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The Enhancement of Hawthorn Leaf Extracts on the Growth and Production of Short Chain Fatty Acids of Two Probiotic Bacteria

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Abstract: Hawthorn leaf extracts were prepared using water, ethanol and methanol and they were evaluated for their effects on the growth and metabolism of *L. acidophilus* and *Bif. infantis* in skim-milk. Ten concentrations of each extracts were used and highest counts of *Bif. infantis* and *L. acidophilus* were related to both concentration and the type of extract. All the three hawthorn leaf extracts increased cell counts over the control with no extracts. The level of 5 mg of (+) Catechin Equivalents (CE) per mL of the growth medium (100 mL) for all hawthorn leaf extracts samples had the most significant effect on the count of *Bif. infants* and *L. acidophilus*. The final values for the Short Chains Fatty Acids (SCFA), especially acetic acid secreted by *L. acidophilus* (2.30 gL^{-1}), were significantly higher than those observed in milk alone. The ethanolic extract of hawthorn had significantly better effect on the growth of *Bif. infantis* and *L. acidophilus* than the other extracts, while water extract exhibited the lowest values. It is concluded that hawthorn leaf extracts showed growth-promoting and prebiotic activity on *Bif. infantis* and *L. acidophilus* and can be added to fermented milk to increase both the quality and the nutritional value of the final milk product and consequently human health.

Key words: Hawthorn leaf extracts, intestinal bacteria, short chain fatty acid production, phenols

INTRODUCTION

Recently, there is an increasing interest in natural extracts for the sake of their positive impact on human organisms (Duda-Chodak *et al*., 2008; Bialonska *et al*., 2009). Moreover, the growing demand for the plant extracts is observed in food and cosmetic industries, because they can be used as natural supplement prolonging the stability and storage life of food products (Grajek *et al*., 2005).

Hawthorn (*Crataegus oxyacantha*) is one of the most important traditional medical plant and has been used as a folk medicine and is widely utilized in food and pharmaceutical preparation mainly because of its beneficial health effects and its low toxicity (Bahorun *et al*., 1996; Liu *et al*., 2010). Hawthorn is a spiny, small tree or bush with white flower and red berries (Hamon, 1988). It is a member of the *Rosaceae* family (Upton, 1999) and is found in Europe, North Africa and Western Asia (Grive, 1971).

Crataegus oxyacantha extracts are widely used in treating cardiovascular diseases such as hypertension, hyperlipidemia and in particular congestive heart failure (Zapatero, 1999; Chang *et al*., 2005; Kwok *et al*., 2010; Miller, 1998), cancer, diabetes and sexual weakness in Arabic traditional medicine (Ljubuncic *et al*., 2005). They may induce anti-ischemia/reperfusion-injury and anti-arrhythmic effect (Chang *et al*., 2002). Consumption of hawthorn altered the digestive enzymes of the stomach and cholesterol metabolism of the liver (Zhu, 1998) and a reduction in blood lipid and cholesterol levels has been reported (Shanthi *et al*., 1994). In addition, they contain abundant amount of antioxidants such as chlorogenic acid, epicatechin, hyperoside and quercetin (Bahorun *et al*., 1996; Liu *et al*., 2010), which may be useful in alleviating the adverse effect associated with Low-Density Lipoprotein (LDL)-cholesterol oxidation in atherosclerosis (Stocker and Keaney, 2004). On the other hand, hawthorn extracts a bound in antioxidant compounds (Kostic *et al*., 2012), which exert antimicrobial (Rauha *et al*., 2000), antiviral (Gabbay *et al*., 2007), antitoxin (Friedman, 2007), or antifungal activity (Newton *et al*., 2002).

The active constituents and the antioxidant effects of the extracts of the leaves and flowers of *C. oxyacantha* have been widely studied. Flavonoids and procyanidins are considered to be the two main active constituents (Chang *et al*., 2002; Cui *et al*., 2006; Bernatoniene *et al*., 2010; Liu *et al*., 2010), which are readily absorbed through the gastrointestinal tract, resulting in significant levels in the circulation system (Vissers *et al*., 2002).

In relation to human, much concern has been focused on phenolic compounds from plant and foods that may modulate microbiota in the intestine by selectively increasing the growth of *bifidobacteria* and *lactobacilli* and decreasing the harmful bacteria such as *Closteridia sp*. (Onoue *et al*., 1997; Molan *et al*., 2009).

Intestinal microbiota (probiotic bacteria) have numerous effects on the immune system and host health. One

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important function is the fermentation of undigested food components leading to the production of metabolites which may exhibit significant health beneficial action, for example, the short chain fatty acid released by probiotic lactic bacteria (Gibson, 2008), production of some digestive enzymes and vitamins, production of antibacterial substances, e.g., organic acids, bacteriocins, lactones and other unidentified substances, removal of carcinogens, improvement of calcium absorption as well as the reduction of faecal enzyme activity (Ouwehand *et al*., 1999; Zubillaga *et al*., 2001; Holzapfel and Schillinge, 2002).

Plants are potential sources of growth modulators of intestinal bacteria because they constitute a rich source of bioactive chemicals and many of them are largely free from harmful adverse effects (Medina *et al*., 2006). Although, There is a great number of articles dealing with the inhibitory effect on different species of bacteria, only few of them refer to the effect on probiotic bacteria (Park *et al*., 2005; Valimaa *et al*., 2007). Accordingly, the aims of this study was to evaluate the influence of hawthorn 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

Hawthorn leaves were randomly and directly picked from hawthorn trees (*Crataegus oxyacantha*). The collected samples were put in plastic bags. The plant material was then dried at room temperature and powdered (20 mesh).

Hawthorn 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-103H, Germany). The mixtures were filtered through whatman no: 4 and then membrane filter (0.45 um). The obtained solid residues of the hawthorn 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 2 h 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^{-1} 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 L^{-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 L^{-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 L^{-1}) and duplicate 0.1 mL aliquots were plated onto MRS agar supplemented with cysteine-HCl (5 g L^{-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 hawthorn leaf extracts: Skimmed milk powder (Regilait, France) was reconstituted in distilled water (100 g L^{-1}) and dispensed into sterile bottles with screw-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 0.2, 0.4, 0.8, 1.6, 2.0, 4.0, 6.0, 10.0 or 20.0 mL into individual bottles of skim-milk and these doses gave concentrations of hawthorn 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, 5.0 and 10.0 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 hawthorn leaf extracts were prepared at the same time. A similar batches of milk and hawthorn leaf extract were employed to monitor the release of SCFA by the selected species at a concentration of 5 mg CE mg^{-1}. This level was the optimum for the growth of both species (see later).

Estimation of growth: Duplicate bottles at each hawthorn 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 hawthorn leaf extract at a concentration of 5 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 amount 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 was used to measure the pH using a digital pH meter Model HI8519 (Hanna Instruments, Germany).

Statistical analysis: All data was expressed as mean ± Standard Deviations (SD) and were tested by one-way analysis of variance using SPSS computer programme (version 10). Differences between the means of treatments were tested using the Least Significant Difference (LSD) test at $p<0.05$.

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	5.50×10^6	4.50×10^5
4	8.50×10^6	2.50×10^6
8	1.20×10^7	9.50×10^6
12	5.00×10^7	2.50×10^7
16	6.50×10^7	6.50×10^7
20	5.50×10^7	4.50×10^7

RESULTS AND DISCUSSION

Table 1 shows the growth of *Bif. infantis* and *L. acidophilus* in skim-milk. Maximum viable cell count was achieved after 16 h incubation and 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 hawthorn leaf extracts.

The total viable counts of *Bif. infantis* and *L. acidophilus* in skimmed milk with different concentrations of the three hawthorn leaf extracts are summarized in Table 2 and 3, respectively. Ten 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 5 mg of (+) Catechin Equivalents per mL of the growth medium (100 mL) for all hawthorn leaf extracts samples had the most significant effect on the count of *Bif. infants* and *L. acidophilus*. At 10 mg (+) Catechin Equivalents per mL of the growth medium for all extracts, the counts of *Bif. infantis* and *L. acidophilus* showed a significant drops than the other lower concentrations. This means that hawthorn extracs at appropriate concentrations could significantly activate the growth of probiotic bacteria, particularly *Bif. infantis* and *L. acidophilus*, in contrast with that previously reported in a study for Duda-Chodak *et al.* (2008), that hawthorn extracts contained no components of bactericidal or bacteriostatic activity against *Lactobacillus casei*.

Table 2: Effect of different concentrations (mg Catechin Equivalents/mL of growth medium) of three hawthorn 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 ($x10^8$)	Methanolic extract ($x10^8$)
0.0	0.73 ± 0.035^{a}	0.75 ± 0.007^{a}	0.73 ± 0.010^{a}
0.1	1.37 ± 0.153^{b}	2.35 ± 0.050^{b}	2.10 ± 0.100^{b}
0.2	1.82 ± 0.175^{c}	2.60 ± 0.050^{cd}	2.35 ± 0.050^{c}
0.4	2.40 ± 0.200^{d}	2.58 ± 0.076^{cd}	2.60 ± 0.050^{de}
0.8	2.67 ± 0.050^{e}	2.80 ± 0.050^{e}	2.68 ± 0.076^{def}
1.0	2.90 ± 0.152^{f}	3.06 ± 0.076^{f}	2.88 ± 0.072^{ef}
2.0	4.23 ± 0.132^{g}	4.72 ± 0.076^{g}	4.50 ± 0.010^{g}
3.0	5.55 ± 0.104^{h}	8.01 ± 0.076^{h}	7.20 ± 0.200^{h}
5.0	6.92 ± 0.015^{i}	9.50 ± 0.100^{i}	8.10 ± 0.264^{i}
10.0	0.49 ± 0.015^{j}	0.64 ± 0.015^{j}	0.46 ± 0.032^{j}

Means within a column with a different super script letter are significantly at ($p\leq0.05$)

Table 3: Effect of different concentrations (mg Catechin Equivalents/mL of growth medium) of three hawthorn 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 ($x10^8$)	Methanolic extract ($x10^8$)
0.0	0.45 ± 0.010^{a}	0.71 ± 0.010^{aj}	0.51 ± 0.010^{a}
0.1	1.14 ± 0.047^{bj}	1.30 ± 0.100^{b}	0.95 ± 0.010^{b}
0.2	1.14 ± 0.050^{c}	1.48 ± 0.076^{c}	1.10 ± 0.100^{c}
0.4	1.53 ± 0.152^{d}	1.80 ± 0.050^{d}	1.35 ± 0.050^{d}
0.8	2.45 ± 0.076^{e}	2.72 ± 0.076^{e}	2.60 ± 0.100^{e}
1.0	2.91 ± 0.076^{f}	3.20 ± 0.100^{f}	3.10 ± 0.100^{f}
2.0	4.23 ± 0.100^{g}	4.70 ± 0.100^{g}	4.50 ± 0.100^{g}
3.0	6.10 ± 0.152^{h}	8.20 ± 0.100^{h}	7.55 ± 0.050^{h}
5.0	6.36 ± 0.068^{i}	8.60 ± 0.100^{i}	8.10 ± 0.100^{i}
10.0	0.77 ± 0.068^{bj}	0.66 ± 0.005^{aj}	0.70 ± 0.010^{j}

Means within a column with a different super script letter are significantly at ($p\leq0.05$)

Our results suggested that hawthorn extracts can be supplemented to food products as a good source of prebiotic material, in addition to the other beneficial effects such as antioxidants and antimicrobial activity, which were previously demonstrated in several studies (Bahorun *et al*., 1996; Liu *et al*., 2010; Kostic *et al*., 2012) and without negative side effects (Lakshmi *et al*., 2012). Most reports suggest that the health effects of hawthorn can be attributed to the antioxidative activities of the fruits and phenolic compounds in combination with vitamin C are considered to be the major antioxidant and bioactive components in hawthorn fruits (Duda-Chodak *et al*., 2008; Kostic *et al*., 2012). Moreover, hawthorn fruit are rich in triterpenoids, organic acids, sugar alcohols and some other components with beneficial effects (Cui *et al*., 2006). So, the bioactivity of hawthorn leaf extracts as a prebiotic could be related to the higher total phenolic contents of these extracts (Duda-Chodak *et al*., 2008). Similar results were, also, obtained by Molan *et al*. (2009) and Haddadin (2010), in which the addition of green tea and olive leaf extracts resulted in a significant increase in the number of *lactobacilli* and *bifidobacteria* to the higher total phenolic contents of these extracts.

On the other hand, ethanolic extract of hawthorn 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*. Previously, Cui *et al*. (2006) suggested that the active phenolics of hawthorn fruit extract were concentrated by ethanolic extraction.

The SCFA compositions of the three extracts of hawthorn leaves were detected and determined by HPLC analysis (Table 4 and 5). The highest amounts found in this study were acetic acid, followed by propionic acid and butyric acid and there were significant differences between their concentration of different extracts and the control. This production patterns of SCFA were similar to the results reported by Haddadin (2010) using olive leaf extract.

The ethanolic extract, also, showed the highest concentration for SCFA, while water extract exhibited the lowest values in both growth media of *Bif. infantis* and *L. acidophilus*. The results showed that the ethanolic

Table 4: Production of short-chain fatty acid at optimal Catechin Equivalents (5 mg 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

Hawthorn extract	Acetic acid	Propionic acid	Butyriic acid	pH
Water extract	1.95 ± 0.055^{ac}	1.20 ± 0.050^{a}	0.87 ± 0.025^{a}	4.49 ± 0.036^{a}
Ethanolic extract	2.15 ± 0.050^{b}	1.32 ± 0.025^{bc}	0.97 ± 0.026^{b}	4.22 ± 0.028^{b}
Methanolic extract	1.96 ± 0.040^{ac}	1.29 ± 0.036^{bc}	0.92 ± 0.025^{c}	4.29 ± 0.015^{c}
Control	0.01 ± 0.000^{d}	0.01 ± 0.000^{d}	0.01 ± 0.000^{d}	4.79 ± 0.015^{d}

Means within a column with a different super script letter are significantly at ($p\leq0.05$)

Table 5: Production of short-chain fatty acid at optimal Catechin Equivalents (5 mg 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

Hawthorn extract	Acetic acid	Propionic acid	Butyriic acid	pH
Water extract	2.12 ± 0.025^{ac}	1.42 ± 0.025^{ac}	1.09 ± 0.036^{a}	4.40 ± 0.010^{a}
Ethanolic extract	2.30 ± 0.050^{b}	1.60 ± 0.050^{b}	1.19 ± 0.050^{bc}	4.20 ± 0.005^{bc}
Methanolic extract	2.18 ± 0.025^{ac}	1.46 ± 0.055^{ac}	1.17 ± 0.055^{bc}	4.20 ± 0.006^{bc}
Control	0.01 ± 0.000^{d}	0.01 ± 0.000^{d}	0.01 ± 0.00^{d}	4.70 ± 0.010^{d}

Means within a column with a different super script letter are significantly at ($p\leq0.05$)

extract of hawthorn leaves seems to contain components that favour the growth and the production of SCFA by *Bif. infantis* and *L. acidophilus*.

A few studies investigated phenolic metabolism of some plant extract using individual strains of bacteria. The data illustrated that specific intestinal bacterial metabolize phenolics to different extent and produce different metabolites which can be either retained by the bacterial cell or released into the media. These metabolites may influence the growth of bacteria producing them and in turn will most likely affect other neighboring microflora species as well (Lee *et al*., 2006). In addition, 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.

In all milk media supplemented with different hawthorn extracts, final pH values were significantly lower than those observed in the control medium (Table 4 and 5). These values may be related to the amounts of lactic acid produced (i.e as the acidity increased, the pH decreased) which is an indication of the activity of the probiotic bacteria (Schaffner and Beuchat, 1986).

In conclusion, this research provides promising results on the growth-promoting and prebiotic activity of all the hawthorn leaf extracts on probiotic bacteria, particularly *Bif. infantis* and *L. acidophilus*. This result can be added to the already known beneficial biological properties of hawthorn plant to the human health, this making this plant even healthier.

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