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Effect of Denak (*Oliveria decumbens* Vent) on Growth and Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* for Production of Probiotic Herbal Milk and Yoghurt

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Abstract: This study was undertaken to determine the suitability of different doses (0, 0.5, 1.0 and 1.5%) of Denak powder (*Oliveria decumbens* Vent) on viability of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in milk and yoghurt during 21 day refrigerated storage for production of probiotic herbal milk and yoghurt. In order to determine the effect of different doses of Denak powder on growth of probiotic bacteria in milk and yoghurt, first lyophilized bacteria *Lactobacillus acidophilus* was added to 1 liter of low fat sterilized milk and was considered as control. Denak powder at the concentrations of 0.5, 1 and 1.5% were added to the samples and incubated until acidity reached 40° Dornic and then left in refrigerator. Similar procedure was applied to the bacteria *Bifidobacterium bifidum*. The results of this experiment indicate the positive correlation between increased bacterial growth and increased Denak concentration. The investigation showed that the yoghurt containing 1% Denak powder had the best for taste, color, and insolubility. The sample with 1.5% Denak powder in milk and yoghurt had greater viscosity than the other samples investigated. The shelf lives of products were determined to be 21 days during which the bacterial count decreased but not less than 10^9. All the results suggest that Denak (*Oliveria decumbens* Vent) promoted the metabolism of lactic acid bacteria in milk and yoghurt. According to these findings, addition of Denak powder to milk and yoghurt can be recommended to take advantage of their beneficial properties on human health attributed to antimicrobial activities.

Key words: Denak, probiotic, *Bifidobacterium bifidum*, *Lactobacillus acidophilus*

INTRODUCTION

Consumers across the world are becoming more interested in foods with health promoting features as they gain more awareness of the links between food and health. Among the functional foods, products containing probiotics are showing promising trends worldwide.

Probiotics such as *Lactobacillus* and *Bifidobacterium* sp. are bacterial members of the normal human intestinal flora that exert several beneficial effects on human health and improve the intestinal microbial balance, resulting in the inhibition of bacterial pathogens, reduction of colon cancer risk, improving the immune system and lowering serum cholesterol levels (Tamime *et al*., 2005; Saarela *et al*., 2002; Isolauri *et al*., 2001).

Recently, the food biotechnology industry has developed a number of commercial products containing a single probiotic strain or bacterial associations of various complexities.

Also, Lactic Acid Bacteria (LAB) and its metabolites have shown to play an important role in improving microbiological quality and shelf-life of many fermented food products. Dairy products have long been consumed by consumers and provide a good example of bio-preservation (Zottola *et al.*, 1994).

Today LAB is a focus of intensive international research for its pivotal role in most fermented foods.

Furthermore, LAB strains synthesize short chain fatty acids, vitamins, and exopolysaccharides (EPS) that are employed in the manufacturing of fermented milk to improve its texture and viscosity (Curk *et al.*, 1996).

Development of dairy products with new products and flavors has potential health benefits thereby increasing sales and consumers satisfaction. Traditional preparation of milk and yoghurt may be beneficial by

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Fig. 1: Denak (*Oliveria decumbens* Vent.)

including other ingredients such as soya protein, vegetables, sweet potato, pumpkin and plum to enhance the flavor as well as the nutritional quality (Joo *et al.*, 2001).

However, traditional medicinal plants such as Denak (*Oliveria decumbens* Vent) has been proved to provide important therapeutic values. The plant *Oliveria decumbens* Vent. (Apiaceae), with the common Persian names of "Mooshkorok", "Den", and "Denak, is found in south and south west parts of Iran and is endemic to Iran. Denak (*Oliveria decumbens* Vent.) belongs to Umbelliferae family and is an endemic plant of Flora Iranica that grows in high temperature areas of south and west of Iran (Fig. 1) (Mozaffarian, 1996).

Denak (*Oliveria decumbens* Vent.) is a relatively less explored plant. The limited researches about therapeutic property of this plant are reported antibacterial and antifungal activity of its essential oil. Recently the study of researchers showed that Denak (*Oliveria decumbens* Vent.) has proper antibacterial effect and can be considered as a new source of antibiotic discovery and development for infection disease treatment purpose (Amin *et al.*, 2005).

In the present study, we investigate first, the ability of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* to grow and survive in presence of Denak (*Oliveria decumbens* Vent.) during 21 day of refrigerated storage and evaluate the organoleptic properties of milk and yoghurt.

MATERIALS AND METHODS

Sample collection and preparation: The plant materials of *O. decumbens Vent* was collected from Kazerun located in Fars province, in the south west of Iran, in July 2012. The flowers of *O. decumbens* Vent were dried under shade at room temperature for 17 days. Low-fat sterilized milk and yoghurt (1.5%) were locally purchased (Kazerun, Iran). Commercially available probiotic cultures of *Lactobacillus acidophilus* LAFTI® L10 and *Bifidobacterium bifidum* LAFTI® B94 were obtained from DSM Food Specialities Australia Pty Ltd. (Moorebank, NSW, Australia). MRS Agar culture medium was used for carrying out the microbial test (MERCK, Germany).

Preparation of probiotic *Bifidobacterium bifidum* milk containing Denak powder (*Oliveria decumbens* Vent) powder at first passage: In order to produce milk containing the probiotic bacterium *Bifidobacterium bifidum*, four containers each containing 1 L of low-fat sterilized milk (1.5% fat) were considered as our four groups. The starter (*Bifidobacterium bifidum*) was added directly to all the containers, followed by adding Denak powder of 0 (Control sample), 0.5, 1 and 1.5% to all the containers, respectively and finally they were placed in the incubator at 38°C. The acidity test was performed approximately every 2 h until reaching 42° Dornic. The samples were then taken out of incubator and transferred to a refrigerator and stored at 2°C. The produced probiotic milk was evaluated once every 7 days by counting the microbes using direct counting method.

Preparation of probiotic *Bifidobacterium bifidum* yoghurt containing Denak powder (*Oliveria decumbens* Vent) at the second passage: To produce *Biidobacterium bifidum* yoghurt in this stage, after providing 4 containers, 1 L of the low-fat sterilized probiotic milk (1.5 % fat) from the control group at first passage and the (1.5%) starter of low-fat yoghurt (1.5%) were added to each container. Different concentrations of Denak powder (0, 0.5, 1, and 1.5%) were added, respectively to the containers and mixed properly so that Denak powder was uniformly dissolved. Afterwards, all the containers were placed in the incubator at 38°C. Approximately every 2 h, the acidity and pH tests were done until acidity reached 90° Dornic. Then, the samples were taken out of the incubator and transferred to a refrigerator and stored at 2°C. The produced probiotic Denak yoghurt was evaluated every 7 days by counting the microbes using direct counting method and after 10 days the yoghurt was evaluated for sensory properties, using questionnaires filled by 15 participants. The respondents were asked to rate the factors of scent, taste and permanence on a scale ranging from very good, good, medium, to weak. The results were analyzed in a statistical descriptive test by SPSS version 17 software.

Preparation of probiotic *Lactobacillus acidophilus* milk containing Denak powder (*Oliveria decumbens* Vent) at first passage: All the same procedures were followed as mentioned above with the difference of using *Lactobacillus acidophilus* instead of *Bifidobacterium bifidum.*

Preparation of probiotic *Lactobacillus acidophilus* yoghurt containing Denak powder (*Oliveria decumbens* Vent) at second passage: All the same procedures were followed as mentioned above with the difference of using *Lactobacillus acidophilus* instead of *Bifidobacterium bifidum*.

Having produced the above-mentioned products, we stored 1000 g of each product in a disposable container placed in a refrigerator for 21 days. During this period, each sample was tested in days 1, 7, 14 and 21 for acidity, pH and sensory properties.

Statistical analysis: All the above experiments were repeated three times with each test carried out in triplicate. SPSS17 was used for one-way analysis of variance for all data, and significant differences ($p<0.05$) among means were determined by the least significant difference test.

RESULTS

Table 1 and 4 show the acidity degrees of Denak milk and yoghurt in *Lactobacillus acidophilus* and *Bifidobacterium bifidum* samples during storage time in the refrigerator. The results of these tables show the positive correlation between increased acidity value and increased Denak concentration which the samples containing 1.5% Denak powder in milk and yoghurt had high acidity value than the other samples investigated.

Table 2 and Table 5 show the growth rates of microbes in Denak milk and yoghurt in *Lactobacillus acidophilus* and *Bifidobacterium bifidum* samples at storage time. The results show that the growth rate of bacteria was increased by increasing the concentration of Denak powder and reached the desired acidity at shorter period.

Table 3 shows the microbial growth on MRS-A cultivation environment of *Lactobacillus acidophilus* Denak milk and yoghurt at refrigerator during 21 day of storage. The samples containing 1.5% Denak powder possessed the highest count of bacteria.

The microbial growth on MRS-A cultivation environment of *Bifidobacterium bifidum* Denak milk and yoghurt at refrigerator during 21 days was poor because *Bifidobacterium bifidum* has good growth on MRS Broth. The microbial growth of *Bifidobacterium bifidum* on MRS

Table 1: The acidity level based on Dornic degree in the Denak *Lactobacillus acidophilus* milk and yoghurt within 21 day storage in the refrigerator

	Acidity level in dornic degree			
	1st day	7th day	14th day	21st day
Denak yoghurt (%)				
0	93	95	99	100
0.5	98	110	121	143
1	109	123	134	158
1.5	117	142	151	187
Denak milk (%)				
0	48	50	53	55
0.5	53	59	70	78
1	60	66	77	87
1.5	69	78	87	103

Table 2: Growth of microbes in the Denak *Lactobacillus acidophilus* milk and yoghurt

	1st day	7th day	14th day	21st day
Denak yoghurt (%)				
0	14.25×10^{10}	9.75×10^{10}	5.5×10^{10}	3×10^{10}
0.50	25×10^{10}	11×10^{10}	19.25×10^{10}	5.75×10^{10}
1	15.5×10^{10}	18.25×10^{10}	10.75×10^{10}	6.5×10^{10}
1.50	40.25×10^{10}	16.25×10^{10}	25.75×10^{10}	9.5×10^{10}
Denak milk (%)				
0	11.5×10^{10}	8×10^{10}	17.5×10^{10}	6.5×10^{10}
0.50	33.5×10^{10}	19.5×10^{10}	26.5×10^{10}	4×10^{10}
1	38.25×10^{10}	39.25×10^{10}	30.25×10^{10}	16.75×10^{10}
1.50	22.5×10^{10}	58.75×10^{10}	28.5×10^{10}	19.5×10^{10}

Table 3: Microbial growth on MRS-A cultivation environment of *Lactobacillus acidophilus* Denak milk and yoghurt at refrigerator during 21 days insolubility

	1st day	7th day	14th day	21st day
Denak yoghurt (%)				
0	25×10^{10}	173×10^{9}	95×10^{9}	15×10^{9}
0.5	45×10^{10}	221×10^{9}	118×10^{9}	75×10^{9}
1	60×10^{1}	465×10^{9}	225×10^{9}	110×10^{9}
1.5	115×10^{10}	655×10^{9}	412×10	143×10^{9}
Denak milk (%)				
0	$10^{9}\times220$	335×10^{9}	55×10^{9}	35×10^{9}
0.5	425×10^{9}	565×10^{9}	180×10^{9}	70×10^{9}
1	95×10^{10}	165×10^{10}	385×10^{9}	105×10^{9}
1.5	215×10^{10}	285×10^{10}	520×10^{9}	215×10^{9}

Table 4: Acidity level based on Dornic degree in the Denak *Bifidobacterium bifidum* milk and yoghurt within 21-day storage in the refrigerator

	Acidity level in dornic degree			
	1st day	7th day	14th day	21st day
Denak yoghurt (%)				
0	92	96	99	106
0.5	100	117	124	148
1	114	129	143	167
1.5	124	138	155	190
Denak milk (%)				
0	48	51	54	57
0.5	54	59	63	78
1	58	69	75	87
1.5	66	80	89	99

Table 5: Growth of microbes in the Denak *Bifidobacterium bifidum* milk and yoghurt

	1st day	7th day	14th day	21st day
Denak yoghurt (%)				
0	8.25×10^{10}	22.5×10^{10}	3.75×10^{10}	1.25×10^{10}
0.5	29.75×10^{10}	33.5×10^{10}	12.25×10^{10}	3.5×10^{10}
1	38.5×10^{10}	22.25×10^{10}	18.75×10^{10}	5×10^{10}
1.5	70.5×10^{10}	40.75×10^{10}	20.5×10^{10}	7.5×10^{10}
Denak milk (%)				
0	$10^{10}\times15.25$	30.25×10^{10}	17.25×10^{10}	10.75×10^{10}
0.5	21.5×10^{10}	55.75×10^{10}	25.75×10^{10}	20.5×10^{10}
1	15×10^{10}	40.5×10^{10}	36.5×10^{10}	30.5×10^{10}
1.5	20.75×10^{10}	25.5×10^{10}	48.5×10^{10}	46×10^{10}

Broth was high. It was observed that *Bifidobacterium bifidum* has high inhibitory activity in MRS Agar during 21 days of storage. These results showed that Denak was suitable for this intestinal bacterium that was kept viable up to the end of fermentation (21 days). All tested *Bifidobacterium bifidum* was capable of growing well on Denak milk and yoghurt without nutrient supplementation.

DISCUSSION

In the present study, the effects of Denak (*Oliveria decumbens* Vent) on the growth and viability of the bacteria *Bifidobacterium bifidum* and *Lactobacillus acidophilus* in probiotic milk and yoghurt were investigated. The acidity, pH and survival of the bacteria in Denak probiotic milk and yoghurt were evaluated at 2 h intervals till reaching 42° Dornic acidity degrees for milk and 90° Dornic degree for yoghurt in the incubator at 38°C. At the first hours of production, the *Lactobacillus acidophilus* milk containing 1 and 1.5% Denak powder reached the acidity of 42° Dornic earliest, followed by 0.5 and 0% milk. Once they reached this acidity level, they were transferred to a refrigerator at 2°C. The storage time in the refrigerator was determined to be 21 days. In direct microbial counting in first day, the highest counts were sequentially in the samples with 0.5, 1 and 1.5% and the controls, indicating the positive correlation between increased bacterial growth and increased Denak concentration. Upon evaluation of the cultured samples on MRS agar media, the same correlation was revealed. The *Lactobacillus acidophilus* yoghurt with 1.5% Denak powder reached the acidity of 90° Dornic earliest, followed by the samples with 1 and 0.5% and the control, Once they reached this acidity level, they were transferred to a refrigerator at 2°C. The storage time in the refrigerator was found to be 21 days. Although the basic feature of the probiotic products consumption is their medicinal effects (bio value), their associated sensory properties are also important. In other words, sensory properties rather than medicinal effects play the most important role in their daily consumptions. Among the probiotic products, fermented ones especially the probiotic yoghurt is popular worldwide for its unique sensory properties (Mortazavian and Sohrabvandi, 2006).

The sensory evaluation was performed by 15 participants for the probiotic *Lactobacillus acidophilus* yoghurt with varying concentrations of Denak powder, after seven days. There were significant differences between the samples ($p>0.05$) and it was shown that the increase of Denak powder gives rise to favorable taste, color, scent and thickness.

The minimum required level of probiotic bacteria to be useful for the consumer's body is 10^7CFU mL^{-1} of living bacteria and the level in the present study was found to be 10^{10}, thus, it could be beneficial for the consumers (Marhamatizadeh *et al*., 2009).

Upon evaluation of the samples on MRS Agar, the *Lactobacillus acidophilus* with Denak powder had the counts equal to logarithmic 10^9 in day 14 and the sample product with 1.5% Denak powder possessed the highest count of bacteria.

Bifidobacterium bifidum milk containing 1.5 and 1% Denak powder reached 42° Dornic acidity earliest than others, followed by the milk with 0.5% and finally the control. Once reached 42° Dornic, the samples were transferred to a refrigerator at 2°C. The permanence of the product in the refrigerator was determined to be 21 days during which the acidity of control sample was lower than other samples.

As revealed in direct microbial counting, the count in day 7 was higher, compared to day 1, for all Denak concentrations, but possessed logarithmic coefficient 10^{10}. The bactericidal and inhibitory effect of low pH was stronger for *Bifidobacterium bifidum* than *Lactobacillus acidophilus* and it seems that during the storage time and enhanced fermentations process, decreased pH caused decreased growth of *Bifidobacterium bifidum*.

At the first hours of production, the *Bifidobacterium bifidum* yoghurt with 1.5 and 1% Denak powder reached 90° Dornic acidity earliest, followed by the yoghurt sample with 0.5% and the control. They were transferred to a refrigerator at 2°C, once reached the 90° Dornic acidity.

The product permanence in the refrigerator was found to be 21 days. No significant difference was observed in the *Bifidobacterium bifidum* yoghurt with Denak powder in terms of color, thickness, taste and scent. The sample with 1.5% was with the highest bacterial counts, as revealed in the evaluation of the samples in direct counting method.

The results of the studies addressing the probiotic bacteria have demonstrated the following: The increased concentration of malt and soya caused increase in the microorganism growth and rising acidity level which in turn resulted in shorter incubation time for the desired acidity. In a study on the effects of soya powder on the growth of the bacteria, *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, in probiotic products, it was demonstrated that the shelf life for the acidity reaching the desired level during incubation decreased for the milk with both bacteria and combined soya and malt, compared to the milk with only soya. As for the yoghurt with both

bacteria, the same results were yielded and incubation time for the yoghurt with malt and soya was decreased (Marhamatizadeh *et al*., 2009, 2011a).

The effect of honey on the growth of the above-mentioned bacteria introduced simultaneously into dairy products and drinks was investigated and the results indicated that yoghurt with only *Lactobacillus acidophilus* tasted sourer than the yoghurt with both bacteria. The products containing *Bifidobacterium bifidum*, compared to those with *Lactobacillus acidophilus*, were with slower growth rate and also tasted less sour and were of longer permanence. They were not of favorable taste when honey concentration increased and the control was of the best taste among all the samples (Marhamatizadeh *et al*., 2010).

In another study addressing the effect of cinnamon on the bacterial growth, it was demonstrated that the increased cinnamon concentration promoted the growth of the bacteria in probiotic milk and yoghur. (Yaghtin, 2010).

In another study addressing that investigated the effect of spearmint on the bacterial growth, it was demonstrated that increased spearmint concentration promoted the growth of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in probiotic milk and yoghurt (Marhamatizadeh *et al.,* 2011b).

In another study addressing the effect of juice on the bacterial growth, it was demonstrated that the increased juice product promoted the growth of the bacteria in probiotic orange and apple (Marhamatizadeh *et al*., 2012a).

In a study that investigated the effect of garlic on bacterial growth and survival, it was observed that increased garlic concentration promoted the growth and viability of probiotic bacteria in milk and yoghurt during refrigerated storage (Marhamatizadeh *et al.,* 2012b).

In another investigation addressing the effect of dill extract on growth and survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, it was represented that dill extract has positive effect on growth and viability of probiotic bacteria in milk and yoghurt during permanence period and finally leaded to produce new fermented dairy product (Marhamatizadeh *et al.,* 2012c).

In experiment researchers investigated the effect of permeate on the growth and survival of the above mentioned bacteria (*Lactobacillus acidophilus* and *Bifidobacterium bifidum*) was indicated that the permeate was suitable support for intestinal bacteria that had kept viable up during 21 days of refrigerated storage and final evaluation of products showed that permeate can be successfully used in the preparation of nutritive probiotic beverages (Marhamatizadeh *et al.,* 2012d).

CONCLUSION

The results of the experiments in this work represent that Denak (*Oliveria decumbens* vent.) has suitable support for these intestinal bacteria for production of probiotic products that were kept viable up to the end of fermentation (21 days). All tested strains were proved a good growth capacity in Denak milk and yoghurt without nutrients added, this being a guarantee on the one hand for the normal evolution of the fermentation and on the other hand for the stability of the final product. Thus it seems that the nutrients are available in acceptable forms and in optimal concentrations in the tested Denak milk and yoghurt. Further, the viability of the probiotics is essential for the quality of the fermented dairy products. The survival of probiotic Denak milk and yoghurt in refrigerated conditions for at least 21 days was in number of greater than 10^9 cfu mL^{-1} which is essential if a product should have probiotic properties. It is important to emphasize that all the products possessed excellent stability during 21 days of storage. The sensory scores of the products were acceptance. From the foregoing results it can be concluded that Denak can be successfully used in formulation of dairy products.

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