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Effect of hydrogen peroxide on antibacterial activities of Canadian honeys

Katrina Brudzynski

Abstract: Honey is recognized as an efficacious topical antimicrobial agent in the treatment of burns and wounds. The antimicrobial activity in some honeys depends on the endogenous hydrogen peroxide content. This study was aimed to determine whether honey's hydrogen peroxide level could serve as a honey-specific, activity-associated biomarker that would allow predicting and assessing the therapeutic effects of honey. Using a broth microdilution assay, I analyzed antibacterial activities of 42 Canadian honeys against two bacterial strains: *Escherichia coli* (ATCC 14948) and *Bacillus subtilis* (ATCC 6633). The MIC_{90} and MIC_{50} were established from the dose–response relationship between antibacterial activities and honey concentrations. The impact of H_2O_2 on antibacterial activity was determined (*i*) by measuring the levels of H_2O_2 before and after its removal by catalase and (*ii*) by correlating the results with levels of antibacterial activities. Canadian honeys demonstrated moderate to high antibacterial activity against both bacterial species. Both MIC_{90} and MIC_{50} revealed that the honeys exhibited a selective growth inhibitory activity against *E. coli*, and this activity was strongly influenced by endogenous H_2O_2 concentrations. *Bacillus subtilis* activity was marginally significantly correlated with H_2O_2 content. The removal of H_2O_2 by catalase reduced the honeys' antibacterial activity, but the enzyme was unable to completely decompose endogenous H_2O_2. The 25%–30% H_2O_2 "leftover" was significantly correlated with the honeys' residual antibacterial activity against *E. coli*. These data indicate that all Canadian honeys exhibited antibacterial activity, with higher selectivity against *E. coli* than *B. subtilis*, and that these antibacterial activities were correlated with hydrogen peroxide production in honeys. Hydrogen peroxide levels in honey, therefore, is a strong predictor of the honey's antibacterial activity.

Key words: honey, antibacterial activity, hydrogen peroxide, catalase, *Escherichia coli*, *Bacillus subtilis*.

Résumé : Le miel est reconnu comme étant un agent antimicrobien topique efficace pour le traitement de brûlures et de blessures. L'activité antimicrobienne de certains miels dépend de leur contenu en peroxyde d'hydrogène endogène. Cette étude avait pour but de déterminer si les niveaux de peroxyde d'hydrogène dans le miel pourraient être employés comme biomarqueurs spécifiques au miel et associés à l'activité qui pourrait permettre de prédire et d'évaluer les effets thérapeutiques du miel. Les activités antibactériennes de 42 miels canadiens furent analysées à l'aide d'un test de microdilution en bouillon avec deux souches bactériennes: *Escherichia coli* (ATCC 14948) et *Bacillus subtilis* (ATCC 6633). Les valeurs de CMI_{90} et CMI_{50} furent déterminées à partir de la relation dose–réponse entre les activités antibactériennes et la concentration des miels. L'impact du H_2O_2 sur l'activité antibactérienne fut déterminée en mesurant les niveaux de H_2O_2 avant et après son élimination par la catalase et en corrélant les résultats avec les niveaux d'activité antibactérienne. Les miels canadiens ont démontré des activités antibactériens de modérées à élevées contre les deux espèces bactériennes. Les valeurs de CMI_{90} et CMI_{50} ont toutes deux révélé que les miels démontraient une activité inhibitrice de la croissance sélective contre *E. coli* et que cette activité était fortement influencée par les concentrations de H_2O_2 endogène. L'activité de *B. subtilis* était marginalement significativement corrélée avec la teneur en H_2O_2. L'élimination de H_2O_2 par la catalase a diminué l'activité antibactérienne des miels mais l'enzyme n'a pu complètement décomposer le H_2O_2 endogène. Les 25 % – 30 % de H_2O_2 restants étaient significativement corrélés avec l'activité antibactérienne résiduelle des miels contre *E. coli*. Ces données indiquent que tous les miels canadiens démontraient une activité antibactérienne avec une sélectivité supérieure contre *E. coli* versus *B. subtilis* et que ces activités antibactériennes étaient corrélées avec la production de peroxyde d'hydrogène dans les miels. Les niveaux de peroxyde d'hydrogène dans le miel sont ainsi des prédicteurs importants de l'activité antibactérienne du miel.

Mots clés : miel, activité antibactérienne, peroxyde d'hydrogène, catalase, *Escherichia coli*, *Bacillus subtilis*.

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Introduction

The growing bacterial resistance to existing antibiotics is becoming an urgent medical issue stimulating the search for new antibiotics to treat bacterial infections (ASM Task Force on Antibiotic Resistance 1995). The need for novel therapies has renewed interest in natural products exhibiting antibacterial properties. Honey has been shown in numerous studies to

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possess growth inhibitory activities against several bacterial strains in vitro (Allen et al. 1991; Molan 1992*a*, 1992*b*; Lusby et al. 2005; Wilkinson and Cavanagh 2005). Due to this antibacterial activity, honey has been successfully developed into an alternative treatment in wound healing (Efem 1988; Subrahmanyam 1996, 1998, 1999). Clinical application of honey has proven to be specifically beneficial in the treatment of wounds that are nonresponsive to conventional therapies, such as diabetic ulcers (Eddy and Gideonsen 2005), and wounds infected with antibiotic-resistant bacteria (Dunford et al. 2000; Lusby et al. 2002).

Manuka (New Zealand) and Medihoney (Australia) honeys, derived from *Leptospermum* spp., are known as the most therapeutically potent honeys. Manuka honey possesses unusual physico-chemical properties: the gel-like consistency (thixotropy) due to its protein content and the presence of the "unique manuka factor" (UMF), originating from the indigenous plant *Leptospermum* spp. (Molan and Russell 1988; Molan 1992*b*). These two characteristic features are thought to be the cause of manuka potent bacteriostatic and (or) bactericidal activities (Molan 1995). Although European and American honeys lack one or both of these factors, they too show bacteriostatic action against a number of pathogens (Torres et al. 2004). This observation suggests that antibacterial activity is a much more common property of honey than has been previously anticipated. In addition, the mechanism of the antibacterial action also seems to be shared by different honeys, since the non-manuka honeys with their average antibacterial activities were shown to completely inhibit the wound-infecting bacteria, including strains of methicilin-resistant *Staphylococcus aureus* (Cooper et al. 2002).

The discovery of hydrogen peroxide (H_2O_2) as an intrinsic compound of honey brought an assumption that H_2O_2 is a main factor underlying the broad antimicrobial activity of honey (White et al. 1963). H_2O_2 is a by-product of glucose oxidation catalyzed by the bee-derived enzyme glucose oxidase. The efficiency of the reaction increases with honey dilution, which facilitates access of the enzyme to its substrate (glucose) and prevents milieu acidification, which inhibits glucose oxidase. The continuous production of H_2O_2 in the diluted honey produces a long-lasting antiseptic effect that is most sought after in fighting infections in wounds. In most honeys, antibacterial activities strongly depend on the presence of this compound (White et al. 1963; Allen et al. 1991; Mundo et al. 2004), with the exception of some manuka honeys, which maintain their growth inhibitory activity even after removal of H_2O_2 (Molan and Russell 1988). The non-peroxide compounds that influence antibacterial activity have also been found in honeys other than manuka honey, including kanuka, heather, lavender, and kamahi (Allen et al. 1991). These nonperoxide compounds can be classified as (*i*) plant-derived compounds, such as flavonoids (pinocembrin, chrysin, galangin, pinobanksin (Bogdanov 1989)), polyphenolic acids (syringic, ferulic, cinnamic, benzoic, and caffeic acids (Russell et al. 1990; Aljadi and Yusoff 2002)), and nonaromatic acids (Bogdanov 1997); (*ii*) pollen-derived catalase (Weston 2000); and (*iii*) proteinaceous compounds (Mundo et al. 2004).

The antibacterial activity of Canadian honeys has not yet been investigated, and its dependence on H_2O_2 production is unknown. To predict and assess the therapeutic effect of Canadian honeys in the treatment of wounds, ulcers, and burns, it is important to establish an activity-associated biomarker. Therefore, the purpose of this project was to determine if the concentration of H_2O_2 in different honeys could forecast the antibacterial activity. Two important methodological choices were made for studying the antibacterial activity of Canadian honeys. Firstly, a broth microdilution assay was chosen over commonly used disk and well diffusion assays because it provides high-throughput and precise measurements of bacterial growth inhibition and allows analysis of kinetics and the dose–response relationship between honey concentration and bacterial growth. Secondly, *Bacillus subtilis* was chosen over *Staphylococcus aureus* as a representative of Gram-positive bacteria, since the *Bacillus* genus is often introduced to honey after its harvest, and since bacillus spores can survive in honey in their metabolically dormant forms for a long time (Snowdon and Cliver 1996). Therefore, antibacterial activity of honey against these microbes is of interest, considering a future therapeutic application of honey.

Materials and methods

Honey samples

Samples of raw honeys were donated by Ontario, New Brunswick, and British Columbia beekeepers. The samples included liquid and crystallized honeys and honeycomb honey from monofloral and polyfloral sources. Honey samples were given numbers at the beginning of experiment and were tested blindly to avoid any bias. Two samples of commercial Active Manuka honeys (Honey New Zealand Ltd., New Zealand, UMF 20+ and 25+) were used as references for antibacterial assays. During the study, samples were kept in their original packaging, in a cool room, and in the dark. Table 1 details the plant source, year of production, and province of 42 honey samples.

Bacterial strains

Bacillus subtilis (ATCC 6633) and *Escherichia coli* (ATCC 14948) were obtained from Ward's Natural Science Ltd. (St. Catharines, Ont., Canada). Bacteria were grown overnight in 5 mL of Mueller–Hinton broth (MHB) in a shaking water bath at 35 °C.

Working inoculum was prepared by diluting overnight cultures with sterile water to match their turbidity to the 0.5 McFarland standard (A_{595} 0.06–0.08). The turbidity of a bacterial culture equal to the 0.5 McFarland standard contains approximately 1×10^8 to 2×10^8 CFU·mL^{-1}. (NCLSS 1996, Jorgensen at al. 1999). The McFarland 0.5-turbidity bacterial suspensions were further diluted 1:10 with MHB for antibacterial assays.

Antibacterial assay

The antibacterial activities of honeys were analyzed by the broth microdilution assay in sterile, polypropylene 96-well microtiter plates (Costar, 3790), according to the guidelines of the National Committee of Laboratory Safety and Standards (NCLSS 1996). Ten millilitres of MHB was inoculated with 1 mL of 1×10^7 CFU·mL^{-1}, and 110 μL aliquots were dispensed to each well of the 96-well plates from rows B to G. Row G contained 110 μL of inoculated MHB without honey and row H contained only 110 μL of MHB (with-

Table 1. Plant source and province of honey samples.

Assigned No.*	Plant source	Location
Spl. 1	Clover	Ontario
Spl. 2	Goldenrod	Ontario
Spl. 3	Light buckwheat	Ontario
Spl. 4	Canola	Ontario
Spl. 5	Loosestrife	Ontario
Spl. 6	Mixed	Ontario
Spl. 7	Mixed	Ontario
Spl. 8	Blueberry	New Brunswick
Spl. 9	Mixed	Ontario
Spl. 10	Blueberry	New Brunswick
Spl. 11	Clover	Ontario
Spl. 12	Mixed	Ontario
Spl. 13	Mixed	Ontario
Spl. 14	Dandelion and locust	Ontario
Spl. 15	Blueberry	Ontario
Spl. 16	Clover	Ontario
Spl. 17	Pumpkin	Ontario
Spl. 18	Buckwheat	Ontario
Spl. 19	Sweet clover	Ontario
Spl. 20	Sweet clover	Ontario
Spl. 21	Dandelion	British Columbia
Spl. 22	Blueberry	British Columbia
Spl. 23	Buckwheat	British Columbia
Spl. 24	Cranberry	British Columbia
Spl. 25	Blackberry	British Columbia
Spl. 26	Fireweed	British Columbia
Spl. 27	Basswood	Ontario
Spl. 28	Acacia	Ontario
Spl. 29	Wildflower	Zambia
Spl. 30	Basswood	Poland
Spl. 31	Buckwheat	Ontario
Spl. 32	Unknown	Ontario
Spl. 33	Clover	Ontario
Spl. 34	Clover and mixed	Ontario
Spl. 35	Goldenrod	Ontario
Spl. 36	Clover and mixed	Ontario
Spl. 37	Star thistle	Ontario
Spl. 38	Mixed clover	Ontario
Spl. 39	Tupelo	Florida
Spl. 40	Wildflower	Ontario
Spl. 41	Mixed	Ontario
Spl. 42	Wildflower	Ontario

*All samples were produced in 2005, except Samples 10, 27, and 40, which were produced in 2004.

out bacteria and honey), serving as sterility control and blank, respectively, for a reader. Honey was diluted twofold with sterile water in a tube and thoroughly mixed to ensure a uniform solution, and 200 μL of the 50% honey solution was dispensed to row A and inoculated with 20 μL of 1×10^7 CFU·mL^{-1} bacterial solution. After mixing, a twofold dilution of honey was made from rows A to F. Each honey was analyzed in triplicate. Plates were incubated with shaking at 35 °C for 18–24 h. The growth of bacteria was measured at A_{595} using a multidetection microplate reader, Synergy TH (Bio-Tek Instruments, Winooski, Vt.). The dose–response curves based on means ± SE (bacterial growth versus honey dilutions) were generated using KC4 software supplied with the reader.

Antibacterial activity was expressed as a minimal inhibitory concentration (MIC) of honey that reduced bacterial growth, measured by the absorbance (A_{595}), by 90% and 50% (MIC_{90} and MIC_{50}, respectively).

Since the serial twofold dilutions of honey were used to produce the dose–response curves in the microtiter plates, the honey concentrations were expressed as both dilutions and concentrations (*v*/*v*), according to the following conversion:

Dilution	Concentration (%)
2	50
4	25
8	12.5
16	6.25
32	3.12
64	1.56

H_2O_2 assay

H_2O_2 in honey was measured with the Amplex® Red Hydrogen Peroxide/Peroxidase Assay kit (Molecular Probes, Invitrogen, Burlington, Ontario, Canada). The assay detects H_2O_2 by utilizing the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxasine), which reacts with H_2O_2 to produce the red fluorescent oxidation product resorufin. The assay was conducted according to the manufacturer's instruction. Fluorescence was measured at an emission wavelength of 590 nm using an excitation wavelength of 530 nm employing the Synergy HT (Bio-Tek) multi-detection microplate reader. The H_2O_2 standard curve was constructed using twofold serial dilutions of 20 μmol·(L stock solution)$^{-1}$. Similarly, honey twofold dilutions from 2 to 32 times (50% to 3.125%) in a phosphate buffer (included in a kit) were prepared just before the assay and were incubated with Amplex Red reagent for 2 h at room temperature in the dark. After incubation, fluorescence was immediately measured with the microplate reader. The blank (row G) was included in each plate and subtracted from the experimental measurements. Data were analyzed using KC4 software.

Catalase treatment

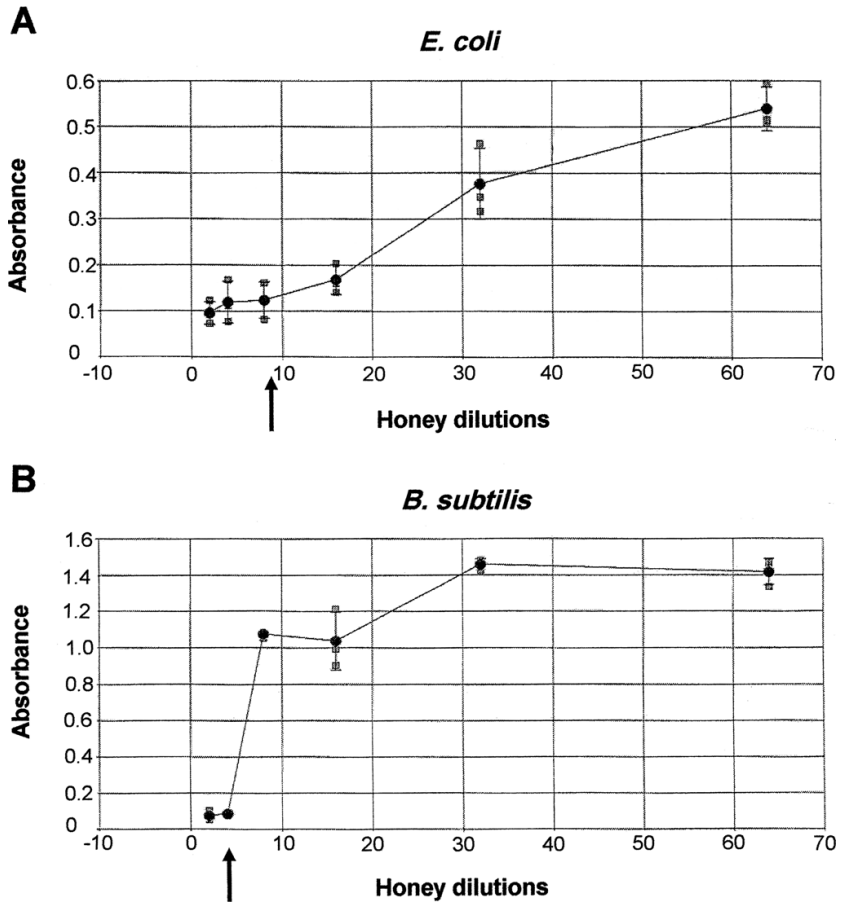
Honeys of known H_2O_2 concentration from Amplex Red assays were treated with catalase (13 800 U·(mg solid)$^{-1}$, Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) at ratio of 1000 U per 1 mL of honey (1:1 diluted with sterile water) for 2 h at room temp. Catalase-treated honey samples were then used in the antibacterial assay to determine the MIC_{90} against *E. coli* and *B. subtilis* and in the Amplex Red assay to determine the effectiveness of H_2O_2 removal from honey.

Results

Canadian honeys possess antibacterial activity against Gram-positive *(B. subtilis)* and Gram-negative *(E. coli)* bacteria

The standard broth microdilution assay was carried out to determine the growth inhibitory activity of 42 honeys against two bacterial species, *E. coli* and *B. subtilis*.

Fig. 1. A typical dose–response curve, represented by Spl. 8, between honey dilutions and bacterial growth measured at A_{595}. Each point represents a mean value of triplicate repetition ± SD. Arrows indicate the mininal inhibitory concentration for 90% of strains. (A) *Escherichia coli*. (B) *Bacillus subtilis*. The A_{595} for controls (inoculated broth, no honey) were 1.285 ± 0.028 and 1.249 ± 0.187 for *E. coli* and *B. subtilis*, respectively.



A dose–response relationship between honey concentration and bacterial growth inhibition was monitored at A_{595} using a multi-detection Synergy HT microplate reader (Fig. 1). The $MICs_{90}$ were established from the dose–response curves as the minimal honey concentration able to reduce the absorbance by 90%. The lack of turbidity in these wells, indicating the inhibition of bacterial growth, was verified again by the visual comparison of their transparency against a blank and (or) control using the white card with contrasting black lines (Jorgensen et al. 1999).

The results of these assays indicated that all honeys exhibited antibacterial activity against both *E. coli* and *B. subtilis*, although the activity varied among samples (Fig. 2). In general, the $MICs_{90}$ showed that Canadian honeys were often more potent against *E. coli* than *B. subtilis* (Fig. 2; Spl. 8, Spl. 10, Spl. 15, etc.). In contrast, the $MICs_{90}$ of Active Manuka honeys (UMF 20 and 25) against *E. coli* and *B. subtilis* were in the same range, indicating the equal potency of manuka against both bacteria. In addition, manuka was consistently the most active honey in all experiments (MIC_{90} 6.25%–3.125% *v*/*v*) (Figs. 2 and 3). Using a MIC_{90} of manuka honey as a reference for the high activity, the distribution of antibacterial activity (anti-*E. coli* activity) among Canadian honeys can be presented as follow: 30.9% of honey samples showed high antibacterial activity (MIC_{90} 12.5%–6.25% *v*/*v*), 4.7% low activity (MIC_{90} 50%–25% *v*/*v*), and the majority of honeys (64.4%) demonstrated moderate activity with the MIC_{90} ranging from 25% to 12.5% (Table 2).

Table 2. Distribution of antibacterial activities in Canadian honeys, based on anti-*Escherichia coli* activity.

	MIC_{90}			
Antibacterial activity	Honey concn. (% *v*/*v*)	Honey dilution	No. of samples	% of total samples
Very high	6.25	16	5	11.9
High	12.5	8	8	19.0
Moderate	25	4	27	64.3
Low	50	2	2	4.76

Note: MIC_{90}, minimal inhibitory concentration for 90% of the strains tested.

Canadian honeys exhibit selective growth inhibitory activity against Gram-negative (*E. coli*) bacterium

The dose–response curves showed that even at a 64-fold dilution, all honey tested retained more than 60% of their antibacterial activity against *E. coli*, while activity against *B. subtilis* was completely lost (Fig. 1). The growth inhibitory activity profiles generated from the dose–response experi-

Fig. 2. Minimal inhibitory concentrations ($MICs_{90}$) for *Escherichia coli* and *Bacillus subtilis* established from the dose–response curves in broth microdilution assays for different honeys. The $MICs_{90}$ are expressed as the honey dilution. In parentheses are the honey concentrations (% *v*/*v*) at given dilutions.

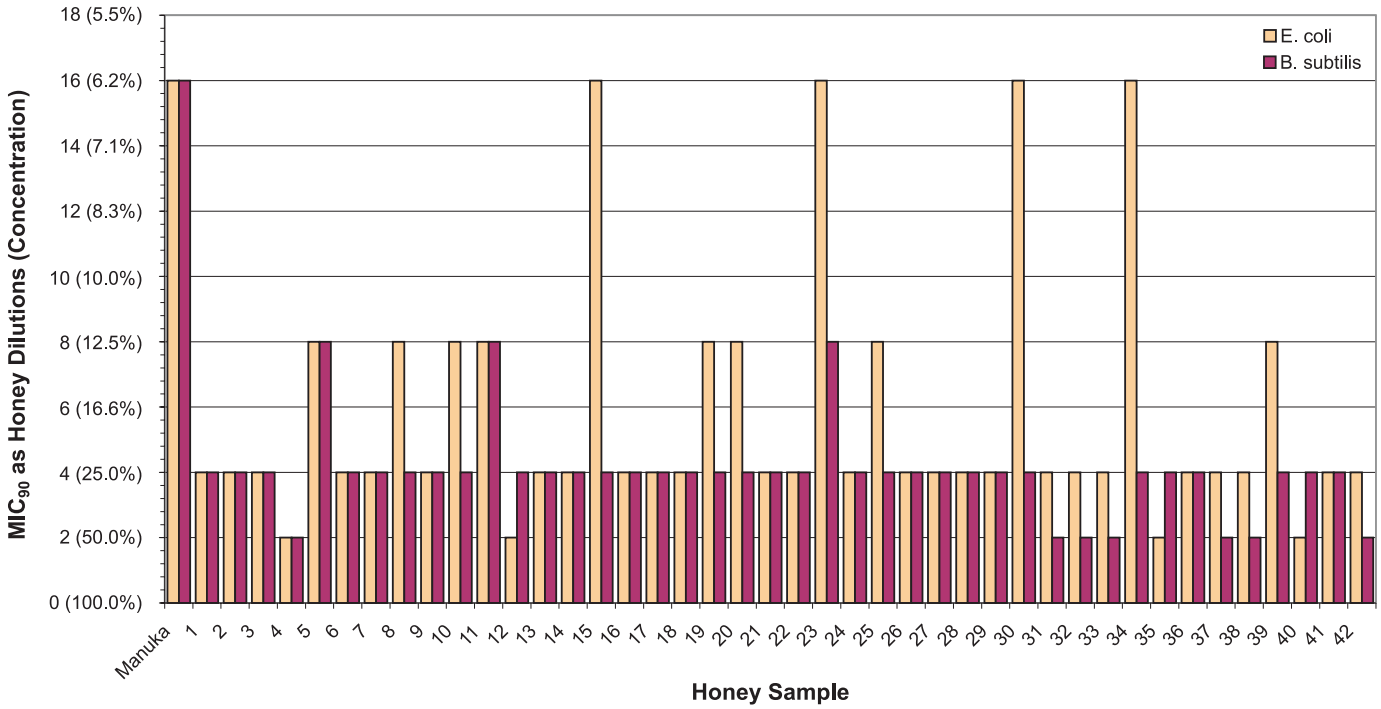
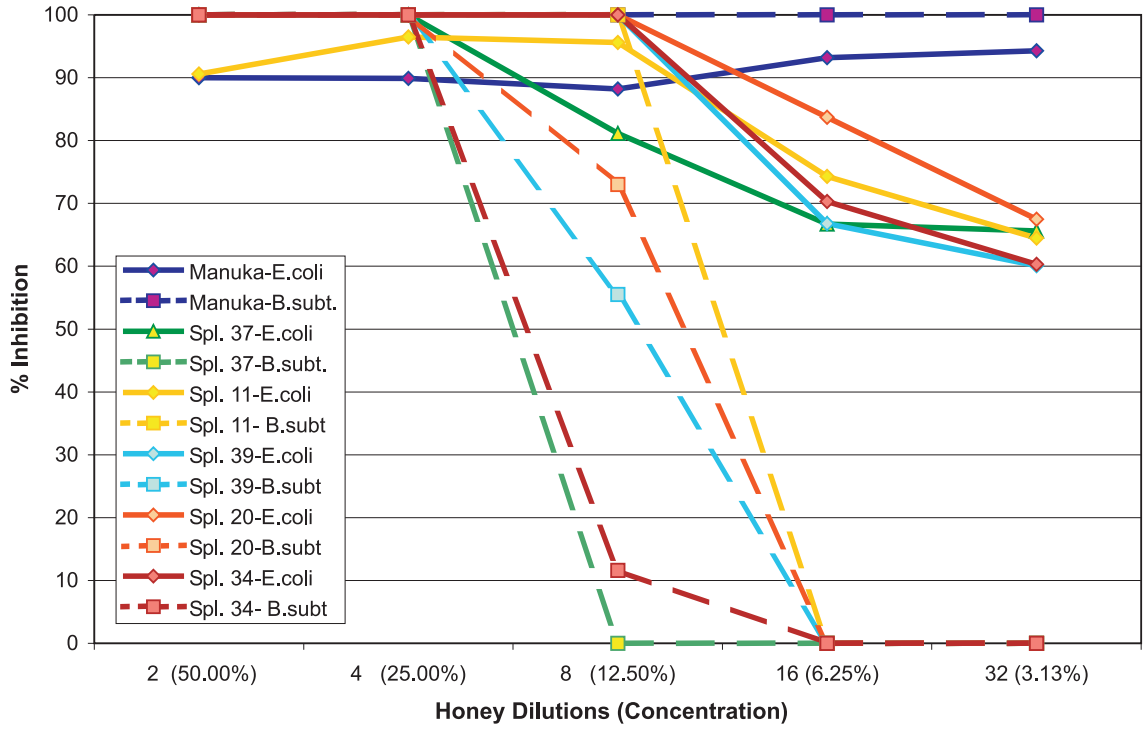


Fig. 3. Comparison of the growth inhibitory activities of individual honeys against *Escherichia coli* and *Bacillus subtilis*.



ments clearly indicated the differences in honey potencies against these two bacterial species (Fig. 3). For the most active honeys, the $MICs_{50}$ for *B. subtilis* ranged from 16% to 8% (*v*/*v*) (6–12.5 dilutions). The complete loss of inhibitory activity was observed at 12.5%–6.25% (8–16 dilutions). In contrast, the same honeys at concentrations of 12.5% to 6.25% were fully active against *E. coli*, indicating a stronger selective inhibitory action of honeys against this Gram-negative bacterium (Fig. 3). Although Fig. 3 represents only a subset of honeys tested, the same distinctive patterns of growth in-

Fig. 4. Representative growth inhibition profiles of individual honeys against *Escherichia coli* at honey serial twofold dilutions from 2 to 4096 expressed in a semi-logarithmic scale.

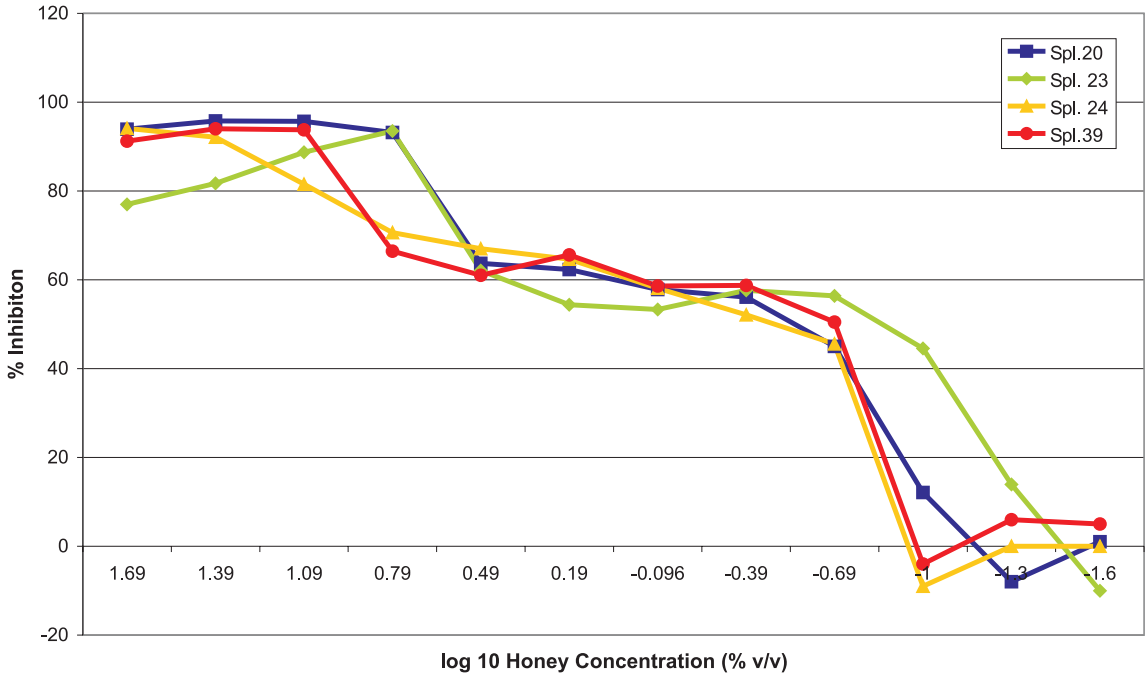


Table 3. Differences in $MICs_{50}$ for *Escherichia coli* and *Bacillus subtilis* for different honeys.

	E. coli		*B. subtilis*	
Honey sample (source)	Honey dilution	% honey concn.	Honey dilution	% honey concn.
Manuka	64	1.56	64	1.56
Spl. 5 (loosestrife)	128	0.78	8	12.5
Spl. 7 (mixed)	128	0.78	4–8	25–12.5
Spl. 8 (blueberry)	128	0.78	4–8	25–12.5
Spl. 10 (blueberry)	1024	0.09	4–8	25–12.5
Spl. 11 (clover)	512	0.19	8–16	12–6.25
Spl. 20 (clover)	256	0.39	8–16	12–6.25
Spl. 23 (buckwheat)	512	0.19	8–16	12–6.25
Spl. 24 (cranberry)	256	0.39	4	25
Spl. 39 (tupelo)	512	0.19	8–16	12–6.25

Note: MIC_{50}, minimal inhibitory concentration for 50% of the strains tested.

hibitions of *E. coli* and *B. subtilis* were observed with all honey samples.

The strong disproportion in honeys potencies against these two bacteria, prompted us to establish a concentration at which honey would reduce the growth of *E. coli* by 50% (MIC_{50}). Therefore, the microdilution assays were repeated with honey at serial twofold dilutions from 2 to 4096. The decrease in honey growth inhibitory activities over the long range of dilutions was surprisingly slow, emphasizing the considerable capacity of honey to inhibit *E. coli* growth (Fig. 4).

The data in Table 3 highlight the drastic differences in honeys MIC_{50} for *E. coli* and *B. subtilis*. These results demonstrate that both $MICs_{90}$ and $MICs_{50}$ are necessary to evaluate antibacterial potencies of honeys. Moreover, the results indicate that the mechanism of cytotoxic action of honey against these bacteria is different.

H_2O_2 concentration in honeys and antibacterial activity

To investigate the impact of H_2O_2 content on the antibacterial activity of honeys, the H_2O_2 concentration in honeys was measured using AmplexRed fluorescent probe in 96-well microtiter plates. Concentrations of H_2O_2 in honeys were calculated from the standard curve. The H_2O_2 content was measured only in monofloral honeys and in honeys collected in the year 2005, since the storage and polyfloral components could influence H_2O_2 levels. The H_2O_2 content was measured at honey serial dilutions of 2–32 times to enable a precise concentration determination and because glucose oxidase is practically inactive in undiluted honey (White et al. 1963). In contrast to the standard curve showing, as expected, a proportional decrease of fluorescence with the H_2O_2 dilutions, a fluorescence of dark honeys actually rose gradually with the dilutions, reaching a peak between the 4th and 8th dilution levels (Fig. 5). Therefore, the fluor-

Fig. 5. Hydrogen peroxide concentrations in different honeys as obtained from the Amplex® Red Hydrogen Peroxide/Peroxidase Assay.

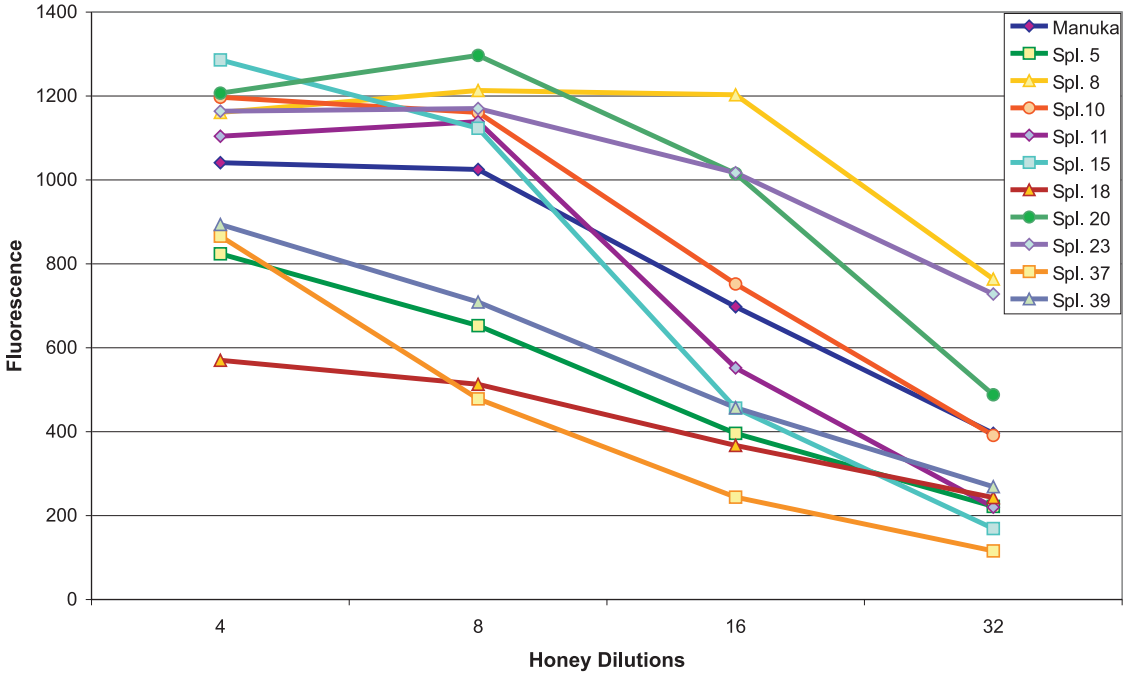


Table 4. Relationship between hydrogen peroxide concentration and MIC_{90} for different honeys.

Honey sample (source)	MIC_{90} *Escherichia coli*	H_2O_2 concn. ($\mu mol \cdot L^{-1} \cdot (mL\ honey)^{-1}$)	MIC_{90} *Bacillus subtilis*
Spl. 4 (canola)	2	48.8	2
Spl. 5 (loosestrife)	8	78.5	8
Spl. 7 (mixed)	4	29.4	4
Spl. 8 (blueberry)	8	238.5	4
Spl. 10 (blueberry)	8	163.5	4
Spl. 11 (clover)	8	190.8	8
Spl. 15 (blueberry)	16	175.6	4
Spl. 18 (buckwheat)	4	75.8	4
Spl. 20 (buckwheat)	8	192.7	4
Spl. 23 (buckwheat)	16	181.0	8
Spl. 33 (comb, clover)	2	110.7	2
Spl. 34 (comb, clover, and wildflowers)	4	119.8	4
Spl. 35 (comb, goldenrod)	4	72.8	4
Spl. 36 (comb, clover)	4	75.3	4
Spl. 37 (comb, star thistle)	4	74.1	2
Spl. 38 (comb, clover, and wildflower)	4	113.1	2
Spl. 39 (tupelo)	8	118.7	4
Spl. 41 (wildflower)	4	128.6	4

Note: MIC_{90}, minimal inhibitory concentration for 90% of the strains tested.

escence values at the 8× dilution were used to calculate the total concentration of H_2O_2 in honeys, taking into account dilution. The H_2O_2 concentration in different honeys varied from 29.4 to 238.5 $\mu mol \cdot L^{-1} \cdot (mL\ honey)^{-1}$ (Table 4). Blueberry, buckwheat, and clover honeys were consistently the highest producers of this compound.

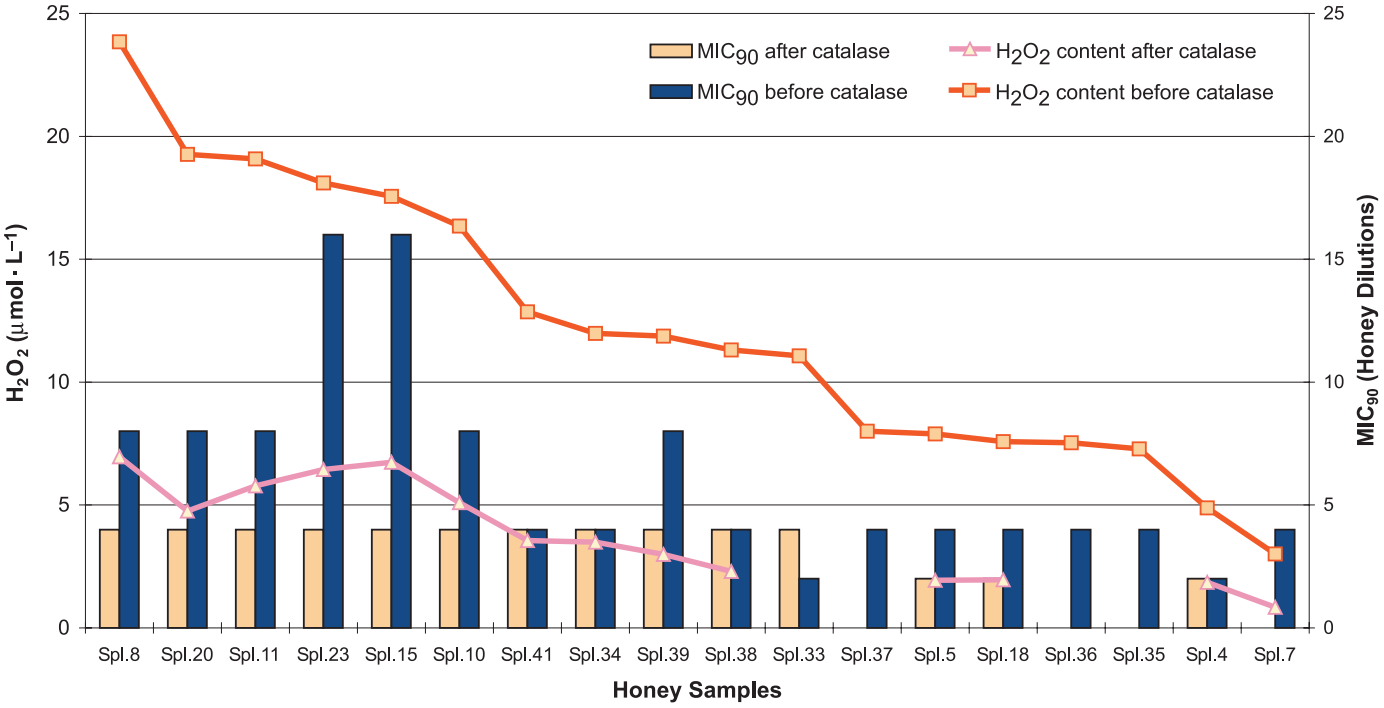
To correlate the antibacterial activity of honeys with their endogenous formation of H_2O_2, the H_2O_2 content of each honey was compared with its $MICs_{90}$ (conducted in parallel assay) against both *E. coli* and *B. subtilis* (Table 4).

The results of a Pearson *r* correlation test revealed that there was a strong correlation between the antibacterial activity against *E. coli* and the total production of H_2O_2 (n = 18, r = 0.661, p = 0.003), but there was a marginally significant correlation between the antibacterial activity against *B. subtilis* and H_2O_2 levels (n = 18, r = 0.467, p = 0.05).

Catalase treatment reduced $MICs_{90}$ of Canadian honeys

To further elucidate the effect of the endogenous H_2O_2 on antibacterial activity of honey, the samples were treated with

Fig. 6. Antibacterial activities of individual honeys expressed as minimal inhibitory concentrations (MIC_{90}) (columns) versus hydrogen peroxide (H_2O_2) concentrations (lines) before and after catalase treatment. Each honey sample was tested in triplicate for its antibacterial activity against *Escherichia coli* and the H_2O_2 content before (in the Web version: blue bars and orange line; in the printed version: black bars and line with squares) and after catalase treatment (in the Web version: tan bars and violet line; in the print version: grey bars and line with triangles). Note that the decrease in H_2O_2 content after catalase treatment parallels the decrease in MIC_{90} expressed as honey dilution (concentration).



the molar excess of catalase (based on the established earlier H_2O_2 concentrations), to remove all of the peroxide from honeys. Each honey sample was incubated with 1000 units of catalase per 1 mL of 50% honey (where 1 unit decomposes 1 µmol·(L H_2O_2)$^{-1}$·min^{-1} over 2 h. The catalase-treated honey samples were used to prepare serial dilutions to measure both H_2O_2 levels (Amplex Red assay) and antibacterial activities (broth microdilution assays). The 20 µmol·(L H_2O_2 stock solution)$^{-1}$ used for the standard curve preparation was also treated with catalase at 1 µunit per 1 µL ratio and included as the controls for the peroxide–peroxidase assays of honeys.

Even though catalase was used in a concentration that exceeded the calculated H_2O_2 content in honey by 500–4000 times, the enzyme was unable to totally remove this compound. As shown in Fig. 6, approximately 25%–30% of H_2O_2 was still present in honeys after catalase treatment. In contrast, catalase at the same concentration completely decomposed H_2O_2 from the standard curve samples. The removal of 70%–75% of H_2O_2 from honeys reduced their antibacterial activities, as indicated by the change of MIC_{90} toward higher honey concentrations in all honey tested (Fig. 6).

The statistical analysis showed again the correlation between the residual H_2O_2 and the reduced antibacterial activity (Pearson r correlation $r = 0.66$, $p < 0.01$). Manuka was not included in the statistical analyses, since its antibacterial activity is not dependent on the presence of H_2O_2 (Molan and Russell 1988).

Discussion

The present study is the first large cohort study done on Canadian honeys, demonstrating honey antibacterial activity and its dependence on H_2O_2 content. All 42 honey samples evaluated by the broth microdilution method showed the growth inhibitory activity against both *E. coli* and *B. subtilis*. The $MICs_{90}$ against *E. coli* indicate that the majority of Canadian honeys (64%) possess a moderate to high (19%) antibacterial activity, ranging from 25% to 12.5%. Only 4.6% of honeys were of low activity (50%–25%) and 11.9% showed very high activity (12.5%–6.25%). Thus, the antibacterial activities in Canadian honeys resemble a Gaussian distribution, where honeys of higher or lower activity could be considered deviations from the normal average.

We have presented in this paper the first evidence that Canadian honeys showed selective activity against Gram-negative *E. coli*. The $MICs_{90}$ of honeys for *E. coli* were found to be 2–3 times higher than for *B. subtilis*. Even more surprising was to uncover dramatic differences in the MIC_{50} between these bacteria, showing a 20- to 100-fold higher potency of honeys against *E. coli*. The inhibition profiles of *E. coli* consistently plateaued over the long range of dilutions, signifying great antibacterial capacity. At the same time, the growth inhibitory activity of honeys against *B. subtilis* disappeared very fast with honey dilution.

The establishment of $MICs_{50}$ appeared to be an important element in the evaluation of antibacterial potency of honeys

and provided additional insight into differences in activities against Gram-positive and Gram-negative bacteria. While $MICs_{90}$ reflected the difference in the bacterial resistance to honey, the $MICs_{50}$ reflected honey's intrinsic activity and pointed to its potentially distinct mechanisms of bacteriostatic and (or) bactericidal action, depending on the bacterial species. The $MICs_{50}$ proved the prolonged antibacterial activity in highly diluted honeys against *E. coli*, and this feature has therapeutic significance. In in vivo evaluations, the $MICs_{50}$ might help in the determination of honey dosage. With this in mind, it will be of great interest to check the honeys MIC_{50} against a broader spectrum of bacterial species.

The honey selectivity to Gram-negative bacteria has been previously reported for angelita honey from Colombia, produced by the stingless bee *Tetragonisca angustula* (Torres et al. 2004), and for several Australian honeys (Lusby et al. 2005; Wilkinson and Cavanagh 2005). In the latter study, the antibacterial activity has been also observed at honey dilutions below 1%. Thus, the selectivity of honeys toward some bacterial species and their potent bacteriostatic capacity at low concentrations is not an exclusive property limited to Canadian honey. One plausible explanation is that this selectivity is conferred by a hypothetical component of honey that is of bee origin because it appears in honeys from different parts of the world (Canadian, Australian, and Colombian honeys) and from different plant sources. This hypothetical component seemed to co-exist with another common factor responsible for nonspecific antibacterial action, H_2O_2.

H_2O_2 is one of the best-known nonspecific contributors to the antibacterial activity of honey (White et al. 1963; Allen et al. 1991; Mundo et al. 2004). Despite this fact, there are a limited amount of studies providing quantitative data on H_2O_2 concentrations that normally occur in honey. The lack of adequate, uniform methodology has led to sometimes conflicting results (White et al. 1963; Bang et al. 2003). To precisely establish honeys H_2O_2 concentrations, the Amplex Red reagent was used, which reacts with 1:1 stoichiometry with H_2O_2 to produce a fluorescent product. Its fluorescence was measured using a microplate reader with built-in software for automatic statistical analyses (see Methods). To our knowledge, this is the first quantitative data on the concentration of H_2O_2 in honeys. The H_2O_2 content in Canadian honeys was found to vary from 30 to 240 $\mu mol \cdot (L\ honey)^{-1}$ (when calculated to 1 mL of undiluted honey). These values are in the range of those reported for the first time by White et al. (1963) in their semiquantitative study. After decoding the numbers assigned to each honey during the study, it appeared that blueberry, buckwheat, and sweet clover honeys were among the highest producers of this compound. The same monofloral honeys showed the highest antibacterial activities against *E. coli* in the microdilution assay (Spl. 23, Spl. 20, Spl. 15, Spl. 8, Spl. 10, Spl. 34, Spl. 11). The statistical analysis revealed a significant correlation between H_2O_2 content and the antibacterial activity, expressed as a MIC_{90} (Pearson correlation, *E. coli*, $r = 0.661$, $p < 0.003$; *B. subtilis*, $r = 0.467$, $p = 0.05$). Thus, our results demonstrate an essential influence of H_2O_2 on the total antibacterial activity of honey. Furthermore, the results validate the use of the endogenous H_2O_2 concentration as a predictive marker of honey antibacterial activity.

The mechanism of H_2O_2 cytotoxicity to bacteria is not yet clear. The observed differences in the MICs of honey against *E. coli* and *B. subtilis* cannot be simply explained by the impeded permeability of H_2O_2 due to the structural differences in the outer membrane and (or) cell wall of Gram-negative and Gram-positive bacteria. Although Gram-positive bacteria are surrounded by a thick peptidoglycan cell wall, its presence does not restrict the penetration of small molecules, such as biocides, hydrophilic nutrients, and antibiotics (Nikaido 1994; Lambert 2002). It has been shown that H_2O_2 rapidly permeates through both types of outer membranes (probably by the porin route), as indicated by decreased bacterial survival after exposure to H_2O_2 (Ma and Eaton 1993; Taormina et al. 2001).

That blueberry, buckwheat, and clover honeys contained the highest amount of H_2O_2 and were among the most active against bacteria indicates also that chemical components of plant origin influence activity and play important role in generating very active therapeutic honeys. Both buckwheat and blueberry are known for the high content of phenolic acids, flavonoids, and anthocyanins (blueberry) possessing antioxidant (Gheldof and Engeseth 2002) as well as prooxidant activities (Cao et al. 1997). In the presence of transitional metals, polyphenols and flavonoids have been shown to be involved in the generation of substantial amounts of H_2O_2 via autoxidation (Cao et al. 1997; Taormina et al. 2001; Akagawa et al. 2003). Therefore, in addition to the glucose – glucose oxidase system as a main source of H_2O_2 production in honeys, plant-derived polyphenols could be the supplementary source of this compound. The results demonstrated that polyphenols-rich honeys (such as blueberry and buckwheat) and glucose-rich honey (such as sweet clover) that efficiently produced H_2O_2 possessed the over-average levels of antibacterial activities. Thus, these monofloral honeys would be desired candidates for a therapeutic application.

The impact of H_2O_2 on antibacterial activity has been further signified by the fact that its removal by catalase diminished the antibacterial activity, as indicated by the reduced MIC_{90} for both *E. coli* and *B. subtilis*, in all honeys tested. Interestingly however, catalase was unable to completely remove H_2O_2, even at an excess of 500–4000 times the micromolar amount of H_2O_2. The 25%–35% of H_2O_2 left in the honey was still considerably large to sustain honey's antibacterial activity, although with the reduced potency. The difficulty with H_2O_2 removal from honey appears to be similar to that observed earlier by White et al. (1963). In their pioneering work on the inhibine (H_2O_2), the authors noted that an unexpectedly high concentration of catalase was necessary to destroy the H_2O_2-dependent antibacterial activity. In view of the results of the present study and that of White et al. (1963), it remains possible that the portion of H_2O_2 produced by honey, including manuka honey, is either not accessible to catalase or its de novo generation exceeds the rate of its degradation by catalase. Nevertheless, this H_2O_2 leftover after catalase treatment correlated significantly with the residual activity against *E. coli*, suggesting that it grants a minimal antibacterial activity in our Gaussian distribution profile.

In summary, the results of the study indicate that the total antibacterial activity of Canadian honey is influenced by at least three factors: (*i*) H_2O_2, (*ii*) plant-originating compound(s) (specifically those derived from buckwheat, blueberry, and

clover), and (*iii*) a hypothetical component conferring selectivity to Gram-negative bacteria. The follow-on project, which is currently in progress, aims to identify compounds in Canadian honeys.

In this study, it has been demonstrated that Canadian buckwheat, blueberry, and sweet clover honeys are good candidates for a therapeutic honey because of their high production of H_2O_2. A strong significant correlation between antibacterial activity and H_2O_2 content makes H_2O_2 a valuable biomarker of the therapeutic potency of honey.

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