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Antibacterial effect of honey against *Escherichia coli*, *Salmonella* Enteritidis and *Listeria monocytogenes*

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1. INTRODUCTION

Honey is a sweet liquid, a supersaturated solution of sugars, mainly glucose and fructose. It can be clear to opaque, light yellow, golden or brown. It also contains numerous of other components like vitamins and minerals, antioxidants, proteins and hydrogen peroxides and other still unknown (Sveriges Biodlares Riksförbund, 2014).

Honey is produced by the honey-bee *Apis mellifera* by the nectar it collects from different flowers. The bee carries the nectar in their honey-stomach, an enlargement of the esophagus, to the bee-hive where it produces the honey mainly by reducing the water-concentration (Sveriges Biodlares Riksförbund, 2014).

Honey have been used by mankind for at least fifteen thousand of years as can be seen in the cave paintings in the Araña cave in Spain. In Egypt it was used for its medicinal effect four thousand years ago. In sacred writings from Babylon one can read about the healing powers of honey. With the discovery of antibiotics the use of honey declined after the Second World War.

Today the use of honey is increasing again partly due to the growing problem of antibiotic resistant bacteria (White, 2005).

Through history, man has used honey for its healing powers without any scientific evidence of its effect. What we need today is more research to better understand the effect of honey and the different fields in which honey can be used (Vásquez and Olofsson, 2008).

The last few decades many researchers have studied honey. Both physical and chemical properties have been examined as well as antimicrobial and antioxidant effect.

The aim of this study was to examine the inhibitory effect of honey against *Escherichia coli*, *Salmonella* Enteritidis and *Listeria monocytogenes*.

2. LITERATURE SURVEY

2.1 Honey

2.1.1 Composition of honey

Honey is composed mostly of different sugars, water and some minerals, vitamins, inhibins, enzymes and pollen. The gross appearance such as taste and color largely depends on which type of flower the honeybees have collected the nectar from. The color can range from white to yellow to almost black. Darker honey contains more minerals. The consistency of the honey depends on how it has been handled as well as its composition of sugars and water. Swedish honey contain low amount of fructose compared to honey from many other countries. Low fructose content makes the honey less fluid and more firm (Sveriges Biodlares Riksförbund, 2014).

The main components of honey:

- 82% carbohydrates, mostly sugars:
    - Monosacharides 69% : fructose 38% and glucose 31%;
    - disaccharides 9%: mostly sucrose and maltose;
    - oligosaccharides 4%
- Water 17%
- Proteins and Amino acids
    - Enzymes: Invertase, Amylase, Catalase, Acid phosphorylase
    - Free amino acids
- Vitamins, minerals and antioxidants
- Organic acids (Loveridge, 2001)

2.1.2 Antimicrobial and antioxidant properties

The antimicrobial properties of honey have been attributed to its high sugar concentration and low water activity, the enzyme peroxidase oxidase and its production of hydrogen peroxide and a third factor the proteinaceous compounds (Mundo et al., 2004) The antioxidant properties is mostly due to its phenolic compounds (Rodríguez et al., 2012)

In a study by Mundo 27 honey samples from different floral sources as well as geographical locations were tested on their growth inhibitory effect on seven food spoilage organisms and

seven food borne pathogens (*Bacillus cereus, Escherichia coli, Listeria monocytogenes*, *Salmonella* enteric ser. Typhimurium and *Staphylococus aureus*). Overlay inhibition assay was used and also a well diffusion assay. The experiments showed variability in the growth inhibition that the different samples of honey had on the bacteria and also differences in the sensibility of this inhibition between 4 different strains of the same bacteria (*S. aureus*). The differences could not be attributed to specific floral or geographical origin. The honey did not prevent mould growth but had good activity against food spoilage bacteria. Non-peroxide antibacterial activity could be detected which indicate that other unidentified components are also responsible for the antimicrobial activity (Mundo et al., 2004).

Molan studied the antibacterial activities of honey in 2006. He concluded that the wide antimicrobial properties seen also in diluted samples of honey against a wide range of bacteria and fungi clearly indicate that the properties depend on more than the high sugar concentration and the low water activity. The antimicrobial properties are also due to the hydrogen peroxide produced through enzymatic reaction, and also in some honeys it is caused by antibacterial substances from plant (Molan, 2006).

14 samples of Mexican honey of different floral origin were tested in an experiment from 2011 on several parameters including the antibacterial properties. The bacteria used in the experiment were *Bacillus cereus*, *Listeria monocytogenes*, *Salmonella* Typhimurium and *Staphylococcus aureus*. The parameters that were measured were increase in lag phase, decreased growth rate and MPD (maximum population density). What they found was that all of the samples of honey had effect on some of the parameters of growth. All of the samples increased the lag phase and reduced growth rate. Some samples decreased MPD. A few samples had complete inhibitory effect on the growth. What further became evident in the experiment was that the floral origin of the honey affected its antibacterial and antioxidant properties. Honey from orange (Citrus sinensis) and eucalyptus (Eucalyptus globules labill) blossom showed the highest concentration of these compounds (Rodríguez et al., 2012).

In another study from 2010 the growth inhibitory effect of 30 samples of Argentinean honey against *Escherichia coli* was studied. The samples of honey came from apiaries situated in two different geographical zones and they had a different floral origin. The honey was divided into groups depending on their floral origin, which was decided through microscopically analysis of the pollen content of the honey. Two experiments with e-coli were performed. One

with incubation of the bacteria in 50% honey solution at 35 ºC for different length of time, maximum 48h and then using PCA to determine bacterial growth. The other experiment tested the non-hydrogen peroxide antimicrobial activity with honey pre-treated by bovine liver catalase. The test was performed with agar well diffusion method where the e-coli was added to the agar and a solution of 50% pre-treated honey was added to the wells which were cut into the agar. The result showed that all of the honey samples had inhibitory effect on the growth of the bacteria. The inhibition appeared to be reversible indicating that the nature of the antimicrobial effect is more bacteriostatic than bacteriocidal. Two of the samples showed complete inhibition of growth at 48h. The experiment testing the non-peroxide activity revealed that most of the antibacterial activity relates to the formation of hydrogen peroxide but still most samples showed some inhibition of growth and some had quite large inhibitory effect. This indicates that also non-peroxide components are an important part of the antimicrobial effect. Two honeys with the same geographical origin showed the highest non-peroxide activity and they were the two which showed complete inhibition of growth of the bacteria in the agar well diffusion test. The author draws the conclusion that these geographical differences might be the effects of a different botanical flora in this region (Fangio et al., 2010).

Another article that also studied honey coming from different floral origin and which also tested the non-peroxide antimicrobial activity was done in Spain. They tested 67 samples of honey on its potential antimicrobial and antioxidative activities against 2 different bacteria namely *Micrococcus luteus* and *Staphylococcus aureus*. Two different tests were performed to measure the antimicrobial properties. First the inhibition by the honey on bacterial growth using disc diffusion method was tested by measuring the inhibition zones. Secondly the non-peroxide antimicrobial capacity was tested by adding bovine liver catalase to the honey. What they found was that 72% of the native honey samples inhibited the growth of the *Staphylococcus aureus*, and 59% inhibited the growth of *Micrococcus luteus*. The authors conclude that after preparation with catalase the antimicrobial activity solely depends on the floral origin of the honey. About half of the samples showed non-peroxide antimicrobial effect after adding catalase. The antioxidative effect was tested by DPPH radical scavenging activity of the honey, and was determined by using spectrophotometry. What they found was that the darkest honey samples presented the highest antioxidant capacity. (Pérez Martin et al., 2008)

In a study from 2012 four varieties of honey; Manuka, Acacia, Lavender and Wild carrot, was tested on their antimicrobial potency against two strains of *Staphylococcus aureus* and one strain of *Pseudomonas aeroginosa*. The test method used was the agar incorporation method. The minimum inhibitory concentration (MIC) was determined. What they found was that the bacteria were unequally sensitive towards the samples of honey. Manuka honey showed the best antimicrobial effect against the two strains of *Staphylococcus* (with a MIC of 6%), while wild carrot honey had the best effect against the strain of *Pseudomonas* (with a MIC of 12%). They also measured the physicochemical properties of the samples. Lavender honey had the lowest pH (3,8). Manuka honey was low in proline and diastase number. Wild carrot honey was highest in proline. Finally the total phenol content and free radical scavenging capacity was tested. Manuka honey had the highest phenolic content (899,09 ±11,75 mg gallic acid/kg) while Lavender honey was the one with the lowest content. The researcher found a very significant correlation between the total polyphenolic content and the free radical scavenging capacity. Their final conclusion of the experiment was that the phenolic compounds play an important role in the antimicrobial properties of honey and that the natural variations in the floral origin of the honey could attribute to the differences in antimicrobial and antioxidant properties (Alzahrani et al., 2012)

Two well known and used medicinal honeys were tested on their antimicrobial properties. The tested samples were Revamil source (RS) honey from the Netherlands and Manuka honey from the UK. They were tested on Bac*illus subtilis, Eschericia coli, Pseudomonas aeruginosa* and methacillin resistant *Staphylococcus aureus*. What was found were not only that they had different effect on the bacteria but also that they executed their antimicrobial effect through different substances. The sample of RS honey killed *Bacillus subtilis*, *Eschericia coli* and *Pseudomonas aeruginosa* within 2 hours whereas the Manuka honey only showed this fast bacteriocid effect on *Bacillus subtilis*. After 24 hours of incubation all of the bacteria including *Staphylococcus aureus* were killed by both samples of honey. They also found out that Manuka honey retained its antimicrobial effect in more diluted samples than the RS honey did. The major factors of the antimicrobial activity of RS honey was Bee defensin-1 and hydrogen peroxide. In Manuka honey these two factors were not found but instead it contained 44 times more methylglyoxal, which also is an antimicrobial factor, than the RS honey did. When the methylglyoxal was neutralized in a test some of the antimicrobial properties remained in the Manuka honey suggesting that there are other unknown factors contributing to the antimicrobial properties (Kwakman et al., 2011).

Another study examined the minimum inhibitory concentration (MIC) of honey against *Staphylococcus aureus*. The MIC was determined by macrodilution and the growth was detected by UV-Vis spectrometer. The study showed that at a concentration higher than 2% the honey inhibited the growth of the bacteria. Component analysis showed that there were differences in the composition of the honey samples that coincided with the geographical origin of the honey. (Miorin et al., 2003).

In 2008 an attempt was made to discover and identify bacteria in and around the honey bees and the honey. The hypothesis was that the bacteria were collected by the bees from flowers they visited when collecting nectar and pollen and that the bacteria could somehow potentially be beneficial for humans. What they instead discovered was a novel lactic acid flora originating from within the honey bee itself. Lactic acid bacteria (LAB) are frequently found on flowers and as a part of the normal bacterial flora of insects. However the flora found in the honey-stomach of the bees and in the fresh honey could not be found on the flowers where the nectar was collected but seemed to originate from the bees themselves. They also discovered that the LAB flora showed variations that coincided with the flower source of the nectar collected by the bee at the particular time. The authors make the conclusion that probably the different nectar sugars affect the resident flora in the stomach so that there is a shift in the number of the different bacteria. This means that which particular bacteria that are highest in number depend on flower source but the bacteria is not originating from the flower. 10 different phylotypes of LAB was found, 3 of which was also found in the fresh honey (Olofsson and Vásquez, 2008).

A further study of the LAB of the honey bee was made in 2012 when a study of the antimicrobial properties of these bacteria against pathogenic gram positive bacteria was made. 32 LAB were isolated from 13 samples of honey. The bacteria that was tested was multiple antibiotic resistant *Staphylococcus aureus*, *Staphylococcus epidermis* and *Bacillus subtilis*. Tests with the LAB were made but also tests using a supernatant with substances produced by the LAB. The results showed that the LAB inhibited the growth of all three bacteria. All samples had an inhibitory effect on *S. aureus*. A lesser effect was shown on the effect on growth of the other two bacteria. All of the supernatants showed antimicrobial properties but to a more varying degree than the LAB samples did. The supernatant inhibited the growth of *S. aureus*. Furthermore was showed that some of the supernatants had antimicrobial properties

that were heat resistant, resistant to low pH and/or resistant to enzymatic degradation (Mohamed et al., 2012)

One of the latest studies in the subject of LAB was made in 2013. It was a continuation by Olofsson and Vásquez on their study from 2008 and was done together with other researchers. This time their aim was to discover and identify the wide range of extra cellular proteins that are produced by the some of the LAB when these bacteria are subjected to microbial stressors. The LAB that were tested were those found in the honey-stomach of the honey bee in 2008. 10 of the 13 bacteria were identified as producing extracellular proteins. Some of the proteins were identified as enzymes, DNA chaperons, S-layer proteins, bacteriocins or lysosomes. Still many remain unknown and with unknown function. The researchers conclude that the different LAB most likely plays important roles in their environment within the honey bee by protecting their host and by participation in the food production of honey. They also conclude that many of these proteins assumable have an antimicrobial function (Butler et al., 2013).

Honey has been tested as a presumptive food additive which could be used for prolonging shelf life of fresh cut fruit salads in a study from 2010. In the experiment samples of fruit was covered by a solution containing 10% of honey and the samples were then incubated at temperatures of 4ºC, 8ºC and 12ºC. After the incubation period PCA was used to do the viable cell count. The experiment showed that honey had a good antimicrobial effect on both mesophilic and psychotrophic bacteria but no real effect on inhibiting the growth of yeast and lactic acid bacteria. It also showed that the temperature affects the growth, with faster growth at the higher temperatures. The authors concluded that honey can be a suitable ingredient to increase shelf life of cut fruit, especially if the temperature is also adjusted (D'Amato et al., 2010).

2.2 The bacteria

2.2.1 *Salmonella* Enteritidis

Belong to the family Enterobacteriaceae. Motile Gram negative rod, up to 3µm in length, facultative anaerobe, mesophilic, a major enteric and systemic pathogen.

The genus *Salmonella* contains more than 2500 serotypes. There are two species; *S. enterica* and *S. bongori*. *S. enterica* has been divided into 6 subspecies. Most species of veterinary importance belong to *S.* enterica subspecies enterica.

Salmonella can infect mammals, birds and reptiles and can be found worldwide. The main route of infection is per os and the way of excretion is by the feces. The bacteria are rather resistant and can survive in damp, shady environment for up to 9 month. It can be found in water, soil on vegetables, and in raw meat.

In the experiment we used *S. enterica* subspecie enterica serotype Enteritidis. It is a pathogen of poultry, humans and many other species. In poultry the infection is often subclinical, in mammals more often it cause clinical disease and in humans it is a common cause of food poisoning. (Quinn et al., 2011).

2.2.2 *Listeria monocytogenes*

Small Gram positive rod, up to 2µm in length, facultative anaerobe, it can grow in a wide range of temperature from 4ºC to 45ºC and tolerate both acidic and alkaline milieu of pH 5,5-9,6. It is mostly considered as being non-pathogenic. The pathogenicity of the bacteria is mostly associated with its replication intracellular in the host.

Listeria can replicate in the environment and is therefore widely spread and common. It spread by direct contact, with feed and water etc.

It is a mostly a hazard for pregnant animals and humans where it cause abortion, and in young as well as immune-compromised individuals where it cause encephalitis (Quinn et al., 2011).

2.2.3 *Echerichia coli*

Together with *Salmonella* it belongs to the family Enterobacteriaceae. It is a Gram negative rod, usually motile, up to 3µm in length, facultative anaerobe, mesophiliic and a major enteric and systemic pathogen.

The species contains many different strains.

E. coli is mostly considered as a normal and important part of the intestinal flora of mammals, colonizing the intestinal tract shortly after birth and persisting throughout life.

Most strains are of low virulence but they can cause opportunistic infections in organs other than the gastrointestinal tract e.g. urinary tract and mammary glands. Virulent strains are not considered as part of the normal flora. They cause different types of enterocolitis. Some entero-haemorrhagic strains are major zoonotic food borne pathogens of humans, causing serious disease (Quinn et al., 2011).

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Honey

The honey that was used in the experiment was a crystallized mixed floral honey. It was from Steninge, Halmstad, situated on the west coast of Sweden. It was collected in the summer of 2012. The honey conforms to the top quality standards set by the Swedish Beekeepers National Association. The parameters that are tested are: purity, consistency, smell and taste, color and water content (must be below 20%) (Sveriges biodlares riksförbund, 2014).

The honey was extracted from the combs using centrifugation. It was never heat treated during the handling by the beekeeper or during the preparation of the samples in the lab but kept at room temperature.

The water content of the sample was measured to 18%.

3.1.2 Bacteria

Three different bacterial strains were used in the experiment. The bacterial strains were maintained on PC agar by subculturing 3 times a month.

- *Salmonella* Enteritidis NCAIM B01216
- *Escherichia coli* NCAIM B01748
- *Listeria monocytogenes* ATCC 19111

3.1.3 Growth medium

As culturing media in the experiment a ½ TSB (Tryptic soy broth) was used.

The broth contained:

- Casein pepton 7,5g
- Soy pepton 1,5g
- d-glucose 1,25g
- NaCl 2,5g
- K_2HPO_4 1,25g
- Distilled water 1000 ml

NaCl pepton was used for suspending the bacteria in when making the serial dilution.
It contained:

- Pepton 1,0 g
- NaCl 8,5 g
- Distilled water 1000 ml

For the plate count PC agar (Plate-count agar) was used.
The agar contained:

- Tryptone 5,0 g
- D-glucose 1,0 g
- Yeast extract 2,5 g
- Agar-agar 14,0 g
- Distilled water 1000 ml

3.1.4 MicroTester

For the measurement a MicroTester system (Micro tester Redox v.2.5.16) using commercial combined redox electrodes over 24 channels was used. The system was developed and patented by the Department of Food Hygiene at Szent István University, Faculty of Veterinary Science together with the Department of Physics and Automatization at Corvinus University, Faculty of Food Science.

The system is composed of a water bath controlled by a thermostat (with an accuracy of ±0,2ºC), 24 test cells of 30 ml test tubes equipped with Schott BlueLine 31 RX redox-electrodes with BNC cables. The measurement unit is connected to an IBM compatible PC via a standard COM port. Windows XP and data collection software developed specially for the test is used.

3.1.5 Other

Cellulose-nitrate membrane filter was used for the sterilization by filtration. Diameter 47mm, pore size 0,45µm, from Labsystem Kft.

3.2 Examination method

The growth of the three different bacteria in the presence of increasing concentration of honey was tested at four different temperatures. The growth of the different bacteria was measured through the change in redox-potential by a MicroTester Redox v.2.5.16. .

3.2.1 The MicroTester

The instrument takes advantage of the typical oxidation-reduction reactions performed by the microorganisms. During the growth and multiplication of the microbes they use biological oxidation to release and gain energy for their growth. During this reaction the microorganisms cause a reduction of their environment due to the production of reducing compounds and the oxygen depletion in the medium.

The changes in the test tubes are continuously measured over all 24 channels and the data is saved. At a certain point, which is easily detected by the instrument, the change in redox potential exceeds the threshold value. This means that the change is significantly bigger than the random changes (e.g. |dE/dt| 0.5 mV/min). This value is the detection criterion. The time it takes to reach this point is called TTD (time to detection). A close relationship is found between the TTD and the logN (initial viable count of the specific bacteria) which is represented by the calibration curve. This means that using the TTD and the calibration curve the microbial count can be estimated.

3.2.2 Reason behind the choice of method:

The use of MicroTester equipment is a simple, cheap and effective way to monitor the growth of bacteria in a liquid media. It gives a precise measurement of the change in redox potential which indicates the point when the bacterial number starts to increase exponentially e.g. the change from lag phase to log phase which is an important parameter when the bacteriostatic effect of a substance is investigated.

The Microtester has not been used for experiments with honey. It is therefore extra interesting to see the results of the experiments and to try to evaluate if it is a good method to use in this kind of experiment.

3.2.3 Preparation of measuring instrument

The 24 electrodes were disinfected in 3% Hydrogen peroxide and then rinsed with 70% Ethanol before inserted aseptically into the test tubes containing ½TSB.

After the experiment was finished the electrodes was rinsed with Sodium hypochlorite 1:10 for 30 min and rinsed in water.

3.2.4 Preparation of samples

Six samples were prepared, five containing honey at the concentration of 1%, 2%, 4%, 6%, 8% and 10% and one negative control not containing honey but containing only ½TSB. The five samples with honey contained 1,2,3,4 and 5 g of honey that were measured on a digital scale with 0,01g sensitivity. The honey samples were then added to sterilized test tubes containing 10 ml 1/2 TSB and the samples were carefully shaken so the honey dissolved. The samples were then sterilized through filtration. 9 ml from each sample were then transferred to the sterilized plastic test-tubes used throughout the rest of the experiment.

The bacterial suspension was prepared by taking one colony of bacteria from the PC agar plate and suspending it into NaCl pepton. A tenfold serial dilution of 5 tubes was made from the bacterial suspension. 1 ml of bacterial suspension from the 3rd tube was then added to the 6 test tubes containing ½TSB and honey.

3.2.5 Performing the measurement

An electrode was inserted aseptically into each test tube. The test tubes were then put in groups of 6 into one of the three water-bath and incubated at 25ºC, 30ºC, 37ºC and 42ºC (except for *Listeria monocytogenes* which was not tested at a temperature of 42ºC). The electrodes were then connected to the measuring device and the redox potential was continually monitored by the computer program for changes. The changes could be continuously monitored in the Figures displayed for each of the test tubes on the monitor.

3.2.6 Determination of initial viable cell concentration and external calibration curve

When the examined microbe is known an external calibration curve can be used by the computer program (see Figure 1). To be able to determine the equation the initial viable concentration of bacteria (N) must be examined. For this the spread plate technique was used. 0.1 ml of bacterial suspension from the 3rd, 4th and 5th tubes of the dilution series was transferred onto PC agar and spread evenly with a glass rod. They were then incubated at 37ºC for 24 hours. After the incubation period the colonies were counted and the initial cell concentration was determined. This data was then put into the computer program monitoring the test. The program then determines the equation of the calibration curve using the information on logN and TTD.

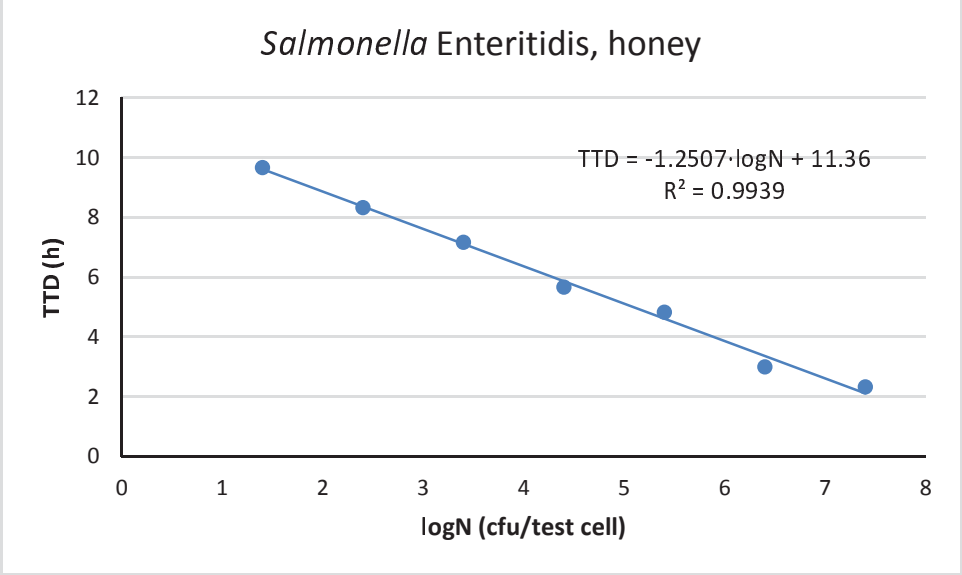

Figure 1 - *Salmonella* Enteritidis – *External calibration curve* (1/2 TSB, T=37 °C)

4. RESULTS

4.1 *Salmonella* Enteritidis

The measured TTD of the redox potential change in the samples of honey and broth, inoculated with *Salmonella* Enteritidis is shown in Table 1. and also in Figure 2.

The initial concentration of bacteria was $2.20 \cdot 10^6$ cfu/ml, logN=6.34

Table 1. – *Salmonella* Enteritidis – TTD

C %	TTD (h)			
	42 °C	37 °C	30 °C	25 °C
0	3	3	4.33	5.67
2	3	2.83	4.33	6.5
4	4	3.83	7.83	8.5
6	6.67	6	8.67	9.5
8	10	8.5	16.5	17.33
10	18.5	11	24	30.33

In almost all samples the TTD became longer as the concentration of honey increased. The only exception was two samples incubated at 42°C where no change was seen between the control and the sample containing 2% honey.

In the control, that did not contain any honey, the TTD was shortest in the samples incubated at 37 °C and 42°C. The time measured was 3 hours. The samples containing honey showed an increased tendency in the TTD as the concentration of honey increased. The samples incubated at 37°C had the shortest TTD compared to other samples of equal concentration of honey. TTD was longest in the samples incubated at 25°C for the control as well as all concentrations of honey.

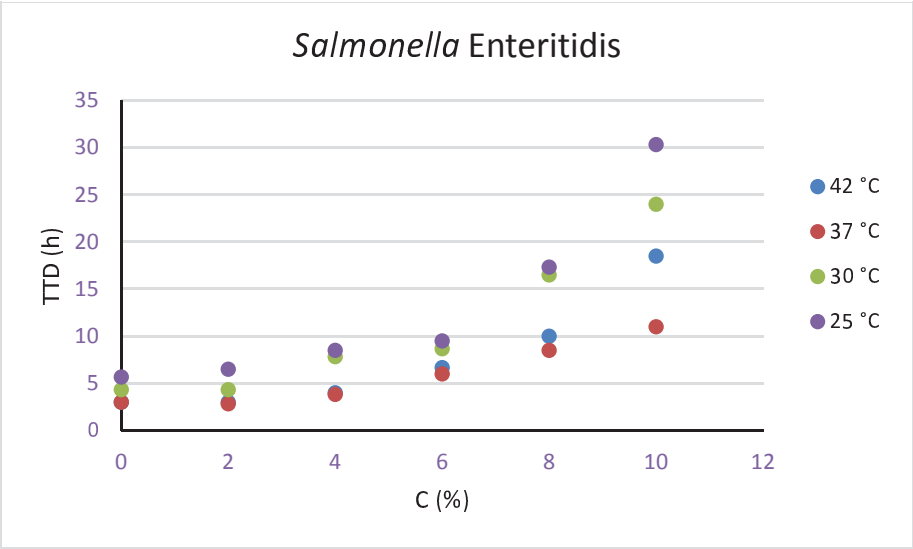

Figure 2. – *Salmonella* Enteritidis – TTD

Comparing the time until detection in the samples incubated at 37 °C with the samples incubated at 25 °C show a non-linear relationship. At 0% honey the TTD in the sample incubated at 25°C is 1.89 times the TTD of the sample incubated at 37°C, at 2% the difference is 2.3 times, at 4% it is 2.22 times, at 6 % it is 1.58, at 8 % it is 2.04 times, and at 10% it is 2.76. The tendency can also be seen in Figure 2 and more clearly in Figure 5.

4.2 *Listeria monocytogenes*

The measured TTD of the redox potential change in the samples of honey and broth, inoculated with *Listeria monocytogenes* is shown in Table 2 and also in Figure 3.

The initial concentration of bacteria was $1.23 \cdot 10^5$ cfu/ml, logN=5.09

Table 2. – *Listeria monocytogenes – TTD*

C %	TTD (h)		
	37 °C	30 °C	25 °C
0	7.5	9.67	12.67
2	8.33	9.5	14.5
4	7	8.5	13.5
6	8.33	9.33	15.83
8	9.39	11.39	22.17
10	15	18.5	28.33

In most samples the TTD became longer as the concentration of honey increased. There were 3 exceptions: two samples incubated at 30°C where a decrease in time was measured between the control and the sample containing 2% honey, two samples incubated at 25 °C where a decrease was measure between 2% and 4% and two samples incubated at 37°C where a decrease was measured between 2% and 4%.

In the control, that did not contain any honey, the TTD was shortest in the samples incubated at 37 °C. The time measured was 7.5 hours. The samples containing honey showed an increased tendency in the TTD as the concentration of honey increased (with exceptions from the samples already mentioned above). The samples incubated at 37°C had the shortest TTD compared to other samples of equal concentration of honey. TTD was longest in the sample incubated at 25°C for the control as well as all concentrations of honey.

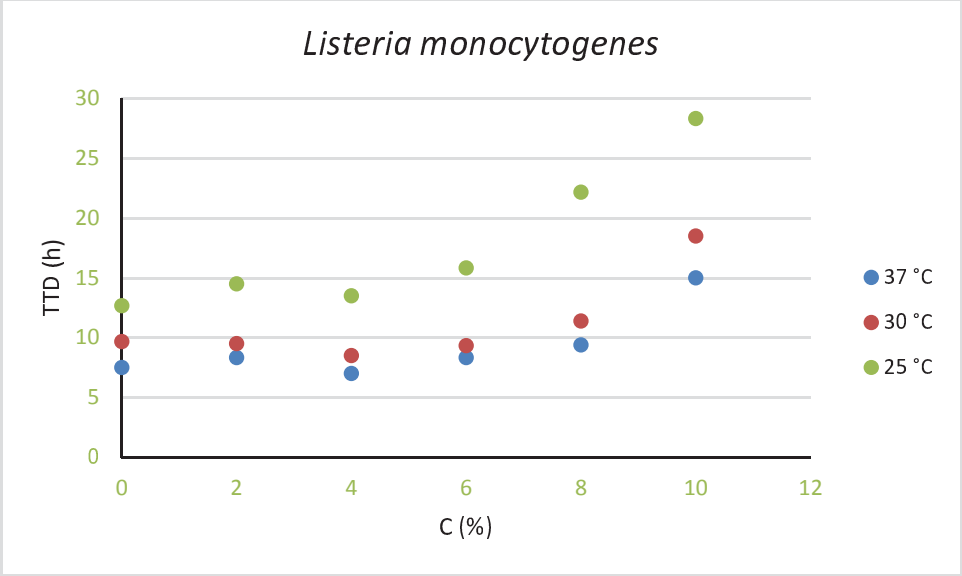

Figure 3. – *Listeria monocytogenes - TTD*

Comparing the TTD in the samples incubated at 37 °C with the samples incubated at 25 °C show a nonlinear difference. At 0% honey the TTD in the sample incubated at 25°C is 1.69 times the TTD of the sample incubated at 37°C, at 2% the difference is 1.74 times, at 4% it is 1.93 times, at 6 % it is 1.9 times, at 8 % it is 2.36 times, and at 10% it is 1.89 times. The tendency can also be seen in Figure 3 and in Figure 6.

4.3 *Escherichia coli*

The measured TTD of the redox potential change in the samples of honey and broth, inoculated with *Escherichia coli* is shown in Table 3 and in Figure 4.

The initial concentration of bacteria was $2.51 \cdot 10^6$ cfu/ml, logN=6.40

Table 3 – Escherichia coli – TTD

C %	TTD (h)			
	42 °C	37 °C	30 °C	25 °C
0	2.83	2.67	5.5	8.67
2	3	2.83	5.5	8.83
4	3.33	3	5.5	9.17
6	4.83	4.5	5.67	10.83
8	6.17	5.17	7.67	12
10	7.5	6.33	9.33	13.67

In almost all samples the TTD became longer as the concentration of honey increased. The exception was three samples incubated at 30°C where no change was seen between the control and the samples containing 2% and 4% of honey.

In the control, that did not contain any honey, the TTD was shortest in the samples incubated at 37 °C. The time measured was 2.67 hours. The samples containing honey showed an increased tendency in the TTD as the concentration of honey increased (with the exceptions from the samples already mentioned above). The samples incubated at 37°C had the shortest TTD compared to other samples of equal concentration of honey. TTD was longest in the sample incubated at 25°C for the control as well as all concentrations of honey.

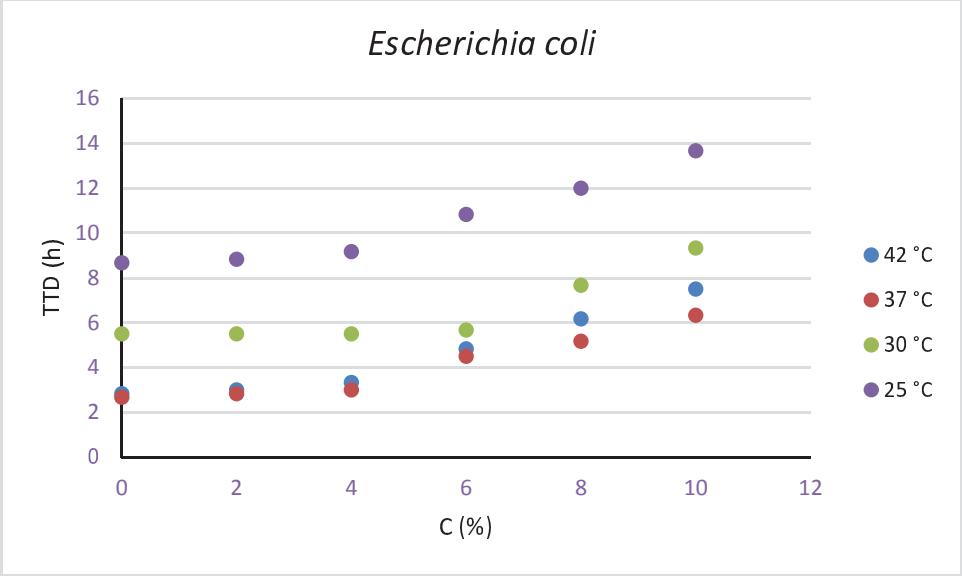

Figure 4. – *Escherichia coli - TTD*

Comparing the time until detection in the samples incubated at 37 °C with the samples incubated at 25 °C show a nonlinear difference. At 0% honey the time until detection in the sample incubated at 25°C is 3.25 times the time until detection of the sample incubated at 37°C, at 2% the difference is 3.12 times, at 4% it is 3.06 times, at 6 % it is 2.41 times, at 8 % it is 2.32 times, and at 10% it is 2.16. The tendency can also be seen in Figure 4 and in Figure 7.

4.4 Inhibitory effect

Considering the high sugar content of the honey, it is important to examine whether the bacteriostatic effect is not the result of the high sugar concentration. Examinations were carried out with *Salmonella* Enteritidis.

1 g honey contains 0.82 g sugar. The detection time of *Salmonella* Enteritidis was determined in 3 different compositions ½ TSB broth:

1. 1 g honey
2. 0.82 g sucrose
3. 0.82 g glucose

The results are summarized in Table 4.

Table 4. Inhibitory effect of honey, sucrose and glucose in ½ TSB (T=37 °C, logN=6.01)

Media	TTD (h)
9 ml 1/2TSB+1 g honey	22.67
9 ml 1/2TSB+0.82 g sucrose	3.67
9 ml 1/2 TSB+0.82 g glucose	4.33
9 ml 1/2 TSB	3.83

The result showed that antimicrobial effect is not the result of the high sugar concentration.

5. DISCUSSION

Honey has been used since ancient times for its taste and medicinal effect. It was used long before the discovery of different microbial pathogens in a light-microscope.

In more recent years the antimicrobial properties of honey has been widely studied. Different antimicrobial factors have been identified like high sugar concentration and low water activity, the enzyme peroxidase oxidase and its production of hydrogen peroxide and a third factor, some proteinaceous compounds, which some researchers now have found to be a unique flora of Lactobacillus bacteria and their products from the honey stomach of the honey-bee.

It is clear that the hydrogen peroxide plays a major role in antimicrobial properties but also that non-hydrogen peroxide compounds, like Bee defensin-1 and methylglyoxal, play an important role. Tested samples of honey have mostly shown bacteriostatic properties but a few samples have had complete inhibitory effect on the growth of some bacteria which indicates bacteriocid effect.

Differences have been discovered in the composition and properties of different honey samples. Much research has been done with this in mind. What they have concluded is that most of the differences can be attributed to the geographically or more recently to the floral origin of the honey.

This experiment showed that with little exceptions the TTD became longer in the presence of honey and that this time increased with increasing concentrations of honey. The exceptions were one example in *Salmonella* where no increase in TTD could be seen between the control sample and the sample containing 2% honey and three occasions in the *Listeria* samples where a slight increase in TTD could be measured between samples of increasing concentrations of honey. These exceptions were only slight deviations from the rest of the results and the overall strong trend was that increasing concentrations of honey increased the TTD. An increase in the TTD indicates that the lag phase was longer, meaning that the growth of the three different bacteria was slower in the presence of honey.

A small difference can be seen in the growth rate between the control samples without honey and the samples containing 2% honey. In both the samples of *Listeria* and the *Eschericia coli* incubated at 37ºC the growth took about 1.1 times longer in the sample containing honey. The samples containing *Salmonella* instead showed the opposite; a small increase in the growth rate in the sample containing 2% honey compared to the control. In the sample of *Salmonella* containing 4% honey and incubated at 37ºC the growth took 1.28 times longer than in the control.

When comparing the control samples with the samples containing 10% honey and incubated at 37 ºC the difference is much bigger. For *Salmonella* the growth took 3.67 times longer in 10% honey than in the control, for *Listeria* it was 2 times longer and for *Escherichia coli* it was 2.37 times longer.

In the samples incubated at 25 ºC the difference is even larger for two of the bacteria. The growth of *Salmonella* took 5.35 times longer at 10% honey than in the control, and for *Listeria* growth took 2.24 times longer. For *Escherichia* growth took 1.58 times longer meaning that the difference in growth-rate at the different concentrations of honey was smaller at this temperature than at 37 ºC.

The temperature clearly had an impact on the growth. The three different bacteria showed preferences regarding the growth at the different temperatures. All three showed the fastest growth when they were incubated at 37ºC. A change (increasing or decreasing) away from this temperature decreased the growth rate. The slowest growth could be seen in the samples incubated at 25 ºC.

When comparing the samples containing Salmonella incubated at 37 ° with the samples incubated at 25 °C a change in the relationship could be seen when comparing samples containing the same concentration of honey at the different temperatures (see Figure 5). A slight tendency could be seen that the difference was increasing as the concentration became higher. This may indicate that the concentration of honey accompanied by an alteration in temperature, away from the optimum for the bacteria, had a synergistic effect in inhibiting growth.

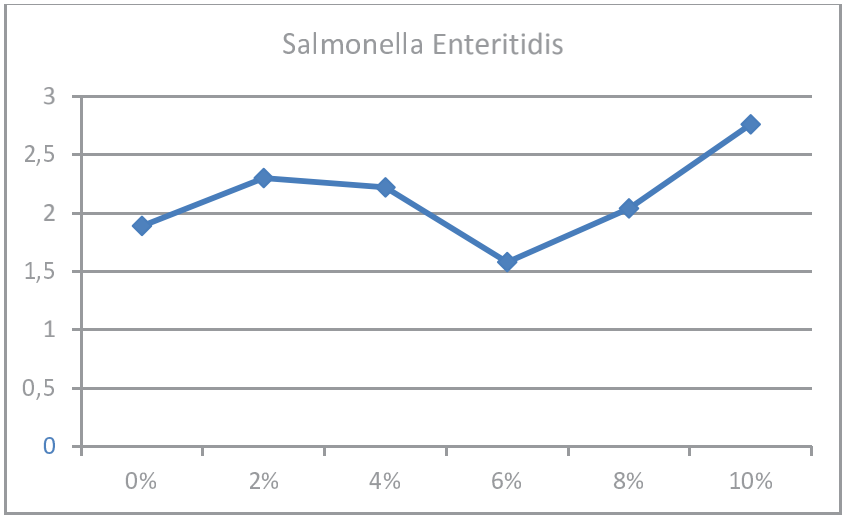

Figure 5. *Salmonell*a Enteritidis – difference in TTD of samples incubated at 25°C compared to samples incubated at 37°C

Also in the tests with *Listeria* a slight increasing tendency can be seen (see Figure 6). Except from the samples with the highest concentration of honey (10%) the difference between 25°C and 37°C is increase as the concentration of honey increase.

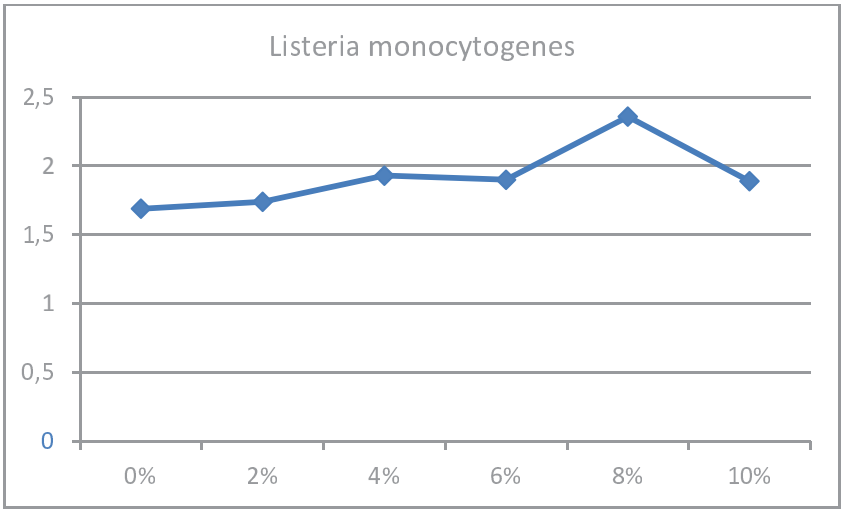

Figure 6. *Listeria monocytogenes* – difference in TTD of samples incubated at 25°C compared to samples incubated at 37°C

Interestingly the test with *Echerichia coli* did not react the same way as the others when making the same comparison between the samples incubated at 25°C and 37°C. The tendency is instead clearly declining (see Figure 7). The difference in TTD gets smaller as the concentration of honey is increasing. Why the same synergistic effect cannot be seen in this test but rather the opposite calls for more research.

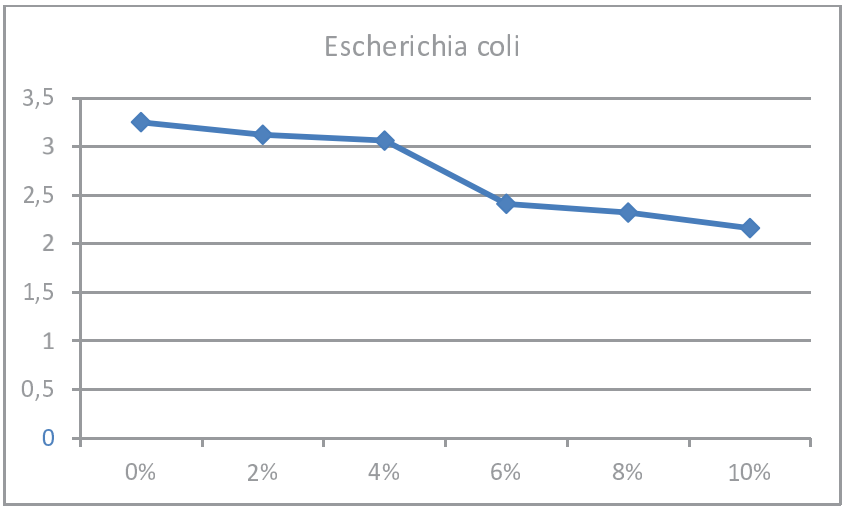

Figure 7. *Escherichia coli* – difference in TTD of samples incubated at 25°C compared to samples incubated at 37°C

This study verified what many other experiments previously have shown that samples of honey mostly have a bacteriostatic effect on the growth of bacteria. Some experiments have found bacteriocidal activities in a few samples of honey but the honey used in this experiment did not show any signs of those characteristics.

The test also confirmed honey as a good candidate to use in food preservation. Previous tests have shown that honey can delay growth of food-borne pathogens and spoilage microbes and thereby prolong shelf-life in fresh cut fruit. The three bacteria in this test are common causes of food-poisoning. The honey clearly delayed the growth of all three bacteria which indicates that it could be used in food preservation to prevent bacteria from reaching amounts high enough to cause food-poisoning.

Many different methods have been used to test the antimicrobial effect of honey. What is especially interesting with this experiment is that the antimicrobial properties of honey have never been tested through redox-potential measurement before. The method is easier and cheaper than many other tests and less time-consuming. The test results are also obtained faster than in many other tests.

The result of the study is very positive and points towards that the micro-tester is a good way to examine the antimicrobial effect of honey in a liquid media. However more tests are needed to confirm the results and verify the accuracy of the method. Especially since it is a rather new technology and the test has never been performed with honey previously. Simultaneous testing of samples of honey and bacteria by the micro-tester and some other method, used frequently in past experiments, would be an interesting continuation of the experiment and useful in the determination on the accuracy of the test-method when it is used with this kind of samples.

Testing the effect of honey at much lower temperatures would also be interesting and give further information on the use of honey as a food preservative that could be used as an additive in the food-industry. It would be especially useful since most research concerning honey is done around room temperature and body temperature.

The aim of the study was to determine if the honey slowed down the growth of the three pathogens and if changing the temperature had a synergistic effect on the growth inhibition. The growth was clearly slower in the presence of honey and the temperature seemed to have a mild synergistic effect on the growth of *Salmonella* Enteritidis and *Listeria monocytogenes* but not on *Escherichia coli.*

6. SUMMARY

In recent years much research has been done on honey especially regarding antimicrobial and antioxidant properties. Many experiments have been performed comparing honey of different floral as well as geographical origin. Differences in physic-chemical properties, antimicrobial properties as well as the content of antioxidants have been observed. Many of the differences have been accredited to the geographical area or the specific floral origin of the honey.

In this study we examined the effect of honey on the growth of *Salmonella* Enteritidis, *Listeria monocytogenes* and *Escherichia coli*. The bacterial growth was detected by the help of MicroTester equipment. The change of the redox-potential was measured at increasing concentrations of honey (0%, 2%, 4%, 6%, 8% and 10%) and incubated at different temperatures (25 °, 30 °C, 37 °C and 42 °C).

The result showed a decrease in the growth rate of *Salmonella* Enteritidis, *Listeria monocytogenes* and *Escherichia coli* when honey was added to the samples. Higher concentrations of honey had more inhibitory effect and change in the temperature away from the preferred also showed inhibitory effect of the growth. The concentration of honey and the temperature seemed to have a synergistic effect in the samples with *Salmonella* and *Listeria monocytogenes* but not in the samples with *E. coli* where the opposite could be seen.

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