



Minimal inhibitory concentrations (MICs) of Chilean honey against infectious bacteria

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Contents

	Preface	4
	Summary	5
1	Introduction	6
2	Materials and Methods	7
	2.1 Pre-test	7
	2.2 MIC dilution assay	7
	2.3 Experimental setup	9
3	Results and discussion	12
	3.1 Minimal Inhibitory Concentrations (MICs)	12
	3.2 Antimicrobial susceptibility	13
	3.3 Discussion	13
4	Conclusions	14

Preface

On June 11th 2020, Paulina Cáceres of the Chilean Consorcio Apícola and Juan Eduardo Henríquez of Apicoop had a discussion with Dennis Schoonman and Hermien van Bokhorst-van de Veen of Wageningen Food & Biobased Research. Paulina and Juan were looking for an institution of international prestige which could be able to make Minimum Inhibitory Concentration tests for Chilean honeys against several bacteria. As they further explained, the main purpose of these tests is to support the antibacterial activity of some Chilean honeys when they arrive to the Chinese market. In the next stage, Apicoop would like to discover the compound, if any, in the Chilean honeys related to their antibacterial activity.

Summary

The minimal inhibitory concentrations (MICs) of 12 different Chilean honey types, provided by Apicoop, were determined against six infectious bacterial species.

Of the standard methods (CLSI standard), the broth dilution assay was applied; using optical density at 595 nm (OD_{595}), bacterial growth was measured in the presence of honey (45.5, 20.7, 9.4, 4.3 and 1.9% [w/w]) after overnight incubation at 35 °C. The observed MICs that inhibited bacterial growth ranged between 1.9 and 45.5% honey.

The most effective honey types, with a detected MIC of 1.9%, were:

- Honey 10-12/PT FC20 and 10-12/PT TC20 against *Acetobacter baumannii*
- Honey 10-12ME0024/53 against *Staphylococcus aureus*

Pseudomonas aeruginosa, *Helicobacter pylori* and *Streptococcus pyogenes* were more resistant against honey, since the majority of the determined MICs were at 9.4 or 20.7% (depending on the honey type).

MICs for *Cutibacterium acnes* (previously known as *Propionibacterium acnes*) could not be determined. This was due to the relatively slow growth of the microorganism in the assay, combined with anaerobic atmosphere conditions and outgrowth of microorganisms naturally present in honey (presumably anaerobic spores) under these conditions.

Notably, the detected bacterial growth inhibition can be, at least partly, caused by a decreased water activity due to the binding of water by honey (due to high sugar content). Further research can be performed on elucidation of the growth inhibiting compound in the honey types.

1 Introduction

This report describes the results of the project on investigating the minimal inhibitory concentration (MIC) of 12 Chilean honey types against six infectious bacterial species. MIC is the lowest concentration of the antimicrobial agent that prevents growth of a microorganism. On request of Apicoop (the client) WFBR tested, as independent research institute, 12 Chilean honey types (Figure 1) and 6 infectious bacterial species, specifically:

- *Streptococcus pyogenes*
- *Helicobacter pylori*
- *Acinetobacter baumannii*
- *Staphylococcus aureus*
- *Pseudomonas aeruginosa*
- *Cutibacterium acnes* (previously known as *Propionibacterium acnes*)

MICs can be determined via several methods. One of the standard methods (CLSI standard) is the broth dilution assay which is a relatively fast high throughput screening assay, for which only a small amount of honey is needed. With this method, growth of a bacteria is detected as a change in turbidity of the media measured via optical density (OD). However, in case the turbidity of a honey type is too high, this broth dilution method will not be applicable. Therefore, WFBR performed a pre-test with one bacterial species and two honey types at the start of the project. The pre-test showed that even at the highest included honey concentration (45.5%), bacterial growth/no growth could be determined using a spectrophotometer (in the case of bacterial growth, the optical density [i.e. turbidity] increases and this can be measured using a plate reader). Based on this, the broth dilution assay was used for the study.

Six bacterial types were inoculated into a liquid growth medium in the presence of different concentrations of de-crystallized honey. Growth was recorded and standardized using a plate reader in micro-dilution using 96-well microtiter plates. As agreed with Apicoop, the tested honey concentrations were 45.5%, 20.7%, 9.4%, 4.3%, and 1.9% with single measurements.

The work performed is a screening of 12 honey types to examine the MIC against several bacterial species. No efforts were performed to investigate a potentially bactericidal effect (i.e. bacterial inactivation) of the honeys nor to the mechanism of inhibition.

With this report, Apicoop can continue to investigate if a specific honey compound is responsible for the antimicrobial properties.



Figure 1 Twelve Chilean honey types.

2 Materials and Methods

Chapter 2 describes the used materials and methods of the pre-test (section 2.1), MIC dilution assay (section 2.2) and experimental setup (section 2.3).

Prior to all experiments, all honey types were de-crystallized by incubation in a water bath for 60 min at 60 °C (Figure 2 and Figure 3).



Figure 2 De-crystallization of the honeys in a water bath.



Figure 3 Honey before (left) and after (right) de-crystallization.

2.1 Pre-test

To check if a broth dilution MIC assay can be used, a pre-test was conducted with *P. aeruginosa* ATCC 9027 and two honey types: 10-12/PT TA20 (with a relatively dark color) and 10-12ME0021/10 (with a relatively light color). The applied method for the MIC test is based on the "Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard; Seventh Edition" standard of the Clinical and Laboratory Standards Institute (CLSI). This is described in section 2.2.

2.2 MIC dilution assay

Bacteria were cultured on Mueller-Hinton agar (MHA; MH of Merck 1.10293 with 1.3% agar) for 24 h at 35 °C. Three to five single colonies were transferred to 5 mL Mueller-Hinton broth (MHB), incubated for 2-3 h at 150 rpm at 35 °C until optical density at 600 nm (OD_{600}) reached an equivalent of a 0.5 McFarland standard (using a Genesys 20 Spectrofotometer). Ten μ L of this culture was added to a well of a 96-wells plate together with 190 μ L MHB containing the honey. Final honey concentrations were

45.5, 20.7, 9.4, 4.3, and 1.9% (w/w). Positive controls (with media and bacterium), blanks (with only media, or media and honey in aforementioned concentrations) and an antibiotic range (with Cefotaxime [Sigma C7912] in final concentrations of 2, 4, 8, 16, 32 and 64 µg/mL) were included. The controls with antibiotic were used to check the general susceptibility of the tested strains. Filled plates were sealed with a breathable thin film (4ti-0518/ST; 4titude, Surrey, UK; which is gas permeable and acts as a moisture barrier seal as well) and incubated in a VWR Incubating Mini Shaker incubator for 18 h at 150 rpm at 35 °C. Growth/no growth of the bacterial culture was determined with a spectrophotometer at 595 nm (using a Tecan Infinite 200 PRO). Growth boundary was set at an OD₅₉₅ increase of more than 0.2. This means that if the measured OD was higher than 0.2 compared to the corresponding blank well (with the same amount of honey), the bacteria is able to grow. See Figure 4 for two 96-wells plates after incubation and Figure 5 for the spectrophotometer.

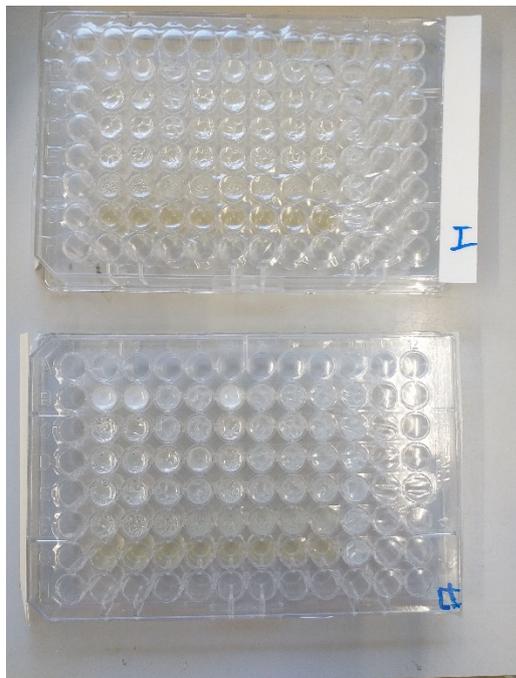


Figure 4 *Ninety six-wells plates (topview) after incubation in the spectrophotometer.*

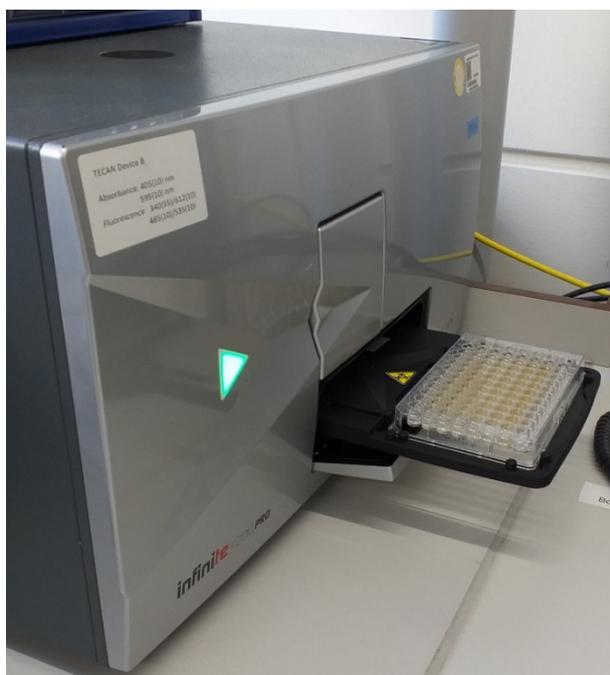


Figure 5 *Spectrophotometer (Tecan) with a 96-wells plate ready to be inserted.*

2.3 Experimental setup

MIC dilution assays with the 12 honey types (Figure 6) and 6 bacteria were performed as described in section 2.2. Growth conditions and medium of each microorganism are listed in Table 1. If needed (see Table 1), a microaerophilic atmosphere was created in anaerobic jars with Anaerocult-C pouches (Merck 116275) and an anaerobic atmosphere was created using Anaerocult-A pouches (Merck 113829) and the 96-wells were closed with their normal lid (i.e. without special seal as used for aerobic conditions as described in section 2.2). When applicable (see Table 1), horse blood was defibrinated, aseptically collected (Biotrading) and lysed. Antibiotic controls were Cefotaxime (sodium salt; Sigma C7912) or Amoxicillin (Sigma A8523) and concentrations were based on the European antibiotic database (<https://mic.eucast.org/Eucast2/>). In case blood was present, which affects the turbidity, the growth boundary was set at an OD₅₉₅ increase of more than 0.05 for *H. pylori* or 0.1 for *S. pyogenes*.

The experimental setup is as shown below (Figure 7) and an example of a series of 96-wells plates is shown in Figure 8.



Figure 6 The 12 labelled Chilean honey types received from Apicoop.

Table 1 Bacteria, culture conditions and MIC assay conditions.

Bacteria (strain)	Atmosphere	Media for culture	Culture conditions and media during MIC assay (in 96-wells plates)
<i>Streptococcus pyogenes</i> (DSM11728/ATCC19615)	Microaerophilic	CA-MH* agar + 5% lysed horse blood	CA-MH* broth + 5% lysed horse blood (35 °C for 24 h)
<i>Acinetobacter baumannii</i> (DSM 30007/ATCC19606)	Aerobic	Mueller-Hinton agar	Mueller-Hinton broth (35 °C for 18 h)
<i>Helicobacter pylori</i> (DSM21031/ATCC43504)	Microaerophilic	Mueller-Hinton agar + 5% lysed horse blood	Mueller-Hinton broth + 5% lysed horse blood (35 °C for 24 h)
<i>Cutibacterium acnes</i> ; previously know as <i>Propionibacterium acnes</i> (DSM1897/ATCC6919)	Anaerobic	Tryptone soy agar	Brain Heart Infusion + 1% cysteine (35 °C for 48-72 h)** Tryptone soya broth (35 °C for up to 72 h)
<i>Staphylococcus aureus</i> (ATCC6538)	Aerobic	Mueller-Hinton agar	Mueller-Hinton broth + NaCl (2% w/v; 0.34 mol/L) (35 °C for 24 h)
<i>Pseudomonas aeruginosa</i> (ATCC9027)	Aerobic	Mueller-Hinton agar	Mueller-Hinton broth (35 °C for 18 h)

*CA-MH = Cation-adjusted Mueller-Hinton (broth or agar) = Mueller-Hinton with final concentrations: 20 to 25 mg of Ca⁺⁺/L and 10 to 12.5 mg of Mg⁺⁺/L.

**According to Blaskovich *et al.* 2019, <https://doi.org/10.1038/s41598-019-50746-4>; the *C. acnes* strain included in this study did not grow under the tested conditions. Testing several other growth media showed that the strain was able to grow on tryptone soya broth (TSB). Therefore TSB was included in the MIC assay.

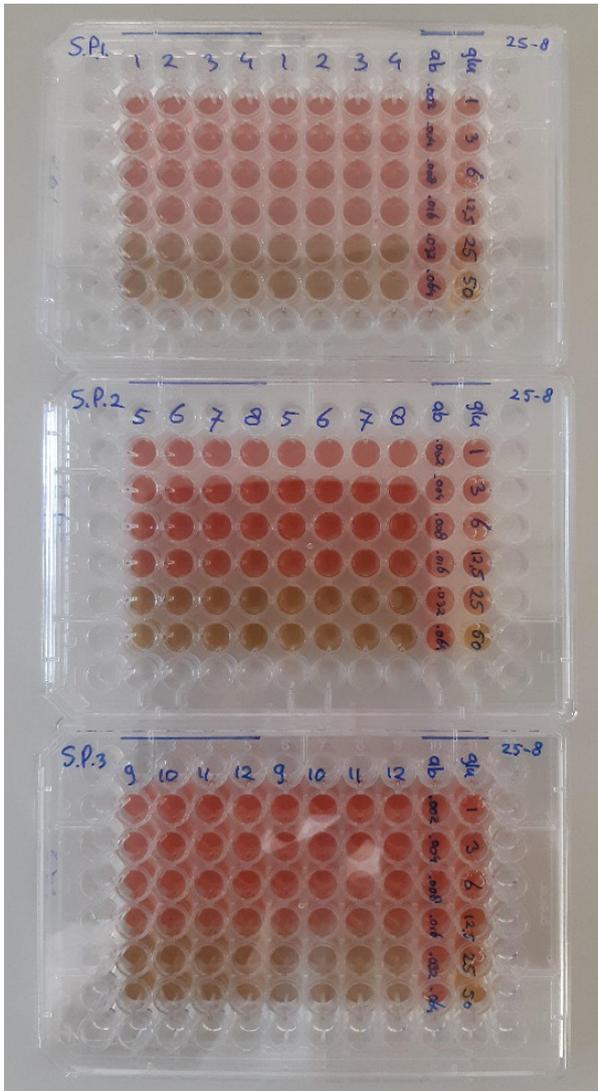


Figure 8 Example of a series of 96-wells plates containing media plus honey, prepared for each bacterial species (*Streptococcus pyogenes* in this example).

3 Results and discussion

3.1 Minimal Inhibitory Concentrations (MICs)

The MICs determined were in the range of 1.9 to 45.5% honey. The measured MICs (the lowest percentages of honey that inhibited bacterial growth) are listed in Table 2.

Most effective honey types against *A. baumannii* were honey 10-12/PT FC20 (number 3) and 10-12/PT TC20 (number 9). They had an MIC of 1.9%, all other honey types had an MIC of 9.4 or 20.7%.

Against *S. aureus*, honey 10-12ME0024/53 (number 8) was effective at 1.9%, most others honey types were effective at 9.4%.

P. aeruginosa was more resistant against honey, since the determined MICs were at 9.4 and 20.7% and this also accounts for *H. pylori* and *S. pyogenes*.

The determined MICs of honey 10-12ME0024/50 (number 7) were consistently 9.4% and had, overall, the lowest MIC detected when taking all bacterial species into account.

Table 2 MICs (%) of the 12 honey types against the tested bacteria.

Honey # and code \ Bacteria	<i>A. baumannii</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>H. pylori</i>	<i>S. pyogenes</i>	<i>C. acnes</i>
1 10-12/PT TE20	20.7	4.3	20.7	20.7*	9.4	NC
2 10-12/PT FA20	20.7	9.4	9.4	20.7*	20.7*	NC
3 10-12/PT FC20	1.9	9.4	4.3	20.7*	20.7*	NC
4 10-12ME0028/01	9.4	9.4	9.4	20.7	9.4	NC
5 10-12ME0021/10	20.7	20.7	20.7	NC	9.4	NC
6 10-12ME0003/04	9.4	9.4	20.7	NC	20.7	NC
7 10-12ME0024/50	9.4	9.4	9.4	NC	9.4	NC
8 10-12ME0024/53	9.4	1.9	9.4	NC	45.5*	NC
9 10-12/PT TC20	1.9	9.4	20.7	9.4	20.7	NC
10 10-12/PT TM20	9.4	4.3	9.4	20.7*	45.5*	NC
11 10-12/PT TA20	9.4	8.4	20.7	20.7*	45.5*	NC
12 10-12ME0010/B	20.7	20.7	9.4	20.7*	20.7*	NC

NC = non-conclusive; due to presence of presumably (semi-)anaerobic bacterial spores in the honey combined with the anaerobic or microaerophilic atmosphere and the relatively slow bacterial growth rates, these MICs could not be determined.

* = MIC might be lower; due to lysed blood in the media and presence of presumably (semi-)anaerobic bacterial spores in the honey, turbidity of some of the inoculated and uninoculated wells was too high.

Due to presence of microorganisms that are naturally present in the honey (presumably anaerobic bacterial spores) combined with the anaerobic atmosphere and the relatively slow growth of *C. acnes*, these MICs could not be determined.

Next to *C. acnes*, for *H. pylori* and *S. pyogenes* some test results were inconclusive. The reason for this is that these two species were tested in medium containing lysed blood (this is required for their growth). Due to the darkness of this medium, but also microaerophilic atmosphere, relatively slow growth rate, and bacteria naturally present in the honey types, the growth inhibition for some honey types could not be determined conclusively and therefore no MIC value could be given in these tests. All other determined *H. pylori* and *S. pyogenes* MICs should be interpreted carefully: actual MICs might be lower.

3.2 Antimicrobial susceptibility

The control wells with antibiotics showed bacterial inhibition of growth. Therefore, the bacterial strains included in this study did not acquire an increased resistance mechanism against the included antibiotics (cefotaxime and amoxicillin; data not shown). See <https://mic.eucast.org/Eucast2/> for further information on MIC distributions for individual microorganisms and antimicrobial agents. As for the antimicrobials, the teste honey types inhibits bacterial growth.

3.3 Discussion

In this project, it was determined that bacterial growth was inhibited in the presence of Chilean honey. Several reasons can explain this observation. It might be that a specific compound in the honey types contributes to this inhibition. An important addition is that honey consists for a large part of sugar (glucose, fructose). Therefore, honey is able to bind water and consequently it lowers the water activity of the solution it is in. In this case, the bound water is not available for bacterial growth. So, this water activity lowering capacity of honey might be, at least partly, causing the bacterial growth inhibition of the honey types.

Further research can be performed on elucidation of the growth inhibiting compound(s) in the honey types. To gain insight in the influence of the water activity on the MIC values due to the sugar of the honey, the water activity of the different honeys can be measured and compared with the MIC capacity of a sugar solution with the same water activity as the honeys.

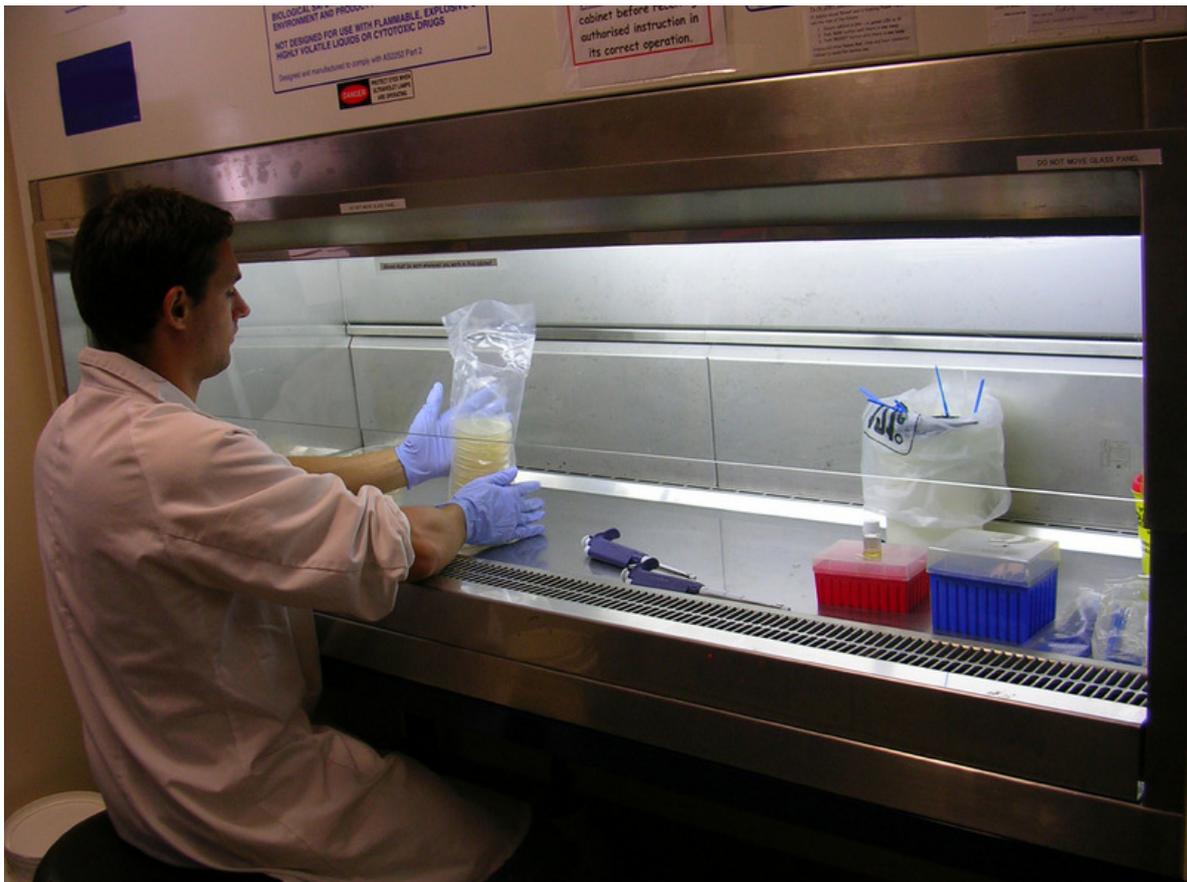


Figure 9 A scientist working in the microbiology lab.

4 Conclusions

Using optical density at 595 nm (OD_{595}), bacterial growth was determined and the observed MICs of the 12 Chilean honey types against six bacterial species were between 1.9 and 45.5%.

Most effective honey types detected, with a MIC of 1.9%, were:

- Honey 10-12/PT FC20 and 10-12/PT TC20 against *Acetobacter baumannii*
- Honey 10-12ME0024/53 against *Staphylococcus aureus*

Overall, when taking all bacterial species into account, the determined MICs of honey 10-12ME0024/50 had the lowest detected MIC (9.4%).

Pseudomonas aeruginosa, *Helicobacter pylori* and *Streptococcus pyogenes* appear to be more resistant against honey, since their determined MICs are higher (overall $\geq 9.4\%$). The determined MICs of *H. pylori* and *S. pyogenes* should be interpreted carefully (it might be that actual MIC's are lower than reported in this report).

Due to the presence of microorganisms that are naturally present in the honey (presumably anaerobic bacterial spores), no MICs could be determined in this project for *Cutibacterium acnes*.

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