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Effect of Honey on the Growth and Metabolism of Two Bacterial Species 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° in reconstituted skim-milk (100 g l^{-1}) and skim-milk with one of three types of honey extracted from hives located in different regions of Jordan. All the honeys increased cell counts over the control with no honey and the final values for SCFA, especially acetic acid secreted by *L. acidophilus* (25.0 g l^{-1}), were significantly higher than those observed in milk alone. Honey from a desert region was most stimulatory to *L. acidophilus*, while one produced from the flora of a mountainous area had most effect on *Bif. infantis*. It is proposed that specific oligosaccharides or other compounds in the honeys were responsible for the contrasted behaviour of the two species and that honey ingested in a human diet might have the same effect on desirable components of the intestinal microflora.

Key words: Intestinal bacteria, honey, growth, fatty acid production

Introduction

For thousands of years, honey has been used to impart sweetness to foods and, over that time, various medicinal and therapeutic properties have been ascribed to it as well (Krell, 1996). In its extracted and purified form, honey is a liquid (or semi-liquid) product with in excess of 80% total solids, the bulk of which is a complex mixture of carbohydrates, including fructose, glucose, maltose and sucrose with, depending on the floras located in the vicinity of the hives, traces of many other sugars (Viñas *et al*., 1997; Al-Jedah *et al*., 2003). In addition, proteins, amino acids, vitamins, organic acids, minerals and numerous minor components have been recorded (Crane, 1990; Caroli *et al*., 1999) and, of potential nutritional importance among the minor constituents, are the oligosaccharides (Al-Qassemi and Robinson, 2003). This group of low molecular weight polysaccharides is of interest because, while they are neither hydrolyzed nor absorbed in the upper part of the gastrointestinal tract (Cummings *et al*., 2001), they may beneficially affect the health of the consumer by selectively stimulating the growth and/or activity of desirable bacteria in the colon (Ustunol, 2000; Chow, 2002; Haddadin *et al*., 2004).

Thus, these essential bacteria, of which *Bifidobacterium spp*. are the most widely studied, dominate the walls of the colon through their unique ability to digest the mucin secreted by cells of the epithelium. In this position, they protect the host by competing for available nutrients and space against bacterial or fungal pathogens, an antagonism that is reinforced by the secretion of organic acids and, perhaps, antimicrobial compounds. In addition, the Short-Chain Fatty Acids (SCFA), mainly acetic, propionic and butyric acids (Nyman, 2002) released during the fermentation of oligosaccharides are absorbed by the human colon in a concentration-dependent manner and are the major respiratory substrates for colonocytes, supplying up to 70% of their energy needs (Topping and Clifton, 2001; Rossi *et al*., 2005).

It has been established that different honeys contain specific oligosaccharides, e.g. isomaltose and melezitose in New Zealand honey (Weston and Brocklebank, 1999) and raffinose in Italian honey (Oddo *et al*., 1995) and it is likely that one or more of these compounds would prove stimulatory to *Bifidobacterium spp*. (Itsaranuwat *et al*., 2003). Equally relevant is the fact that the lactobacilli in the small intestine, e.g. *Lactobacillus acidophilus*, could well be affected advantageously by the sugars and/or other components in honey (Qiao *et al*., 2002; Rossi *et al*., 2005).

However, although it has been shown that different strains of bifidobacteria respond in a specific way to the addition of a given type of honey to a growth medium (Ustinol, 2000), the reaction of one strain of bacterium to a range of locally available honeys merits investigation. The aim of this project was, therefore, to determine the extent to which three different honeys produced in Jordan would stimulate the growth and/or SCFA secretion of two intestinal bacteria that had been isolated previously from infants living in Amman (Haddadin *et al*., 2004).

Materials and Methods

Three samples of honey from different regions of Jordan were provided by the Jordan University Apiary-Faculty of Agriculture. Sample (1) was from hives located in the mountains around Al Saroo, sample (2)

was produced by bees scouring the flora of the Jordanian desert at Al-Azraq and sample (3) was taken from hives on the campus of the University of Jordan in Amman.

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: CM361, Unipath Ltd., Basingstoke, Hants., UK) at the Food Technology and Nutrition Department, University of Jordan. Prior to use in the experimental programme, 50 mL sterile MRS broth (Code: CM359, pH 6.5±0.2) with cysteine-HCl (5 g l^{-1}) in Duran bottles were inoculated with a loopful of culture and incubated at 37°C for 12 h in an anaerobic jar. Once activated, each culture was maintained by sub-culturing weekly by adding an inoculum (0.5 mL of the previous culture) to MRS Broth (50 mL) with incubation at 37°C for 12 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 honey: Skimmed milk powder (Regilait, France) was reconstituted in distilled water (100 g l^{-1}) and dispensed as aliquots of 100 mL into sterile bottles with screw-caps. The bottles of milk were then heat-treated at 73°C for 30 min in a water bath. Before adding honey to the heat-treated milk, stock solutions of each honey were prepared by weighing sub-samples (300 g) into beakers (750 mL) followed by 300 mL of hot, deionized distilled water. After thorough stirring to disperse the honey, each suspension (approx. 450 mL) was vacuum-filtered through, in sequence, a coarse cotton pad, a No. 40 filter paper (Whatman, England) and a membrane filter (0.45 µm). Final sterilization was achieved with a micro-filtration unit using a sterile cellulose-ester membrane (0.20 µm, ADVANTEC MFS, Japan) fitted to a syringe that dosed the diluted honey directly into the bottles of skim-milk.

The rates of addition were 1.5, 3.75, 7.5, 11.0 or 15 mL into individual bottles of skim-milk and these doses gave approximate concentrations of honey of 1.0, 2.5, 5.0, 7.5 and 10.0 g 100 ml^{-1}; the slight dilution effect of the skim-milk solids was not considered to be important. Control bottles of skim-milk without honey were prepared at the same time.

A similar batches of milk and honey were employed to monitor the release of SCFA by the selected species, except that the only concentration of the honeys used was 7.5 g 100 ml^{-1}. This level was not the optimum for growth of *L. acidophilus* (see later) but, as maximum SCFA production was being sought, the use of the higher concentration was deemed to be appropriate.

Estimation of growth: Duplicate bottles at each honey concentration 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; 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 with each honey at a concentration of 7.5 g 100 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; duplicate bottles 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 Systems, Japan) was equipped with a manual 20 µL loop injector, a variable wavelength ultraviolet/visible detector (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-R2AX, Japan). Analyses were performed isocratically at a flow rate of 1.0 mL min^{-1} and a temperature of 25°C. The column used was a 150×4.6 mm Hypurity Advance (Thermo Quest, Hypersil Division, USA). The mobile phase was prepared by mixing H_3PO_4 (10 g 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

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 of milk

Time (h)	*B. infantis*	*L. acidophilus*
0.0	1.00×10^{7}	6.31×10^{5}
4	2.82×10^{7}	3.98×10^{6}
8	3.16×10^{7}	3.98×10^{7}
12	6.16×10^{7}	7.94×10^{7}
16	3.80×10^{7}	2.51×10^{7}
20	3.47×10^{7}	1.99×10^{7}

Table 2: Effect of different concentrations (g 100 ml^{-1} of growth medium) of three types of honey on the growth of *Bif. infantis* over a period of 16 h; all figures as cfu ml^{-1} and means of duplicate samples from two bottles of milk

Concentration of honey	Honey (1)	Honey (2)	Honey (3)
0.0	5.49×10^{8d}	4.89×10^{8b}	1.97×10^{8d}
1.0	1.05×10^{9b}	5.06×10^{8b}	4.07×10^{8c}
2.5	1.02×10^{9b}	4.26×10^{8b}	9.12×10^{8ab}
5.0	9.66×10^{8b}	5.75×10^{8ab}	8.22×10^{8ab}
7.5	1.26×10^{9a}	7.24×10^{8a}	1.14×10^{9a}
10.0	7.94×10^{8c}	5.01×10^{8b}	5.95×10^{8bc}

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

Table 3: Effect of different concentrations (g 100 ml^{-1} of growth medium) of three types of honey on the growth of *L. acidophilus* over a period of 16 h; all figures as cfu ml^{-1} and means of duplicate samples from two bottles of milk

Concentration of honey	Honey (1)	Honey (2)	Honey (3)
0.0	1.84×10^{8cd}	1.58×10^{8c}	1.53×10^{8bc}
1.0	3.23×10^{8a}	1.53×10^{8c}	2.09×10^{8ab}
2.5	2.99×10^{8ab}	6.53×10^{8a}	2.75×10^{8a}
5.0	2.68×10^{8abc}	2.57×10^{8b}	2.04×10^{8ab}
7.5	1.97×10^{8bcd}	3.47×10^{8b}	1.91×10^{8ab}
10.0	1.55×10^{8d}	1.68×10^{8c}	1.15×10^{8c}

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

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 HI 8519 (Hanna Instruments, Germany) at 23°C.

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 counts to be achieved after 12 h incubation. However, as the secretion of SCFA was considered as potentially the most important effect of the addition of honey, it was decided that all the test cultures could be incubated for 16 h without appreciable loss of cell numbers.

The total viable counts of *Bif. infantis* and *L. acidophilus* in skimmed milk with different concentrations of the three kinds of honey are summarized in Table 2 and 3, respectively. Six concentrations of each honey were used in this research and highest counts of *Bif. infantis* and *L. acidophilus* were related to both concentration and origin of the honey. The level of 7.5 g 100 ml^{-1} for all honey samples had the most significant effect on the counts of *Bif. infantis*, but lower concentrations had the best effect on the growth of *L. acidophilus*. Concentrations of 2.5 g 100 ml^{-1} gave the highest counts with honeys (2) and (3), while a concentration of 1.0 g 100 ml^{-1} (honey 1) had a significant growth promoting effect on *L. acidophilus* (Table 3).

These results are in general agreement with those reported by Ustunol (2000), who investigated the effect of 1, 3 and 5% honey on the growth and viability of commercially available strains of Bifidobacterium in milk. He found that, with most of the strains, the highest concentration (5%) had the most pronounced effect, but the impact was dependent on the strain. Since this effect of the honey was strain specific, it is not surprising that the response to the honeys used in the present study was generic specific. Whether *Bif. infantis* needs higher amounts of specific sugars or oligosaccharides for cell reproduction than *L. acidophilus* was not examined further and it cannot be ruled out that *L. acidophilus* is more susceptible to the antibacterial effect (s) of honey (Caroli *et al.*, 1999).

What the statistical analysis did reveal was that honey (1) from the mountains of Al Saroo had a significantly better effect on the growth of *Bif. infantis* than honey (2) but, although honey (2) at 2.5 g 100 ml^{-1} did support a higher count of *L. acidophilus*, the contrast between the honeys was less noticeable. Given the difference in floras over which the bees scavenged, a greater contrast might have been expected, but certainly honey (1) seems to contain certain components that favour the growth of *Bif. infantis*.

The SCFA produced during fermentation are determined by the substrates available, their fermentability and the rate of breakdown (Parrett and Edwards, 1997) and the

Table 4: Effect of three types of honey (7.5 g 100 ml^{-1} of growth medium) on the production of short-chain fatty acids by *Bif. infantis* over a period of 16 h; all figures as g l^{-1}±standard deviation and means of duplicate samples from two bottles of milk

Substrate	Acetic acid	Propionic acid	Butyric acid	pH
Honey (1)	6.6±0.074^{a}	2.2±0.053^{a}	2.2±0.039^{a}	4.6^{b}
Honey (2)	4.9±0.057^{a}	0.9±0.008^{a}	0.9±0.050^{b}	4.2^{a}
Honey (3)	4.5±0.225^{a}	0.8±0.054^{a}	0.6±0.002^{c}	4.6^{b}
Control	<0.002^{b}	<0.002^{b}	<0.002^{d}	4.6^{b}

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

Table 5: Effect of three types of honey (7.5 g 100 ml^{-1} of growth medium) on the production of short-chain fatty acids by *L. acidophilus* over a period of 16 h; all figures as g l^{-1}±standard deviation and means of duplicate samples from two bottles of milk

Substrate	Acetic acid	Propionic acid	Butyric acid	pH
Honey (1)	7.4±0.167^{c}	3.7±0.124^{a}	7.5±0.306^{a}	4.3^{a}
Honey (2)	25.0±1.40^{a}	3.9±0.124^{a}	3.3±0.173^{b}	4.6^{b}
Honey (3)	10.5±0.565^{b}	2.2±0.233^{b}	1.1±0.079^{c}	4.2^{a}
Control	<0.002^{d}	<0.002^{c}	<0.002^{d}	4.7^{b}

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

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 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 no significant difference between the honeys with respect to the concentration of acetic acid, but there was a significant difference between the treatments and the control; the same conclusion applied to propionic acid. However, the fermented milk with honey (1) contained the highest amount of butyric acid, followed by the milk with honey (2); the milk with honey (1) also supported the highest overall quantity of acids. In the samples with *L. acidophilus*, there were significant differences between the three milks (Table 5), with honey (2) supporting both the highest cell count and total quantity of SCFA. The high level of acetic acid was especially notable for an organism that is, at least in milk, homofermenative, i.e. the values of SCFA in the control milks were below the level of detection. This contrast suggests that, in the human intestine, the metabolism of *L. acidophilus* may be very different from that observed in a yoghurt culture, for example, where lactose is the principal fermentable substrate.

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 honey (2) had a significantly lower pH than the control, suggesting that an appreciable amount of lactic acid had been produced.

In the case of *L. acidophilus*, the milks with honeys (1) and (3) had significantly lower pH and these values may be related to the amounts of lactic acid produced.

Overall, it is clear that all the honeys beneficially influenced the growth and metabolism of these two organisms of intestinal origin and it might be reasonable to assume that honey ingested by a consumer would have a similar effect on the native populations of these species in the lower intestine. Obviously some of the stimulatory compounds could be absorbed during transit of the honey through the upper intestine of the host but, if oligosaccharides were the principal activators of the test bacteria, then honey may well have the beneficial role ascribed to it by Al-Qassemi and Robinson (2003).

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