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# Effect of Olive Leaf Extracts on the Growth and Metabolism of Two Probiotic Bacteria of Intestinal Origin

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**Abstract:** The increase in viable cell numbers and the production of Short-Chain Fatty Acids (SCFA) by *Bifidobacterium infantis* and *lactobacillus acidophilus*-both of human intestinal origin-were measured over 16 h at 37°C in reconstituted skim-milk (100 g $L^{-1}$) and skim-milk with one of three olive leaf extracts (water, ethanolic and methanolic extracts). The olive leaves were collected from a well known olive tree variety in Jordan (Nabali). All the three olive leaf extracts increased cell counts over the control with no extracts and the final values for SCFA, especially acetic acid secreted by *L. acidophilus* (1.84 $gL^{-1}$), were significantly higher than those observed in milk alone. The ethanolic extract of olive leaves showed highest effect on cell count and SCFA production for both bacterial species. It is proposed that the polyphenol compounds in olive leaf extracts were responsible for the stimulation of probiotic bacteria growth and metabolism and that olive leaf extracts 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 research is the first attempt to produce fermented milk with olive leaf extracts.

**Key words:** Intestinal bacteria, olive leaf extracts, growth, fatty acid production, polyphenolics

## INTRODUCTION

Olive leaves are a copious by-products deriving from olive tree cultivation and olive mills. Large a mounts of leaves are principally generated during pruning of the trees and harvesting and working of the olives (De Leonardis *et al*., 2008). The industrial use of olive leaves is limited to animal feed and phytotherpy (Martin Garcia *et al*., 2003).

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; Briante *et al*., 2002) properties, atherosclerosis inhibition and hypotensive action (Khayyal *et al*., 2002; Somova *et al*., 2003; Fehri *et al*., 1994; Quiles *et al*., 2000) 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).

Beneficial properties of olive leaf extracts are further enhanced by the bioavailability of their polyphenolic constituents, which are readily absorbed through the gastrointestinal tract, resulting in significant levels in the circulation system (Visioli and Galli, 2000; Vissers *et al*., 2002). In relation to human, much concern has been focused on phenolic compounds from plants and foods that may modulate microbiota in the intestine by selectively increasing that growth of *bifidobacteria* and *lactobacilli* and decreasing that harmful bacteria such as clostridia.

The olive leaf polyphenol composition is similar to that of olive oil. Oleuropein and other secoiridoids are the principle compounds, while simple phenols, enclosed hydroxytyrosol, are present but in lower amounts (Tuck and Hayball, 2002). Olive leaves contain flaronoids such as: rutin flavonol, Luteolin-7-glucoside (Pereira *et al*., 2007) The olive leave extracts was shown to have an antioxidant capacity 400% higher than vitamin C and almost double that of green tea or grape seed extract (Ryan and Robards, 1998).

In the consequence of the high incidence of civilization diseases, science and industry direct their interest to ward production of food products, which beyond the normal nutritional function, deliver health benefits. These products are called functional foods and the probiotics are an example (Fuller, 1991; Robesfroid, 2000). In recent years, the developed countries have arrived at a new food formulations, derived from the combination of nutraceutical compounds and probiotic micro-organisms and formulations are setting quite a trend (De Leonardis *et al*., 2008). The growing popularity of functional food causes increasing interest in raw materials, which can raise the prohealth value of food when supplemented (Duda-Chodak *et al*., 2008).

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 (Kim *et al*., 2004; Medina *et al*., 2006; Mobe *et al*., 1999; Nagayama *et al*., 2002). Some researchers indicate that polyphenolics compounds from different plant extracts may have, also, negative effect on bacteria which are desirable for human health. Ligstroside, one of the polyphenolic compounds present in virgin olive oil, showed the strong bactericidal activity against a broad spectrum of micro-organisms both Gram-positive and Gram-negative (Duda-Chodak *et al*., 2008). Molan *et al*. (2009), found that green tea extract increased significantly the number of *Lactobacilli* and *Bifidobacterium*. However, Medina *et al*. (2006), found that olive oil was effective toward foodborne pathogens, intestinal microflora as well as positively acting micro-organisms like *L. acidophilus* and *Bif. bifidum*. The phenolic compound catechin had no influence on growth of *Clostridium* sp., but stimulated *Lactobacilli* and *Bifidobacterium* (Lee *et al*., 2006).

Only few studies refer to the effect of polyphenolic plant rich extract on probiotic bacteria. Accordingly, the knowledge of the interaction between particular micro-organisms and plant extracts rich in polyphenolic compounds is indispensable for appropriate utilization of those extracts. The doubts appear especially in the situation when food containing probiotic bacteria is supplemented with plant raw material rich in polyphenols. Duda-Chodak *et al*. (2008) reported that the probiotic yoghurt supplementation with plant materials should be proceeded by careful studies about their influence on the bacteria. Accordingly, the objective of study was to evaluate the influence of olive leaf extracts (water, ethanolic and methanolic extracts) on the growth and metabolism of *L. acidophilus* and *Bif. infantis* that had been isolated previously from infants living in Amman (Haddadin *et al*., 2004).

## MATERIALS AND METHODS

In November 2009, olive leaves were randomly and directly picked from an olive tree (*Nabali* variety). The trees have 10 years old, not irrigated and no phytosanitary treatments had been applied in the last year. The leaves were collected at the operator height around the whole perimeter of each tree. The collected samples were put in plastic bags. The plant material was then dried at room temperature and powdered (20 mesh).

**Olive leaf extracts preparation:** Ground powdered leaves were extracted in distilled water, ethanol (70% v/v) and methanol (70% v/v) at 20% (w/v) concentration. The mixtures were mixed on rotary shaker (New Brunswick Scientific, USA) for two hours and then for 15 min in ultrasonic bath (Bandelin Electronic-RK-103 H, Germany). The mixtures were filtered through whatman no: 4 and then membrane filter (0.45 um). The obtained solid residues of the olive leaf extracts, after solvents evaporation, were redissolved in 50 ml distilled water to give 50 mg $mL^{-1}$ expressed as (+) Catechin Equivalents (CE).

**Determination of total phenolics:** The concentration of phenolics in the extracts was determined by the method of Singleton *et al*. (1999) and results were expressed as (+) Catechin Equivalents (CE). Samples (0.5 ml) were mixed with 2.5 ml of 0.2 N Folin-Ciocalteu reagent (Sigma-Aldrich) for 5 min then 2 ml of 7.5% sodium carbonate were added. After standing for 2h at room temperature, the Absorbance was measured at 760 nm using UV/visible spectrophotometer (Jasco-V-530, Japan). The estimation of total phenolics compounds in the extract was carried out in triplicate. The concentration between 0-200 µg/ml was used as standard to produce the calibration curve.

**Bacterial strains:** The bacterial isolates had been identified previously as *Bifidobacterium infantis* and *Lactobacillus acidophilus* (Awaisheh *et al*., 2004) and these were stored at 4°C on slants of MRS Agar (Code: CM 361, Unipath Ltd., Basing stoke, Hants., UK) at the Nutrition and Food Technology department, University of Jordan. Prior to use in the experimental programme, 50 ml sterile MRS broth (Code: CM 359, PH 6.50±0.20) with cysteine-HCl (5 g $\ell^{-1}$) in Duran bottles were inoculated with a loopful of culture and incubated at 37°C for 16 h in anaerobic jar. Once activated, each culture was maintained by subculturing weekly by adding an inoculum (0.5 ml of the previous culture) to MRS broth (50 ml) with incubation at 37°C for 16 h.

**Optimum growth time for the cultures:** To determine the optimal incubation time in relation to the total viable count of the two species, batches of 500 ml of reconstituted (100 g $L^{-1}$) skimmed milk powder (Regilait, France) were dispensed into Duran bottles and heat treated at 73°C for 30 min. After cooling to 37°C, duplicate bottles of skimmed milk were inoculated with freshly prepared cultures of *Bif. Infantis* or *L. acidophilus* (20 ml $\ell^{-1}$) and the bottles incubated at 37°C. Samples were taken to determine the total viable count at the beginning of the experimental period and then after 4, 8, 12, 16 and 20 h of incubation. On each occasion, serial dilutions (down to $10^{-7}$) of the fermented milk were completed in test tubes of sterile peptone (9 ml, 1.0 g $\ell^{-1}$) and duplicate 0.1 ml aliquots were plated onto MRS Agar supplemented with cysteine-HCl (5 g $\ell^{-1}$) and incubated at 37°C for 48 h in anaerobic jars. The results were recorded as colony-forming units (cfu) per ml of milk.

**Preparation of milk with different olive leaf extracts:** Skimmed milk powder (Regilait, France) was reconstituted in distilled water (100 g $L^{-1}$) and dispensed into sterile bottles with serew-caps. The bottles of milk were then heat treated at 73°C for 30 min in a water bath. The extracts, all with a same concentration of 50 mg $mL^{-1}$ of (+) catechin equivalents, were sterilized by micro-filtration unit using a sterile cellulose-ester membrane (0.2 µm-Advantec MFS, Japan) fitted to a syringe that dosed the required a mount of each extract into the bottle of skim-milk. The rates of addition were 0.20, 0.40, 0.80, 1.60, 2.00, 4.00, 6.00 or 10 ml into individual bottles of skim-milk and these doses gave concentrations of olive leaf extracts (water, ethanolic and methanolic), expressed as (+) catechin equivalents, of 0.1, 0.2, 0.4, 0.8, 1.0, 2.0, 3.0 and 5.0 mg $mL^{-1}$ of growth medium. The volume of added extract was part of the total volume of growth medium (i.e 100 mL). Control bottles of skim-milk without olive leaf extracts were prepared at the same time. A similar batches of milk and olive leaf extract were employed to monitor the release of SCFA by the selected species at a concentration of 3 mg CE $mg^{-1}$. This level was the optimum for the growth of both species (see later).

**Estimation of growth:** Duplicate bottles at each olive leaf extracts concentration were inoculated with either *Bif. infantis* or *L. acidophilus* (1 ml aliquots of an active MRS broth culture) and incubated at 37°C for 16 h in anaerobic jars; duplicate bottles of the control milk were treated similarly. After incubation, serial dilutions (down to $10^{-7}$) were made as described above and the results were recorded as cfu per ml of milk.

**Production of Short-Chain Fatty Acids (SCFA):** Duplicate bottles of milk for each olive leaf extract at a concentration of 3 mg-CE $mL^{-1}$ were inoculated with either *Bif. infantis* or *L. acidophilus* (1.0 ml aliquots of an active MRS broth culture) and incubated at 37°C for 16 h in anaerobic jars; duplicated bottle of the control milk were treated similarly. The short chain fatty acids in the fermented milks were measured using the method proposed by Marsili *et al.* (1981). High Performance Liquid Chromatography (HPLC) was used. The chromatographic system (Jasco System, Japan) was equipped with a manual 20 µL Loop injector, a variable wavelength ultraviolet/visible dector (Jasco Model 875, Japan) and an insulated column oven (Jasco Model 865, Japan). Column effluents were monitored at a wavelength of 210 nm and quantification was based on peak height measurements using an integrator recorder (Shimadzu-C-R6A, Japan). Analyses were performed isocratically at a flow rate of 1.0 mL $min^{-1}$ and temperature of 25°C. The column used was a 150 x 4.6 mm Hypurity Advance (Thermo Quest, Hypersil Division, USA). The mobile phase was prepared by mixing $H_3PO_4$ (10g $L^{-1}$) with HPLC Grade methanol at a ratio of 95: 5. The mobile phase was micro-filtered using a PTFE membrane (0.2 µm) and then degassed by sonication and helium purging.

Acetic, propionic and butyric acids (Sigma, USA) were used as standards. Stock solutions of different concentrations of each acid were prepared, namely 100, 200, 300, 600 and 1000 mg $L^{-1}$. Each concentration was injected in duplicate to obtain its retention time and area under the curve. The coefficient of correlation (r), regression equation and standard curves for each acid were calculated using Microsoft office excel 2003. The test of significance of coefficient of correlation (r) values was carried out at 0.01 probability. The recovery percent of each acid was determined by adding a known a mount of each acid to a sample of fermented milk and, after mixing, taking 5 mL of the test mixture.

This sample was then centrifuged for 10 min. at 4000 rpm and the supernatant micro-filtered and analyzed using the HPLC. The same procedure was applied to the experimental samples.

**Measurement of pH:** A sub-sample (10 ml) of the each fermented milk use used to measure the pH using a digital pH meter Model HI8519 (Hanna Instruments, Germany).

**Statistical analysis:** The General Linear Model (GLM) produced by the Statistical Analysis System (SAS) version 7 (SAS® System for Microsoft® Windows® 2001), was used to analyze the data. Differences between the means of treatments were tested using the Least Significant Difference (LSD) test at $p<0.05$.

## RESULTS AND DISCUSSION

The growth of *Bif infantis* and *L. acidophilus* in skim-milk is shown in Table 1 and the trend was for the maximum viable cell count to be achieved after 16 h incubation. It was decided that all the test cultures could be incubated for 16 h, as the secretion of SCFA was considered as potentially the most important effect of the addition of olive leaf extracts.

Table 1: Growth of *Bif. Infantis* and *L. acidophilus* in reconstituted skim milk (100 g $L^{-1}$) at 37°C and sampled at the times indicated; all figures as CFU $ml^{-1}$ and means of duplicate samples from two bottles

| Time | *Bif. infantis* | *L. acidophilus* |
|---|---|---|
| 0.0 | $7.50 \times 10^6$ | $6.50 \times 10^5$ |
| 4 | $2.50 \times 10^7$ | $3.50 \times 10^6$ |
| 8 | $3.25 \times 10^7$ | $2.50 \times 10^7$ |
| 12 | $4.00 \times 10^7$ | $6.50 \times 10^7$ |
| 16 | $5.10 \times 10^7$ | $7.80 \times 10^7$ |
| 20 | $4.40 \times 10^7$ | $2.80 \times 10^7$ |

Table 2: Effects of different concentrations (mg catechin equivalents/ml of growth medium) of three olive leaf extracts on the growth of *Bif. infantis* over a period of 16 h; all figures as cfu $ml^{-1}$ and means±SD of triplicate samples from three bottles of milk

| Concentration of extract (mg CE $ml^{-1}$) | Water extract ($x10^8$) | Ethanolic extract ($x10^8$) | Methanol extract ($x10^8$) |
|---|---|---|---|
| 0.0 | $0.48±0.015^{ab}$ | $0.65±0.050^{abcl}$ | $0.68±0.015^{abc}$ |
| 0.1 | $0.65±0.010^{ab}$ | $0.82±0.010^{abc}$ | $0.75±0.050^{abc}$ |
| 0.2 | $0.92±0.060^{c}$ | $0.65±0.474^{abcl}$ | $0.81±0.011^{abc}$ |
| 0.4 | $1.77±0.251^{de}$ | $1.65±0.050^{d}$ | $1.10±0.100^{d}$ |
| 0.8 | $1.75±0.050^{de}$ | $2.15±0.217^{e}$ | $2.10±0.100^{ef}$ |
| 1.0 | $2.07±0.104^{f}$ | $2.80±0.100^{f}$ | $2.30±0.173^{ef}$ |
| 2.0 | $2.89±0.130^{gh}$ | $4.06±0.115^{gh}$ | $3.46±0.35^{g}$ |
| 3.0 | $2.91±0.076^{gh}$ | $4.20±0.100^{gh}$ | $3.79±0.085^{ha}$ |
| 5.0 | $0.15±0.010^{j}$ | $0.41±0.020^{acl}$ | $0.12±0.002^{l}$ |

• Means within a column with a different superscript letter are significantly different at ($p \leq 0.05$)

Table 3: Effects of different concentrations (mg catechin equivalents/ml of growth medium) of three olive leaf extracts on the growth of *L. acidophilus* over a period of 16 h; all figures as cfu $ml^{-1}$ and means±SD of triplicate samples from three bottles of milk

| Concentration of extract (mg CE $ml^{-1}$) | Water extract ($x10^8$) | Ethanolic extract ($x10^8$) | Methanol extract ($x10^8$) |
|---|---|---|---|
| 0.0 | $0.37±0.010^{al}$ | $0.44±0.045^{a}$ | $0.32±0.020^{a}$ |
| 0.1 | $0.86±0.076^{b}$ | $0.90±0.050^{b}$ | $0.74±0.032^{bc}$ |
| 0.2 | $1.10±0.100^{c}$ | $0.13±0.020^{c}$ | $0.93±0.025^{bc}$ |
| 0.4 | $2.06±0.152^{d}$ | $2.41±0.085^{d}$ | $1.40±0.050^{d}$ |
| 0.8 | $2.43±0.076^{e}$ | $2.84±0.050^{e}$ | $2.45±0.150^{e}$ |
| 1.0 | $3.00±0.200^{fg}$ | $3.68±0.170^{f}$ | $3.06±0.096^{f}$ |
| 2.0 | $3.13±0.057^{fg}$ | $3.94±0.150^{g}$ | $3.41±0.175^{g}$ |
| 3.0 | $3.64±0.083^{h}$ | $4.30±0.200^{h}$ | $3.80±0.086^{h}$ |
| 5.0 | $0.36±0.006^{al}$ | $5.53±0.208^{l}$ | $6.56±0.493^{l}$ |

• Means within a column with a different superscript letter are significantly different at ($p \leq 0.05$)

The total viable counts of *Bif. infantis* and *L. acidophilus* in skimmed milk with different concentrations of the three olive leaf extracts are summarized in Table 2 and 3, respectively. Nine concentrations of each extracts were used in this research and highest counts of *Bif. infantis* and *L. acidophilus* were related to both concentration and the type of extract. The level of 3 mg of (+) Catechin Equivalents (CE) per ml of the growth medium (100 mL) for all olive leaf extracts samples had the most significant effect on the count of *Bif. infants* and *L. acidophilus*. At 5 mg (+) catechin equivalents $mL^{-1}$ of the growth medium for all extracts, the counts of *Bif. infantis* and *L. acidophilus* showed a significant drops than the other lower concentrations. These results are in agreement with those reported by De Leonardis *et al.* (2008), in which the antimicrobial activities of olive leaf extract showed no inhibition on *streptococcars thermophilus* and *Lactobacillus delbruechii spp bulgaricus* up to concentration of 3.2 mg $ml^{-1}$ (as hydroxytyrosil) in the growth medurm.

They concluded that olive leaf extracts can be added as an integrator or antioxidant to fermented milk to increase both the quality and the nutritional value of the final milk product, without inducing any negative effects on the viability of the lactic acid bacteria. Similar results were, also, obtained by Molan *et al.* (2009), in which the addition of green tea extract resulted in a significant increase in the number of *lactobacilli* and *bifidobacteria*. This prebiotic activity of olive leaf extracts may be related to the higher total phenolic contents of these extracts (Molan *et al.*, 2009).

This prebiotic activity of olive leaf extracts, as non-carbohydrate prebiotics, is needed to stimulate the growth of probiotic bacteria without side effects such the enhancement of fructooligosaccharides to the growth of non-probiotic bacteria such as *Clostridium perfringens* and *Eubacterium biforme* (Bello *et al.*, 2001).

The mechanism by which phenolic extracts increased the growth of probiotic bacteria is not known. Molan *et al.* (2009) presented a possible partial explanation for this enhancing effects is the ability of polyphenols, in green tea extract, to act as antioxidant and antiradical agent to modulate the oxidative stress in the medium generated by the metabolic activities and consequently provide a better environment for the growth and multiplication of these bacteria. The importance of this research, is in the production of functional dairy products with an acceptable sensory properties and containing 300 mg CE per 100 ml milk. This product will enhance gut health and decrease putrefactive products and increased organic acids by lowering pH (Hara, 1997).

The statistical analysis did reveal that ethanolic extract of olive leaves had significantly better effect on the growth of *Bif. infantis* and *L. acidophilus* than the other extracts. This effect may be due to the ability of ethanolic extract to contain certain components that support the growth of *Bif. infantis* and *L. acidophilus*.

Table 4: Production of short-chain fatty at optimal catechin equivalents (3 ml CE $ml^{-1}$ of growth medium) of three extracts by *Bif. infantis* over a period of 16 h; all figures as g $L^{-1}$ and means±SD of triplicate samples from three bottles of milk

| Olive leaf extract | Acetic acid | Propionic acid | Butyric acid | pH |
|---|---|---|---|---|
| Water extract | 0.95±0.045$^{a}$ | 0.65±0.045$^{a}$ | 0.50±0.050$^{a}$ | 4.60±0.100$^{abc}$ |
| Ethanolic extract | 1.78±0.047$^{b}$ | 0.94±0.045$^{b}$ | 0.75±0.032$^{b}$ | 4.50±0.050$^{abc}$ |
| Methanolic extract | 1.31±0.030$^{c}$ | 0.80±0.050$^{c}$ | 0.62±0.0493$^{c}$ | 4.50±0.050$^{abc}$ |
| Control | 0.010±0.000$^{d}$ | 0.010±0.000$^{d}$ | 0.010±0.000$^{d}$ | 4.80±0.100$^{d}$ |

• Means within a column with a different superscript letter are significantly different at ($p \leq 0.05$)

Table 5: Production of short-chain fatty at optimal catechin equivalents (3 ml CE $ml^{-1}$ of growth medium) of three extracts by *L. acidophilus* over a period of 16 h; all figures as g $L^{-1}$ and means±SD of triplicate samples from three bottles of milk

| Olive leaf extract | Acetic acid | Propionic acid | Butyric acid | pH |
|---|---|---|---|---|
| Water extract | 1.10±0.050$^{a}$ | 0.94±0.040$^{a}$ | 0.73±0.032$^{abc}$ | 4.61±0.065$^{a}$ |
| Ethanolic extract | 1.84±0.041$^{b}$ | 1.11±0.032$^{bc}$ | 0.96±0.040$^{b}$ | 4.30±0.100$^{bc}$ |
| Methanolic extract | 1.54±0.081$^{c}$ | 1.05±0.066$^{bc}$ | 0.81±0.037$^{c}$ | 4.40±0.100$^{bc}$ |
| Control | 0.010±0.000$^{d}$ | 0.010±0.000$^{d}$ | 0.010±0.000$^{d}$ | 4.800±0.100$^{d}$ |

• Means within a column with a different superscript letter are significantly different at ($p \leq 0.05$)

The SCFA produced during fermentation are determined by the substrates available, their fermentability and the rate of breakdown (Parrett and Edwards, 1997) and the highest amounts found in this study were acetic acid, followed by propionic acid and butyric acid. This pattern is in with agreement with the results reported by Haddadin *et al*. (2007) and Topping and Clifton (2001), in which the concentrations of acetic, propionic and butyric acids were in the order: acetate > propionate = butyrate.

Regarding the metabolism of *Bif. infantis* (Table 4), There was significant difference between the different extracts with respect to the concentration of acetic, propionic and butyric acids and the control. The ethanolic extracts of the fermented milk contained the highest amount of all SCFA, followed by the methanolic extract and water extract. The ethanolic extract, also, supporting both the highest quantity of butyric acid and cell count. In the samples with *L. acidophilus*, there were significant differences between the three extracts with respect to the concentration of acetic acid, also, there was a significant difference between the extracts and the control (Table 5). The ethanolic extract showed the highest concentration for propionic and butyric acids and cell counts. In the milk samples containing *L. acidophilus*, there was a significant difference between the extracts and the control. The values of SCFA in the control milk were below the level of detection. The results showed that the ethanolic extract of olive leaves seems to contain components that favour the growth and the production of SCFA by *Bif. infantis* and *L. acidophilus*. Cornu *et al*. (1984) suggested that phenolic compounds in any plant extract could have an activating or inhibiting effect on probiotic bacteria, according to their constitution and concentration and the bacterial strain. The growth rate stimulation by phenolic compounds and the increase in cell density during the later stage of cell incubation could be related to their ability to metabolize these phenolic compounds (Stead, 1994; Vivas *et al*., 1997; Reguant *et al*., 2000).

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 total acidity (see Table 4 and 5). The milk fermented with *Bif. infantis* in the presence of olive leaf extracts had a significant lower pH than the control, suggesting an appreciable amount of lactic acid had been produced. In the case of *L. acidophilus,* the milks with olive leaf extract had significantly lower pH than the control (Table 5) and these values may be related to the amounts of lactic acid produced. These results of lower pH values in the milk with olive leaf extracts could be attributed to the phenolic compounds of the extracts, which are known to serve as an oxygen scavengers and to reduce the redox potential of the growth media, as probiotic bacteria grow better in the absence of oxygen (Alberto *et al*., 2004).

Overall, it is clear that all the olive leaf extracts beneficially influenced the growth and the metabolism of these two organisms of intestinal origin and it might be reasonable to assume that olive leaf extracts ingested by a consumer would have a similar effect on the native population of these species in the lower intestine. As a consequence of this research, it is expected that the twin beneficial attributes of polyphenolic rich extracts and probiotics of a fermented milk will create a market potential for a group of new health based functional food. If polyphenolics were the principal activators of the test bacteria, then olive leaf extracts may well have the beneficial role in promoting probiotic bacteria and inhibiting harmful bacteria such as *Clostridium* and *Escherichia coli*, thus maintaining optimal human health.

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