

# Antibacterial activity of bee honey and its therapeutic usefulness against *Escherichia coli* O157:H7 and *Salmonella typhimurium* infection

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## Summary

The authors studied the effect of storage period and heat on the physical and chemical properties of honey and proceeded to study the antibacterial effect of honey on *Escherichia coli* and *Salmonella typhimurium*. In samples of honey (Egyptian clover honey) that were heat-treated and stored over a long period of time, water content decreased, hydroxymethyl furfural (HMF) was produced and increased in concentration, and enzyme activity decreased. Colour, measured in optical density, was markedly affected in honey samples stored over long periods of time, as was the refractive index, but electrical conductivity remained unaffected by storage or heating. Similarly, the storage period had no effect on pH value.

To study the therapeutic effect of honey on *E.coli* and *S. typhimurium*, 25 isolates of *E. coli* O157:H7 (18.5%) and 49 isolates of *S. typhimurium* (36.2%) were isolated from 135 samples taken from children and calves (30 stool samples from children and 105 samples from calf organs and faecal swabs). Most *E. coli* O157:H7 and *S. typhimurium* isolates were highly resistant to most antibiotic discs.

*In vitro*, the antibacterial effect of honey was more pronounced on *E. coli* O157:H7 than on *S. typhimurium*. Water content, pH value, HMF and the presence of H<sub>2</sub>O<sub>2</sub> all played an important role in the potency of clover honey as an antibacterial agent.

*In vivo*, mice were used as a model for studying the parenteral usefulness of honey as an antibacterial agent against both pathogens. The antibacterial activity of honey that had been stored over a long period of time decreased and high concentrations of honey proved more effective as antibacterial agents. In this study there was lower mortality among mice treated with honey but the parenteral application of honey and its therapeutic properties require further investigation.

## Keywords

Antibacterial – Bee honey – *Escherichia coli* O157 – Honey – *Salmonella typhimurium*.

## Introduction

Honey has been used as a medicine in many cultures for a long time (13, 34, 43, 45) and is still used in folk medicine. In more recent times, the use of honey as a therapeutic substance has been rediscovered by the medical profession and it is gaining acceptance as an antibacterial treatment of topical infections resulting from burns and wounds (1, 4, 32, 33). It has also been found to be effective in treating bacterial gastroenteritis in infants (24, 26, 29, 35).

The enzyme content of fresh honey is one of the characteristics that make it beneficial to human health, but processing, heating and prolonged storage can lower enzyme activity (59). In addition, some types of honey form bacteria-killing hydrogen peroxide when diluted, but storage conditions can also have a detrimental effect on this process. However, honey has other important beneficial characteristics that are less influenced by storage conditions (14).

This paper examines the therapeutic and antibacterial effects of different honey samples (stored for different lengths of time) on *Escherichia coli* O157:H7 and *Salmonella typhimurium*. The effects were examined *in vivo* using experimentally infected mice and *in vitro* using isolates taken from stool samples and faecal swabs from calves and children in Egypt. Collibacillosis and salmonellosis are diseases with major economic consequences not only in Egypt, but throughout the world (65) and *S. typhimurium* and *E. coli* O157:H7 are etiological agents of infections in children and calves which cause high morbidity and frequent mortality in cattle of all ages (53). In addition, according to the Centers for Disease Control and Prevention, every year in the United States of America, at least 200 deaths and 20,000 illnesses are attributed to Shiga-toxigenic *E. coli* O157:H7 (42). Similarly, in 2000, 22,799 cases of salmonellosis were reported to the Sanitary Epidemiological Station in Poland. Most children affected by *S. typhimurium* were under five years of age (25).

## Material and methods

### Animals

Eighty-five calves of different ages suffering from diarrhoea were obtained from private farms in Egypt. Ten of the eighty-five calves were received dead.

### Children

The data used in the study were the results of tests performed at different private clinical laboratories on thirty

children suffering from diarrhoea. All of the children were under five years of age.

### Experimental laboratory animals

Two hundred and sixty specific-pathogen-free mice, with an average body weight of 25 g, were obtained from the Plant Protection Research Institute (Cairo, Egypt) and kept under observation for one week by collecting faecal samples and examining them bacteriologically for the presence of salmonella or *E. coli* pathogens. Eighty of them were used for measuring the safety dose of honey, and the rest were used as models for experimental infection and treatment.

### Samples

#### Faecal samples

Thirty stool samples from diarrhoeic children were collected and seventy-five faecal swabs were collected from diarrhoeic calves.

#### Internal organs

Thirty samples of liver, intestine and spleen (ten samples of each) were obtained from ten dead diarrhoeic calves. The same organs were collected from dead experimental infected mice.

#### Honey samples

Four samples of honey were collected (A, B, C and D). All the samples were Egyptian clover honey, but each one had been stored for a different period of time, with A being the oldest and D being the freshest. The different storage periods are listed in Table I.

**Table I**  
**Storage time of the honey samples**

Sample	Year of collection	Period of storage till 2000
A	1979	21 years
B	1984	16 years
C	1988	12 years
D	2000	7 months

### Physical and chemical analysis of the honey samples

The four samples of honey (A, B, C and D) were analysed for the following parameters.

### Water content and refractive index

The refractive index of the samples was measured using a refractometer set to a constant temperature of 20°C. Moisture content was then determined by converting these readings to percent moisture using the procedure of the Association of Official Analytical Chemists (AOAC) (62).

### Colour (optical density)

Ten grams of each honey sample were diluted with 100 ml of distilled water and centrifuged for 10 min using the method described by White (59). At 3,000 xg the absorbancy of the filtrate supernatant was measured at 350 nm using distilled water as a blank.

### Specific electrical conductivity

Electrical conductivity was measured as described by Vorwohl *et al.* (57). The reading was taken at a standard temperature of 20°C using the following formula:

$$K_{20} = K_t / 1 + \alpha (t_2 - 20)$$

$K_{20}$  = correct reading for electrical conductivity at temperature  $t_2$  (above or below 20°C)

$\alpha$  = constant factor 0.0261

The results are expressed as:  $10^{-4}$  S/cm.

### pH value

The pH value was measured using a pH meter as described by Egan *et al.* (21)

### Detection of hydroxymethyl furfural

Hydroxymethyl furfural (HMF) was detected using a technique based on the method described by Winkler (63). Five grams of honey were dissolved, without heating, in oxygen free distilled water and transferred to a 125 ml graduated flask and diluted to volume with oxygen free distilled water. Two millilitres of honey solution was pipetted into two tubes and 5 ml of P-touidine solution was added to each. Into one test tube, 1 ml of water was pipetted and into the other 1 ml of barbituric acid solution was added; both mixtures were then shaken. Absorbance was read using a spectrophotometer against a blank at a wave length of 550 nm. Calculation: Mg/100 g hydroxymethyl furfural = absorbance/test  $\times$  192 (2).

### Detection of glucose oxidase

As described by White and Subers (61), for each of the honey samples, a solution was prepared by taking 5 g of honey, adding 5 ml of buffer (0.4% phosphate buffer with pH 6.5) and diluting to volume in a 25 ml flask using distilled water. Ten millilitres of the buffered honey solution were then mixed with 10 ml of distilled water in a capped bottle, warmed in a bath and placed in a roller drum at 20 rpm for 1 h at 37°C.

In the meantime, 3 test tubes were prepared, two of which contained 2 ml of distilled water and 6 ml of reagent. (The reagent was prepared as follows: 10 mg of o-dianisidine [3,3 – dimethoxybenzidine] were dissolved in 2 ml of 95% ethyl alcohol in a 200 ml flask, 5 ml of 0.4 phosphate buffer pH 6.5 was added and the solution was diluted to volume with distilled water.) The third test tube contained the blank (2 mg of peroxidase dissolved in 50 ml of 0.01 M phosphate buffer pH 6.5).

An appropriate volume of the incubated honey solution was added to the 3 test tubes (0.1 ml to 2 ml, depending on the peroxide content of the honey solution). The contents were mixed and the absorbance of the solution was measured at 400 nm 5 min to 10 min later. The results of the assay were expressed as follows: mg  $H_2O_2$ /g honey/hour.

### Isolation and identification of *Escherichia coli* O157:H7 and *Salmonella typhimurium*

To isolate *E. coli* O157, the collected samples from children, calves and mice were cultured on MacConkey agar, eosin methylene blue media and Sorbitol MacConkey agar after being enriched in Moosel's enteric enrichment broth for 24 h at 37°C. To isolate *S. typhimurium* the samples were cultured on XLD media and Salmonella-Shigella agar after being enriched in Selinite F broth for 18 h at 37°C (8, 18, 23).

The suspected colonies were subjected to biochemical identification as described in previous studies (7, 16, 31, 43), using an oxidase test, a catalase test, an indol test, a methyl red test, the Vogus Proskaur test, production of  $H_2S$  on triple sugar iron media, a urease test, a citrate test, and a sugar fermentation test.

Serological identification of *E. coli* O157:H7 and *S. typhimurium* was carried out according to a previously developed procedure (15) using polyvalent and monovalent diagnostic *E. coli* antisera, polyvalent salmonella antisera and monovalent O and H antisera in agglutination tests.

### Antimicrobial susceptibility testing

The disc diffusion technique was used as previously described by Dustmann (19), using different types of antimicrobials. All isolates (25 *E. coli* O157:H7 and 46 *S. typhimurium*) were inoculated into Müller Hinton broth and incubated for 18 h to 24 h, the density was then adjusted to  $1.5 \times 10^8$  using Macferlan tubes.

### Preparation of honey suspensions for the disc diffusion test

The four samples of stored honey (A, B, C and D), were each diluted in sterile distilled water to obtain 5%, 10% and 20% concentrations.

### Antimicrobial susceptibility to honey in the disc diffusion test

The disc diffusion test was carried out as described by Mirsa and Wamota, and Helms *et al.* (27, 30, 35). Eight millimetre diameter-filter paper was saturated with 0.1 ml of each of the honey suspensions, which was equivalent to 5 mg, 10 mg and 20 mg of honey per disc. The density of the isolates was the same as that used in the antimicrobial susceptibility testing of the various chemotherapeutic agents.

### Minimum inhibitory concentrations of stored honey against the isolated organisms

The broth dilution technique was used to ascertain the minimum inhibitory concentration (MIC) of the honey samples. The test was carried out as described by Heuvelink *et al.* (28). A suspension of the organism was adjusted to  $1.5 \times 10^8$  organism/ml and further diluted to 1:200 in Müller Hinton broth. Five millilitres each of Müller Hinton broth was pipetted into ten sterile screw capped test tubes. A weight of 100 mg/ml of the honey was dissolved completely in the first tube. A serial dilution of honey, with a dilution factor of half was established. Tube number 10 served as a positive growth control containing Müller Hinton broth and bacterial inoculum only, and an additional tube containing broth only was used as a negative control. A volume of 0.1 ml of the bacterial suspension ( $7.5 \times 10^5$  organism/ml) was added to each tube. The tubes were incubated at 37°C for 18 h and visually examined for evidence of turbidity. The lowest

concentration of honey in the series that inhibited the growth of the organism was taken to be the MIC, expressed in mg/ml.

### Experimental infection of mice

One hundred and eighty mice were divided into two groups of ninety (groups A and B). Each group was further subdivided into six subgroups of fifteen mice ( $A_1$ - $A_6$  and  $B_1$ - $B_6$ ). Subgroups  $A_1$ - $A_5$  were subcutaneously injected in the neck with 1 ml ( $7.5 \times 10^5$  organism/ml) of *E. coli* O157:H7, while subgroup  $A_6$  was left uninfected (control). Groups  $B_1$ - $B_5$  were experimentally infected with the same dose and the same concentration of *S. typhimurium* and group  $B_6$  were kept without infection as a control. Most of the *E. coli* challenged mice had developed diarrhoea after two to three days of challenge and mice infected with *S. typhimurium* also developed diarrhoea after a similar period. The treatment was applied by subcutaneously injecting 1 ml of the honey samples in the neck for five to ten days. Groups  $A_1$  to  $A_4$  and  $B_1$  to  $B_4$  were treated with honey samples A, B, C or D and groups  $A_5$  to  $A_6$  and  $B_5$  to  $B_6$  were left without treatment (control groups). The morbidity and mortality of the mice were recorded.

## Results

### Chemical properties of the honey samples

The results of the honey analysis test can be seen in Table II.

### Water content

The water content of the fresh sample (sample D, collected in 2000) was in the normal range of fresh honey (19.5%). The other three samples that had been stored for longer periods (12 years, 16 years and 21 years) showed a

**Table II**  
Effect of different periods of storage on some physical and chemical properties of four Egyptian clover honey samples

Physical and chemical properties	A	B	C	D	LSD at 0.05
Water content percentage	13.8 ±* 1.104	14.0 ± 0.98	14.6 ± 1.168	19.5 ± 1.56	± 0.052
Electrical conductivity 10 <sup>-4</sup> S/cm	3.25 ± 0.26	2.76 ± 0.221	2.35 ± 0.165	2.05 ± 0.185	±0.20
Colour as optical density at 400 nm	3.532 ± 0.321	3.025 ± 0.221	2.175 ± 0.196	0.175 ± 0.014	±0.15
Refractive index at 20°C	1.5023 ± 0.120	1.5018 ± 0.120	1.5002 ± 0.110	1.4880 ± 0.110	±0.01
Refractive index at 40°C	1.4978 ± 0.119	1.4973 ± 0.118	1.4957 ± 0.119	1.4853 ± 0.119	±0.01
pH value (1 – 14)	4.6	4.1	4.9	5.2	
Hydroxymethyl furfural mg/kg	295.25 ± 23.62	180.36 ± 14.43	125.75 ± 8.80	5.65 ± 0.45	±50.2
Glucose oxidase mgH <sub>2</sub> O <sub>2</sub> /g honey/L	0.00	0.00	0.00	95.50	

LSD: least significant difference established using analysis of variance (ANOVA) according to Snedecor and Cochran (50)

\* mean ± standard error

significant decrease in water content. Ghazali and Sin (24) reported a slight decrease in water content when honey was stored at different temperatures ranging between 4°C and 50°C, while Vorwohl *et al.* (57) and Nour *et al.* (41), indicated that honey usually loses water content when stored in a country with relatively low air humidity, such as Egypt.

### Refractive index

The RI was slightly reduced at 40°C compared to at 20°C. These results could be due to the honey being stored in conditions of relatively low air humidity (41).

### Colour

The colour, measured in optical density, of samples A, B and C was markedly affected by long storage periods. These samples showed higher values of optical density at 400 nm. Sample D had the lowest value of optical density (0.175) which was in the range of fresh honey. Nour (40) showed that the range of colour, measured in optical density, for fresh clover honey was between 0.118 and 0.245. Wooton *et al.* (64) found that colour changed at markedly different rates during storage at temperatures of 43°C, 50°C and 80°C. Ghazali and Sin (24) and Nour *et al.* (41) added that honey darkened more quickly at 50°C after it had been stored for eighteen weeks; the degree of darkening was about six times that of honey stored at 28°C.

### Specific electrical conductivity

Specific electrical conductivity was not affected when honey was stored for different periods. However, sample D had a low level of conductivity ( $2.5 \times 10^{-4}$  S/cm) and sample A had a high level of conductivity ( $3.250 \times 10^{-4}$  S/cm). (Sample A was a mixture of clover honey and eucalyptus honey, which may explain why it had higher conductivity.) The rest of the samples had conductivity levels in the normal range for clover honey. These results are in agreement with Nour (40) and Nour *et al.* (41), where the range of conductivity levels for clover honey was 2.03 to  $4.83 \times 10^{-4}$  S/cm. Vorwhol (56) added that honeys of the same floral origin have approximately the same conductivity level, even if they have been harvested in different years and originate from different geographic regions and different climates.

### pH value

The pH of the four samples of honey were found to be in the normal range for fresh honey, thus demonstrating that storage period had no effect on the pH values of different samples.

### Hydroxymethyl furfural production

Table II also shows the effect of storage periods on HMF accumulation in stored honey samples. Hydroxymethyl furfural is produced when some of the sugars in honey,

such as glucose and fructose, begin to break down, especially when stored at high temperatures over long periods of time. A high HMF content has been used as an indicator of improper storage temperature, or as an indication that the honey has become adulterated with invert sugar produced by acid hydrolysis at high temperatures. The results indicate that sample D showed the lowest HMF content (5.65 mg/kg). However, the other samples with longer storage periods had much higher levels than the maximum level accepted in Europe (4.0 mg/kg). These results are in agreement with Schade *et al.* (49) who studied the increase of HMF in four samples stored for between thirteen and fifteen months at 20°C; they recorded an increase in HMF of 3.3 mg/100 g in one sample. Nour (40) examined sixty fresh honey samples collected directly from the beekeeper, the HMF was from 0.11 mg/kg to 1.27 mg/kg; however, after being stored for six months the HMF increased gradually from 0.31 mg/kg to 1.02 mg/kg.

### Glucose oxidase activity

The enzyme activity of the honey was greatly affected during storage. Samples A, B and C showed no activity of enzyme glucose oxidase. On the other hand, sample D contained 95.5 mg H<sub>2</sub>O<sub>2</sub>/g honey/h. These results are in agreement with White (58); Crane (15); Nour (40) and Nour *et al.* (41), who all reported a slight decrease in enzyme activity when honey was stored at room temperature.

## Isolation and identification of *Escherichia coli* O157:H7 and *Salmonella typhimurium*

The rate of isolation of *E. coli* O157:H7 and *S. typhimurium* from samples from children and calves are recorded in Table III. The frequency rate of *E. coli* O157:H7 was high in the stool samples of children and the intestines of dead calves (16.6% and 30% respectively). The rate of

**Table III**  
Prevalence rates of *Escherichia coli* O157:H7 and *Salmonella typhimurium* in diarrhoeic children and calves

Samples	<i>E. coli</i> O157:H7		<i>S. typhimurium</i>	
	Number	%	Number	%
Stool samples from children (30)	5	16.6	10	33.3
Calves				
Faecal swabs (75)	13	17.3	20	26.7
Liver (10)	2	20	9	90
Spleen (10)	2	20	5	50
Intestine (10)	3	30	5	50
Total calf samples (105)	20	19.04	39	37.1
<b>Total no. of samples (135)</b>	<b>25</b>	<b>18.5</b>	<b>49</b>	<b>36.2</b>



*S. typhimurium* isolation was 33.3% in children and 90% in liver of the dead calves. The overall rates of *E. coli* O157:H7 and *S. typhimurium* isolation were 18.5% and 36.2% respectively.

### The disc diffusion test

The results of the disc diffusion test of different chemotherapeutic agents revealed that most isolated strains of *E. coli* O157:H7 and *S. typhimurium* were highly resistant to the majority of antimicrobials used (Table IV). The diameters of the inhibition zones of the different honey samples are illustrated in Table V. Honey sample D gave the maximum inhibition zone diameter against *E. coli* O157:H7 and *S. typhimurium* at all the different dilutions. Sample A (which had been stored over the longest period of time) gave the minimum inhibition zone diameter against *E. coli* O157:H7. The MIC against *E. coli* O157:H7 and *S. typhimurium* decreased as the storage time of the honey increased (Table VI). Sample D of honey (stored for the shortest period of time) showed the highest MIC against both isolates (1.5 mg/ml and 3.1 mg/ml, respectively).

### Experimental infection of mice and the therapeutic effects of honey stored for different lengths of time

Morbidity among mice infected with *S. typhimurium* decreased slightly when treated with sample B but otherwise remained unchanged. Morbidity among mice infected with *E. coli* was the same when they were treated

with samples A and D, higher when treated with sample C and lower when treated with sample B. For both *E. coli* and *S. typhimurium* mortality was high in mice treated with sample A and lower in groups treated with samples B, C and D (Table VII).

## Discussion

*Salmonella typhimurium* and *E. coli* O157:H7 play an important role as an epidemiological causative agent of diarrhoea in children and calves and the misuse of different antimicrobial agents has resulted in the appearance of highly resistant strains of these bacteria.

The prevalence rates of *E. coli* O157:H7 isolated from calves and children were 19.04% and 16.6% respectively and those of *S. typhimurium* were 37.1% and 33.3% respectively (Table III). This prevalence is higher than that reported by Koneman *et al.* (31), Alstad *et al.* (5), Mirsa and Wamota (35), Champman *et al.* (13), Sanderson *et al.* (48), Henvelink *et al.* (28), Fey *et al.* (22), Vernozy-Rozand *et al.* (55), Saha *et al.* (47), Gonera (25) and Park *et al.* (42). This high prevalence in calves and children may be attributed to the misuse of different antimicrobials without the advice of physicians or veterinarians. This explanation is further supported by the results of the antimicrobial disc diffusion tests. Table IV shows the high resistance rate of most isolated strains of *E. coli* O157:H7 and *S. typhimurium* against the majority of antimicrobial agents. Mirsa and Wamota (35); Adesiyun and Kaminjolo (3), Bacon *et al.* (7) and Helms *et al.* (27) have also reported a high resistance

**Table IV**  
The results of a disc diffusion test of chemotherapeutic agents against *Escherichia coli* O157:H7 and *Salmonella typhimurium*

Chemotherapeutic agents	<i>E. coli</i> O157:H7 (25 isolates)								<i>S. typhimurium</i> (49 isolates)							
	Children (5)				Calves (20)				Children (10)				Calves (39)			
	Sensitive		Resistant		Sensitive		Resistant		Sensitive		Resistant		Sensitive		Resistant	
	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%
Amikacin	1	20	4	80	4	20	16	80	2	20	8	80	5	12.8	34	87.2
Ampicillin	1	20	4	80	3	15	17	85	1	10	9	90	3	7.7	36	92.3
Carbenicillin	0	0	5	100	2	10	18	90	0	0	10	100	4	10.3	35	89.7
Cefotaxime	1	20	4	80	3	15	17	85	3	30	7	70	9	23	30	77.0
Chloramphenicol	0	0	5	100	0	0	20	100	0	0	10	100	2	5.1	37	94.9
Colistin sulphate	0	0	5	100	0	0	20	100	0	0	10	100	1	2.6	38	97.4
Erythromycin	0	0	5	100	1	5	19	95	1	10	9	90	1	2.6	38	97.4
Gentamycin	1	20	4	80	2	10	18	90	2	20	8	80	8	20.5	31	79.5
Kanamycin	0	0	5	100	0	0	20	100	0	0	10	100	1	2.6	38	97.4
Neomycin	0	0	5	100	0	0	20	100	2	20	8	80	3	7.6	36	92.3
Nitrofurantoin	0	0	5	100	0	0	20	100	3	30	7	70	7	17.9	32	82.0
Oxytetracyclin	0	0	5	100	1	5	19	95	0	0	10	100	1	2.6	38	97.4
Streptomycin	0	0	5	100	0	0	20	100	0	0	10	100	0	0	39	100
Trimethoprim	0	0	5	100	0	0	20	100	0	0	10	100	0	0	39	100

of *S. serovar typhimurium* and *E. coli* O157:H7 to different antimicrobials.

### The antimicrobial activity of honey

The antimicrobial activity of honey was studied both *in vivo* and *in vitro*. The diameters of the inhibition zones and the MIC of the different honey samples were studied and the results are illustrated in Tables V and VI; the longer the storage period, the greater the decrease in antimicrobial

**Table V**  
The mean diameter of the inhibition zones of the four honey samples (in millimetres) against *Escherichia coli* O157:H7 and *Salmonella typhimurium*

Honey sample	Concentration of honey					
	<i>E. coli</i> O157:H7			<i>S. typhimurium</i>		
	5%	10%	20%	5%	10%	20%
A	14	16	18	0	0	0
B	12	17	18	0	0	12
C	14	16	20	0	14	15
D	19	22	24	0	15	20

**Table VI**  
The mean minimum inhibition concentrations of the different honey samples against *Escherichia coli* O157:H7 and *Salmonella typhimurium*

Honey sample	Concentration of honey in mg/ml	
	<i>E. coli</i> O157:H7 (35 isolates tested)	<i>S. typhimurium</i> (56 isolates tested)
A	100	100
B	25	50
C	6.25	12.5
D	1.5	3.1

**Table VII**  
The morbidity and mortality rates after parenteral use of honey in mice infected with *Escherichia coli* O157:H7 and *Salmonella typhimurium*

Honey sample	Total Morbidity mice	Group I (15 mice) infected with <i>E. coli</i> O157:H7				Group II (15 mice) infected with <i>S. typhimurium</i>			
		Mortality		Mortality		Morbidity		Mortality	
		Number	%	Number	%	Number	%	Number	%
A	30	13	86.6	8	53.3	14	93.3	14	93.3
B	30	12	80	5	33.3	13	86.6	13	86.6
C	30	14	93.3	3	20	14	93.3	11	73.3
D	30	13	86.6	0	0	14	93.3	6	40
Control group (a)	30	13	86.6	13	86.6	14	93.3	14	93.3
Control group (b)	30	0	0	0	0	0	0	0	0

a) 15 mice infected with *E. coli* O157:H7 and 15 mice infected with *S. typhimurium*

b) mice not infected or treated

activity against *S. typhimurium* and *E. coli* O157:H7. The concentration of honey had an impact on antibacterial activity as observed in Table V: in the disc diffusion test a 5% concentration of the four honey samples produced no antibacterial effect on *S. typhimurium* and a small inhibition zone against *E. coli* O157:H7 when compared to honey concentrations of 10% and 20%. Duisberg and Warnecke (18) reported low antimicrobial activity when honey was exposed to heat and light. Khristov and Mladenov (30) and Chambonnand (12) found that the concentration of honey needed for complete inhibition of *E. coli* growth was 20%. Nabbdank and Skareek (39) and Taormina *et al.* (52) reported that the concentration of honey needed for complete inhibition of *S. typhimurium* growth was  $\leq 25\%$ . The sensitivity of the microorganism itself plays an important role in its susceptibility to honey as an antibacterial agent, i.e. *S. typhimurium* were more resistant than *E. coli* O157:H7 (Tables V and VI). Molan has reported the relative sensitivity of various species to antimicrobials depending on the virulence of the isolated microorganism (36).

The disc diffusion method is mainly a qualitative test for detecting the susceptibility of bacteria to antimicrobial substances, however, the MIC reflects the quantity needed for bacterial inhibition. The MIC of honey sample A was 100 mg/ml for both *E. coli* O157:H7 and *S. typhimurium* (Table VI). This result confirms that storage and elevated temperatures reduce the antibacterial activity of honey. This could be attributed to the presence of HMF and the inactivation of glucose oxidase, however, in old honey samples, it could be due to the decrease in water content (13.8) (Table II). This result is in agreement with Thiamann (53), Radwan *et al.* (44) and White (58) who reported that the pH value of honey is acidic and this acidity is due primarily to the content of gluconolactone/gluconic acid present as a result of enzyme action in the ripening nectar. Thiamann (53) recorded that the optimum growth of *E. coli* and *Salmonella* normally occurs at a pH of 7.2 and 7.4. Molan (36, 37) explained

that the dilution of honey under experimental conditions changes or neutralises the pH of the honey, thus changing the antibacterial activity. However, Rychurch and Dolezol (46), Linder (32), Daghighie *et al.* (17) and Bogdanov *et al.* (11) found no correlation between antibacterial activity and the pH level. *In vitro*, the high antibacterial effect of honey sample D in the disc diffusion test and the low MIC may be attributable to the presence of glucose oxidase, which is activated by dilution in water resulting in the production of H<sub>2</sub>O<sub>2</sub> which is toxic to bacteria. Stinson *et al.* (51), White and Subers (61), Dustmann (19), Bogdanov (9), Armstrong and Otis (6) and Bogdanov (10) reported that glucose oxidase enzymes were destroyed by storage or heating or exposure to light. This observation explains why honey that had been stored over longer periods of time had a lower antibacterial effect. Although there was no glucose oxidase in honey samples A, B and C (Table II), they still had some antibacterial effect on both isolates (Tables V and VI). This could be due to the presence of another substance other than the presence or absence of glucose peroxidase. These results were in agreement with Molan (37).

The *in vitro* study proved that there was antibacterial activity in the honey samples; this was followed by a study of this activity *in vivo*, using mice. Table VII shows the morbidity and the mortality rates of mice infected with *E. coli* O157:H7 and *S. typhimurium* and treated with different honey samples after testing the safety dose of honey for subcutaneous injection (Table VIII). The mice treated with honey sample A showed high morbidity and mortality for both *E. coli* O157:H7 (86.6% morbidity and 53.3% mortality) and *S. typhimurium* (93.3% morbidity and 93.3% mortality). However, the *E. coli* challenge group treated with honey sample D had 86.6% morbidity and 0% mortality. The *S. typhimurium* group treated with sample D had 93.3% morbidity and 40% mortality. This proved that the longer the period of storage the greater the drop in the antibacterial activity *in vivo*.

**Table VIII**  
**The safety dose of honey for subcutaneous injection**

Honey sample	Total number of mice tested	Number of dead mice in each subgroup <sup>a)</sup>			
		(2 ml)	(1.5 ml)	(1 ml)	(0.5 ml)
A	20	5	3	0	0
B	20	5	2	0	0
C	20	5	3	0	0
D	20	5	1	0	0

a) Each group of 20 was divided into 4 subgroups of 5 and each subgroup was injected with a different dose of honey

## Conclusion

The exact explanation for the antibacterial activity of honey is not known, but it is clear that the higher the concentration of honey the greater its usefulness as an antibacterial agent. It is also evident that the antibacterial effect decreases over time and that different species of bacteria differ in their susceptibility to honey.

Although there is evidence of antibacterial activity from the use of honey in the topical treatment of infected wounds, further consideration needs to be given to its parenteral application and therapeutic properties in order to optimise the use of this product in clinical and systemic infections.

It should be noted that the success of the parenteral route to control *E. coli* O157:H7 and *S. typhimurium* infection in mice may be attributed to more than just antibacterial activity: it could be due to the tetracycline residues in honey and to the fact that honey stimulates the proliferation of lymphocytes and phagocytes, thus activating the immune response to infection (1, 38, 54).





## L'activité antibactérienne du miel d'abeille et son intérêt thérapeutique contre les infections à *Escherichia coli* O157:H7 et à *Salmonella typhimurium*

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### Résumé

Les auteurs ont étudié les effets de la durée de stockage et du traitement thermique du miel sur ses propriétés physico-chimiques. Par ailleurs, ils se sont penchés sur l'action thérapeutique du miel par rapport à *Escherichia coli* et *Salmonella typhimurium*. Le stockage de longue durée d'échantillons de miel (miel de trèfle égyptien) traités à la chaleur s'est accompagné d'une réduction de la teneur en eau, de la production et de l'augmentation de la concentration en hydroxyméthyl-furfural (HMF), ainsi que d'un ralentissement de l'activité enzymatique. À l'issue d'une conservation prolongée, la couleur des échantillons de miel, mesurée en densité optique, avait subi une forte dégradation, de même que l'indice de réfraction. En revanche, la conductivité électrique n'a été affectée ni par le stockage ni par le traitement thermique ; le stockage n'avait pas non plus modifié le pH.

Dans le but d'étudier l'action thérapeutique du miel sur *E. coli* et *S. typhimurium*, 25 isolats d'*E. coli* O157:H7 (18,5 %) et 49 isolats de *S. typhimurium* (36,2 %) ont été obtenus à partir de 135 prélèvements effectués chez des enfants et des veaux (30 échantillons de selles d'enfants et 105 prélèvements d'organes de veaux et écouvillonnages de fèces). La plupart des isolats d'*E. coli* O157:H7 et de *S. typhimurium* se sont montrés très résistants à la plupart des disques antibiotiques.

L'action thérapeutique du miel *in vitro* était plus marquée sur *E. coli* O157:H7 que sur *S. typhimurium*. La teneur en eau, le pH, le HMF et la présence d' $H_2O_2$  ont tous contribué de manière significative à l'efficacité antibactérienne du miel de trèfle. Des modèles murins ont permis d'étudier *in vivo* l'utilité du miel administré par voie parentérale comme agent antibactérien envers ces deux agents pathogènes. L'activité antibactérienne du miel avait diminué après une longue période de stockage ; par ailleurs, il s'est révélé particulièrement efficace comme antibactérien à fortes doses. Les auteurs ont constaté un taux de mortalité plus faible parmi les souris ayant bénéficié du traitement au miel. Toutefois, l'administration parentérale et les vertus thérapeutiques du miel requièrent des études complémentaires.

### Mots-clés

Antibactérien – *Escherichia coli* O157 – Miel – Miel d'abeille – *Salmonella typhimurium*.



## Actividad antibacteriana y utilidad terapéutica de la miel de abeja contra las infecciones por *Escherichia coli* O157:H7 y *Salmonella typhimurium*

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### Resumen

Tras analizar la influencia del calor y el tiempo de almacenamiento en las propiedades físicas y químicas de la miel, los autores estudiaron sus efectos terapéuticos contra *Escherichia coli* y *Salmonella typhimurium*. En muestras de miel (miel de trébol egipcio) tratadas con calor y almacenadas durante largo tiempo decrecía el contenido hídrico, se generaba hidroximetil-furfural (HMF), cuya concentración aumentaba, y disminuía la actividad enzimática. Las medidas de densidad óptica daban fe de un acusado cambio cromático en las muestras de miel conservadas durante mucho tiempo, cambio que afectaba igualmente al índice de refracción. No así la conductividad eléctrica, insensible al calor y al almacenamiento. Análogamente, el periodo de conservación no tenía efecto alguno sobre el pH.

Para estudiar los efectos terapéuticos de la miel contra *E. coli* y *S. typhimurium* se tomaron 25 colonias puras de *E. coli* O157:H7 (18,5%) y 49 de *S. typhimurium* (36,2%) aisladas a partir de 135 muestras extraídas a niños y terneras (30 muestras de heces infantiles y 105 muestras de órganos e hisopados fecales de ternera). La mayoría de las cepas aisladas de *E. coli* O157:H7 y *S. typhimurium* presentaban elevada resistencia a gran parte de los discos de antibióticos.

*In vitro*, el efecto terapéutico de la miel resultaba más acusado en *E. coli* O157:H7 que en *S. typhimurium*. El contenido hídrico, el valor del pH, el nivel de HMF y la presencia de H<sub>2</sub>O<sub>2</sub> influían considerablemente en la potencia de la miel de trébol como agente antibacteriano.

En las pruebas efectuadas *in vivo* se utilizaron ratones como modelo para estudiar la utilidad de la administración de miel por vía parenteral para combatir ambos patógenos. Tras un largo periodo de almacenamiento, la actividad antibacteriana de la miel decaía y se precisaban concentraciones elevadas para que resultara un antibacteriano eficaz. Aunque en ese estudio se registró un bajo nivel de mortalidad de ratones tratados con miel, es conveniente estudiar más a fondo la aplicación parenteral y las propiedades terapéuticas de la miel.

### Palabras clave

Antibacteriano – *Escherichia coli* O157 – Miel – Miel de abeja – *Salmonella typhimurium*.



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