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Study of different levels of *Cannabis Sativa* extract on quality properties and viability of probiotic bacteria in Aloe vera yoghurt

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ABSTRACT

In recent decades, the use of natural ingredients such as herbs and spices to increase shelf life, nutritional value and variety in the taste of food has increased. Consumption of probiotic products, especially yogurt, is common among people because of their effect on the health of consumers. In this study, the effect of different levels of cannabis extract (0, 5 and 10%) on physicochemical properties and survival of *Lactobacillus acidophilus* bacteria in aloe vera yogurt was investigated. Yogurt samples were evaluated for pH, acidity, serum separation, sensory properties, rheological properties and viability of probiotic bacteria during storage. The results showed that storage time had a significant effect on pH and acidity and with increasing time, pH decreased and acidity increased. Samples containing 5% cannabis extract showed the lowest serum separation and control showed the highest serum separation at the end of storage. It was found that cannabis extract had a positive effect on the growth of *Lactobacillus acidophilus* compared to the control, although with increasing the concentration of the extract from 5 to 10%, survival of probiotic bacteria was reduced. In sensory evaluation, yogurt containing 5% cannabis extract received the highest flavor, odor and general acceptance.

1.Introduction

Probiotics are described as live microorganisms that, when consumed in adequate amounts, confer health benefits on the host. Traditionally, probiotics are added to yogurt and other fermented dairy products [1]. There is a great desire to develop functional probiotic dairy products containing plant-based ingredients because all age groups are interested in their taste and flavor. In addition to being tasty. Natural plant materials contain various bioactive compounds with antioxidant activity such as vitamins A, C, and E [2], phenolic compounds, etc., and recent studies have shown that they increase the total antioxidant capacity of foods containing them .[۳]

One of these indigenous crops of Iran is cannabis (Cannabis Sativa L.), which can play a role in the production of healthy food products. The area under cultivation of this crop in Iran is 816 hectares, and its main producing areas are Markazi and Isfahan provinces [4]. Cannabis is an annual, biennial plant that has played an important role in the production of food, medicine, and fiber since ancient times [5]. In Europe, cannabis and its by-products are used in the construction, cosmetic, and animal feed industries. The protein content of oil cannabis varieties is 26.5% [6], the protein of fiber varieties is 26.6%, and the protein of its meal is estimated to be 33.6% to 43.1% based on dry matter [7]. Cannabis seeds contain significant amounts of sulfur-containing amino acids compared to soybeans, but except for arginine, the amount of other amino acids in cannabis is lower than in soybeans. The ratio of essential amino acids to total amino acids in cannabis protein (45.16%) is higher than in soy protein (42.72%). The in vitro digestibility (pepsin and trypsin digestion method) of cannabis protein is higher than that of soy protein and is more suitable for human consumption than soy protein [8]. Cannabis is commonly referred to as an oil crop, with oil accounting for about 30% of the seed weight [5], but researchers have found that the seed oil content varies between 26.3% and 37.5%, that these variations being related to genotype, year, and genotype-year interactions.[۹]

Since no study has been conducted so far on the preparation of probiotic aloe vera yogurt containing cannabis, the aim of this study is to produce probiotic aloe vera yogurt using different concentrations of aqueous cannabis extract and to investigate its effect on the qualitative, rheological, and viability properties of probiotic bacteria in this product.

2-Materials and Methods

The materials used in the study included Lactobacillus acidophilus bacteria (La-5) and yogurt starter (CY340) (Christensen Hansen, Denmark), phenolphthalein reagent (Sigma, USA) and sodium hydroxide (Merck, Germany). All materials used were obtained from reputable manufacturers.

2-1-Preparation of cannabis extract

First, the cleaned and dried cannabis seeds were powdered using an electric grinder. In the next step, 10 ml of sterile distilled water was added for each gram of dry seed powder. Then, the mixture was placed in a water bath at 40°C for 24 hours. Finally, using a centrifuge (2000 rpm, 15 minutes), an aqueous extract was obtained, which was actually the clear supernatant phase.[۱۰]

2-2 -Preparation of Aloe Vera Gel

After preparing the Aloe Vera leaves, the leaves were washed and the serrated edges of the leaves were cut using a sharp knife. The upper layer of the leaf was removed lengthwise and the gel was carefully separated from the leaf. The gel pieces were finely ground in a blender and passed through

a sterile metal sieve with a mesh of 20 to form a homogeneous solution. The resulting gel was pasteurized at 65°C for 15 minutes. In this study, Aloe Vera extract with a concentration of 10% was used.

2-3 -Preparation of Probiotic Yogurt Containing Cannabis Extract

To prepare cannabis yogurt, milk (1% fat) was heated to 60°C. Then 10% Aloe Vera gel and different levels of cannabis extract (0, 5 and 10%) were added to it and completely homogenized. The mixture was heated at 90°C for 5 minutes and then cooled to the inoculation temperature (40°C). Then, 1% yogurt starter and 0.2% probiotic bacteria Lactobacillus acidophilus were added and thoroughly mixed to obtain a uniform mixture. The samples were incubated at 40°C until reaching pH 4.4 and then placed in a refrigerator at 5°C.[۱۱]

2-4 -Tests performed on yogurt samples

2-4-1 -Determination of acidity

The titratable acidity (in terms of lactic acid) of yogurt samples was determined using the titration method using 0.1 M sodium hydroxide solution and 0.3 ml of phenolphthalein until reaching a pink color .[۱۱]

Titratable acidity percentage = (volume of sodium hydroxide consumed × N/9 × 90/sample weight × 100)

2-4-2 -pH determination

The pH of the yogurt samples was determined using a digital pH meter. The pH meter was calibrated using buffer solutions at pH 4 and pH 7.

2-4-3 -Viscosity measurement

The apparent viscosity of the aloe vera yogurt samples containing cannabis extract was measured at room temperature using a Brookfield digital viscometer. The samples were subjected to shear rates of 0.1 to 100/s for the upward curve. The viscosity was expressed in centipoise.[۱۲]

2-4-4 -Determination of sensory characteristics

Samples of aloe vera yogurt containing cannabis extract were evaluated for sensory characteristics one day after production and on the 30th day of storage time by a group of 20 trained evaluators. The evaluated characteristics included color, taste, odor, texture and overall acceptance. A 5-point hedonic scale was used to evaluate sensory characteristics, which included [excellent (5), satisfactory (4), acceptable (3), unacceptable (2), and inedible (1)] [13]. The average of the data from the first and 30th day was reported .

2-4-5 -Serum separation (syneresis)

20 g of yogurt samples were poured into test tubes and centrifuged for 10 minutes at 2200 rpm. After centrifugation, the amount of separated serum was weighed and the percentage of water loss was calculated from the following equation:[۱٤]

Serum separation (%) = separated serum weight/yogurt weight ×100

2-4-6 -Examination of the viability of probiotic bacteria in yogurt samples

The number of surviving cells of probiotic bacteria Lactobacillus acidophilus in each of the yogurt samples was determined immediately after preparation and during 21 days of storage in the refrigerator (once every 7 days). The bacterial count of the samples was determined as follows :

10 g of the yogurt sample was added to 90 ml of physiological serum, mixed thoroughly and diluted to a dilution of 10-9. Then, 0.1 ml of this solution was cultured in MRS agar medium. The plates were incubated in an anaerobic jar for 72 h at 37 °C [15]. The number of probiotic bacteria per gram of yogurt was calculated using the Iranian standard formula and in terms of log CFU/g.

2-5- Statistical analysis

In this study, all tests were performed in three replicates. The data from the experiments were analyzed using a

completely randomized design in a factorial format using SPSS:20 software. Means comparison was performed using Duncan's test at a probability level of 5% .

3 -Results and discussion

3-1 -Effect of different levels of cannabis on pH and acidity of probiotic aloe vera yogurt samples

Table 1 shows the pH values of aloe vera yogurt samples containing probiotic bacteria along with different levels of cannabis extract from the first to the 21st day. According to the table, it can be seen that the difference in pH value between the three studied treatments on the first day was not significant (P>0.05). On the 21st day, all treatments had a significant difference with the pH of the control sample, and the highest pH value was observed in the sample containing 5% cannabis extract. In general, with increasing the percentage of extract in the formulation from 0 to 5%, the pH value increased, and with a further increase to 10%, the pH of the yogurt samples decreased.

Table 1- Effect of Canabis extracts on pH of aloe vera yogurt samples

Treatments	1st day	7th day	14th day	21st day
control	$4.43\pm0.1^{Aa*}$	4.21 ± 0.02^{Bb}	4.03 ± 0.05^{Cb}	3.83 ± 0.05^{Dc}
5% Canabis extract	4.44 ± 0.05^{Aa}	4.34 ± 0.1^{ABa}	4.28 ± 0.04^{Ba}	4.23 ± 0.05^{Ba}
10% Canabis extract	4.49 ± 0.09^{Aa}	4.02 ± 1^{ABc}	4.08 ± 0.07^{Bb}	4.04 ± 0.1^{Bb}

*Non-homonymous capital letters indicate a significant difference between maintenance days (p<0.05)
*Non-homonymous lower case letters indicate a significant difference between treatments (p<0.05)

Table 2 shows the acidity of aloe vera yogurt samples containing different levels of cannabis extract. The acidity increased with increasing storage period. The studied treatments showed a significant effect on the acidity, and the highest and lowest acidity levels at the end of the storage period were observed in the control and 5% cannabis extract treatments, respectively.

Table 2- Effect of Canabis extracts on acicdity of aloe vera yogurt samples

Treatments	1st day	7th day	14th day	21st day
control	$0.91\pm0.01^{Ca*}$	0.98 ± 0.05^{BCa}	1.01 ± 0.01^{Ba}	1.23 ± 0.05^{Aa}
5% Canabis extract	0.84 ± 0.05^{Ba}	0.87 ± 0.01^{Bb}	0.89 ± 0.01^{Bb}	0.92 ± 0.02^{Ac}
10% Canabis extract	0.84 ± 0.03^{Ba}	0.91 ± 0.05^{Ba}	1.04 ± 0.03^{Aa}	1.08 ± 0.03^{Ab}

*Non-homonymous capital letters indicate a significant difference between maintenance days (p<0.05)
*Non-homonymous lower case letters indicate a significant difference between treatments (p<0.05)

*Non-homonymous lower case letters indicate a significant difference between treatments (p<0.05)

The trend of pH decrease and acidity increase in the control treatment was greater than the treatments containing cannabis extract, and the lowest changes were observed in the treatment containing 5% cannabis extract. Yogurt, due to the growth of Lactobacillus bacteria and the conversion of milk lactose to lactic acid, will have a lower pH over time and its acidity will increase, which will make the yogurt sourer. Adding cannabis extract, especially at a concentration of 5%, has controlled the acidity and pH in yogurt. Thus, it can be concluded that cannabis extract prevents excessive acidity increase due to its anti-acid compounds, and this is due to the effect of cannabis extract on lactic acid bacteria. Antimicrobial compounds of cannabis include phenolic compounds, mainly caffeoyl tyramine and cannabidiol [16]. Lotfizadeh Dehkordi et al. (2013) also showed in their study of the effect of Tragopogon dubius extract on the properties

of probiotic yogurt that adding Tragopogon dubius extract controlled the increase in acidity and decreased pH.[۱۷]

3-2 -Effect of different levels of cannabis extract on the serum separation (syneresis) of probiotic aloe vera yogurt samples

One of the main problems of yogurt and other acidic dairy products is their two-phase formation and syneresis during storage time, which is caused by low pH and its effect on protein precipitation. The results of this study showed that the serum separation rate of yogurt samples increased significantly during storage (P<0.05). Yogurt samples containing 5% cannabis extract showed the lowest serum separation rate and the control showed the highest serum separation or syneresis rate at the end of the storage time. By increasing the concentration of cannabis extract to 5% on all days of storage, the syneresis rate decreased, and beyond this level, although the syneresis rate was numerically lower than that of the control, no significant trend was observed (P>0.05) (Table 3).

Table 3- Effect of Canabis extracts on serum separation (%) of aloe vera yogurt samples

Treatments	1st day	7th day	14th day	21st day
control	$20.1\pm1.7^{Da*}$	25.3 ± 1.5^{Ca}	31.5 ± 2.3^{Ba}	34.3 ± 1.3^{Aa}
5% Canabis extract	12.8 ± 2.2^{Dc}	16.7 ± 2.5^{Cc}	21.8 ± 2.1^{Bc}	25.2 ± 2.1^{Ac}
10% Canabis extract	18.9 ± 1.5^{Dab}	23.4 ± 1.4^{Cab}	29.4 ± 1.5^{ABab}	32.6 ± 2.1^{Aab}

*Non-homonymous capital letters indicate a significant difference between maintenance days (p<0.05)
*Non-homonymous lower case letters indicate a significant difference between treatments (p<0.05)

3-3 -The effect of different levels of cannabis extract on the sensory properties of probiotic aloe vera yogurt samples

Sensory properties are one of the basic factors in the acceptance of many products and obtaining satisfaction from their consumption. Table 4 shows the effect of different treatments on the sensory properties of probiotic yogurt samples. According to the results of the evaluation of the sensory properties, it was observed that treatments containing 5% cannabis extract received the highest taste score and the treatment containing 10% cannabis extract received the lowest score.

Table 4- Effect of Canabis extracts on sensory properties of aloe vera yogurt samples

Treatments	Taste	Odor	color	Texture	Overall acceptance
control	4.8^{ab*}	4.8^{ab}	5.0^{a}	4.7^{ab}	4.7^{ab}
5% Canabis extract	5.0^{a}	5.0^{a}	4.8^{ab}	4.9^{a}	4.9^{a}
10% Canabis extract	4.5^{b}	4.8^{ab}	4.3^{c}	4.5^{b}	4.4^{b}

*In each column, non-homonymous lower case letters indicate a significant difference between treatments (p<0.05)

The studied treatments did not show a significant effect on the odor of the probiotic yogurt samples. Numerically, the treatment containing 5% cannabis extract had the highest odor score and the control containing 10% cannabis extract had the lowest odor score.

As the concentration of cannabis extract in the aloe vera yogurt formulation increased, the color score decreased. Some evaluators reported a discolored brown appearance for yogurts containing 10% cannabis extract. The highest and lowest color scores were for the control and the treatment containing 10% cannabis extract, respectively. The studied treatments affected the texture of the samples, and yogurt samples containing 5% cannabis extract having the highest score and treatments containing 10% cannabis extract having the lowest texture score. Some evaluators reported a slimy and watery

texture for the 10% cannabis extract concentration.

The highest overall acceptance score was for treatments containing 5% cannabis extract, which did not show a significant difference from the control (although the control scored higher numerically), and the lowest overall acceptance was for the treatment containing 10% cannabis extract.

There was a significant difference in the taste and sensory characteristics of the different yogurt samples, especially the treatments with higher amounts of extract compared to the control. Adding more than 5% cannabis extract reduced the sensory scores compared to the control. In a study, it was found that in yogurt samples containing probiotic bacteria, increasing the concentration of thyme did not give the yogurt a desirable taste, and in all samples, yogurt containing 0.03% thyme and the control had the best taste. Increasing more than this amount of thyme reduced the sensory characteristics scores of the yogurt .[۱۸]

3-4 -Effect of different levels of cannabis extract on rheological properties of probiotic aloe vera yogurt samples

Figure 1 shows the effect of shear rate on the viscosity of different treatments. It is clear from the figure that all samples follow the shear-thinning or pseudoplastic power law well, which is quite clear with respect to the apparent viscosity. Apparent viscosity should always be expressed along with the shear rate used to calculate it, otherwise it will be meaningless. For a shear-thinning (pseudoplastic) fluid, the apparent viscosity decreases with increasing shear rate, and this behavior is the reason for the fluid's name.

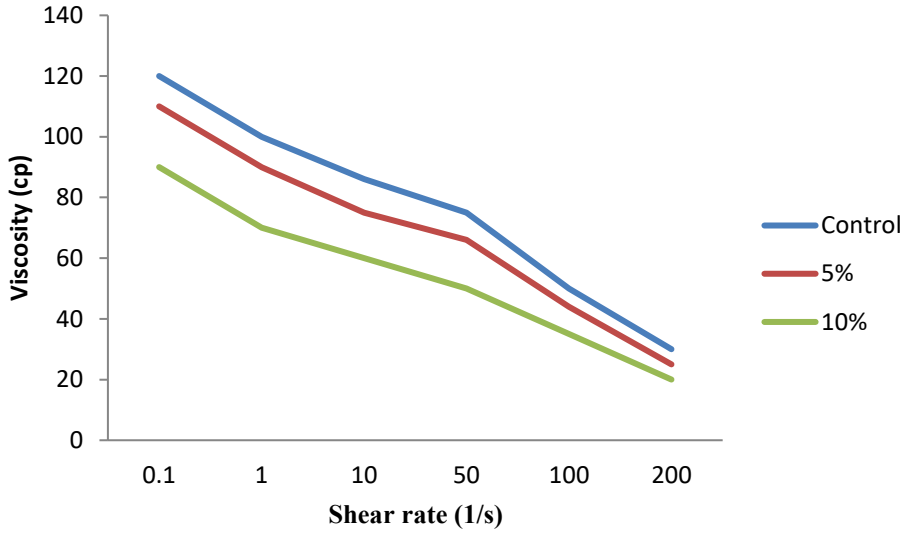

Figure 1- Shear rate-viscosity relationship of Aloe vera yogurt samples containing Cannabis extract

The data related to the parameters k, n and viscosity obtained from the power law model fitting on the flow charts of yogurt samples containing different concentrations of cannabis extract are reported in Table 5. With increasing extract percentage, k and viscosity decreased and n increased.

Table 5- Rheological factors of Aloe vera yogurt samples with Cannabis extracts

Treatments	Apparent viscosity (cp)	Flow behavior index (n)	Consistency index (k)	R^2
control	750±25[a*]	0.41±0.1[c]	121±12[a]	0.996
5% Canabis extract	640±15[b]	0.53±0.2[b]	102±8[b]	0.985
10% Canabis extract	550±20[c]	0.67±0.1[a]	88±10[a]	0.995

*In each column, non-homonymous lower case letters indicate a significant difference between treatments ($p<0.05$)

By adding cannabis extract to yogurt samples, viscosity decreased, which is due to the wateriness of the extracts and the watery texture of the yogurt. In the study by Lotfizadeh Dehkordi et al., adding cannabis extract to yogurt samples did not have a negative effect on viscosity and to some extent increased viscosity compared to the control [17]. Contrary to our results, in the study by Marhamatizadeh et al., the higher the concentration of malt extract, the higher the consistency and viscosity of the yogurt produced, such that the control yogurt had the lowest consistency and the yogurt containing 6% malt extract had the highest consistency [19]. In the present study, the samples showed shear thinning behavior, which was consistent with the study by Karajian and Salari (2011) [20]. For a shear thinning liquid (pseudoplastic), the apparent viscosity decreases with increasing shear rate, and this behavior is the reason for the name fluid. This behavior occurs because molecules are arranged irregularly at low shear rates and are only partially aligned, which leads to high viscosity. When the shear rate increases, the alignment of molecules increases, resulting in increased internal friction and a decrease in apparent viscosity.[۲۱]

In a study, the viscosity was reduced by adding dill extract, with the highest viscosity being that of control yogurt. Yogurt is a protein gel in which denatured soluble proteins form a porous, network-like structure by bonding to casein, which is capable of retaining water. It seems that adding the extract to yogurt weakens this network and reduces viscosity. In addition, viscosity is affected by the activity of starter bacteria and acidity.[۲۲]

3-5 -The effect of different levels of cannabis extract on the viability of probiotic bacteria in aloe vera yogurt samples

The therapeutic effects of probiotic bacteria require that they be alive and active, therefore the number of viable probiotic cells per gram or milliliter of the product at the time of consumption is very important and includes the essential value of the product. Thus, considering the necessity of having a certain number of probiotic bacteria at the time of consumption of the product, the effect of adding different amounts of cannabis extract on the viability of Lactobacillus acidophilus was investigated. Table 6 shows the trend of changes in the number of viable cells of these bacteria in the yogurt samples studied during the storage time. According to the results obtained, the highest microbial count for all treatments was on the first day (immediately after fermentation and preparation of yogurt) and the number of viable probiotic bacteria cells in all samples decreased significantly with time. On the first day of storage, the highest survival rate was observed in yogurt samples containing 10% cannabis extract. On other days of storage, as the percentage of cannabis extract in the yogurt formulation increased to 5%, the number of Lactobacillus acidophilus increased and then showed a decreasing trend. At the end of the storage time, the highest number of viable probiotic bacteria was observed in yogurt samples containing 5% cannabis extract.

Table 6 - The effect of Cannabis extract on the survival of probiotic bacteria (log CFU/g) in aloe vera yogurt samples

Treatments	1st day	7th day	14th day	21st day
control	$8.46\pm0.1^{Ac*}$	7.82 ± 0.2^{Bc}	6.32 ± 0.1^{Cc}	5.67 ± 0.2^{Dc}
5% Canabis extract	8.81 ± 0.3^{Ab}	$8.74\pm.1^{Ba}$	7.91 ± 0.3^{Ca}	7.8 ± 0.5^{Da}
10% Canabis extract	8.85 ± 0.5^{Aa}	8.66 ± 0.1^{Bb}	7.53 ± 0.5^{Cb}	7.66 ± 0.5^{Db}

*Non-homonymous capital letters indicate a significant difference between maintenance days ($p<0.05$)
*Non-homonymous lower case letters indicate a significant difference between treatments ($p<0.05$)

In the present study, the number of Lactobacillus acidophilus probiotic bacteria in all samples decreased during refrigerated storage from day 1 to day 21, and the decrease in the number of these bacteria was more evident with increasing cannabis extract concentration from 5 to 10%. This indicates that cannabis extract at high concentrations reduces the survival of probiotic bacteria in yogurt. The results of a study showed that, apart from the negative effect on pathogens, cannabis seed extract did not show any specific antimicrobial activity against Bifidobacterium and Lactobacillus species [23]. In agreement with our results in a study, a slight but significant increase in growth was observed for Lactobacillus paracasei, Lactobacillus plantarum, and Bifidobacterium longum with the use of cannabis extract.[٢٤]

In this regard, Akbari et al. investigated the effect of water-alcoholic extract of Artemisia vulgaris on the growth of Lactobacillus acidophilus and Bifidobacterium lactis in probiotic yogurt and reported that higher concentrations of Artemisia vulgaris extract reduced the viability of probiotic bacteria. However, in none of the treatments containing Artemisia vulgaris extract did the number of bacteria reach less than 106 bacteria per gram [25]. Other factors affecting the reduction in the number of probiotic bacteria during refrigeration include changes in acidity, production of metabolites such as organic acids and hydrogen peroxide by yogurt-specific bacteria, and limited access to nutrients in the environment [26, 27]. According to the findings of Zaika and Kissinger, the use of some essential oils and plant extracts such as oregano, cumin, and mint had an inhibitory effect on acid-producing bacteria at the concentrations used and reduced acid production during the storage period compared to the control treatment [28]. In the study of Sarabi-Jamab and Niazmand (2009), the viability of Lactobacillus acidophilus in bioyogurt containing different concentrations of Mentha piperita and Ziziphora clinopodioides was significantly reduced after 7 days of storage at 4 °C [29]. In a study, Marhamati Zadeh et al. investigated the effect of different levels of malt extract (0%, 2%, 4% and 6%) on the growth of probiotic bacteria in milk and yogurt and showed that with increasing the concentration of malt extract, the growth of bacteria was faster and the time to reach the desired acidity was shorter. With increasing the concentration of malt extract, the growth rate of probiotics increased [19]. In the present study, increasing the concentration of the extract to 5% improved the survival of probiotic bacteria.

4-Conclusion

In general, the results of this study showed that adding 5% cannabis extract to the probiotic yogurt formulation maintained the physicochemical properties, sensory acceptance, and improved the viability of probiotic bacteria in the yogurt samples until the end of the storage time.

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چکیده

در دهه‌های اخیر، استفاده از ترکیبات طبیعی از جمله گیاهان داروئی و ادویه‌ها به منظور افزایش زمان نگهداری، ارزش تغذیه‌ای و تنوع در طعم ومزه مواد غذایی افزایش یافته‌است. مصرف فرآورده‌های پروبیوتیک به‌ویژه ماست به دلیل تاثیر آنها بر سلامت مصرف‌کنندگان بین مردم رایج است. در این مطالعه اثر سطوح مختلف عصاره شاهدانه (۰، ۵ و ۱۰ درصد) بر ویژگی‌های فیزیکوشیمیایی و زنده مانی باکتری‌های پروبیوتیک *لاکتوباسیلوس اسیدوفیلوس* در ماست آلوئه‌ورا بررسی شد. نمونه‌های ماست از نظر pH ، اسیدیته، درصد آب اندازی، خواص حسی، خواص رئولوژیکی و زنده‌مانی باکتری‌های پروبیوتیک در طول دوره نگهداری مورد بررسی قرار گرفتند. نتایج نشان داد که زمان، تاثیر معنی‌داری بر pH و اسیدیته داشت وبا افزایش زمان، pHکاهش و اسیدیته افزایش یافت. نمونه‌های حاوی ۵ درصد عصاره شاهدانه کمترین میزان جدا شدن سرم و نمونه شاهد بیشترین درصد جدا شدن سرم یا آب‌اندازی) را در پایان دوره نگهداری نشان دادند. مشخص شد که عصاره‌ی شاهدانه اثر مثبت بر روی رشد باکتری‌های *لاکتوباسیلوس اسیدوفیلوس* نسبت به شاهد داشت، هرچند با افزایش غلظت عصاره از ۵ به ۱۰ درصد زنده‌مانی کاهش یافت. در ارزیابی حسی، ماست حاوی ۵ درصد عصاره شاهدانه بیشترین امتیازات طعم، بو و پذیرش کلی را دریافت کرد.