agave tequilana* (AEAT) on viability of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* was investigated. No reduction of cell viability was seen in any treatment groups of *L. acidophilus* during the storage time. A statistically significant increase in *L. acidophilus* count was found in synbiotic yogurt samples containing 1 and 1.5% concentrations of the extract at day 21 of storage compared to the first day ($p<0.05$). The viability of *B. bifidum* remained unchanged in control probiotic yogurt during the storage, while there was a viability increase of 1, 1.2 and 2.4 log cycles in synbiotic yogurt samples containing 0.5, 1 and 1.5% concentrations of the extract during the storage, respectively. The final pH at day 21 ranged from 3.98-4.15. The pH of all yogurt samples was decreased as a function of time, while no statistically difference in pH was seen between different groups ($p>0.05$). All the yogurt samples revealed an increase in titratable acidity as a function of storage time. In conclusion, as the results of this study indicated improvement of probiotic bacteria in yogurt incorporated with AEAT, its potential application as a functional food formulation is recommended.

1. Introduction

In recent years, consumers have developed an increasing knowledge and belief of the role of food and nutrition for health and well-being. The growing consumer interest in healthy eating has become a primary driver for the food industry to investigate and design food products that possess additional functional/health properties over common nutritional values. Probiotic food products are among the most popular functional foods marketed worldwide. According to FAO/WHO (1) definition, probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit to the host". The great consumer acceptance of probiotic foods is due to the fact that probiotic microorganisms demonstrate different health benefits to the human by improving lactose digestion, preventing intestinal infections, preventing cancers, modulating the immune system, and lowering cholesterol (2). To exhibit their health benefits, the probiotic bacteria must survive during food processing and storage as well as gastrointestinal transition to reach alive their site of action in adequate number (3). It has been suggested that the probiotic products should contain at least 10^6 CFU/100 g or 10^9 per dried capsule to transfer probiotic effects to the host (4). Incorporation of prebiotic compounds to probiotic food, which resulted in synbiotic, is a choice practice that not only increases the growth and/or survival of probiotic bacteria in food preparations, but also could confer special health benefits including improved bioavailability of minerals such as calcium, magnesium and iron, increased activity of beneficial live active cultures, and inhibition of harmful bacteria in the digestive tract (2). Prebiotics have been defined as selective non-digestible carbohydrate food sources that promote the proliferation of *Bifidobacteria* and *Lactobacilli* (5). The most important prebiotics are inulin and oligofructoses, which are soluble and fermentable fibers (6). Yogurt is the most popular fermented dairy product market in the world due to its therapeutic, nutritional, and sensory properties. From a nutritional point of view, yogurt is a nutrient-dense food, as it contains protein, riboflavin, vitamins B6 and B12, and calcium. Yogurt and fermented milk products

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are among the best carriers for functional food ingredients including probiotics, prebiotics, and antioxidants such as polyphenols and carotenoids (7). *Agave tequilana* is a plant native to Mexico, which has been commonly used to obtain Tequila, a widely known alcoholic beverage (8). Several biological activities, including antibacterial, antioxidant, and anti-inflammatory properties of this plant are reported (9). It is reported that some agave species demonstrated antioxidant activity (10). The prebiotic effect of agave fructans has been well known by several researchers (11-15). The aim of this study was to evaluate the effect of aqueous extract of *A. tequilana* on the survival of probiotic *Lactobacillus acidophilus* and *Bifidobacterium bifidum* during storage.

2. Material and methods

2.1. Preparation of aqueous extract of A. tequilana

The *A. tequilana* was prepared from plant garden in Tehran province of Iran. All the leaves were cut into small pieces and dried for two weeks in the shade at environmental temperature. It was then comminuted using a mechanical grinder (Moulinex, Paris, France). For extract preparation, 50 g of the grinded plant was soaked in 450 mL water let shaking for 48 h at 250 rpm, followed by filtration through filter paper of Whatman No. 1, then vaporized at 50 °C using a rotary evaporator (Buchi Rotavapor R-114, Switzerland) and further dried at 40 °C. The extract powder was refrigerated at 4 °C till running the experiments (16).

2.2. Preparation of probiotic bacteria

The probiotic bacteria including *L. acidophilus* (La5) and *B. bifidum* (Bb-12) prepared from CHR Hansen (Horsholm, Denmark) was used in this study. Freeze-dried bacteria were added to the sterile MRS-Broth medium and incubated for 48 h at 37 ° C in aerobic condition and anaerobic jar for *L. acidophilus* and *B. bifidum*, respectively. Bacterial cultures were harvested by centrifugation at 4000 ×g at 4 °C for 10 min and washed twice with sterile saline and collected by centrifugation. A bacterial suspension with optical density (OD) of 0.1 at 600 nm was prepared and the cell numbers was determined using the surface plate count technique through preparing serial dilutions and plating on MRS agar. The plates were then incubated at 37 °C for 3 days in aerobic and anaerobic conditions for *L. acidophilus* and *B. bifidum*, respectively, as mentioned above. The bacterial number was calculated by counting bacterial colonies.

2.3. Production of yogurt

Standardized milk containing 1.5% fat and 12% dry matter was heat-treated at 85 °C for 15 min, then cooled down to 45 °C. Direct vat yogurt starter culture containing *Lactobacillus delbrueckii sub-species bulgaricus* and *Streptococcus thermophiles* (CHR Hansen, Horsholm, Denmark) was added to the milk according to the manufacture instruction. For preparation of probiotic yogurt, 1% (v/v) probiotic bacterial suspension with 10^8 CFU/mL density was inoculated. Aqueous extract of *A. tequilana* (AEAT) at final concentrations of 0.5, 1, and 1.5 % (w/v) was added to the milk to prepare synbiotic yogurt. Plain yogurt without probiotic bacteria and AEAT were prepared as a control. The yogurt samples were incubated at 42 °C until the pH reached 4.5. The fermentation was stopped by cooling the yogurt to 4 °C. The prepared yogurt samples were stored at 4 °C until analyzing at days 1, 7, 14, and 21.

2.4. Measurement of pH and titratable acidity

The pH of the yogurt samples was measured using a digital pH meter (Jenway 3320, England). The titratable acidity (TA) was determined by the titration method. Ten grams yogurt sample was mixed thoroughly with 90 mL of distilled water and titrated using 0.1 M NaOH solution in the presence of Phenolphthalein as an indicator. Titratable acidity was expressed as % lactic acid.

2.5. Evaluation of probiotic viability

The viable probiotic bacteria were quantified through the surface plate count method. Ten grams of each yogurt sample were diluted with 90 ml of 0.1% peptone water. Preparation of serial dilutions, plating, and incubation conditions method were done as mentioned above. MRS bile agar and MRS agar containing 0.05% cysteine hydrochloride and 0.3% sodium propionate were used for selective enumeration of *L. acidophilus* and *B. bifidum*, respectively.

2.6. Statistical analysis

All experiments were separately repeated three times. The data were expressed as means ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) using SPSS 20 (Chicago, IL, USA) followed by Duncan's *post hoc* mean separation. Statistical significance was set at $p<0.05$.

3. Results and discussion

The results of *L. acidophilus* viability in probiotic yogurt containing different concentrations of AEAT were demonstrated in Fig. 1. No reduction of cell viability was seen in any treatment groups during the storage time. A statistically significant increase in *L. acidophilus* count was found in synbiotic yogurt samples containing 1 and 1.5% concentrations of the extract at day 21 of storage compared to the first day ($p<0.05$). Generally, there were no significant differences in *L. acidophilus* count between different treatment groups at each given day during the storage time ($p<0.05$). The results of *B. bifidum* count in probiotic yogurt containing different concentrations of AEAT were shown in Fig. 2. The viability of *B. bifidum* remained unchanged in control probiotic yogurt during the storage, while there was a viability increase of 1, 1.2, and 2.4 log cycles in synbiotic yogurt samples containing 0.5, 1, and 1.5% concentrations of the extract during the storage, respectively.

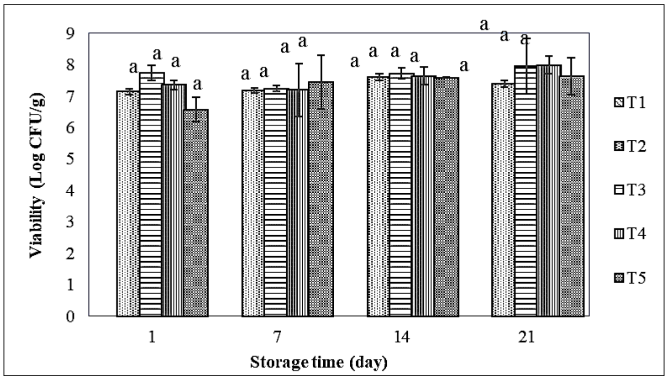

Fig. 1. Viability of *Lactobacillus acidophilus* in synbiotic yogurt containing aqueous extract of *Agave tequilana* (AEAT) during storage. T_1: plain yogurt, T_2: probiotic yogurt, T_3: synbiotic yogurt containing 0.5% AEAT, T_4: synbiotic yogurt containing 1% AEAT, T_5: synbiotic yogurt containing 1.5% AEAT. Different letters show statistical significant between different treatments at each day ($p<0.05$).

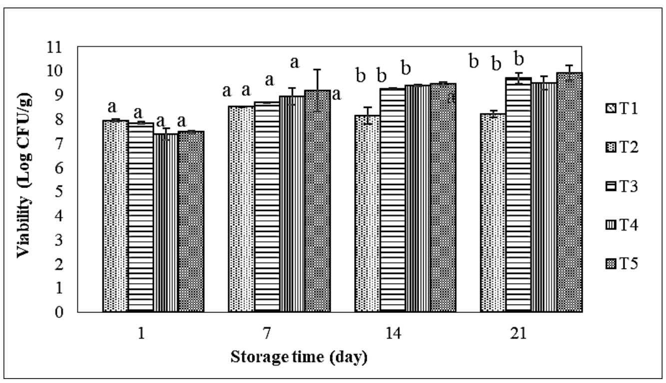

Fig. 2. Viability of *Bifidobacterium bifidum* in synbiotic yogurt containing aqueous extract of *Agave tequilana* (AEAT) during storage. T_1: plain yogurt, T_2: probiotic yogurt, T_3: synbiotic yogurt containing 0.5% AEAT, T_4: synbiotic yogurt containing 1% AEAT, T_5: synbiotic yogurt containing 1.5% AEAT. Different letters show statistical significant between different treatments at each day ($p<0.05$).

The results of this study revealed that the viability of the probiotic bacteria was increased due to the incorporation of aqueous extract of *A. tequilana* to probiotic yogurt. This improving effect may be justified by the prebiotic activity of fructans present in this plant as reported earlier (17). López and Urías-Silvas (11) found that fructans of agave induced growth of *L. casei* and *B. lactis* even more remarkably than inulin. Moreover, the prebiotic effects of different compounds present in agave composition including fructans and fibers have been demonstrated *in vivo* both in animal (12) and human (13, 15) models. Furthermore, it is reported that some *lactobacillus* species could metabolize phenolic compounds (16). According to Tabasco et al. (19), the susceptibility of *Lactobacilli* and *Bifidobacteria* to phenolic compounds depended on the bacterial strains, and chemical structure and concentration of the phenolic compound. In general, the probiotic count of yogurt samples investigated at day 21 was higher than 10^8 CFU/g, which was more than the minimum concentration recommended for probiotic products (4). The pH values of yogurt samples are shown in Fig. 3. The final pH at day 21 ranged from 3.98-4.15. The pH of all yogurt samples was decreased as a function of time, while no statistical difference in pH was seen between different groups ($p<0.05$). The titratable acidity of different yogurt samples is demonstrated in Fig 4.

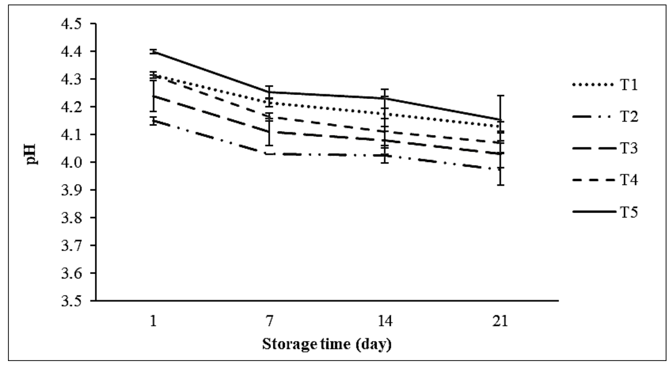

Fig. 3. The pH changes of synbiotic yogurt containing aqueous extract of *Agave tequilana* (AEAT) during storage. T_1: plain yogurt, T_2: probiotic yogurt, T_3: synbiotic yogurt containing 0.5% AEAT, T_4: synbiotic yogurt containing 1% AEAT, T_5: synbiotic yogurt containing 1.5% AEAT.

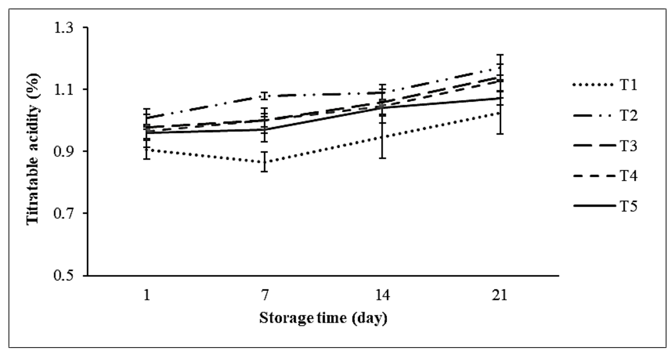

Fig. 4. Changes of titratable acidity (%) in synbiotic yogurt containing aqueous extract of *Agave tequilana* (AEAT) during storage. T_1: plain yogurt, T_2: probiotic yogurt, T_3: synbiotic yogurt containing 0.5% AEAT, T_4: synbiotic yogurt containing 1% AEAT, T_5: synbiotic yogurt containing 1.5% AEAT.

All the yogurt samples revealed an increase in titratable acidity as a function of storage time. The lowest and highest final acidity were recorded for plain yogurt and probiotic control yogurt, respectively. There was no statistically significant difference in acidity between synbiotic yogurt containing different concentrations of *A. tequilana* extract ($p<0.05$). The results of the present study are correlated with earlier researches which showed an increase in acidity of yogurt during storage (20-23).

4. Conclusion

In this study, we presented a novel synbiotic functional yogurt by incorporating aqueous extract of *A. tequilana* to probiotic yogurt. The results of this study showed a significant increase in viability of the probiotic bacteria was seen in synbiotic yogurts. Generally, our results proposed the use of this yogurt formulation as a functional product for improving consumer health

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