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Effect of Olive Leaf Extract on Growth and Viability of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* for Production of Probiotic Milk and Yoghurt

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ABSTRACT: The present study was designed to investigate the effect of olive leaf extract on Growth and viability of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in milk and yoghurt during 21 days refrigerated storage. In order to determine the effect of different doses of olive leaf extract on growth and viability 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. Olive leaf extract at the concentrations of 0.2, 0.4 and 0.6% 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 produced samples were then examined in terms of pH, acidity and microbial count during the incubation period, and permanence. The number of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in olive leaf milk and yoghurt were significantly higher than those in the control milk and yoghurt. The results indicate the positive correlation between increased bacterial growth and increased olive leaf concentration. In the samples containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, it was observed that increased concentrations of olive leaf extract create a favorable taste in milk and yoghurt. The samples with 0.6% olive leaf in milk and yoghurt had greater viscosity than the other samples investigated. This research will create a market potential for a range of new health based food to maintain optimal human health. This experiment is the first attempt to produce fermented dairy product with olive leaf extract.

***Keywords*:** Olive Leaf Extract, Probiotic, Bifidobacterium bifidum, Lactobacillus acidophilus

INTRODUCTION

During the last decade, fundamental studies opened a new field of research dealing with bioactive or biogenic substances derived from or synthesized in foods through fermentation (Gobbetti et al., 2010).Overall, functional foods or beverages are fortified through addition of exogenous functional compounds or using microorganisms that produce biogenic compounds or have probiotic features. Currently, fermented milk and yoghurt are the most diffuse functional in the market.

Olive leaf (*Olea europaea L*.) is known to be resistant in nature to microorganisms and insect attack, and much research has focused on the antimicrobial activity of compounds contained in olive and olive oil. Phenolic compounds including oleuropein (Fig. 1), tyrosol, hydroxytyrosol, caffeic acid, gallic acid, syringic acid, p- coumaric acid and luteolin, isolated from olive leaves, have been shown to inhibit or delay the rate growth of a range of microorganisms (Korukluoglu et al.,2008). Furthermore, olive leaf extract and its individual constituents are considered safe and non-toxic for human and animal consumption. (Abaza et al.,2008; Ritchason,1999).

Olive leaves contain high quantities of phenol substances very similar to those present in olives and their derived products (De Leonardis et al., 2008). There is compelling scientific evidence that olive leaf polyphenols are bioactive compounds. Olive leaves or their specific organic, show antiviral (Lee-Huang et al., 2003), antimicrobial (Bisignano et al., 1999) antioxidant and anti-inflammatory (Mann et al., 1999) properties, atherosclerosis inhibition and hypotensive action (Khayyal et al., 2002; Somova et al., 2003) and anti-carcinogenic properties that lead to the prevention of some cancers (Owen et al., 2004) and finally, stimulation of the thyroid (Al-Qarawi et al., 2002).

Different results were obtained regarding the effects of plant extracts rich in polyphenolic compounds on the growth of probiotic bacteria and other microorganisms. It was proved that plant extracts can inhibit the growth of food associated pathogens and micro-organisms responsible for food spoiling, as well as intestinal microflora, both pathogenic and physiological (Medina et al., 2006; Nagayamaet al., 2002).

Only few studies refer to the effect of polyphenolic plant rich extract on probiotic bacteria. Accordingly, the objective of this study was to evaluate the effect of olive leaf extract (0,0.2,0.4,0.6 %) on growth and viability of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* and functional properties of milk and yoghurt during refrigerated storage.

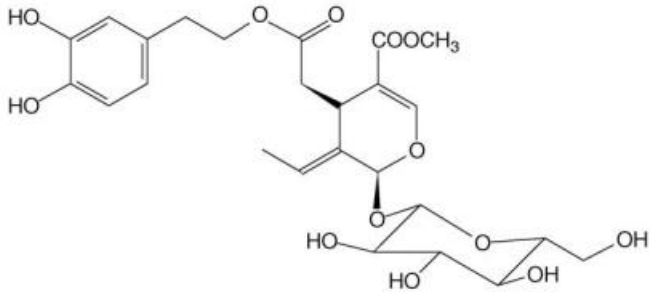

Figure 1. Chemical structure of oleuropein, the major component of the olive leaf extract.

MATERIALS AND METHODS

Materials

In July 2012, olive leaves were randomly and directly picked from an olive tree. The collected samples were put in plastic bags. The plant material was then dried at room temperature and powdered (20 mesh). 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).

Olive Leaf Extract Preparation

Olive Leaf Extract was prepared by mixing olive leaves powder with ethanol (96%) in the ratio of 20:400 by soxhlet system. The extraction lasted for three hours and ethanol was evaporated on rotary evaporator (Heidolph model no Laboro TA4000). Then the obtain extract was percolated through a bed of activated carbon (1 g of activated carbon for every 100 mL of extract). The filtered sample transferred to vacuum oven for four days to concentrate the olive leaf extract and after this time the extract kept for further use in a cold (4°C) and dry place. Rotary evaporator was used to separate olive leaf extract into its components based on their respective volatilities, through the process of evaporation and condensation.

Methods

Preparation of probiotic Bifidobacterium Bifidum milk containing Olive Leaf Extract (OLE) at first passage

In order to produce milk containing the probiotic bacterium *Bifidobacterium bifidum*, four containers each containing 1 liter of low-fat sterilized milk (1.5% fat) were considered as our four groups. The starter (*Bifidobacterium bifidum*) was added to all the containers, followed by adding olive leaf extract of 0 (Control sample), 0.2, 0.4, and 0.6% to all the containers, respectively and finally they were placed in the incubator at 38°C. The acidity test was performed approximately every 2 hours 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 Olive Leaf Extract (OLE) at the second passage

To produce *Bifidobacterium bifidum* yoghurt in this stage, after providing 4 containers, 1 liter 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 olive leaf extract (0, 0.2, 0.4, and 0.6%) were added respectively to the containers and mixed properly so that olive leaf extract was uniformly dissolved. Afterwards, all the containers were placed in the incubator at 38°C. Approximately every 2 hours, 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 olive leaf yoghurt was evaluated every 7 days by counting the microbes using direct counting method and after 7 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 Olive Leaf Extract (OLE) 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 Olive Leaf Extract (OLE) 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 gram 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 AND DISCUSSION

Results

Table 1 and Table 4 show the acidity degrees of olive leaf extract 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 values and increased olive leaf extract concentration which the samples containing 0.6% olive leaf extract in milk and yoghurt had high acidity value than the other sample investigated.

Table 2 and Table 5 show the growth rates of microbes in olive leaf extract 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 Olive leaf extract and reached the desired acidity at shorter period.

Table 3 shows the microbial growth on MRS-A cultivation environment of *Lactobacillus acidophilus* olive leaf extract milk and yoghurt at refrigerator during 21 day of storage. The samples containing 0.6% olive leaf extract powder possessed the highest count of bacteria.

The microbial growth on MRS-A cultivation environment of *Bifidobacterium bifidum* Olive leaf 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 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 Olive leaf extract was suitable for this intestinal bacterium that was kept viable up to the end of fermentation (21days). All tested *Bifidobacterium bifidum* was capable of growing well on Olive leaf extract milk and yoghurt without nutrient supplementation.

Table 1. The acidity level based on Dornic degree in milk and yoghurt containing *Lactobacillus acidophilus* with Olive Leaf Extract(OLE) within 21 days storage in the refrigerator.

	Acidity level in Dornic degree								
OLE milk	first day	7^{th} day	14^{th} day	21^{th} Day	OLE yoghurt	first day	7^{th} Day	14^{th} day	21^{th} day
0%	43	45	43	43	0%	91	94	93	93
0.2%	45	49	48	51	0.2%	93	95	95	94
0.4%	48	53	56	59	0.4%	95	98	99	97
0.6%	48	59	76	80	0.6%	97	110	117	110

Table 2. Growth of microbes in milk and yoghurt containing *Lactobacillus acidophilus* with Olive Leaf Extract(OLE) within 21days storage in the refrigerator

(OLE) milk	first day	7^{th} day	14^{th} day	21^{th} Day	(OLE) yoghurt	first day	7^{th} Day	14^{th} day	21^{th} day
0%	9.5×10^{10}	11×10^{10}	17.5×10^{10}	6.5×10^{10}	0%	11.25×10^{10}	14.25×10^{10}	7.5×10^{10}	4.25×10^{10}
0.2%	25.5×10^{10}	22.5×10^{10}	28.5×10^{10}	11×10^{10}	0.2%	17.5×10^{10}	11.5×10^{10}	13.25×10^{10}	7.75×10^{10}
0.4%	32.25×10^{10}	39.25×10^{10}	45.25×10^{10}	16.75×10^{10}	0.4%	22.5×10^{10}	24.25×10^{10}	24.75×10^{10}	13.5×10^{10}
0.6%	25.5×10^{10}	50.75×10^{10}	52.5×10^{10}	24.5×10^{10}	0.6%	31.25×10^{10}	39.25×10^{10}	41.75×10^{10}	27.5×10^{10}

Table 3. The microbial growth on MRS-A cultivation environment of *Lactobacillus acidophilus* Olive Leaf Extract milk and yoghurt at refrigerator during 21 days insolubility.

(OLE) milk	first day	7^{th} day	14^{th} day	21^{th} Day	(OLE) yoghurt	first day	7^{th} Day	14^{th} day	21^{th} day
0%	$10^9\times110$	225×10^9	45×10^9	15×10^9	0%	35×10^{10}	103×10^9	95×10^9	15×10^9
0.2%	425×10^9	465×10^9	110×10^9	75×10^9	0.2%	35×10^{10}	180×10^9	118×10^9	79×10^9
0.4%	75×10^{10}	160×10^{10}	275×10^9	125×10^9	0.4%	45×10^{10}	365×10^9	225×10^9	120×10^9
0.6%	110×10^{10}	205×10^{10}	310×10^9	175×10^9	0.6%	105×10^{10}	420×10^9	312×10^9	143×10^9

Table 4. The acidity level based on Dornic degree in milk and yoghurt containing *Bifidobacterium bifidum* with Olive Leaf Extract(OLE) within 21days storage in the refrigerator

	Acidity level in Dornic degree								
(OLE) milk	first day	7^{th} day	14^{th} day	21^{th} Day	(OLE) yoghurt	first day	7^{th} Day	14^{th} day	21^{th} day
0%	44	44	43	49	0%	92	94	97	94
0.2%	45	46	45	72	0.2%	94	96	94	94
0.4%	47	49	47	81	0.4%	96	100	103	98
0.6%	48	53	51	90	0.6%	100	105	110	110

Table 5. Growth of microbes in milk and yoghurt *Bifidobacterium bifidum* with Olive Leaf Extract(OLE) within 21days storage in the refrigerator

(OLE) milk	first day	7^{th} day	14^{th} day	21^{th} Day	(OLE) yoghurt	first day	7^{th} Day	14^{th} day	21^{th} day
0%	10^{10}×11.25	15.25×10^{10}	17.25×10^{10}	9.75×10^{10}	0%	13.25×10^{10}	22.5×10^{10}	19.75×10^{10}	7.25×10^{10}
0.2%	21. 5× 10^{10}	32.75×10^{10}	21.75×10^{10}	19.5×10^{10}	0.2%	17.75×10^{10}	31.5×10^{10}	39.25×10^{10}	9.5×10^{10}
0.4%	23×10^{10}	41.5×10^{10}	48.5×10^{10}	27.5×10^{10}	0.4%	32.5×10^{10}	29.25×10^{10}	42.75×10^{10}	22×10^{10}
0.6%	27.75×10^{10}	52.5×10^{10}	59.5×10^{10}	41×10^{10}	0.6%	39.5×10^{10}	47.75×10^{10}	57.5×10^{10}	31.5×10^{10}

Discussion

In the present study, the effects of Olive leaf extract (*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 Olive leaf extract probiotic milk and yoghurt were evaluated at 2 hours 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 0.4 and 0.6% Olive leaf extract reached the acidity of 42°Dornic earliest, followed by 0.2, 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 0.2, 0.4, & 0.6% and the controls, indicating the positive correlation between increased bacterial growth and increased Olive leaf extract concentration. Upon evaluation of the cultured samples on MRS agar media, the same correlation was revealed. The *Lactobacillus acidophilus* yoghurt with 0.6% Olive leaf extract reached the acidity of 90°Dornic earliest, followed by the samples with 0.4, & 0.2% 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 21days.

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 Olive leaf extract, after seven days. There were significant differences between the samples ($p < 0.05$) and it was shown that the increase of olive leaf extract 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 Olive leaf extract powder had the counts equal to logarithmic 10^9 in day 14, and the sample product with 1.5% Olive leaf extract powder possessed the highest count of bacteria.

The milk containing *Bifidobacterium bifidum* with 0.4 and 0.6 % Olive leaf extract reached 42°Dornic acidity earliest than others, followed by the milk with 0.2% 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.

The release of lactic acid is an indication of the activity of the probiotic bacteria (Ustunol, 2000) and the pH of all the cultures was monitored to provide an indication of The milk and yoghurt fermented with *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in the presence of olive leaf extract had a significant lower pH than the control, suggesting an appreciable amount of lactic acid had been produced. These results of lower pH values in the milk containing *Lactobacillus acidophilus* with olive leaf extract could be attributed to the phenolic compounds of the extract, which are known to serve as an oxygen scavenger and to reduce the redox potential of the growth media, as probiotic bacteria grow better in the absence of oxygen.

As revealed in direct microbial counting, the count in day 14^{th} was higher, compared to day 1, for all Olive leaf extract 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 0.4 and 0.6% Olive leaf extract reached 90°Dornic acidity earliest, followed by the yoghurt sample with 0.2% 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 Olive leaf extract in terms of color, thickness, taste and scent. The sample with 0.6% 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; 2011)

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 yoghurt. (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*.,* 2011)

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., 2012)
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*.,* 2012)

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., 2012)

In experiment that 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*.,* 2012)

CONCLUSION

The results of this experiment represent that olive (*Olea europaea L*.) leaf extract (OLE) has suitable support for these intestinal bacteria for production of probiotic products that were kept viable up to the end of fermentation (21days). It is important to emphasize that all the products possessed excellent stability during 21 days of storage. All tested strains were proved a good growth capacity in olive leaf extract 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 polyphenol compounds in olive leaf extract were responsible for the stimulation of probiotic bacteria growth and survival and that can ingested in human diet might have the same effect on desirable components of the intestinal microflora. The results of the research will be used in functional food development and food preservation purposes. This research will create a market potential for a range of new health based food to maintain optimal human health. This experiment is the first attempt to produce fermented dairy product with olive leaf extract. According to these findings, addition of olive leaf extract to milk and

yoghurt can be recommended to take advantage of their beneficial properties on human health attributed to antimicrobial activities.

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