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Evaluating alternative antibiotics for control of European Foulbrood disease

A report for the Rural Industries Research and Development Corporation

by
Dr Stephen Doughty
Dr Joanne Luck
Mr Russell Goodman

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Researcher Contact Details
Dr Stephen Doughty
PIRVic Knoxfield
Department of Primary Industries
Private Bag 15
Ferntree Gully Delivery Centre
Victoria 3156

Phone: (03) 9210 9222
Fax: (03) 9800 3521
Email: stephen.doughty@dpi.vic.gov.au

In submitting this report, the researcher has agreed to RIRDC publishing this material in its edited form.

RIRDC Contact Details
Rural Industries Research and Development Corporation
Level 1, AMA House
42 Macquarie Street
BARTON ACT 2600
PO Box 4776
KINGSTON ACT 2604

Phone: 02 6272 4819
Fax: 02 6272 5877
Email: rirdc@rirdc.gov.au.
Website: http://www.rirdc.gov.au

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Foreword

European Foulbrood disease of honeybees is an endemic disease in the commercial apiaries of South-eastern Australia. The current treatment method is to apply the antibiotic oxytetracycline.

This antibiotic can result in long lasting antibiotic residues of honey produced and stored in treated hives. Detection of oxytetracycline contamination in Australian honey by our export partners would have serious consequences for the Australian apiary industry.

The objectives of this project were to identify an alternative antibiotic for the control of European Foulbrood. The selection criteria were that a successful alternative antibiotic should be effective against European Foulbrood, it should degrade quickly and leave no residue in honey.

This project identified six candidate antibiotics that were effective against the causative bacteria of European Foulbrood. Following analysis of these antibiotics, ampicillin and amoxicillin were selected as promising alternatives to oxytetracycline.

This project was funded from honeybee industry revenue, matched by funds provided by the Australian Government, with additional funds provided by the Victoria State Government (Department of Primary Industries).

This report, an addition to RIRDC's diverse range of over 1000 research publications, forms part of our Honeybee R&D program, which aims to improve the productivity and profitability of the Australian beekeeping industry.

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Simon Hearn
Managing Director
Rural Industries Research and Development Corporation

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Abbreviations

AFB	American Foulbrood
Amox	Amoxicillin
Amp	Ampicillin
APVMA	Australian Pesticides and Veterinary Medicines Authority
Bac	Bacitracin
BLD	Basic Larval Diet
D	Dead
DDA	Disc Diffusion Assay
DNA	Deoxyribose Nucleic Acid
dNTPs	DNA Tri-Phosphates
EFB	European Foulbrood
ELISA	Enzyme Linked Immuno-Sorbent Assay
EMAI	Elizabeth Macarthur Agricultural Institute
EU	European Union
g	Gravity
gm	Gram
Gram	Gramicidin
HPLC	High Performance Liquid Chromatography
kPa	Kilo-Pascal
L	Live
LGS	Larval Gut Smear
lt	Litre
M	Molar
µg	Micro-Gram
mg	Milli-Gram
MIC	Minimum Inhibitory Concentration
MID	Minimum Inhibitory Dilution
ml	Milli-Litre
µl	Micro-Litre
mm	Milli-Metre
MRL	Maximum Residue Limit
MSDS	Material Safety Data Sheet
nm	Nano-Metre
NSW	New South Wales
^{O}C	Degrees Celsius
OD	Optical Density
OTC	Oxytetracycline
PCR	Polymerase Chain Reaction
pmol	Pico-Moles
ppb	Parts Per Billion
rpm	Revolutions Per Minute
rRNA	Ribosomal Ribose Nucleic Acid
sec	Seconds
Tri/Trimeth	Trimethoprim
v/v	Volume per Volume
VIC	Victoria

Contents

Executive Summary

European Foulbrood disease of honeybees is an endemic disease in the commercial apiaries of South-eastern Australia. The current treatment method is to apply the antibiotic oxytetracycline. This antibiotic can result in long lasting antibiotic residues of honey produced and stored in treated hives. Detection of oxytetracycline contamination in Australian honey by our export partners would have serious consequences for the Australian apiary industry.

Antibiotics were selected from all antibiotics registered for use by the Australian Pesticides and Veterinary Medicines Authority. From this listing, antibiotic types with rapid breakdown rates, which were likely to be effective against *Melissococcus pluton*, were identified. Candidate antibiotics from these types were obtained for laboratory testing.

Antibiotic	**Type**
Ampicillin	ß-lactam
Amoxicillin	ß-lactam
Trimethoprim	Enzyme inhibitor
Gramicidin	Antimicrobial peptide
Nisin	Antimicrobial peptide
Monensin	Ionophore
Oxytetracycline[1]	Tetracycline

[1] - current treatment, included as a control antibiotic.

The selected antibiotics were tested for their effectiveness in stopping *M. pluton* growth in laboratory culture. Initial experimentation used a disc diffusion plate assay to examine the efficacy of the selected antibiotics against *M. pluton*. Subsequent assays were performed using a liquid culture 'minimum inhibitory concentration' assay. This work identified ampicillin as highly effective against *M. pluton*, with several other being equally effective as the current treatment; oxytetracycline.

To ensure that application of the candidate antibiotics ampicillin and amoxicillin was not detrimental to the growth of *Apis mellifera* larvae, *in-vitro* larvae rearing experiments were performed. Honeybee larvae extracted from an EFB free hive were grown in the laboratory. They were fed a synthetic larval diet supplemented with selected antibiotics, including the current treatment; oxytetracycline. The addition of these antibiotics to the larval diet did not appear to affect larval viability

The comparative rate of degradation, of the candidate antibiotics, in honey was assessed using a bio-assay on antibiotic spiked honey samples. Most of the tested antibiotics showed little breakdown over a 21 day period, except for ampicillin and amoxicillin. Long term testing of the ß-lactam antibiotics indicated they degrade rapidly, with their effectiveness reducing to background honeys levels over a 40 to 65 day period, when stored at 35 ^{o}C.

This project has identified two suitable candidate antibiotics; ampicillin and amoxicillin. They fulfil the selection criteria set in the project objectives, as they are both highly effective at controlling *M. pluton in-vitro* and they are rapidly degraded in honey. Further work should be performed to examine their effectiveness at controlling European Foulbrood in an infected hive situation.

1 Introduction and objectives

1.1 Review of Literature

1.1.1 Introduction

The two main bacterial pathogens of commercial honeybees (*Apis mellifera*) are; American foulbrood and European foulbrood:

American foulbrood (AFB) is caused by *Paenibacillus larvae* subsp. l*arvae*. It is a notifiable disease in mainland Australian states and is primarily controlled by colony destruction.

European foulbrood (EFB) is caused by *Melissococcus pluton*, it is endemic in all eastern Australian states and is currently controlled by antibiotic therapy.

However, the European Union (EU) does not license any antibiotics for the control of brood diseases, thus requiring honey imports to be free of all antibiotic residues (Martin, 2002). Since the EU is a major importer of Australian honey, there is a need to develop alternative therapeutic controls against EFB.

1.1.2 European Foul Brood

1.1.2.1 Introduction

EFB is a seasonal disease caused by *M. pluton* (Shimanuki *et.al*., 1992). Outbreaks generally occur at times of poor nectar flow, such as mid to late spring. This is when the colony is rearing an increased amount of brood under nutrient resource limited conditions. The disease affects the brood (larvae) and can result in low colony populations, causing low (and slow) build up of surplus honey (Morse and Flottum, 1997).

1.1.2.2 Pathology

M. pluton, the primary causative agent of EFB, infects the mid-gut of developing larvae (Bailey, 1960). The source of bacteria is believed to be mainly from contaminated nurse bees or from bacteria left in the cell from previous infections (Bailey and Ball, 1991). The bacteria rapidly multiply in the anaerobic mid-gut of the larvae, invading the peritrophic membrane and the intestinal epithelium (Tarr, 1938). The bacteria starve their host of nutrients, resulting in larval death or following pupation, stunted adult bees.

Larvae weakened by EFB also deposit the bacteria on their cell walls when defecating prior to pupating (Figure 1).

Following initial infection of a larvae with *M. pluton*, several opportunistic pathogens can become secondary invaders, including; *Paenibacillus alvei*, *Enterococcus faecalis* and *Enterococcus faecium* (Bailey and Ball, 1991).

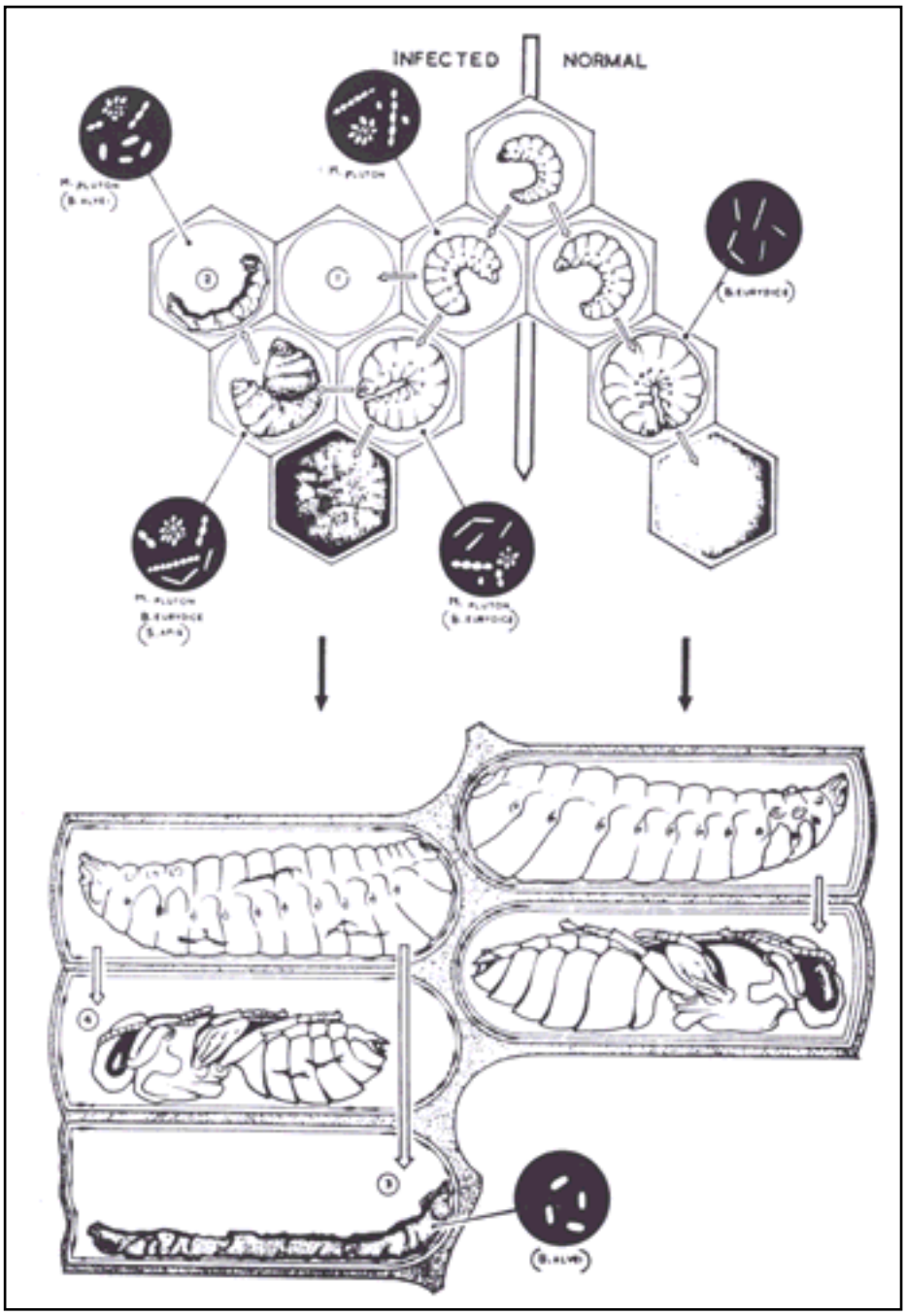


Figure 1: Infection pathway of *M. pluton*. (sourced from Bailey and Ball, 1991)

1) Infected larvae are detected and ejected from the hive.

2) Infected larvae die before they are detected.

3) Infected larvae are capped but the larvae die before pupating.

4) Infected larvae pupate forming undersized adults

Paths 2, 3 & 4 result in *M. pluton* contamination of the cells

1.1.2.3 Detection

The diagnosis of EFB is established by the recognition of clinical symptoms of disease at the hive and the subsequent confirmation of *M. pluton* in diseased material in the laboratory.

Field diagnostics

Larvae with EFB turn yellow/brown in colour, then die twisted against the side or bottom of the cell. In severe cases, the brood combs with exhibit a 'pepperbox' appearance with infected cells interspersed with health brood. The cell caps of infected larvae can become concave or punctured, compared to health brood, which have convex cell caps (Morse and Flottum, 1997).

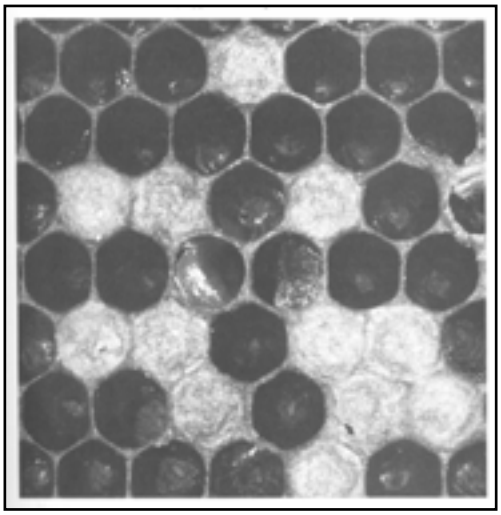


Figure 2 – Typical appearance of a *M. pluton* infected brood comb (Morse and Flottum, 1997)

Laboratory diagnostics

ELISA

Pinnock and Featherstone (1984) were the first to develop an ELISA method for the detection of *M. pluton*. They raised α-*M. pluton* antibodies in rabbits and hens using a rapid course of immunisations with a *M. pluton* bacterin. The antibodies were used at an unrecorded dilution in a sandwich ELISA. The detection limit of this test is approximately 10^5 cells/ml. This allowed Pinnock and Featherstone to identify *M. pluton* in apparently healthy bee colonies.

Microscopic analysis

Microscopic analysis at x1000 magnification of carbol fuschin stained larval smears is a common method used for routine diagnosis of EFB (Hornitzky and Wilson, 1989). Larvae are considered to have EFB when smears contain lanceolate cocci, although the presence of *Enterococcus faecalis* and *Paenibacillus alvei* in putative diseased smears are considered presumptive evidence of EFB (Bailey and Ball, 1991).

Polymerase chain reaction (PCR)

PCR techniques are becoming more prevalent in pathology, as a quick and specific test for the presence of pathogens. Two *M. pluton* specific PCR techniques have been published. Goven *et.al.* (1998) developed a single step PCR using primers specific to *M. pluton* 16s rRNA published sequence. This PCR enabled the amplification of a specific product from pure *M. pluton* culture and crudely lysed larvae. A hemi-nested PCR protocol was developed by Djordjevic *et.al.* (1998). This used a two-step PCR, where the PCR product from the first reaction (specific to *M. pluton* and *E. faecalis*) was used as template in a second, *M. pluton* specific, reaction. This assay was reported to be highly sensitive against culture grown *M. pluton.*

Other techniques including scanning electron microscopy and biochemical tests can also be used for the detection of *M. pluton* in diseased samples (Alippi, 1991).

1.1.2.4 Treatment

The only treatment licensed for use in controlling EFB in Australia is the veterinary drug oxytetracycline (OTC). OTC is a broad-spectrum bacteriostatic antibiotic that affects the 16S and 18S rRNAs of both Gram positive and Gram negative bacteria. OTC resistance has been observed in *Paenibacillus larvae*, however, to date no resistance by *M. pluton* has been published (Waite *et.al.*, 2003), (Hornitzky and Smith, 1999b).

The current control method relies on OTC being ingested by the larvae where it acts on the *M. pluton* present in the larval gut. However, the infection cycle starts with the nurse bee infecting the larvae through contaminated food (Bailey and Ball, 1991) and primarily occurs at times when the larvae are under nutrient stress

Oxytetracycline is an excellent treatment for EFB as ingestion of the antibiotic by nurse bees results in secretion of OTC containing food to the larvae (Hornitzky *et.al*., 1988). However, OTC residues can also be readily found in honey and royal jelly of treated colonies (Matsuka and Nakamura, 1990).

Early work indicated that tetracycline degraded readily in honey in less than 4 weeks (Gochnauer and Bland, 1974). However, more recent work has identified OTC residues in honey up to 6 - 9 weeks following treatment (Matsuka and Nakamura, 1990), (Gilliam *et.al*., 1979), with a residual breakdown product being identifiable indefinitely.

A number of husbandry techniques can be used to minimise the risk of OTC contaminating honey. These include ensuring a plentiful supply of nectar in spring, when the colony is starting to increase in size and the brood may be under nutrient stress.

Since the current detection limit for OTC is approximately 10 – 20 parts per billion (ppb) (Martin, 2002) (possibly down to 1 – 2 ppb (Planken, 2003)), any honey from treated hives can potentially have detectable OTC or OTC breakdown products. If OTC was found in EU imported Australian honey, it could potentially result in the closure of that market to Australian producers. Thus there is a need to either; for the EU to agree on and institute a realistic maximum residue limit (MRL) for OTC in honey, or identify an alternative therapy for the control of EFB.

1.1.3 Alternative Control Measures

Any alternative to OTC ideally needs to fulfil the following requirements:

1 - be effective against *M. pluton, in vitro* and *in vivo*

2 - have a short half-life and degrade to an undetectable residue

3 - leave no detectable residue in honey following treatment

4 - be licensed for use within Australia (and the EU)

5 - require minimal handling by the apiarist during treatment.

1.1.3.1 Conventional antibiotic therapy

Prior to the adoption of OTC as a standard treatment for EFB, a number of antibiotics were tested. These included; erythromycin, streptomycin and penicillin (Wilson and Moffett, 1957). Erythromycin was shown very effective against EFB, while streptomycin and penicillin had limited effectiveness against the disease.

In addition to OTC, several antibiotics have been trialed for the control of AFB in North America, however, they are of limited use against EFB. These include tylosin (Peng *et.al*., 1996) and sulphathiazole (Haseman, 1946).

Recently research had been undertaken, examining the value of ampicillin (Nakajima *et.al*., 1997) and mirosamicin (Nakajima *et.al*., 1998) as chemi-therapeutic agents against AFB. Ampicillin is very effective against *P. larvae in vitro* and was readily inactivated in honey (below detection level in 28 days). However, it tracked poorly into the developing larvae in a colony situation. Unfortunately the experimentation did not include a test infection of the colony, so no disease control data is available. While penicillin was previously found ineffective against EFB, It is well worth examining other beta-lactam antibiotics for activity against *M. pluton*.

Treatment of colonies with mirosamicin, while tracking well into larvae, resulted in long lasting honey residues. Antibiotic levels were still at unacceptable levels 20 days post treatment.

Commonly used veterinary and medical antibiotics can be grouped based on their structural types. Different types have varying half-lives, as listed in table 1. Alternative antibiotics suitable for testing against *M. pluton* should include members of the more readily degrading broad-spectrum β-lactams, and macrolides. These may have shorter half-lives in honey, however, many are currently used in medical applications and so concerns may be raised about increasing bacterial resistance to these antibiotics (van den Bogaard and Stobberingh, 1999)

Other antibiotics of interest are those currently used as animal feed supplements.

Flavomycin: A member of the Flavophospholipol family, it is used worldwide in the dairy, poultry and pork industries (Bolder *et.al*., 1999). Flavomycin is effective against Gram positive bacteria and is widely used in ruminant animals to help gut flora maintenance (van der Merwe *et.al*., 2001).

Monensin: An antibacterial ionophore again which is widely used in ruminant animals and has been shown to be active against bovine strains of *Enterococcus faecium* (Laukova *et.al*., 1995). Testing of monensin in sheep indicated that use of the antibiotic did not appear to induce resistance in susceptible rumen bacterial populations (Rogers *et.al*., 1997).

1.1.3.2 Antimicrobial peptides

Several peptide antibiotics have been used worldwide including the ionophores; nisin, bacitracin, gramicidin, polymyxin b and virginiamycin. Bacitracin and virginiamycin have been banned as animal feed supplements in EU, since they (or analogues) are currently used in human treatments (EU Council Regulation No. 2821/98). Polymyxin b (only effective against Gram negative bacteria) and gramicidin are in medicinal use, however gramicidin is only used in topical applications because of its haemolytic activity (Kondejewski *et.al*., 1999). Nisin is a commonly used food preservative used worldwide, particularly in the cheese industry (Delves-Broughton *et.al*., 1996). Its use is supported and encouraged by the EU (Flair-Flow Europe project no. 387/01/SME3 - Biopreservation of Foods Using Nisin). Therefore, the two peptide antibiotics suitable for testing for anti-*M. pluton* activity are nisin and gramicidin.

1.2 Objectives

The project objectives are:

To determine the potential of antibiotics, other than oxytetracycline (OTC), to control the bacterial honey bee brood disease, European Foulbrood (*Melissococcus pluton*) (EFB).

To identify suitable efficacious antibiotics that quickly degrade and leave no residues in honey extracted from treated hives.

2 Methodology

2.1 Strains

Two *M. pluton* bacterial strains were used during this project (table 2.1).

Table 2.1 *M. pluton* bacterial strains.

M. pluton 96/A727/1	Supplied by Dr M. Hornitzky (EMAI)
M. pluton LGS-A-5	Field isolated strain (Melton, Victoria)

2.2 In-vitro culturing of *M. pluton*

M. pluton was grown in the laboratory using the method described by Bailey (Bailey, 1957) and Hornitzky and Smith (Hornitzky and Smith, 1998a). Broth media was made using the following recipe (table 2.2):

Table 2.2 EFB media components required for 1 lt of media.

Yeast Extract (OXOID L21)	10 gm
D-Glucose (AJAX-APS 783-500g)	10 gm
Starch (AJAX-APS 526-500g)	10 gm
KH_2PO_4 (AJAX-APS 391-500g)	13.6 gm
L-cysteine (SIGMA C-7352)	400 mg
Distilled water	To 900 ml

Once components are dissolved, the pH of the media was adjusted to 6.7 with 5 M KOH (AJAX-APS 405-500g). The media was made up to 1lt with distilled water and autoclaved at 121 ^{o}C (110-115 kPa) for 25 minutes.

For solid media, 15 gm of Technical Agar No. 3 (OXOID L13) was added prior to autoclaving.

Prior to use, Nalidixic acid (Aldrich 15,854-2) was added to final concentration of 3 µg/ml. Nalidixic acid stock (12 mg/ml) was made up in 0.2 M NaOH and filter sterilised.

Standard incubation conditions:

Broth and plate cultures were incubated at 35 ^{o}C in an anaerobic jar, using the anaerogen gas pack system (OXOID AN35). Agar plates were incubated inverted and broth cultures were incubated with the vessel lid loose.

2.2.1 Modification of broth culture to suit microtitre plate testing.

The existing media recipe contains starch, which results in an initial turbidity in the media prior to culturing. Starch was replaced with sucrose (AJAX-APS 530-2KG) at the same concentration. *M. pluton* appeared to grow in the modified media at an enhanced rate compared to starch containing media, at the expense of a small increase in acid production.

Sucrose was used in place of starch for all subsequent solid and liquid media.

2.3 Microscopic analysis and Grams straining

Gram staining was performed for routine examination of culture material.

For bacterial colonies, a sterile loop was used to remove a small amount of bacterial material from the plate. This was mixed with 25 µl of sterile distilled water on a microscope slide and left to dry. The dried culture material was heat fixed by passing through a Bunsen burner flame three times.

For liquid culture material, 250 µl of broth culture was pelleted by centrifugation in a microtube. The bacterial pellet was resuspended in 25 µl of sterile distilled water and spread across the slide. Following drying of the culture it was heat treated as above.

Gram staining was performed using the following steps:

1 – Crystal violet stain 90 seconds.

2 – Water rinse 10 seconds.

3 – Iodine fix 90 seconds.

4 – 95% ethanol rinse 15 seconds.

5 – Safranin stain 90 seconds.

6 – Water rinse 90 seconds.

Following the final rinse, the slides were dried in a 37 ^{o}C incubator, and examined by microscope.

2.4 Polymerase Chain Reaction (PCR) detection of M. pluton

PCR amplification of the *M. pluton* 16s rRNA gene was performed as described by Govan *et.al.* (Govan *et.al*., 1998). Red Hot DNA Polymerase (ABgene AB-0406/a) enzyme and buffers were routinely used.

Template:

Test samples were isolated using the DNeasy Tissue Kit (Qiagen 69504).

Controls used were:

M. pluton genomic DNA positive control.

Enterococcus faecalis genomic DNA negative control.

Nuclease free water negative control.

Primers:

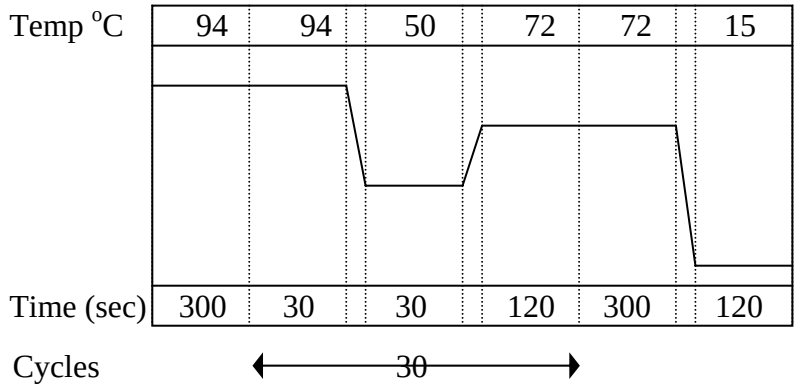
MP16SF: GAAGAGGAGTTAAAAGGCGC

MP16SR: TTATCTCAAGGCGTTCAAAGG

Table 2.3 Reaction mixture.

Reagent	Volume µl
Template	1.0
MP16SF 20 pmol/µl	1.0
MP16SR 20 pmol/µl	1.0
1 mM dNTPs	2.5
10x Buffer	2.5
25 mM $MgCl_2$	1.5
Taq enzyme	0.3
Nuclease free water	15.2
Total	25.0

Figure 2.1 Cycle conditions



Following PCR, samples were analysed on a 1% (w/v) agarose gel containing 1 µg/ml ethidium bromide (SIGMA E1385). The presence of a 829 base pair product indicated the presence of *M. pluton* DNA in the template material.

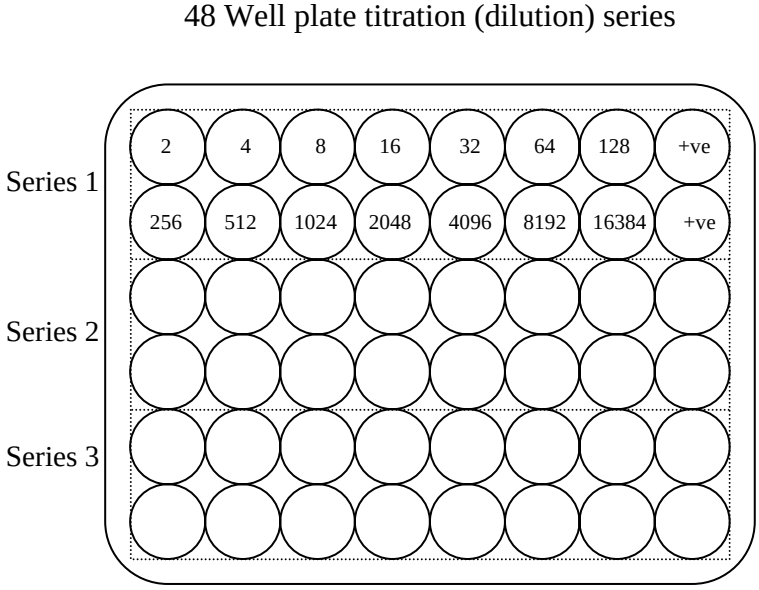
2.5 Disc diffusion assay system

The estimation of antibiotic sensitivity was initially performed using a disc diffusion system. Briefly, EFB media agar plates were made containing 2 % agar (rather than the standard 1.5%). Onto these was spread 100 µl of *M. pluton* culture, grown to stationary phase in EFB broth media (4 to 5 day old cultures). The plates were dried in an incubator, following which, small discs of Whatman No. 1 filter paper containing a defined amount of antibiotic were gently placed on the surface of the agar. The plates were inverted and incubated under standard conditions. Following 3 – 5 days incubation, the diameter of the 'zone of inhibition' surrounding the antibiotic discs was measured and recorded.

2.6 Liquid based MIC assay under standard conditions

Minimum inhibitory concentrations of antibiotics to *M. pluton* were estimated using a miniaturised version of the tube titration system. Briefly, broth titrations of antibiotics were performed in 48 well (Greiner-One 677180) tissue culture plates (figure 2.2).

Figure 2.2 48 well plate layout, including dilution series and positive control wells.



For 48 well microtitre plates, 500 µl of EFB broth was added to each well of the plate and the plate was marked out into three titration series of 14 wells or six titration series of 7 wells. 500 µl of antibiotic containing EFB media, at double the initial required concentration, was mixed with the media in the first well of the series. 500 µl of this was transferred to the second well in the series, with mixing. Serial titration of the antibiotic was continued in this manner, until the final well in the series, at which point the 500 µl of media removed from the well was discarded.

Thirty microlitres of late log to early stationary phase broth culture of *M. pluton* was added to each well. The well contents were mixed by shaking the plate on an orbital shaker for 1 minute at 150 rpm, and the plates incubated under standard conditions for 3 – 4 days.

Following the incubation, the plates were analysed by either:

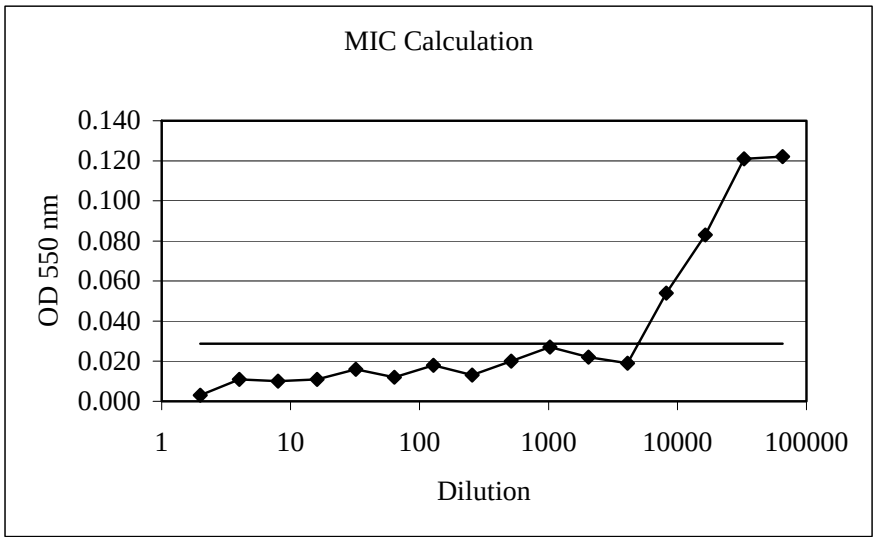
Visual examination of the plate wells for bacterial growth. The MIC value is calculated from the dilution of the last well in the titration series that contains no bacterial growth. If the dilution of the last (bacteria free) well is 1/512, and the initial antibiotic concentration was 1mg/ml, then the MIC is the concentration of the antibiotic in that well (1 mg/ml ÷ 512 = 1.95 µg/ml).

Spectrographic analysis of the culture turbidity in each well at 550 nm. For 48 well plates, the optical density (absorbance) of 200 µl of media from each well was measured in a spectrophotometer (Eppendorf Biophotometer), using an cuvette with a 1 cm path length (Eppendorf Uvette 0030 106.300). The optical density values were graphed for each dilution (logarithmic scale) and a threshold value determined for each data series (10 % of the difference between the mean minimum value and the mean maximum value). The highest dilution with an optical density below the threshold was used to determine the MIC value (figure 2.3).

Figure 2.3 Example of MIC calculation

Neat antibiotic concentration: 1.0 mg/ml

Dilution	OD 550 nm
2	0.003
4	0.011
8	0.010
16	0.011
32	**0.016**
64	**0.012**
128	**0.018**
256	**0.013**
512	**0.020**
1024	**0.027**
2048	**0.022**
4096	**0.019**
8192	0.054
16384	0.083
32768	**0.121**
65536	**0.122**



Mean maximum value (bold data): 0.122

Mean minimum value (bold data): 0.018

Difference: 0.122 – 0.018 = 0.104, 10% of difference: 0.010

Threshold (line): 0.018 + 0.010 = 0.028

MIC = Neat antibiotic concentration ÷ highest dilution below threshold

MIC = 1.0 mg/ml ÷ 2048 = 1.22 µg/ml

2.7 In-vitro larval rearing

Larval rearing assays were performed in the manner described by Peng *et.al.* (1992) with some modifications (McKee, 2003). Fresh royal jelly was supplied by One World Enterprises. It was aliquoted into 70 ml specimen jars (Biolab LBS32004Y), with approximately 35 ml per jar. The royal jelly was freeze dried in a freeze drier (Dynavac) over a 2 day period. The resulting material was ground to powder with a mortar and pestle and stored frozen at –20 ^{o}C until required.

Table 2.4 The Basic Larval Diet (BLD) was prepared using the following recipe:

Freeze dried royal jelly	4.2 gm
Glucose (AJAX-APS 783-500g)	0.6 gm
Fructose (BDH 284335T)	0.6 gm
Yeast Extract (Difco 212750)	0.2 gm
Sterile distilled water	14.4 ml

The BLD components were mixed thoroughly by vortexing and stored at 4^{o}C, unused BLD was discarded after one month.

In vitro larval rearing was performed by grafting one-day-old bee larvae from brood comb, onto the meniscus of 5 ml of BLD in a 50 mm diameter sterile petri dish (Bunzl). Grafting was done using a larval grafting tool, or a bent matchstick. The larvae were incubated at 35 ^{o}C, in a humidified incubator and were examined every 12 hours for viability.

After 2 – 3 days growth, the live larvae were transferred to individual wells of a sterile, 96 well U bottomed plate (Nunc 163320), containing 20 µl of BLD, which was then incubated at 35 ^{o}C in a humidified incubator. Larvae were observed and fed 20 µl of BLD every 12 hours, for 2 days, after which they were fed 30 µl of BLD until 8 days post grafting. The number of live larvae was then assessed and the plates frozen for further analysis.

2.8 Honey residue assay

Residue testing of various antibiotics in honey was performed by microbial inhibition analysis, using *M. pluton* as an indicator organism. Briefly, antibiotic was mixed into honey (warmed to 40 ^{o}C) using a Pronto Frother (Housewares International CCW-2008), to produce a homogenous solution with a final antibiotic concentration of 1 mg/ml. Each antibiotic was tested in triplicate. The honey was then equilibrated at the required temperature and a time zero sample removed and frozen at –20 ^{o}C. Sampling occurred at regular intervals (approximately weekly) through out the course of the experiment.

The weight of each sample was recorded and used to calculate the sample volume (assuming the density at 22 – 24 ^{o}C was 1.42 gm/ml). Samples were diluted to 25 % (v/v) in sterile distilled water prior to analysis.

Example calculation:

Sample weight: 0.450 gm

Honey density (at 20 ^{o}C): 1.42 gm/ml

Therefore sample volume = 0.450 ÷ 1.42 = 0.317 ml

Add 0.317 x 3 ml = 0.951 ml of sterile distilled water to make 25 % (v/v) honey solution.

The analysis method was performed in the same manner as the 96 well microtitre plate liquid assay used for MIC testing of antibiotics (see above). For residue testing analysis, the last dilution (minimum inhibitory dilution - MID) below the threshold was used to estimate the concentration of the antibiotic remaining in the honey sample relative to the MIC value calculated from the day zero samples (figure 2.4).

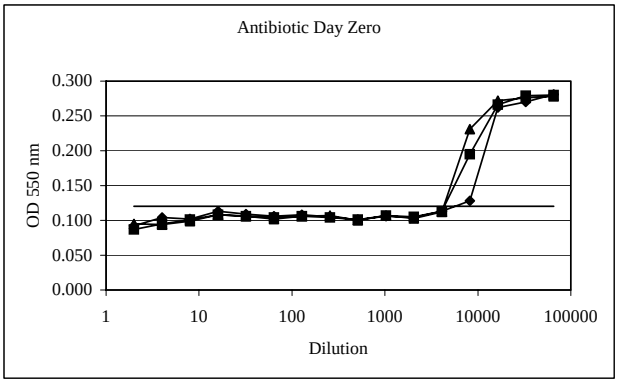
Figure 2.4 Example of residue analysis calculation

Initial antibiotic concentration in honey: 1.0 mg/ml

Antibiotic concentration in 25 % honey: 250 µg/ml

Day zero triplicate samples

Dilution	Sample 1	Sample 2	Sample 3
2	0.092	0.087	0.095
4	0.104	0.095	0.094
8	0.102	0.101	0.099
16	0.113	0.108	0.108
32	0.109	0.106	0.106
64	0.106	0.104	0.102
128	0.108	0.106	0.106
256	0.105	0.104	0.107
512	0.101	0.101	0.100
1024	0.106	0.107	0.107
2048	0.104	0.105	0.103
4096	0.113	0.113	0.112
8192	0.128	0.195	0.231
16384	0.262	0.266	0.272
32768	0.270	0.279	0.276
65536	0.281	0.280	0.278



Mean maximum value: 0.274

Mean minimum value: 0.103

Difference: 0.274 – 0.103 = 0.171, 10% of difference: 0.017

Threshold (line): 0.103 + 0.017 = 0.120

Last dilution before threshold (MID): 4096

Concentration of antibiotic at day zero in this dilution:

= Initial concentration ÷ dilution value

= 250 µg/ml ÷4096 = 61 ng/ml

Therefore, the *M. pluton* MIC value under these conditions is 61 ng/ml, for this particular antibiotic. Subsequent samples of honey may contain decreased antibiotic concentrations, as the antibiotic degrades over time. This will result in a decrease in the minimum inhibitory dilution value, which can be used to estimate the residual antibiotic concentration

Example calculation

MIC of antibiotic under experimental conditions: 61 ng/ml

MID of antibiotic at day 21 of honey storage: 512

Therefore, the residual concentration of antibiotic is: MIC x MID

= 61 ng/ml x 512 = 31.2 µg/ml

Sampling can occur until the MID value of an antibiotic is equal or less than the MID value for the honey + water negative control.

3 Isolation and Culturing of *Melissococcus pluton*.

3.1 Introduction

Prior to this project, *M. pluton* had not been reliably cultured in this laboratory. The first step of this project was to establish the culture requirements and to obtain monoculture strains of *M. pluton*. The first literature source for *M. pluton* in-vitro growth was Bailey (1957), however the adapted method by Hornitzky (1998) was used in this laboratory, with some modifications.

Two independent sources of *M. pluton* were obtained for this project. Dr M. Hornitzky from the Elizabeth Macarther Agricultural Institute (EMAI, Camden, NSW) kindly supplied several *M. pluton* strains collected during the course of an epidemiological study. One of these, 96/A727/1, a NSW strain, was used for subsequent work in this project. A field isolated strain (LGS-A-5), from an infected hive in Melton, Victoria was also obtained and subcultured to a monoculture.

There are two *M. pluton* PCR methods described in the literature. One is a less sensitive one-step method, using primers against 16S rRNA gene sequences specific for *M. pluton* (Govan *et.al*., 1998). The more sensitive PCR test uses a hemi-nested procedure (Djordjevic *et.al*., 1998). This involves two PCR steps, where the product of the first step is used as template in the second reaction. The extra sensitivity of the latter method, detecting 1 – 10 bacteria/ml, was not required for testing laboratory cultures, so the Govan *et.al.*(1998) method was established.

3.2 Results

3.2.1 Laboratory culture of *M. pluton* strain 96/A727/1

Vial 96/A727/1 was opened and the contents re-hydrated with 500 µl of sterile distilled water. After mixing the contents with a pasteur pipette, approximately half of the re-hydrated culture was spread-plated onto EFB agar. The remainder was diluted to ~500 µl with sterile 80 % glycerol and frozen at – 70 ^{o}C. The spread-plated material was incubated under standard conditions for 7 day, following which, the dominant colony type was subcultured on secondary and tertiary plates until a monoculture was obtained. This was examined by PCR and light microscopy and found to be *M. pluton* (data not shown).

3.2.2 Field Isolation of *M. pluton.*

Cultures of *M. pluton* were isolated from an EFB infected hive (Melton, Victoria). A diseased brood comb was removed from the hive and transported back to the laboratory. Five suspected diseased larvae (labelled A to E) were removed from their cells with 70% ethanol sterilised forceps and spatula. The diseased larvae were placed on a microscope slide and pulled apart, to expose the mid-gut. A sterile swab stick was used to collect material from the gut (Larval Gut Smear - LGS), which was struck on to EFB agar plates, in duplicate. The agar plates were incubated under standard conditions for 7 days.

Following incubation, five suspected *M. pluton* colonies were subcultured from plate LGS-A onto fresh EFB agar plates, which were incubated as before. Well-separated colonies from LGS-A plate 5, suspected of being *M. pluton*, were subcultured onto a third series of plates and incubated as standard.

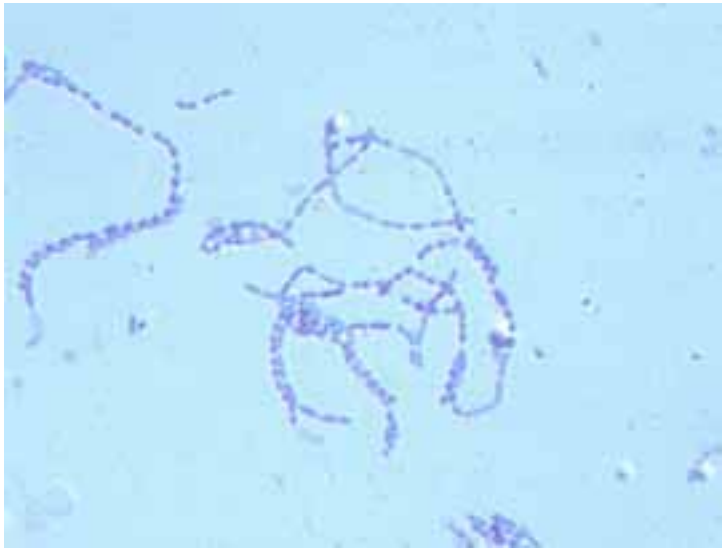
PCR (results not shown) and light microscopy was used to analyse the monoculture colonies on plate LGS-A-5.

3.2.2.1 Light microscopy of LGS-A-5.

LGS-A-5 colony material was transferred from the tertiary isolation plate to 200 µl of sterile distilled water and mixed vigorously, to disperse the bacterial cells. This material was then pelleted in a microfuge (14,000 rpm (13,000 *g*), 1 minute) and the water removed. The pellet was resuspended in 30 µl of water, and spread onto the surface of a microscope slide using a micropipette. The colony material was allowed to dry onto the slide and stained using Grams stain, as described in the materials

and methods. The slide was examined on a .Leica Lietz DM RBE microscope and an example field of view was captured using a Leica DC100 digital camera attachment and Paint Shop Pro version 6.02 (Jasc software).

Figure 3.2 Light field view of Gram stained *M. pluton* LGS-A-5 (S. Doughty 2003).



M. pluton has a distinct 'string of pearls' appearance when viewed in this manner.

3.3 Conclusions

Establishment of *M. pluton* culturing was initially difficult, as the published culture requirements are subject to some interpretation. We were unable to grow *M. pluton* using OXOID yeast extract, until the recipe was supplemented with 0.1% L-cysteine. However, the use of DIFCO yeast extract negates the need for cysteine supplementation. The initial media recipe also used 1% starch (glucose polymer), which caused turbidity in the media, due to incomplete solubilisation of the starch. Starch was substituted with sucrose (glucose – fructose dimer), with no apparent detriment.

Field isolation of *M. pluton* can be an unreliable procedure as a number of hive-borne bacteria, such as *Enterococcus faecalis* and *Paenibacillus alvei*, can also grow on the same media. The inclusion of nalidixic acid in the media can help inhibit their growth, but does not appear to stop it entirely. Some of these contaminating species are faster growing than *M. pluton*, and *P. alvei* has swarming motility, however, they can be distinguished by colony morphology and by being facultative anaerobes, rather than strict anaerobes.

4 Investigating alternative antibiotics for the control of EFB

4.1 Introduction

A comprehensive review of literature was undertaken to identify antibiotics that may be suitable for use in controlling EFB.

If a no-residue antibiotic is not identified, then an alternative should have a readily available testing procedure, to easily detect residue contamination.

The candidate antibiotic should currently be registered for use in Australia as a veterinary medicine. All antibiotics currently used in Australia are registered with the Commonwealth and specific State authorities. The Commonwealth authority, the Australian Pesticides and Veterinary Medicines Authority (APVMA) is the National Registration Authority for Agricultural and Veterinary Chemicals. The APVMA web site (http://www.apvma.gov.au/) provides a database (PUBCRIS) of all registered products, including antibiotics, which can be searched by category, active constituent or product name.

Additional minor selection criteria are that the alternative antibiotic should not pose a health risk to the apiarists. Although this is outside the scope of this project, some means of achieving this for potentially harmful antibiotics would be through reducing the amount of material required, or through reduced handling of the material via pre-formulation or packaging of the antibiotic in its released form. It should also be of equal or less cost to the apiarist, as the acceptance of a new control agent would be hampered if it costs considerably more to the end user.

4.2 Results

Table 3.1 details the type and lifespan of several commonly used antibiotics, and their effectiveness against bacteria of the Gram positive genus; *Micrococcus*, (Table 4.1).

The beta-lactam and cephalosporin antibiotics are natural or semi-synthetic analogues of penicillin antibiotics and are commonly used in human and veterinary medicine. They are listed as having the fastest decay rates, although they appear to be less effective against Micrococcus, that other listed antibiotics. The current treatment against EFB, OTC, is listed as have a moderately long lifespan.

Table 4.1 Types and estimated lifespan of various antibiotics (compiled by Dr Christian Saywell, State Chemistry Laboratory, Werribee, Victoria and Mr Russell Goodman, DPI, Victoria).

Type	Antibiotic	Lifespan	*Micrococcus* ML8 MIC (µg/ml)	*Micrococcus* ML6 MIC (µg/ml)
B-lactams	Penicillin G	Short lived	>40	>40
	Amoxicillin		40	>40
	Ampicillin		37	>40
	Cloxacillin		-	19??
Cephalosporins	Ceftiofur		32	35
	Cephalonium		23	28
	Cepheroxium		-	16
Macrolides (plus others)	Erythromycin	Moderately lasting	29	17
	Lincomycin		18	-
	Tylosin		21	18
	Tilmicosin		28	-
	Oleadomycin		-	-
Tetracyclines	Tetracycline		-	Si
	Oxytetracycline		-	Si
	Chlorotetracycline		-	17
Aminoglycosides	Neomycin	Long lasting	16	-
	Dihydro-Streptomycin		16	-
	Streptomycin		17	-
	Gentamycin		19	-
	Spectinomycin		-	-

From the antibiotics listed in Table 4.1, several have been used to control EFB symptoms. Penicillin G was found to be ineffective against *M. pluton*, however, Erythromycin (in the United States), Tylosin and Oxytetracycline have all been used effectively for the control of EFB.

4.2.1 Antibiotics considered not suitable for further analysis

The list of antibiotics registered for use in veterinary medicine by the APVMA and Chemical Standards Victoria contains a wide range of antibiotics (Table 4.3). Many are not of use due to being ineffective against *M. pluton* type bacteria, or being predicted to result in a long residue lifespan. The present study has identified the following types of antibiotics (Table 4.2) as not suitable for the following reasons:

Table 4.2 Antibiotics not considered suitable for EFB control.

5-nitro-imidazole	Anti-protozoan antibiotic
Aminoglycosides	Long lasting residues
Antimicrobial peptides Bacitracin and Virginiamycin	Banned from use in EU countries
Anti-β-lactamase	Only effective in conjunction with a β-lactam antibiotic, against bacteria producing β-lactamase
β-lactams (narrow spectrum)	Previously shown to be ineffective against *M. pluton*
Cephalosporins	More effective against Gram negative than Gram positive bacteria
Everninomicins	Variant of aminoglycosides, this having long lasting residues
Flavophospholipols	*Enterococcus* (related to *Melissococcus*) species are intrinsically resistant to this antibiotic
Fusidic acid	Specific anti-staphylococcal antibiotic
Ionophores	Not effective against Gram positive bacteria
Lincosamide	Variant of macrolides
Macrolides	Moderate lasting residue
Quinolines	Mainly effective against Gram negative bacteria
Sulfonamides	Concerns about residue in honey from current and past use for AFB control in Europe
Tetracyclines	Current treatment for EFB.

Table 4.3 Antibiotics that are registered by the Australian Pesticide and Veterinary Medicine Authority

Antibiotic	**Class**
Carnidazole	5-nitro-imidazole
Apramycin	Aminoglycoside
Dihydrostreptomycin sulfate	Aminoglycoside
Framycetin sufate	Aminoglycoside
Gentamicin	Aminoglycoside
Neomycin sulfate	Aminoglycoside
Spectinomycin sulfate	Aminoglycoside
Streptomycin sulfate	Aminoglycoside
Bacitracin	Antimicrobial peptide
Gramicidin	Antimicrobial peptide
Polymyxin b sulfate	Antimicrobial peptide
Thiostrepton	Antimicrobial peptide
Virginiamycin	Antimicrobial peptide complex
Clavulanic acid	Anti-β-lactamase
Amoxycillin	β-lactam - Broad
Ampicillin	β-lactam - Broad
B procaine penicillin	β-lactam - Narrow
Benethamine penicillin	β-lactam - Narrow
Benzathine penicillin	β-lactam - Narrow
Benzylpenicillin sodium	β-lactam - Narrow
Cloxacillin as the sodium salt	β-lactam - Narrow
Penethamate hydriodide	β-lactam - Narrow
Procaine penicillin	β-lactam - Narrow
Cefadroxil	Cephalosporin
Ceftiofur	Cephalosporin
Cefuroxime	Cephalosporin

Cephalexin	Cephalosporin
Cephalonium dihydrate	Cephalosporin
Chloramphenicol	Cephalosporin
Novobiocin sodium	Enzyme inhibitor
Trimethoprim	Enzyme inhibitor
Avilamycin	Everninomicin
Flavomycin	Flavophospholipol
Flavophospholipol	Flavophospholipol
Fucidic acid diethanolamine	Fusidic acid
Lasalocid sodium	Ionophore
Maduramicin ammonium	Ionophore
Narasin	Ionophore
Semduramicin sodium	Ionophore
Lincomycin	Lincosamide
Clindamycin	Macrolide
Erythromycin	Macrolide
Kitasamycin	Macrolide
Milbemycin oxime	Macrolide
Nystatin	Macrolide
Oleandomycin	Macrolide
Spiramycin	Macrolide
Tiamulin	Macrolide
Tilmicosin	Macrolide
Tylosin	Macrolide
Difloxacin hydrochloride	Quinolines
Enrofloxacin	Quinolines
Orbifloxacin	Quinolines
Sulfadimidine	Sulfanilamide
Sulfamerazine	Sulfanilamide
Sulfatroxazole	Sulfanilamide
Phthalylsulfathiazole	Sulfathiazole
Mafenide	Sulfonamide
Sulfadiazine	Sulfonamide
Sulfadoxine	Sulfonamide
Chlortetracycline hydrochloride	Tetracycline
Doxycycline hydrochloride	Tetracycline
Oxytetracycline	Tetracycline
Tetracycline hydrochloride	Tetracycline

4.2.2 Antibiotics considered suitable for further analysis

Antibiotics that were considered suitable for further analysis were of the following types:

1 - β-lactams – (broad spectrum) – active against Gram positive bacteria and short lifespan

2 - antimicrobial peptides – active against Gram positive bacteria

3 - enzyme inhibitors – some are active against Enterobacter type bacteria (related to *M. pluton*).

From these types, antibiotics were selected for further testing (Table 4.4):

Table 4.4 Antibiotics selected for further testing as alternatives to OTC.

Antibiotic	Type	Source
Ampicillin	β-lactam - Broad	SIGMA A-0166
Amoxicillin[2]	β-lactam – Broad	CSL Moxacin
Gramicidin	Antimicrobial peptide	SIGMA G-5002
Nisin	Antimicrobial peptide	SIGMA N-5764
Trimethoprim	Enzyme inhibitor	SIGMA T-7883
Oxytetracycline	Tetracycline	SIGMA O-5875
Bacitracin[1]	Antimicrobial peptide	SIGMA B-0125
Monensin[1]	Ionophore	SIGMA M-5273
Erythromycin[1]	Macrolide	SIGMA E-5389

[1] - included to compare their effectiveness against existing treatment.
[2] - Amoxicillin was not identified until later in the project.

The listed antibiotics were purchased from the listed supplier, or were already present in the laboratory.

4.3 Human health issues

Examination of the material safety data sheets (MSDS) for the selected antibiotics indicated some potentially serious health consequences from prolonged use or misuse. Nisin, trimethoprim and bacitracin are all listed as non-hazardous irritants. Ampicillin and amoxicillin have the potential to cause an anaphylactic reaction in sensitive individuals, while gramicidin can cause chemical meningitis with prolonged exposure. Erythromycin can cause intestinal upset and respiratory irritation and is a reproductive hazard (teratogen) with chronic exposure. Monensin is listed as being toxic, causing spastic paralysis and ataxia. The current treatment OTC is listed as non-hazardous, however chronic exposure can have carcinogenic, mutagenic and teratogenic effects and it is listed as a reproductive hazard.

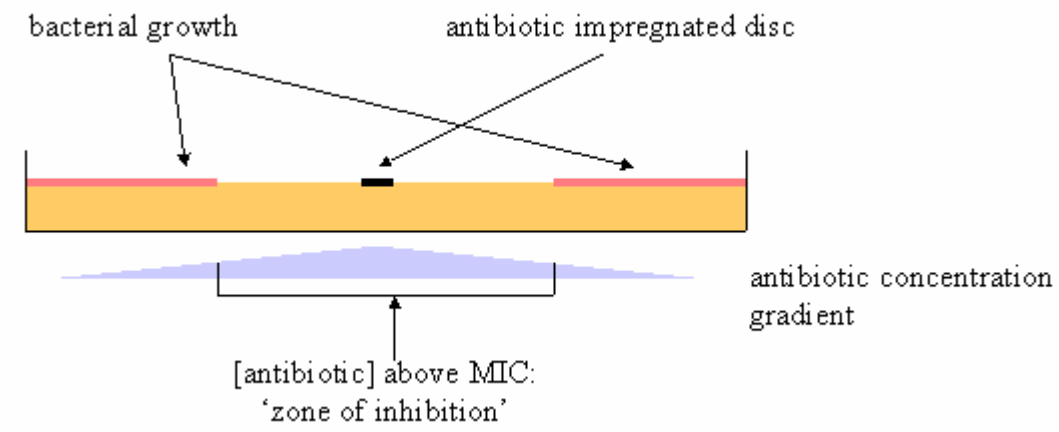
5 Minimum inhibitory concentrations of antibiotics to *Melissococcus pluton*

5.1 Introduction

In order to examine the anti-microbial effectiveness of the selected antibiotics against *M. pluton*, agar plate based disc diffusion assays (DDA) and liquid broth minimum inhibition assays (MIC) were performed.

DDAs work on the principle of an antibiotic diffusing through agar media in a culture plate. This produces an antibiotic concentration gradient away from the inoculation site (usually an antibiotic impregnated disc). To visualise the effect this concentration gradient has on the tested bacteria, the agar surface of the plate is spread plated with testing organism, to produce a bacterial lawn, when incubated under the correct conditions. With an antibiotic concentration gradient present in the agar media, the bacteria will only be able to grow where the antibiotic concentration is below the minimum inhibitory concentration, leaving a 'zone of inhibition' surrounding the antibiotic impregnated disc.

Figure 5.1 Cross section view of a disc diffusion assay.



DDAs are excellent methods for assessing bacterial sensitivity of several bacterial samples to a single antibiotic. However, they are not ideal for comparison between antibiotics, because the diffusion rate of the various antibiotics through the agar affects the size of the zone of inhibition. Since the agar medium is essentially a water based medium, antibiotics such as ampicillin, which are highly water soluble, will diffuse faster and farther through the agar, than a water insoluble antibiotic such as gramicidin.

To overcome this, an alternative method was employed. Liquid broth MIC analysis involves growing the testing bacteria in liquid medium, in a series of aliquots, each containing a different, known antibiotic concentration. The lowest antibiotic concentration in the series, that inhibits bacterial growth, is deemed the minimum inhibitory concentration (MIC). It this situation, the assay was performed in a microtitre plate (48 or 96 wells), and antibiotic titrated across the wells using pre-determined or serial dilution. Serial dilution results in the halving of the antibiotic concentration between wells; if the first well contains 1 mg/ml antibiotic, the next well will contain 500 µg/ml, the next will contain 250 µg/ml, etc.

The DDA method was used in the initial analysis, as this gave a quick estimation of the antibiotics effectiveness against *M. pluton*. MIC analysis was then performed to produce a quantitative ranking of the various antibiotics ability to inhibit *M. pluton* growth *in-vitro*.

5.2 Results

5.2.1 Disc Diffusion Assay

Disc diffusion analysis was performed on the antibiotics listed in table 5.1.

Table 5.1 Antibiotics and their stock concentrations and diluents.

Antibiotic	Stock (mg/ml)	Diluent
Ampicillin	6	H_2O
Erythromycin	6	50% ethanol
Bacitracin	25	H_2O
Monensin	10	95% ethanol
Gramicidin	25	DMSO
Nisin §	2.5	0.02 M HCl 0.75% NaCl
Oxytetracycline	6	50% ethanol

§ Nisin is supplied as 2.5% nisin in milk solids.

Antibiotics were pipetted onto paper discs at two different (high and low) concentrations. These were placed on (duplicate) plates containing seeded lawns of *M. pluton* 96/A727/1, and incubated under standard conditions. Following incubation for five days, the zones of inhibition surrounding the antibiotic discs were measured (table 5.2).

Figure 5.1 DDA plate of Bacitracin.

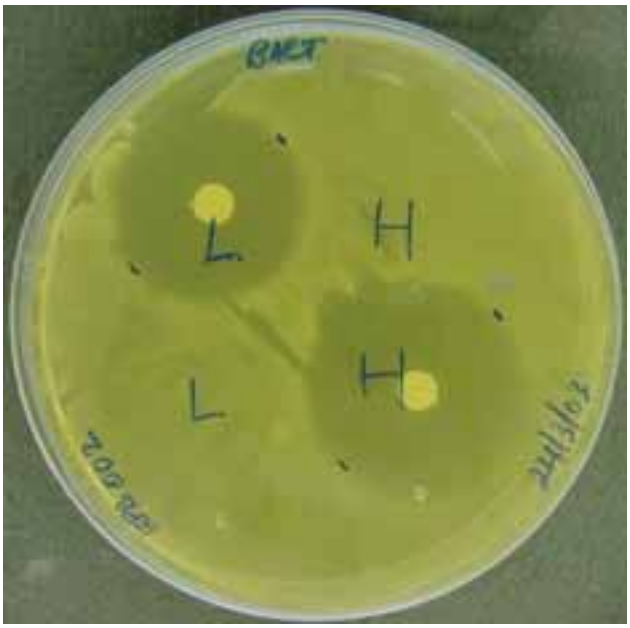


Table 5.2 Results of DDA plate assays.

Antibiotic	Amount added to discs (µg)		Zone of inhibition (mm)	
	Low	High	Low	High
Ampicillin	10	30	Whole plate	Whole plate
Erythromycin	10	30	25, 26	28, 28
Bacitracin	32	125	28, 28	32, 32
Monensin	10	50	0, 0	0, 0
Gramicidin	25	125	6, 6	7, 7
Nisin	2.5	12.5	7, 6	13, 15
Oxytetracycline	10	30	0, 0	0, 0

It is interesting to note that OTC and Monensin did not result in distinguishable zones of inhibition, where as Ampicillin caused clearing on the entire plate surface. To provide a better quantitative valuation of the various antibiotics effectiveness, MIC analysis was performed.

5.2.2 Minimum inhibitory concentration assay

MIC experiments were performed in 48 well plates and visually scored.

5.2.2.1 Experiment 1: MIC of oxytetracycline and nisin.

Oxytetracycline stock was diluted to 40 µg/ml on EFB media and serially diluted 1 in 2 across a 7 well dilution series, in triplicate. Nisin stock was diluted to 10 µg/ml (400 µg/ml total solids) and serially diluted across the triplicate series. 20 µl of *M. pluton* 96/A727/1 culture was added to each well, including 6 control wells, and the plate incubated under standard conditions for 3 days. Bacterial growth was assessed visually, and scored using the following chart (table 5.3)

Table 5.3 Scoring values for MIC plate assay

Score	Growth
-	No growth
+/-	Slight turbidity
+	Low growth
++	Medium growth
+++	High growth

Table 5.4 Oxytetracycline and nisin MIC assay scores.

[µg/ml]	20	10	5	2.5	1.25	0.625	0.3125	control
OTC 1	-	-	-	-	-	+++	+++	+++
OTC 2	-	-	-	-	-	+++	+++	+++
OTC 3	-	-	-	-	+/-	+++	+++	+++
Nisin 1	+/-	+	+++	+++	+++	+++	+++	+++
Nisin 2	+/-	+	+++	+++	+++	+++	+++	+++
Nisin 3	+/-	+	+++	+++	+++	+++	+++	+++
[µg/ml]	5	2.5	1.25	0.625	0.3125	0.156	0.078	control

From table 5.4 it is possible to calculate the MIC of oxytetracycline for *M. pluton* as between 2.5 and 1.25 µg/ml, under these conditions. The MIC of nisin cannot be calculated, however it is greater than 5 µg/ml.

5.2.2.2 Experiment 2: MIC of gramicidin and nisin.

Gramicidin stock was made up to 100 µg/ml in EFB media and nisin to 100 µg/ml (4 mg/ml total solids). Both antibiotics were serially diluted across the 48 well plate in triplicate. *M. pluton* 96/A727/1 culture (20 µl/well) was added to all wells and the plate incubated under standard conditions. The results are shown in table 5.5

Table 5.5 Gramicidin and nisin MIC assay scores.

[µg/ml]	50	25	12.5	6.25	3.125	1.563	0.781	control
Gram 1	-	-	-	-	-	-	-	+++
Gram 2	-	-	-	-	-	-	-	+++
Gram 3	-	-	-	-	-	-	-	+++
Nisin 1	-	+	+++	+++	+++	+++	+++	+++
Nisin 2	-	+	+++	+++	+++	+++	+++	+++
Nisin 3	-	+	+++	+++	+++	+++	+++	+++
[µg/ml]	**50**	**25**	**12.5**	**6.25**	**3.125**	**1.563**	**0.781**	**control**

From this data it is now possible to estimate the MIC of nisin at between 50 and 25 µg/ml. The MIC of gramicidin is less than 0.8 µg/ml, but further dilutions need to be tested to accurately determine the MIC.

5.2.2.3 Experiment 3: MIC of ampicillin and bacitracin.

Ampicillin and bacitracin stocks were both made up to 100 µg/ml in EFB media. Both antibiotics were serially diluted across the 48 well plate in triplicate. *M. pluton* 96/A727/1 culture (20 µl/well) was added to all wells and the plate incubated under standard conditions. The results are shown in table 5.6

Table 5.6 Ampicillin and bacitracin MIC assay scores.

[µg/ml]	50	25	12.5	6.25	3.125	1.563	0.781	control
Amp 1	-	-	-	-	-	-	-	+++
Amp 2	-	-	-	-	-	-	-	+++
Amp 3	-	-	-	-	-	-	-	+++
Bac 1	-	-	-	-	-	+++	+++	+++
Bac 2	-	-	-	-	-	+++	+++	+++
Bac 3	-	-	-	-	-	+++	+++	+++
[µg/ml]	**50**	**25**	**12.5**	**6.25**	**3.125**	**1.563**	**0.781**	**control**

The MIC of Ampicillin is less than 0.8 µg/ml and the MIC is between 3.12 and 1.56 µg/ml.

5.2.2.4 Experiment 4: MIC of ampicillin and trimethoprim.

Ampicillin and trimethoprim stocks were both made up to 2 µg/ml and 400 µg/ml respectively, in EFB media. Both antibiotics were serially diluted across the 48 well plate in triplicate. *M. pluton* 96/A727/1 culture (20 µl/well) was added to all wells and the plate incubated under standard conditions. The results are shown in table 5.7

Table 5.7 Ampicillin and trimethoprim MIC assay scores.

[ng/ml]	1000	500	250	125	62.5	31.25	15.63	control
Amp 1	-	-	-	-	-	+	+++	+++
Amp 2	-	-	-	-	-	+/-	+++	+++
Amp 3	-	-	-	-	-	+	+++	+++
Tri 1	-	-	-	-	-	-	-	+++
Tri 2	-	-	-	-	-	-	-	+++
Tri 3	-	-	-	-	-	-	-	+++
[µg/ml]	**200**	**100**	**50**	**25**	**12.5**	**6.25**	**3.125**	**control**

Further dilutions of Ampicillin have allowed the calculation of the MIC at between 62 and 31 ng/ml. The MIC of trimethoprim cannot be calculated, but is less than 3.12 µg/ml.

5.2.2.5 Experiment 5: MIC of ampicillin (second experiment).

Further analysis of the MIC of ampicillin to *M. pluton* was performed. In this situation, two rows of 7 wells were used for serial dilutions, starting at 2.5 µg/ml (from a 5 µg/ml stock). *M. pluton* 96/A727/1 was added to each well (20 µl) prior to incubation. Results are shown below in table 5.8.

Table 5.8 Ampicillin MIC assay scores.

[ng/ml]	2500	1250	625	313	156	78	39	control
1°Amp 1	-	-	-	-	-	-	-	+++
1°Amp 2	-	-	-	-	-	-	-	+++
1°Amp 3	-	-	-	-	-	-	-	+++
2°Amp 1	+	++	+++	+++	+++	+++	+++	+++
2°Amp 2	+	++	+++	+++	+++	+++	+++	+++
2°Amp 3	+	++	+++	+++	+++	+++	+++	+++
[ng/ml]	20	10	5	2.5	1.2	0.6	0.3	control

From this it is possible to calculate the MIC of ampicillin (under these conditions) as 40 ng/ml.

5.2.2.6 Experiment 6: MIC of gramicidin (second experiment).

The second gramicidin MIC assay used the same method as the ampicillin experiment. Gramicidin stock was prepared at 5 µg/ml, and serially diluted across 14 wells. *M. pluton* 96/A727/1 was added to each well (20 µl) prior to incubation. Results are shown below in table 5.9.

Table 5.9 Gramicidin MIC assay scores.

[ng/ml]	2500	1250	625	313	156	78	39	control
1°Gram 1	-	-	+/-	+/-	+/-	+	++	+++
1°Gram 2	-	-	+/-	+/-	+/-	+	++	+++
1°Gram 3	-	-	+/-	+/-	+/-	+	++	+++
2°Gram 1	+++	+++	+++	+++	+++	+++	+++	+++
2°Gram 2	++	+++	+++	+++	+++	+++	+++	+++
2°Gram 3	+++	+++	+++	+++	+++	+++	+++	+++
[ng/ml]	**20**	**10**	**5**	**2.5**	**1.2**	**0.6**	**0.3**	**control**

From the two gramicidin assays it is possible to calculate the gramicidin MIC as between and 0.780 µg/ml and 0.625 µg/ml.

The data from the six experiments is collated in table 5.10.

Table 5.10 Calculated MIC values for tested antibiotics.

Antibiotic	MIC value
Ampicillin	40 ng/ml
Bacitracin	3.215 µg/ml
Gramicidin	0.8 µg/ml
Nisin	50 µg/ml
Trimethoprim	< 3.125 µg/ml
Oxytetracycline	2.5 µg/ml

5.3 Discussion

Monensin and erythromycin were removed from the list of selected antibiotics, following DDA experiments. Monensin was not considered further, because of it lack of inhibition in the assay, and because of health concerns with its toxic effects. The inability of monensin to inhibit *M. pluton* growth may have been due to its poor solubility in water, resulting in minimal diffusion of the antibiotic through the agar medium. It may also have been due to this type of antibiotic (ionophores) mainly being effective against protozoan rather than bacterial infection.

From the MIC analysis, *M. pluton* is 100 times more sensitive to ampicillin (MIC – 40 ng/ml), than other tested antibiotics. Amoxicillin was identified as a suitable candidate after this section of experimentation was completed. However, it is anticipated that the MIC value of amoxicillin would be similar to ampicillin, as these are closely related antibiotics. The MIC value for OTC (between 1.25 and 2.5 µg/ml) agrees with other reports in the literature, which calculate the MIC at between 1 and 2 µg/ml (Hornitzky and Smith, 1999a).

6 Sensitivity of *Apis mellifera* larvae to selected antibiotics

6.1 Introduction

For an antibiotic to be considered suitable for EFB control, it must not have any deleterious effects on the organism it is protecting. This study investigates the effects of three antibiotics (ampicillin, amoxicillin and oxytetracycline) on the larval stages of the European honey bee (*Apis mellifera*). To test this, larvae were grown *in-vitro*, using a modification of the procedure described by Peng *et.al.* (1992). Once transferred to individual wells of a microtitre plate, the larvae were fed food containing 20 times the estimated MIC concentration of antibiotics. This was continued until the larvae started to pre-pupate. The number of live versus dead larvae at pre-pupation was used to estimate the level of antibiotic toxicity.

6.2 Results

6.2.1 Experiment 1 – Test of growth environment.

Basic larval diet (BLD) was prepared using freeze dried royal jelly and additional components (see methodology). Twelve larvae, estimated at 36 – 48 hours old, were extracted from brood comb and floated on the meniscus of 5 ml of BLD (figure 6.1). Two days after extraction, the larvae were transferred to individual wells of a "U-bottomed" microtitre plate containing 20 µl of BLD (figure 6.2). They were fed 30 µl of BLD at 0900 hours and 18:00 hours each day, for 3 days. Following this the larvae were visually scored for viability and frozen at –20 ^{o}C (Table 6.1).

Figure 6.1 Petri dish containing larvae in BLD.

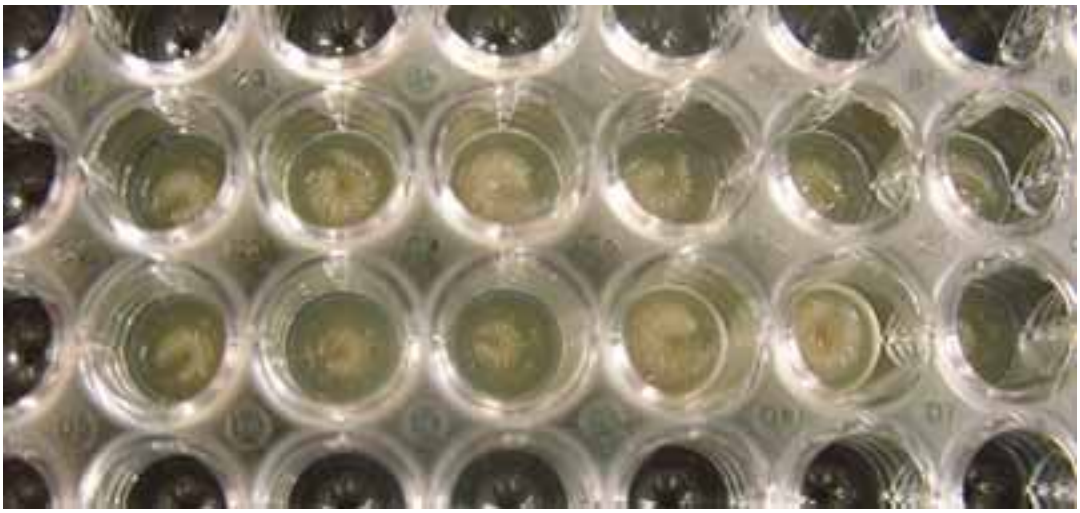
Table 6.1 Viability of larvae following *in-vitro* culturing.

	1	**2**	**3**	**4**	**5**	**6**
Row 1	D	L	L	L	L	L
Row 2	D	D	D	L	L	D

L – Live, D – Dead

Out of 12 larvae, 7 survived *in-vitro* culture (58%).

Figure 6.2 Larvae in microtitre plate wells.



6.2.2 Experiment 2 – Preliminary antibiotic sensitivity to ampicillin, amoxicillin and oxytetracycline.

BLD was prepared from freshly freeze-dried royal jelly as described previously. Forty-five larvae (estimated 24 – 36 hours old) were extracted from brood comb and floated on 7.5 ml of BLD. Following incubation for 2 days, 40 larvae were transferred to U bottomed wells in a microtitre plate, in 4 groups of 10. These were fed 25 µl of BLD at approximately 12 hourly intervals, for one day. They were then fed BLD containing antibiotics at 20 times MIC for 4 days (Table 6.2).

Table 6.2 Antibiotic concentrations fed to larvae in BLD.

Antibiotic	MIC value	Antibiotic Concentration in BLD
Control (No antibiotic)	Zero	Zero
Ampicillin	40 ng/ml	800 ng/ml
Amoxicillin	40 ng/ml[1]	800 ng/ml
Oxytetracycline	2.5 µg/ml	25 µg/ml

[1] - Estimated MIC based on similarity to ampicillin.

Following incubation, the larvae were scored for *in-vitro* growth (Table 6.3).

Table 6.3 Viability of larvae following *in-vitro* culturing with antibiotic containing BLD.

	1	2	3	4	5	6	7	8	9	10	% Live
Control - BLD	L	L	D	L	L	L	L	L	D	D	70
BLD + Ampicillin	L	L	L	L	D	D	L	L	L	D	70
BLD + Amoxicillin	L	D	D	L	L	L	L	L	L	L	80
BLD + Oxytetracycline	L	D	D	D	L	L	L	D	L	L	60

6.2.3 Experiment 3 – Antibiotic sensitivity to ampicillin, amoxicillin and oxytetracycline.

This was a duplicate of experiment 2, with more larvae per group, and more stringent control of larvae selection and feeding. Sixty-eight, 24 – 36 hour old, larvae were extracted and transferred to 7.5 ml of BLD. Following two days incubation, 48 larvae of similar size were transferred to microtitre plate wells, in four groups of 12. An additional 10, size-matched, larvae were also transferred to the plate All larvae were fed 25 µl of BLD at 1030 hours and 1800 hours on the day of transfer. They were then fed 25 µl BLD containing various antibiotics (Table 6.2), at 0600 hours and 1800 hours for 4 days. On the fifth day post transfer, the larvae were fed 25 µl BLD plus antibiotics at 0600 hours, then frozen at 1700 hours, for scoring.

Table 6.4 Viability of larvae following *in-vitro* culturing with antibiotic containing BLD.

	1	2	3	4	5	6	7	8	9	10	11	12	% Live
Control - BLD	L	L	L	L	L	L	L	L	L	L	L	L	100
BLD + Ampicillin	L	L	L	L	L	L	L	L	L	L	L	L	100
BLD + Amoxicillin	L	L	L	L	L	L	L	L	L	L	L	L	100
BLD + Oxytetracycline	L	L	L	L	L	L	L	L	L	L	L	L	100
Control - BLD	L	L	L	L	L	D	D	L	L	L	-	-	80

6.3 Discussion

Preliminary larval rearing experiments indicated that the addition of antibiotics to larval food has a minimal effect on larval viability, up to the pre-pupation stage. The variability between experiment two and three may be due to the increased stringency of handling; larvae were fed at exactly 12-hour intervals and all larval and microtitre plate handling was as gentle as possible.

Due to time constraints, the larvae were unable to be grown through to pupation. It is expected that the viability values would decrease, the longer the larvae were incubated *in-vitro*. A further experiment that would provide useful information would be to experimentally infect larvae with *M. pluton*, and examine larval viability in the presence or absence of antibiotic in their food. It is expected that antibiotic fed to larvae would protect them from infection, if the antibiotic concentrations in the larval mid-gut were maintained above the MIC of the particular antibiotic to *M. pluton*.

Scaling up these experiments would be necessary to obtain further validation that the three antibiotics are not detrimental to the health of the larvae. Due to a limited number of available larvae to work and the short-term nature of the project, this preliminary investigation was conducted to obtain an indication of the effects of these selected antibiotics on larval health.

7 Residue testing of antibiotics in honey

7.1 Introduction

One of the major objectives of the project was to identify an antibiotic that degraded quickly in honey, resulting in no residue. To that end, the degradation rate of several antibiotics in honey was investigated. Two experiments were performed, each at a different temperature. In the first experiment, antibiotic amended honeys were incubated at room temperature (22 – 26 ^{o}C) to mimic the temperature of stored, extracted honey prior to processing. In the second experiment, they were incubated at 35 ^{o}C to mimic the temperature of honey stored in the hive.

The testing of honey for oxytetracycline residue is normally performed using disc diffusion assays with *Bacillus cereus* reporter bacteria, or by High Performance Liquid Chromatography (HPLC). HPLC tests are also available to detect trimethoprim residues in meat. Tests for ampicillin and amoxicillin residues in milk are readily available, mostly using laboratory or slide-based immunochemistry methods. However, no residue detection methods have been identified in Australia, for gramicidin or nisin, from any source.

Due to the difficulty of identifying suitable quantitative residue testing procedures, a common MIC style test was developed. Initial experimentation using *B. cereus* indicated that this accepted reporter bacterial species was not sufficiently sensitive to the tested antibiotics, other than oxytetracycline. To overcome this, *M. pluton* was used as a reporter. This had the advantage of using a modification of the already established MIC technique. The microtitre plate system was further miniaturised from a 48 well plate to a 96 well plate, with each well containing a 100 µl culture. This allowed scoring by spectrographic analysis with an ELISA plate reader. This laboratory's ELISA plate reader (Labsystems Multiskan MS) allowed the collection of spectrographic data directly onto a PC using Labsystems own proprietary software (Genesis microplate collection and analysis software). This data was then loaded into Microsoft Excel for collating and further analysis.

Since honey has some inherent antibacterial properties, the MIC values of the honey containing antibiotics were compared to honey containing sterile distilled water, as a control. This control identified the background level of bacterial inhibition. During the course of the experiment, the antibiotics in the honey samples were observed to degrade. This degradation was quantified by the MIC of each sample taken over time. Once the MIC value of an antibiotic sample was equal to the MIC value of the water control, the antibiotic was regarded as having completely lost its effectiveness to inhibit disease. In this situation, the antibiotic may have completely degraded, or it may have been chemically modified into a non-effective, but still detectable form.

7.2 Results

7.2.1 Experiment 1 – Residues in honey stored at room temperature.

Honey, free of oxytetracycline residue, was sourced from a local honey supplier. This was aliquoted into specimen jars, at 50 ± 0.5 gm per aliquot. Assuming a room temperature density of 1.42 gm/ml for honey, this (50 gm ÷ 1.42 gm/ml) equated to 35.2 ml per aliquot. To each of these, antibiotics were added to a final concentration of 1 mg/ml (Table 7.1). One aliquot was prepared for each antibiotic. The honey was cooled following the addition of the antibiotics, then equilibrated at room temperature in a dark cupboard.

Table 7.1 Honey plus antibiotic aliquots.

Antibiotic	Stock	Volume added
Ampicillin	100 mg/ml	352 µl
Amoxicillin	92 mg/ml	383 µl
Trimethoprim	40 mg/ml	880 µl
Gramicidin	25 mg/ml	1.4 ml
Oxytetracycline	100 mg/ml	352 µl
No antibiotic control	NA	NA

Samples were removed from the honey mixtures at approximately weekly intervals. Each sample was weighed (table 7.2), diluted to 25 % honey (v/v) with sterile distilled water and frozen at –20 ^{o}C.

Table 7.2 Mass (gm) of each sample taken over 104 days of sample collection.

Day	Amp	Amox	Trimeth	Gram	OTC	Control
0	1.42	1.45	1.48	1.41	1.43	1.42
7	1.46	1.46	1.40	1.40	1.35	1.45
14	1.43	1.41	1.35	1.44	1.25	1.40
21	1.43	1.31	1.3	1.35	1.33	1.38
28	1.44	1.44	1.37	1.41	1.41	1.39
35	1.33	1.34	1.39	1.38	1.33	1.43
42	1.205	1.23	1.265	1.337	1.381	1.295
49	1.306	1.301	1.309	1.325	1.387	1.328
56	1.315	1.390	1.401	1.339	1.226	1.381
66	1.358	1.363	1.394	1.384	1.401	1.330
91	1.172	1.122	1.197	1.360	1.185	1.269
104	0.354	0.400	0.387	0.359	0.396	0.364

The antibiotic concentration of selected samples was examined using the microtitre plate MIC assay system.

7.2.1.1 MIC assay of samples day 0 and 21.

Samples taken from the antibiotic containing honey aliquots on days 0 and 21 were titrated by serial dilution across 16 wells of a microtitre plate (three plates in total). 20 µl of late log *M. pluton* 96/A727/1 culture was added to each well and the plates was incubated under standard conditions for three days.

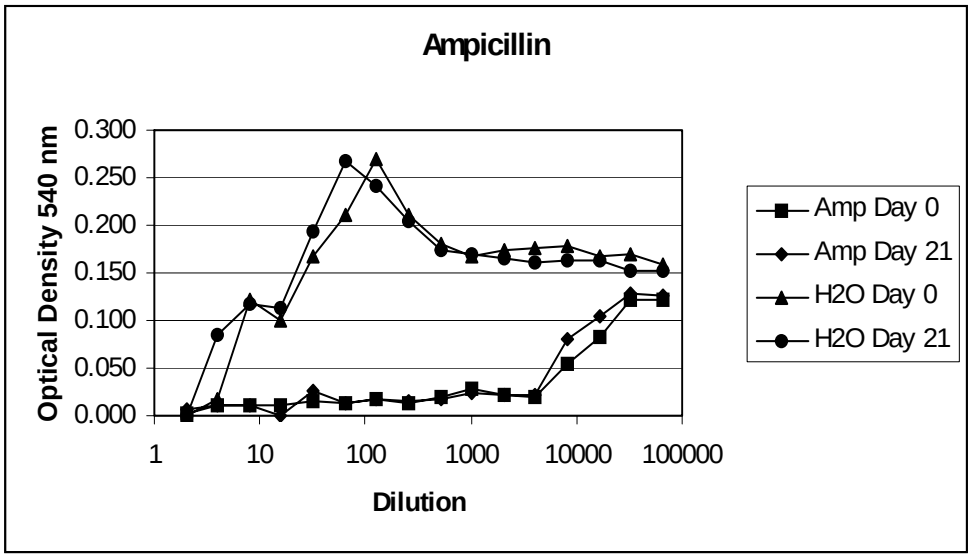
Prior to analysis of the plates, the well contents were thoroughly mixed to resuspend bacterial growth that was sedimented on the bottom of the well. This was done using a Ratek orbital mixer (Ratek OM11), at 240 rpm for 5 minutes.

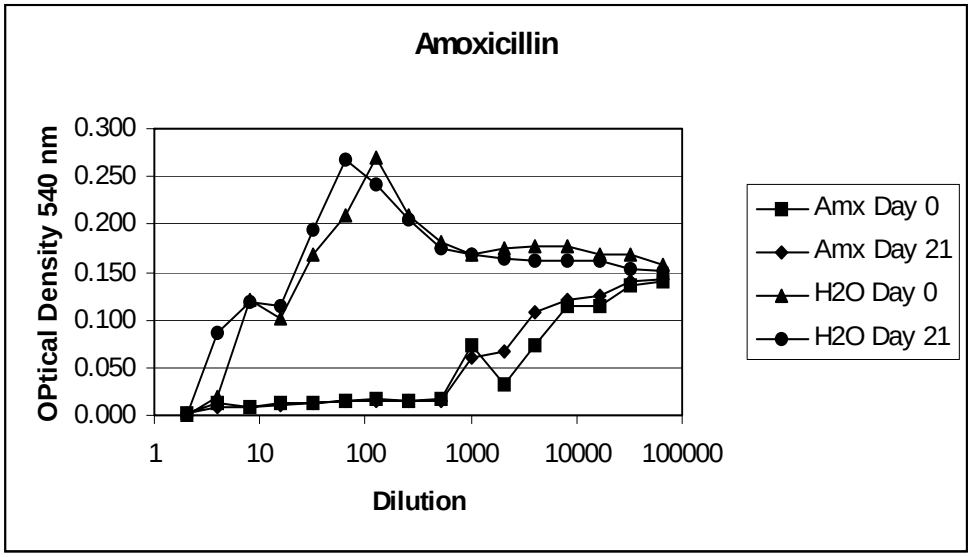
The turbidity (optical density) of each well culture was assayed at 540 nm on the microplate reader. This data was collected by a PC connected to the microplate reader and analysed using Microsoft Excel.

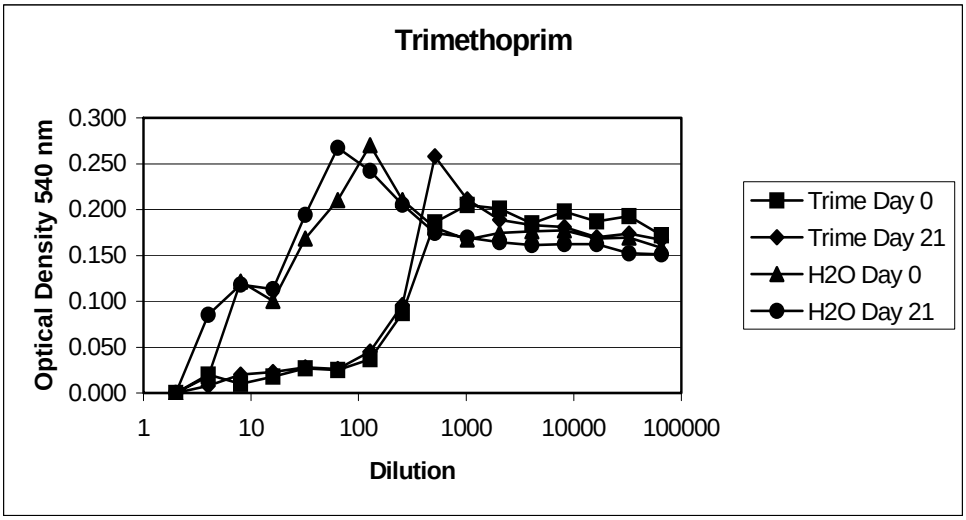
Table 7.3 Optical density values at 540 nm

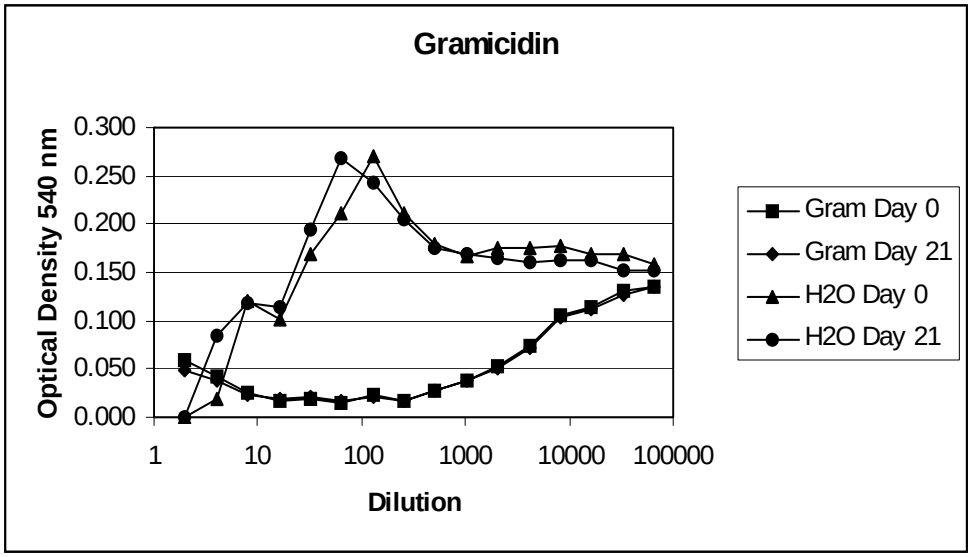
Dil[n]	Amp Day 0	Amp Day 21	Amox Day 0	Amox Day 21	Trimeth Day 0	Trimeth Day 21	Gram Day 0	Gram Day 21	OTC Day 0	OTC Day 21	H_2O Day 0	H_2O Day 21
2	0.003	0.006	0.002	0.003	0.000	0.000	0.059	0.049	0.008	0.006	0.000	0.000
4	0.011	0.010	0.013	0.009	0.020	0.008	0.043	0.038	0.011	0.013	0.018	0.085
8	0.010	0.010	0.008	0.008	0.010	0.020	0.026	0.023	0.009	0.019	0.121	0.118
16	0.011	0.000	0.014	0.011	0.018	0.023	0.017	0.018	0.014	0.019	0.100	0.113
32	0.016	0.027	0.014	0.013	0.027	0.028	0.020	0.021	0.023	0.021	0.168	0.194
64	0.012	0.012	0.015	0.015	0.025	0.026	0.015	0.016	0.013	0.014	0.210	0.267
128	0.018	0.018	0.018	0.016	0.037	0.045	0.023	0.022	0.021	0.024	0.270	0.242
256	0.013	0.016	0.016	0.015	0.087	0.096	0.017	0.016	0.036	0.045	0.210	0.205
512	0.020	0.018	0.018	0.016	0.186	0.258	0.028	0.028	0.059	0.068	0.180	0.174
1024	0.028	0.023	0.074	0.060	0.205	0.211	0.039	0.038	0.139	0.148	0.167	0.169
2048	0.022	0.021	0.032	0.067	0.201	0.189	0.052	0.051	0.177	0.168	0.174	0.164
4096	0.019	0.021	0.074	0.108	0.185	0.183	0.075	0.072	0.169	0.166	0.176	0.161
8192	0.054	0.080	0.115	0.121	0.198	0.181	0.106	0.104	0.189	0.173	0.177	0.162
16384	0.083	0.104	0.115	0.126	0.187	0.170	0.114	0.113	0.178	0.164	0.168	0.162
32768	0.121	0.129	0.135	0.141	0.193	0.174	0.131	0.126	0.186	0.172	0.169	0.152
65536	0.122	0.127	0.140	0.143	0.172	0.167	0.136	0.135	0.146	0.157	0.158	0.151

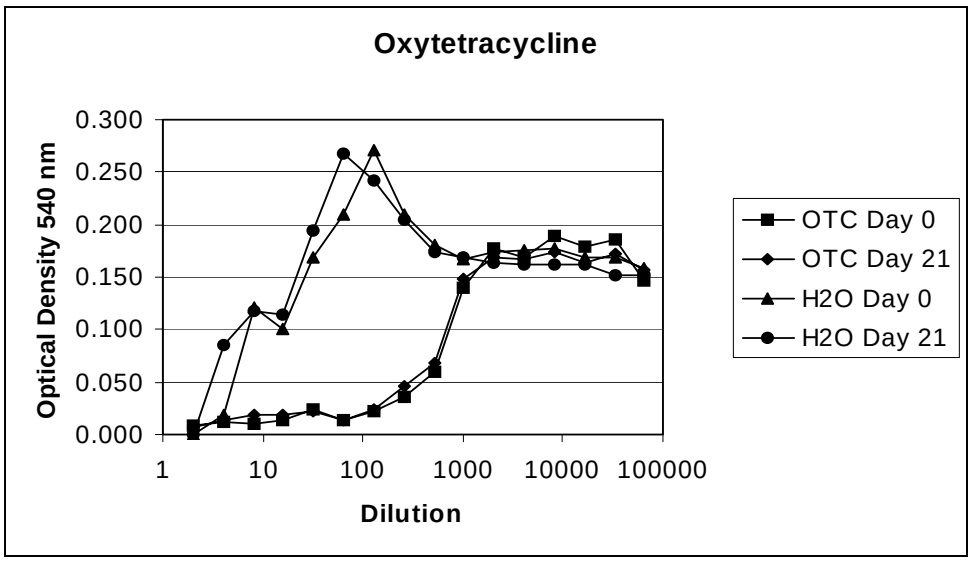
Figure 7.1 Optical density at 540 nm of cultures at each dilution






Trimethoprim
Optical Density 540 nm
0.300
0.250
0.200
0.150
0.100
0.050
0.000
1
10
100
1000
10000
100000
Dilution
Trime Day 0
Trime Day 21
H2O Day 0
H2O Day 21


Gramicidin
Optical Density 540 nm
0.300
0.250
0.200
0.150
0.100
0.050
0.000
1
10
100
1000
10000
100000
Dilution
Gram Day 0
Gram Day 21
H2O Day 0
H2O Day 21


Oxytetracycline
Optical Density 540 nm
0.300
0.250
0.200
0.150
0.100
0.050
0.000
1
10
100
1000
10000
100000
Dilution
OTC Day 0
OTC Day 21
H2O Day 0
H2O Day 21

7.2.2 Experiment 2 – Residues in honey stored at 35 °C.

Honey from the same container used in experiment 1 was added to specimen containers at 10 gm ± 0.5 gm per container. The equates to an ambient temperature volume of (10 gm ÷ 1.42 gm/ml) 7.04 ml per container. Antibiotics were added to the containers to a final concentration of 1 mg/ml and thoroughly mixed using a handheld cappuccino "frother". Each antibiotic was assayed in triplicate, using three independently prepared preparations. The containers were incubated in a light-proof, 35°C incubator. The containers were equilibrated at room temperature prior to each sampling.

Table 7.4 Honey plus antibiotic aliquots.

Antibiotic	Weight (gm)			Volume (ml)			Stock (mg/ml)	Stock Volume (µl)		
	1	2	3	1	2	3		1	2	3
Amp	14.25	14.23	14.21	10.035	10.021	10.007	100	100	100	100
Amox	14.25	14.28	14.25	10.035	10.056	10.035	92	109	109	109
Trimeth	14.20	14.27	14.29	10.000	10.049	10.063	40	250	251	252
Gram	14.24	14.27	14.28	10.028	10.049	10.056	25	401	402	402
Nisin	14.30	14.30	14.24	10.070	10.070	10.028	250	40	40	40
OTC	14.24	14.26	14.26	10.028	10.042	10.042	100	100	100	100

Samples were removed from the incubated honey/antibiotic mixtures at approximately weekly intervals. They were weighed (table 7.5) and diluted to 25% (v/v) honey in sterile water before being frozen at –20 °C awaiting further analysis.

Table 7.5 Sample weights (gm) for 35 °C honey plus antibiotic assay.

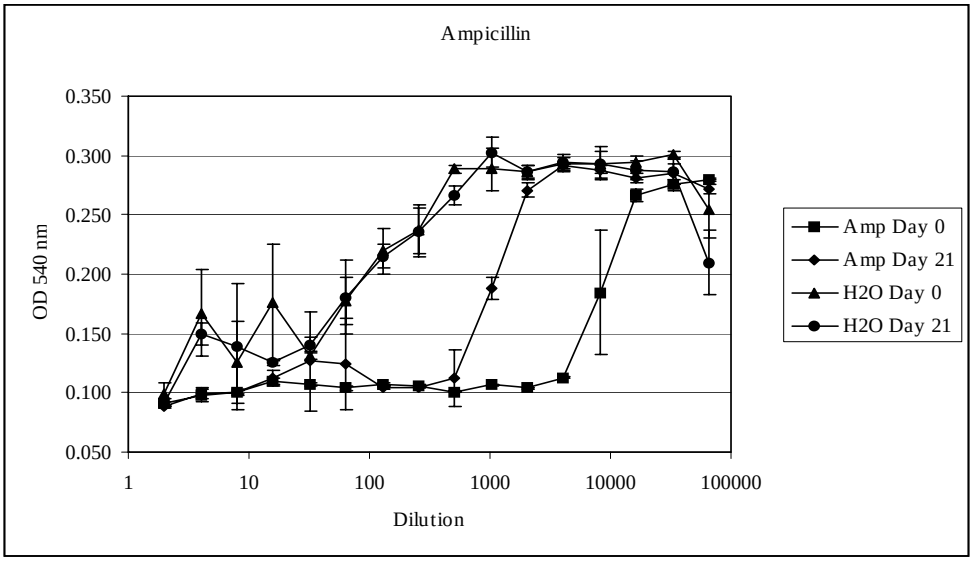
Day	No.	Amp	Amox	Trimeth	Gram	Nisin	OTC	H_2O
	1	0.453	0.451	0.438	0.438	0.437	0.435	0.425
0	2	0.430	0.456	0.423	0.432	0.420	0.422	0.437
	3	0.400	0.430	0.422	0.442	0.466	0.462	0.461
	1	0.409	0.413	0.417	0.445	0.457	0.412	0.430
9	2	0.415	0.439	0.425	0.393	0.461	0.409	0.453
	3	0.400	0.460	0.445	0.406	0.451	0.425	0.452
	1	0.351	0.450	0.414	0.400	0.391	0.402	0.432
14	2	0.370	0.391	0.432	0.396	0.408	0.422	0.430
	3	0.350	0.420	0.390	0.408	0.415	0.410	0.432
	1	0.423	0.365	0.352	0.370	0.376	0.388	0.347
21	2	0.347	0.373	0.335	0.380	0.361	0.347	0.365
	3	0.365	0.363	0.370	0.391	0.367	0.362	0.375
	1	0.355	0.394	0.375	0.390	0.419	0.465	0.380
29	2	0.393	0.365	0.374	0.410	0.437	0.379	0.410
	3	0.379	0.404	0.402	0.443	0.404	0.439	0.395
	1	0.355	0.364	0.345	0.323	0.346	0.355	0.355
35	2	0.343	0.397	0.391	0.398	0.338	0.379	0.399
	3	0.362	0.350	0.340	0.340	0.360	0.400	0.398
	1	0.361	0.369	0.368	0.371	0.402	0.407	0.346
42	2	0.385	0.382	0.382	0.391	0.387	0.377	0.331
	3	0.384	0.398	0.350	0.348	0.364	0.362	0.350
	1	0.413	0.423	0.343	0.389	0.441	0.428	0.412
65	2	0.372	0.399	0.354	0.409	0.386	0.373	0.376
	3	0.432	0.381	0.408	0.410	0.403	0.360	0.402
	1	0.402	0.378	0.324	0.347	0.357	0.368	0.375
70	2	0.360	0.360	0.345	0.385	0.372	0.405	0.369
	3	0.398	0.366	0.379	0.398	0.398	0.345	0.401
	1	0.522	0.511	0.543	0.512	0.510	0.519	0.522
91	2	0.522	0.506	0.547	0.522	0.549	0.551	0.532
	3	0.511	0.533	0.516	0.527	0.519	0.510	0.513

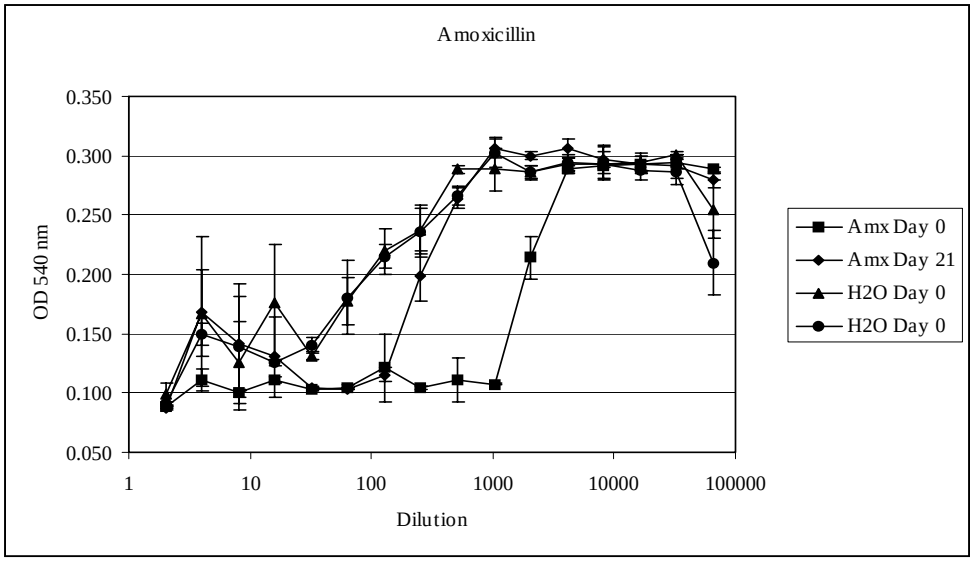
7.2.2.1 MIC assay of samples day 0 and day 21.

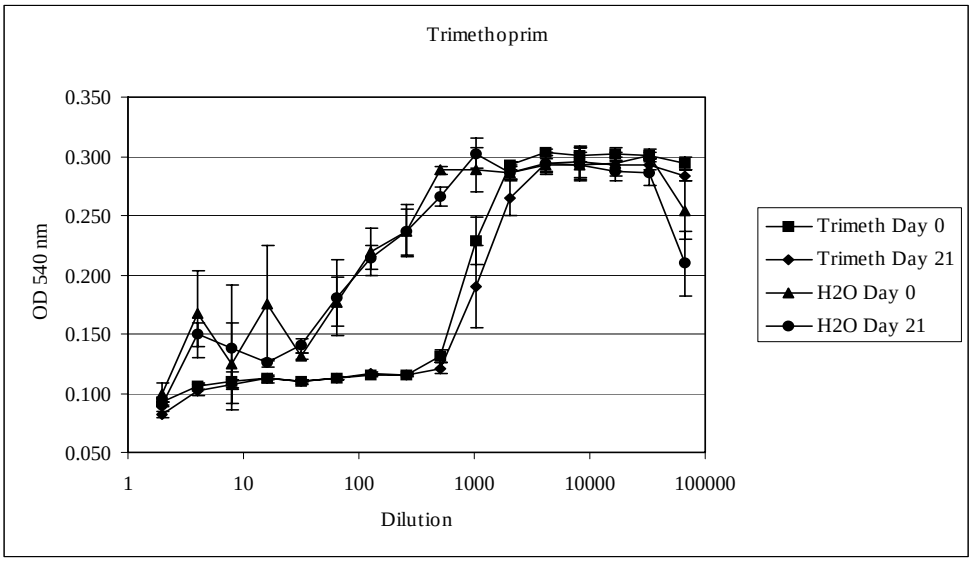
Samples taken from the antibiotic containing honey aliquots on days 0 and 21 were titrated by serial dilution across 16 wells of a microtitre plate (seven plates in total). 20 µl of late log *M. pluton* 96/A727/1 culture was added to each well and the plates was incubated under standard conditions for three days. Prior to analysis of the plates, the well contents were thoroughly mixed to resuspend bacterial growth that was sedimented on the bottom of the well. This was done using a Ratek orbital mixer (Ratek OM11), at 240 rpm for 5 minutes.

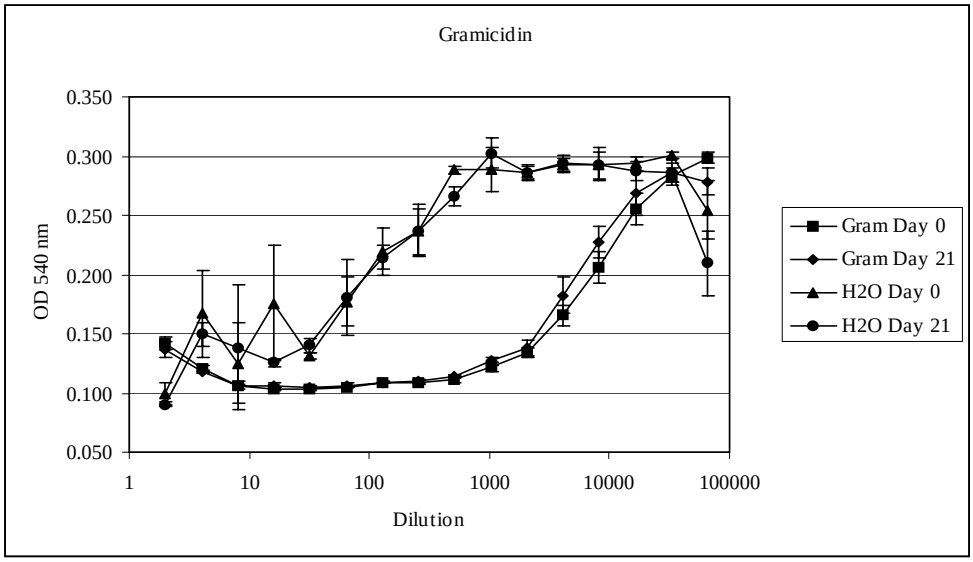
The turbidity (optical density) of each well culture was assayed at 540 nm and analysed using Microsoft Excel. The mean and standard deviation of the triplicate sample's absorbance values were plotted versus the dilution values.

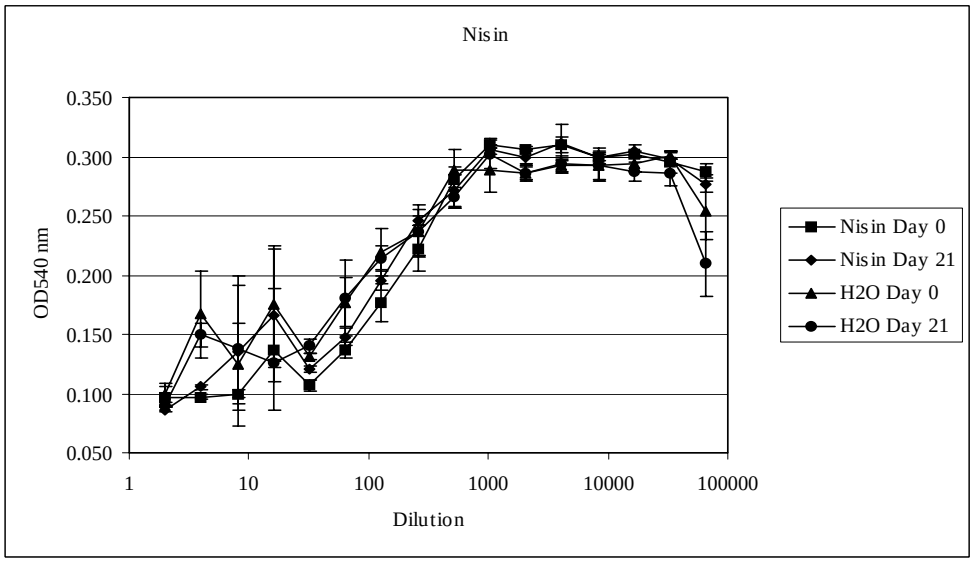
Figure 7.2 Optical density at 540 nm of cultures at each dilution

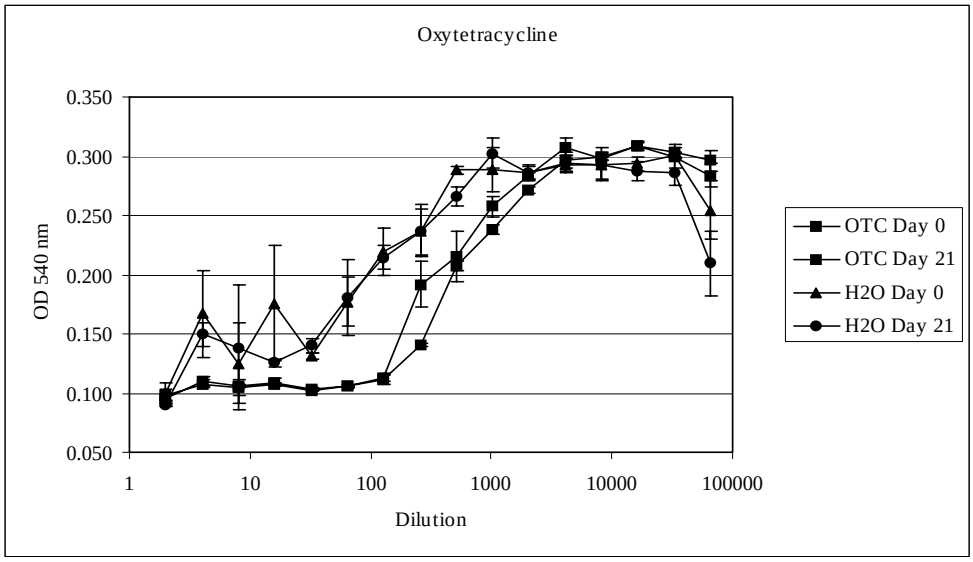







Gramicidin
0.350
0.300
0.250
0.200
0.150
0.100
0.050
OD 540 nm
1
10
100
1000
10000
100000
Dilution
Gram Day 0
Gram Day 21
H2O Day 0
H2O Day 21


Nisin
0.350
0.300
0.250
0.200
0.150
0.100
0.050
OD540 nm
1
10
100
1000
10000
100000
Dilution
Nisin Day 0
Nisin Day 21
H2O Day 0
H2O Day 21

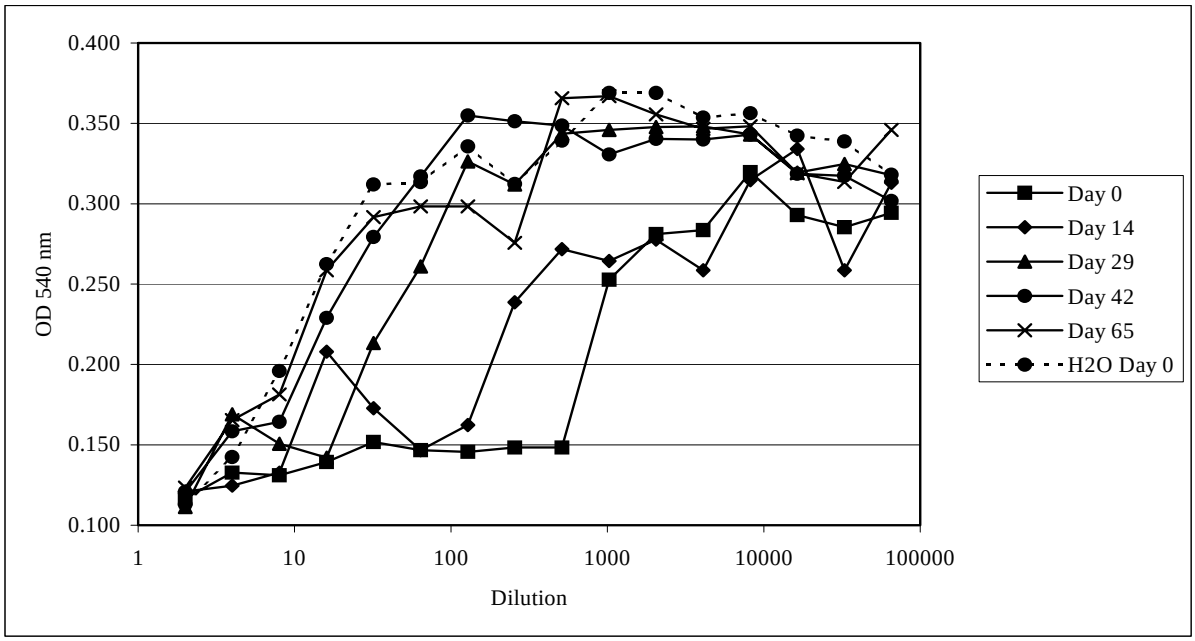

Oxytetracycline
0.350
0.300
0.250
0.200
0.150
0.100
0.050
OD 540 nm
1
10
100
1000
10000
100000
Dilution
OTC Day 0
OTC Day 21
H2O Day 0
H2O Day 21

Analysis of these results indicated that ampicillin and amoxicillin degraded significantly quicker that the other tested antibiotics. Further testing was performed on these antibiotics only.

7.2.2.2 MIC assay of amoxicillin samples between days 0 and 65.

Samples taken from honey containing amoxicillin, were analysed by MIC assay. The triplicate samples from days; 0, 14, 29, 42 and 65 were titrated by serial dilution across a microtitre plate (3 plates in total). 20 µl of *M. pluton* 96/A727/1 culture was added and the plates were incubated under standard conditions for 3 days. Following incubation, the culture turbidity was analysed and the results collated.

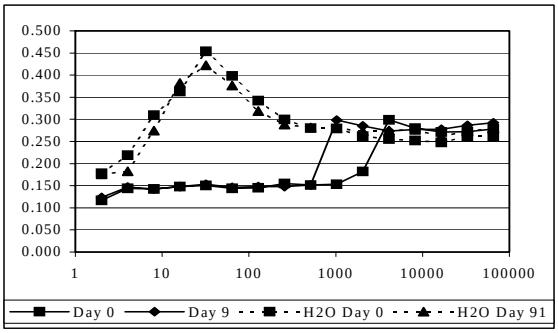
Figure 7.3 Mean optical density values (at 540 nm) for amoxicillin samples.

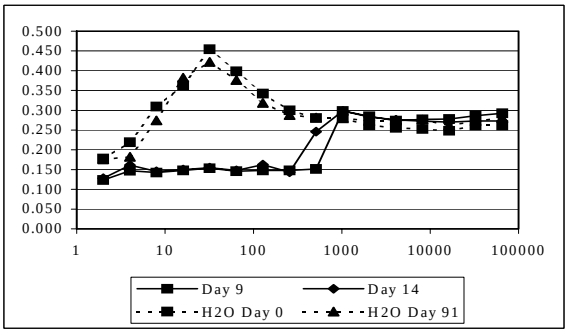


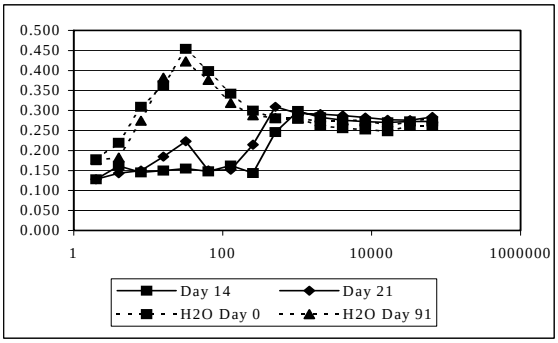
7.2.2.3 MIC assay of ampicillin and amoxicillin samples between days 0 and 91.

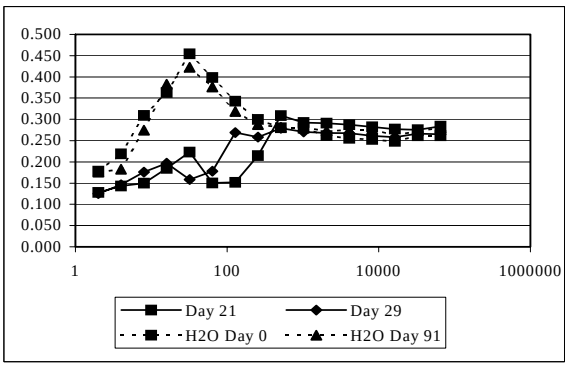
Ampicillin and amoxicillin triplicate samples for days; 0, 9, 14, 21, 29, 35, 42, 65, 70 and 91 were analysed by MIC assay. Samples were titrated by serial dilution across a microtitre plate (12 plates in total) and 20 µl of *M. pluton* 96/A727/1 culture added to each well. The plates were incubated under standard conditions for 5 days, following which, the culture turbidity of each well was assessed and the results collated.

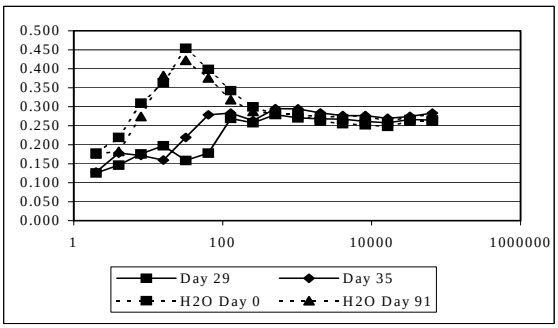
Figure 7.4 Mean optical density (at 540 nm) of ampicillin samples.

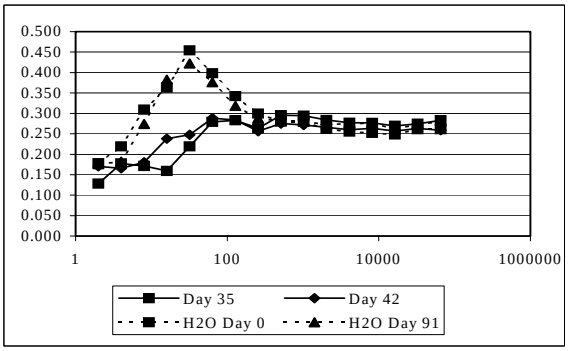


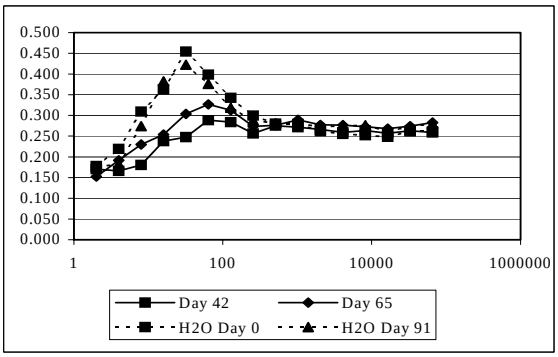


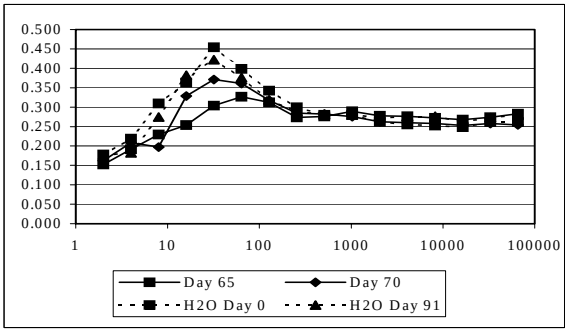












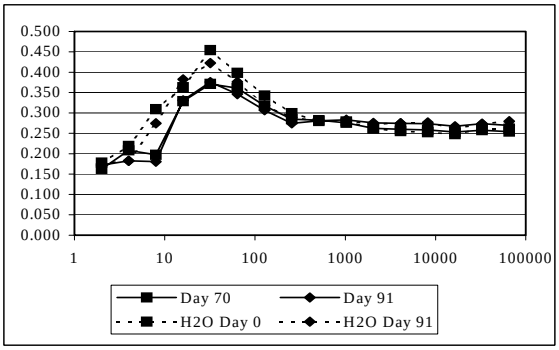
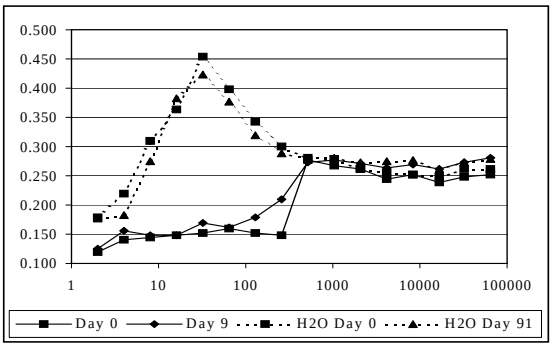
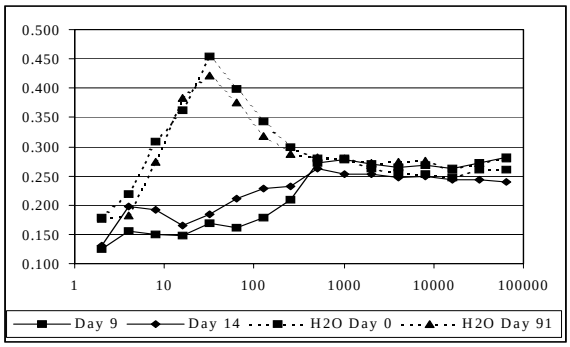
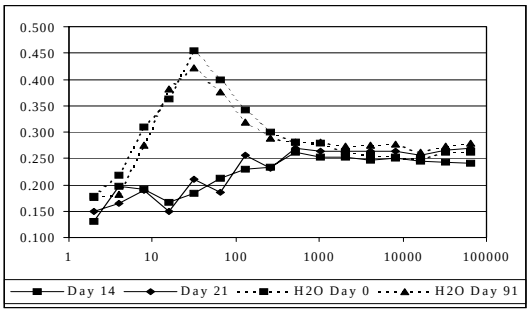
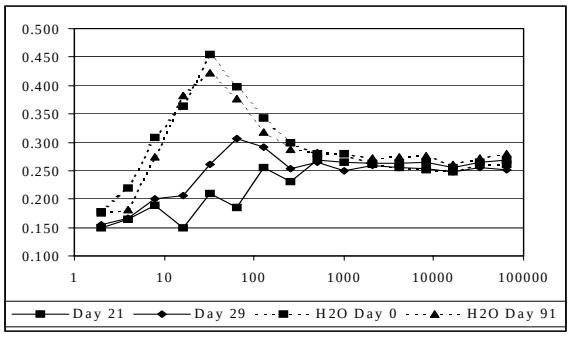


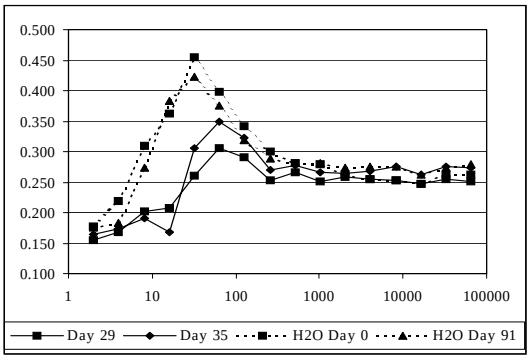
Figure 7.5 Mean optical density (at 540 nm) of amoxicillin samples.

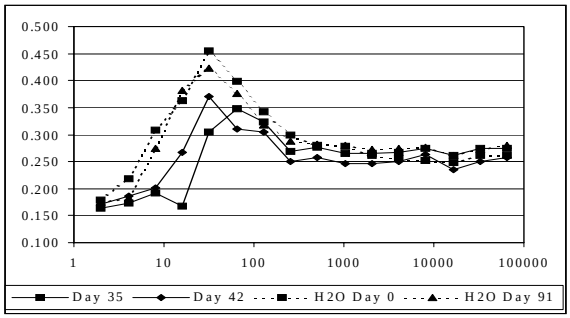


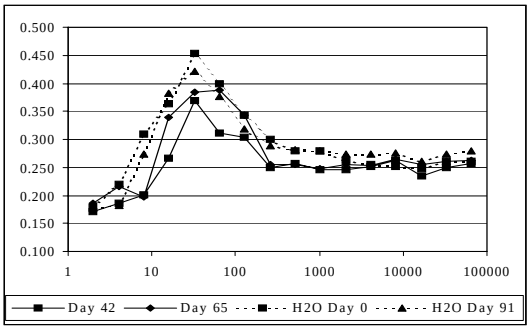


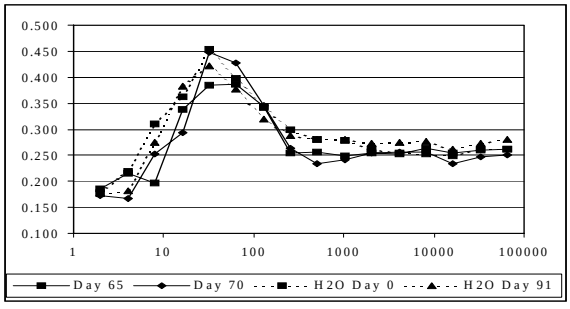


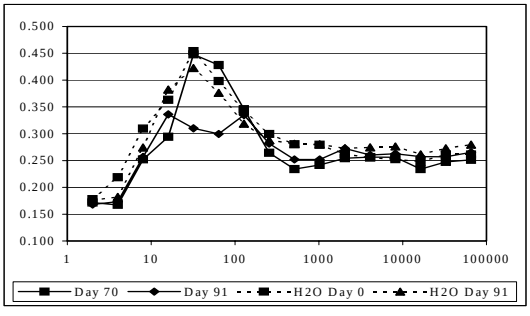












7.2.2.4 SNAP beta-lactam test kit analysis

One of the current field-test assays for the detection of beta-lactam based antibiotics in milk is the IDEXX SNAP test kit. This is an enzyme linked receptor binding assay that can detect a range of beta lactam and cephalosporin antibiotics, including ampicillin and amoxicillin. The test is calibrated to differentiate between samples that are above or below 4-5 parts per billion (µg/ml) for the antibiotics (EU and FDA maximum residue limits for these antibiotics in milk).

Ampicillin triplicate samples from days 21, 65 and 91 were analysed by SNAP test. 450 µl of each 25 % (v/v) honey sample was mixed with the reagent pellet and incubated for 5 minutes at 45 ^{o}C in a heated waterbath. This mixture was then added to the sample well of a pre-heated SNAP device. Once the solution had started to dissolve the dye in the activation circle, the SNAP activator was depressed and the reaction incubated at 45 ^{o}C for 7 minutes. All results were photographed within 10 minutes of the incubation period.

Figure 7.6 Interpretation of SNAP results (from IDEXX SNAP brochure).

NEGATIVE RESULT	**POSITIVE RESULT**
Sample spot is darker than or equal to the control spot	Sample spot is lighter than the control spot

Figure 7.7 SNAP results for ampicillin triplicate samples for days 21, 65 and 91. Refer to figure 7.6 for results interpretation.

The SNAP tests did not product a consistent result. Some reactions (65-1 and 65-2) did not produce a colourimetric response, while others produced differing results for different samples of the same time point (21-1, 21-2 and 21-3 and 91-1 and 91-2).

7.3 Discussion

7.3.1 Room temperature residue test.

The MIC type assay used on the honey and antibiotic mixtures was able to give a clear indication of the dilution at which the antibiotic was no longer able to inhibit bacterial growth. The control samples (honey plus sterile distilled water) also inhibited growth of *M. pluton*, however, this may be due to the dilution of essential nutrients in the first wells, as well as the inherent anti-bacterial properties of honey.

The minimum inhibitory dilutions (MID) for the day zero and day 21 samples were identical for all of the samples, indicated that breakdown of the antibiotics at room temperature was occurring very slowly. Honey samples were collected until day 104 and are available for future analysis, however, no further MIC assays were performed because of the lack of degradation and this experiment only used a single sample for each assay point. The subsequent experiment, storing honey at 35 ^{o}C used triplicate samples allowing some statistical analysis to be performed on the data.

7.3.2 35 ^{o}C residue test.

Samples were removed from the honey antibiotic mixture over 91 days of incubation. In the initial MIC assay of day zero and day 21 samples, there is no evidence of antibiotic degradation for gramicidin or trimethoprim samples. The oxytetracycline and nisin samples show a small difference in MIC profiles, however, the MIC results for the honey aliquots containing ampicillin and amoxicillin indicate considerable degradation of the antibiotics over the 21 day incubation period. Further experimentation was performed with only ampicillin and amoxicillin, as these appeared to be the most promising candidates for an alternative to oxytetracycline.

Assessment of the amoxicillin samples from day zero to day 65 indicated that by day 42 there is no apparent difference between the MIC profiles of the amoxicillin sample and the water day zero control. While immunochemical analysis would be required to confirm this result, it is noteworthy that this degradation has occurred from an initial concentration of 1 mg/ml, which is a far higher concentration than would be expected to be found in a treated hive. This is equivalent to an entire one gram oxytetracycline treatment sequestering into 1 litre (1.42 kg) of honey in the hive.

Further assessment of amoxicillin and ampicillin was performed on samples from day zero to day 91 of the incubation. The amoxicillin MIC results of this experiment confirmed the previous results. By day 42, the MIC profile of the amoxicillin sample is similar to the MIC profile of the water control samples. The ampicillin sample does not appear to have totally degraded, as there still appears to be some residual activity beyond the level assessed for the waster control.

7.3.3 SNAP test.

The SNAP test appears to interact poorly with the 25% honey sample, resulting in inconsistent reactions. Further experimentation with this is likely to solve this problem. For instance, if the high sugar content of the sample is causing the interference, honey diluted to 10% rather than 25% may give a more consistent result.

8 Discussion

All of the antibiotics licensed for veterinary medical use in Australia were examined to identify suitable candidates as alternative control means for EFB. Many antibiotics were rejected as being unsuitable, since they were not effective against *M. pluton*, were banned from use in the European Union, or were likely to result in long lasting residues.

Candidate antibiotics were selected from the remaining antibiotic types and their effectiveness against *M. pluton* tested. Initial DDA and MIC tests indicated that *M. pluton* was highly susceptible to beta lactam antibiotics.

The degradation rate of the selected antibiotics in honey was examined. Initial testing indicated amoxicillin and ampicillin degraded at a much higher rate than the other test antibiotics.

These antibiotics were tested for toxic effects on *Apis mellifera* larvae. While there was some discrepancy in the results between experiments, the *in vitro* reared larvae showed no ill effects from being fed a diet high in the two beta lactam antibiotics.

Further work is required to analyse the protection provided by the addition of ampicillin and amoxicillin to hives in EFB prone areas.

Ampicillin and amoxicillin have several advantages. There are readily available diagnostic tests for their detection, ranging from quick and easy "kitchen bench" type tests, to extremely sensitive and quantitative laboratory tests. The Charm 6600 analyser in capable of detecting ampicillin and amoxicillin to 50 and 100 parts per billion respectively. The antibiotics are widely used in veterinary medicine and their physio-chemical behaviour is widely documented.

The disadvantages of ampicillin and amoxicillin are that bacterial resistance can occur with widespread use. They are also currently in human medical use and exposure can cause allergic reactions in susceptible people. Exposure to ampicillin and amoxicillin by a susceptible individual may result in skin rashes, diarrhoea or anaphylactic shock.

While ampicillin and amoxicillin appear to be the best candidates for an oxytetracycline alternative, their misuse may result in antibiotic residue in exported honey, in the same manner as oxytetracycline. Ideally, a non-antibiotic alternative would be used to control EFB, one that did not result in residue contamination with usage. Two such methods are; bacteriophage therapy and quorum quenching.

Bacteriophage treatment

Bacteriophages are a class of bacteria infecting viruses (Adams, 1959). They have been used as therapeutic agents since the 1920's (Carlton, 1999), however their use declined with the advent of cheap and readily available antibiotics (in western countries) (Stone, 2002). They are ubiquitous organisms, being found in all natural environments (McNeil, 2002). Monovalent bacteriophages are highly specific for their host (active against one species or even one serotype of bacteria). They also have the additional advantage of being self-replicative.

Temperate bacteriophages specific for *P. larvae* have been identified in several bacterial strains (Gochnauer and L'Arrivee, 1969, Drobnikova, 1982), and claims have made of using phage therapy to control AFB outbreaks (Gochnauer, 1970).

A similar bacteriophage based therapeutic approach could be used to control *M. pluton* infections within bee colonies. Australian isolates of *M. pluton* are genetically homogeneous (Djordjevic *et.al*., 1999). Thus, a single bacteriophage strain with natural or induced lytic ability may be able to kill *M. pluton* populations in a hive environment. The *P. larvae* bacteriophages were acid labile and so were not expected to remain viable long in larval food or honey (Gochnauer, 1970). If this is the case with *M. pluton* bacteriophages, there may be a small window of opportunity for infection but importantly, there would be negligible residual contamination of honey and bee products.

Quorum quenching

Many species of bacteria are able to sense the cell density of their population. This ability (quorum sensing) is used as a sensor to control gene activation (Gochnauer, 1970, Miller, 2001). In Gram positive bacteria, autoinducing peptides are used as the signalling molecule (Sturme *et.al*., 2002), whereas in Gram negative bacteria acyl-homoserine lactone is used (Miller and Bassler, 2001).

Recently, researchers have examined the ability of several bacteria to disrupt the quorum sensing system of competing bacteria (quorum quenching). This quenching is done by either enzymatic degradation of the signalling molecule (Dong *et.al*., 2001,2002) or by providing an analogous molecule that interferes with the natural signalling system (Manefield *et.al*., 1999, Bauer, 2002).

A quorum sensing system has recently been identified in *Enterococcus faecalis*, a closely related bacteria to *M. pluton* (Nakayama *et.al*., 2001). A gene (*luxS*) encoding a key protein involved in quorum sensing signal production (Taga *et.al*., 2001, Schauder, 2001) has also been identified in *M. pluton* in this laboratory.

Quorum quenching may be of use in controlling EFB diseases outbreaks in honeybee colonies, however the actual signalling molecule used by *M. pluton* needs to be identified and a suitable disruption method or analogous compound identified.

9 Recommendations

This study has identified two candidates for the control of EFB: The beta lactam antibiotics; ampicillin and amoxicillin. These antibiotics should be scrutinised for their ability to protect *in vitro* reared larvae from infection by *M. pluton* and their effect on EFB in an *in-vivo* hive situations.

However due to a similar situation arising with residual contamination of honey, which would be lessened, but not eliminated, with these antibiotics, novel non-residual bacterial control methods like bacteriophage therapy and quorum quenching should be pursued.

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