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308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

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Effect of Propolis on Two Bacterial Species with Probiotic Potential

M.S.Y. Haddadin, I. Nazer, Sara' Jamal Abu Raddad and R.K. Robinson
Department of Food Technology and Nutrition, University of Jordan, Amman, Jordan

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) and in skim-milk with different concentrations of propolis extracted from hives located at the University of Jordan. Increasing levels of propolis from 100 - 800 mg/100 ml of milk inhibited significantly the growth of *Bif. infantis* over the control with no propolis, but even 800 mg/100 ml had a stimulatory effect on *L. acidophilus*. The final values for acetic (12.1 g/l), propionic (2.7 g/l) and butyric (1.4 g/l) acids secreted by *Bif. infantis* were much higher than those observed in milk alone, while *L. acidophilus* released detectable amounts of propionic and butyric acids. As these SCFA are essential for health of the human colon, it is suggested that propolis taken as a medicine could, in spite of the adverse effect on the growth of *Bif. infantis*, offer an additional benefit as a stimulant of the metabolism of the intestinal microflora.

Key words: Probiotic bacteria, propolis, growth and fatty acid production

Introduction

After honey, propolis is one of the most important products of any bee hive, for it is a compound used by bees to make a protective shield at the entrance of hive (Munstest and Zygmut, 2001). In addition, it is used to fill any structural cracks in the hive, to attach the corners of frames to the grooves in the hive and also to polish the cells of the honeycomb. Generally, propolis is composed of resin and vegetable balsam (50%), wax (30%), aromatic oils (10%), pollen (5%) and various other substances depending upon the vegetation of the area (Kosonocka, 1990; Moreno *et al*., 1999; Banskota *et al*., 2000; Pereira *et al*., 2000; Bankova *et al*., 2000; Bankova *et al*., 2002).

Aside from its value in strengthening the structure of the hive, the antiseptic properties of propolis may well be important in ensuring that the corpses of any insects that die within a hive do not cause disease (Munstest and Zygmut, 2001). Indeed, the ancient Egyptians are alleged to have exploited the anti-putrefactive properties of propolis to embalm cadavers and its medicinal properties were known to both Greek and Roman physicians. In particular, it was employed as an antiseptic in wound treatment and as a mouth disinfectant, uses that were perpetuated in Europe during the Middle Ages and among Arab physicians (Castaldo and Capasso, 2002). Modern herbalists recommend propolis for its anti-bacterial, anti-fungal, anti-viral and anti-inflammatory properties, as a means of increasing the body's natural resistance to infections and for use against the cause of gastric ulcers, *Helicobacter pylori* (Schmidt and Buchmann, 1999; Kumazawa *et al*., 2003; Mishima *et al*., 2005). Applied externally, propolis can relieve various types of dermatitis caused by bacteria and fungi (Castaldo and Capasso, 2002).

The precise reason(s) for the antimicrobial effect does not appear to have been established, but it must be emphasized that propolis has a complex chemical composition (Abd El Hady and Hegazi, 2002; Koo *et al*., 2002). For example, Abd El Hady and Hegazi (2002) found 104 different chemicals in three samples of propolis collected from different areas in Egypt and these were grouped into seven categories: Aliphatic acids, aromatic acids, esters, di- and triterpenes, flavonoids, sugars and miscellaneous. Walker and Crane (1987) listed 149 compounds isolated from samples collected from around the world and again acids and flavonoids were well represented. It is not surprising, therefore, that propolis has anti-microbial properties but, if it can inhibit the growth of gastric pathogens like *H. pylori*, what effect might routine medication be having on the normal intestinal microflora and/or probiotic bacteria ingested for their alleged therapeutic properties (Itsaranuwat *et al*., 2003).

The aim of this project was, therefore, to grow two typical intestinal/probiotic bacteria, namely *Lactobacillus acidophilus* and *Bifidobacterium infantis* in milk containing different concentrations of propolis and establish whether propolis would affect their growth and/or metabolism. Obviously the behaviour of the bacteria in milk in the presence of propolis may not reflect their reaction in the intestine, but at least the tests might indicate if medicines containing propolis could effect the intestinal microflora or co-ingested probiotic bacteria.

Corresponding Author: M.S.Y. Haddadin, Department of Food Technology and Nutrition, University of Jordan, Amman, Jordan

Materials and Methods

Source of Propolis: Propolis was obtained from the Faculty of Agriculture apiary, University of Jordan by placing mesh screens above the combs in six separate hives. After three months, the screens were removed and placed in a deep-freeze at - 20°C. The frozen propolis was then scraped off the screens and placed in an open dish to dry. Once the surface water had evaporated, the pieces of propolis were ground to a fine powder using a Moulinex blender; the powder was stored in a sealed jar until needed.

To dissolve the propolis, 5g of powder were added to 50ml of 70% ethanol and gently shaken at room temperature for 24 hours. The alcoholic solution was then evaporated under vacuum at 50°C to give a resinous residue (Abd El Hady and Hegazi, 2002). This material was difficult to dissolve in milk and to solve this problem, 95 ml of distilled water containing pre-dispersed Gum Guar (1.61g) was added to the flask. The suspension of guar gum and the propolis extract were mixed manually in the first instance and then blended with a hand-held, high-speed mixer to give a 'solution' that could be dispensed volumetrically. For the purposes of the experiment, it was assumed that the entire weight of propolis had been incorporated into the guar suspension and hence that the concentration was 50 mg of propolis /ml.

Probiotic micro-organisms: The cultures of *Bif. infantis* and *L. acidophilus* had been previously isolated from the stools of breast-fed infants living in Amman and maintained on slopes of MRS Agar (Code: CM361, Unipath Ltd., Basingstoke, Hants., UK). The cultures were routinely sub-cultured every three months and stored at 4°C after initial growth of the inoculum at 37°C. The isolates were identified physiologically and biochemically according to Bergey's Manual of Determinative Bacteriology (Kandler and Weis, 1986) and Prokaryotes (Hammes *et al.*, 1992) and their ability to grow in milk had been established by Awaisheh *et al.* (2004). For use in the experimental programme, batches of 100 ml of reconstituted (100 g/l) skim-milk powder (Regilait, France) were dispensed into screw-cap bottles and heat-treated at 73°C for 30 min. After cooling to 37°C, duplicate bottles of skim-milk were inoculated with loopfuls of freshly-prepared cultures of *Bif. infantis* or *L. acidophilus*. The bottles were then incubated at 37°C for 12 h (Haddadin *et al.*, 2007), followed by storage at 4°C until used.

Preparation of milk with propolis: Different volumes of the propolis extract (namely 2, 4, 8, 12 and 16 ml) were added to the appropriate volumes of reconstituted skim-milk to give final volumes of 100 ml; the concentrations of propolis were calculated to be 100, 200, 400, 600 and 800 mg/100 ml, respectively. After careful mixing, the milks were pasteurized at 63°C for 30 min in screw-cap bottles. Control samples of milk without propolis were prepared at the same time. This lower level of heat treatment was selected to reduce the counts of bacteria that might compete with the probiotic species, while at the same time limiting, as far as possible, any significant loss of volatile components from the propolis.

Estimation of growth: Duplicate bottles at each propolis concentration were inoculated with either *Bif. infantis* or *L. acidophilus* (1.0 ml aliquots of a culture in skim-milk) 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 in sterile peptone water (CM9, 15 g/l) and aliquots (0.1 ml) were spread onto pre-poured plates of MRS Agar; the results were recorded as colony-forming units (cfu) per ml of milk.

Production of Short Chain Fatty Acids (SCFA): Based on the results of the growth experiment, duplicate bottles of milk with propolis concentrations of 100 mg/100 ml or 800 mg/100 ml were inoculated with *Bif. infantis* and *L. acidophilus*, respectively, - employing 1.0 ml aliquots of an active skim-milk culture and incubated at 37°C for 16 h in anaerobic jars; duplicate bottles of the control milk were treated similarly. The levels of acetic, propionic and butyric acids in the 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 20µl 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 quantitation 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 and a 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 (10 g/l) 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 (Haddadin *et al.*, 2004). A value of < 0.002 in Table 2 indicates that the true figure was below the level of detection.

Measurement of pH: A sub-sample (10 ml) from each bottle was used to measure the pH using a digital pH meter Model HI 8519 (Hanna Instruments, Germany) at 23°C.

Table 1: Effect of different concentrations (mg/100 ml of growth medium) of propolis on the growth of *Bif. infantis* and *L.acidophilus* over a period of 16 h; all figures as cfu/ml and means of duplicate samples from two bottles of milk

Weight of propolis (mg/100 ml)	*Bif. infantis*	*L. acidophilus*
0.0	$4.54x108^{a}$	$6.92x106^{e}$
100	$3.69x108^{b}$	$9.48x106^{d}$
200	$2.95x108^{c}$	$1.39x107^{c}$
400	$2.14x108^{d}$	$7.13x107^{b}$
600	$1.80x108^{d}$	$8.63x107^{ab}$
800	$1.10x108^{e}$	$9.68x107^{a}$

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

Statistical analysis: The General Linear Model (GLM) produced by the statistical analysis system (SAS) version 7 (SAS, 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

Six concentrations of propolis were used, namely 0, 100, 200, 400, 600 and 800 mg/100 ml of milk and the effect of these concentrations on the growth of *Bif. infantis* and *L. acidophilus* over 16 h at 37°C is shown in Table 1. What was noticeable was that impact of propolis on both species was concentration dependent and while the propolis extract had an adverse effect on the growth of *Bif. infantis* - the control had a significantly higher count, the same extract was stimulatory to *L. acidophilus*. Thus, the count of *L. acidophilus* in the presence of 800 mg of propolis was significantly higher than in the control without propolis, but the cause of this enhanced growth was not established. However, Abd El Hady and Hegazi (2002) indicated that various sugars could be extracted from propolis and it may that such materials were being utilised by *L. acidophilus*.

On the other hand, the strong antibacterial properties of propolis appear to have affected *Bif. infantis* more than *L. acidophilus* and a decline in comparison with the control was even found in milk containing 100 mg of propolis. This finding could be relevant for those using propolis as a medicine because, according to the Propolis Information Bureau, the average daily intake for the curing of throat infections should be 2 g of propolis; higher doses are recommended for deep-seated conditions like arthritis. Obviously it was not possible in this preliminary study to isolate the compounds in propolis that inhibited *Bif. infantis* or to surmise whether they might pass intact along the digestive tract in a human and reach the population of bifidobacteria in the colon. Nevertheless, with proposed intakes of 2 - 3 g/day, some exposure of the native bifidobacterial flora is feasible, along with a decline in viable counts. If a typical treatment time for curing a sore throat is around two weeks, then any fall in the population of bifidobacteria would probably pass unnoticed by the patient, but it is interesting to speculate whether loose stools or mild diarrhoea accompanies long term usage of propolis extracts. Such an effect should, of course, be simple to remedy by the co-ingestion of a yoghurt containing a high count of a suitable species/strain of *Bifidobacterium* (Itsaranuwat *et al.*, 2003) and it may be that patients who take natural remedies like propolis consume probiotic milks as a matter of course; the Propolis Information Bureau list no side-effects on their web-site.

Table 2: Effect of propolis (100 mg / 100 ml of growth medium for *Bif. infantis* and 800 mg/100 ml for *L. acidophilus*) on the production of short-chain fatty acids over a period of 16 h; all figures as g / l ± standard deviation and means of duplicate samples from two bottles of milk

Organism	Acetic acid	Propionic acid	Butyric acid	pH
Bif. infantis	12.1±0.260	2.7± 0.108	1.4±0.096	4.6^{b}
Control	< 0.002	< 0.002	< 0.002	4.6^{b}
L. acidophilus	< 0.002	0.6± 0.029	0.9±0.022	4.2^{a}
Control	< 0.002	< 0.002	< 0.002	4.6^{b}

Although propolis had an adverse effect on the growth of the bifidobacteria, it did have a desirable influence on the release of acetic, propionic and butyric acids (see Table 2). In humans, these fatty acids are generated during the fermentation of various substrates in the colon and they play an essential role in maintaining its healthy status (Topping and Clifton, 2001). The fact that a comparatively low concentration of propolis (1.0 mg/ml) stimulated such a dramatic secretion of these acids by *Bif. infantis* suggests that patients using propolis might, despite a possible decline in the counts of bifidobacteria, be deriving a considerable benefit in addition to the intended medical one. Indeed, the desirable impact of propolis on the metabolism of *Bif. infantis* indicates that it might be considered, perhaps at a level that does not affect growth, as a prebiotic. The results in Table 2 show also that propolis, even at the apparently optimum level of 8.0 mg/ml, did not alter the metabolism of *L. acidophilus* to the same extent, even though the low pH of the test milk suggests that the normal respiratory pathways leading to lactic acid may have been stimulated by a factor in propolis.

While propolis is highly regarded as a medicine with anti-bacterial properties, these results indicate that certain components could be having a positive impact on some of the species within the intestinal microflora. Certainly *L. acidophilus* responded well to exposure to propolis and the enhanced secretion of SCFA by *Bif. infantis* could be rated as an extremely welcome outcome. As mentioned earlier, there is no evidence that the components active in these experiments would reach the lower intestine of a human intact, but the fact that they could provide a further benefit for those taking propolis to cure a disease is an attractive prospect.

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