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Fatty acids
– an alternative control strategy for honeybee diseases

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by Michael Hornitzky

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Foreword

There are two major bacterial diseases of honeybees in Australia; European foulbrood (EFB) caused by *Melissococcus pluton* and American foulbrood (AFB) caused by *Paenibacillus larvae* subsp. *larvae*.

EFB causes significant economic loss to the beekeeping industry in Australia and around the world. In Australia the impact of EFB on the bee farming industry necessitated the introduction of hive treatment in Australia in 1977 with the antibiotic oxytetracyline hydrochloride (OTC). In a recent RIRDC funded study [DAN136A; "Oxytetracycline sensitivity, diversity and study of *M. pluton* (European foulbrood)"] 104-*M. pluton* isolates from New South Wales, Victoria, Queensland, South Australia and Victoria were all demonstrated to be sensitive to low concentrations of OTC. However, recent advances in the detection of very low concentrations of OTC have resulted in the detection of OTC in honey following hive treatment with this antibiotic when the honey was tested after the recommended withholding period. These findings may have a serious impact on the marketing of honey as it is considered to be a natural product, and as such, an unadulterated food. Alterative methods for the control of EFB are needed if problems with residues are to be eliminated.

AFB is considered by many to be the most serious disease of honeybees in Australia. In many countries OTC is used in the treatment of this infection. However, in recent years OTC resistant *P. l. larvae* have been reported from the USA, Canada and Argentina. On mainland Australia OTC is not recommended for the treatment of AFB which is controlled by the destruction of infected hives or by the irradiation of hive material with Cobalt 60 once the bees have been killed.

Fatty acids are important in the development, nutrition and reproduction of honeybees. As control agents, fatty acids would be safe and environmentally-sound as well as non-toxic to man. They are actual foods and in the case of unsaturated fatty acids are essential for growth, development and health. Interestingly, many of the fatty acids also have bactericidal properties; however, little work has been carried out on their effect on honeybee bacterial pathogens.

The aim of this study was to determine the sensitivity of *M. pluton* and *P. l. larvae* isolates, collected from diverse geographical areas in Australia, to a range of fatty acids.

This project was funded from industry revenue, which is matched, by funds provided by the Federal Government.

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Executive Summary

European foulbrood and American foulbrood are two of the most important diseases of honeybees in Australia. The recent emergence of oxytetracycline hydrochloride (OTC) resistant strains of *Paenibacillus larvae* subsp. *larvae* (the cause of American foulbrood) in the USA, Canada and Argentina and the detection of OTC residues in honey following hive treatment with this antibiotic has foreshadowed the need for alternative control strategies to the OTC treatment of diseased honeybee colonies.

As control agents fatty acids are safe and environmentally-sound and have also been demonstrated to have antifungal and bactericidal properties. In this study the activity of 28 fatty acids was tested against 15 *P. l. larvae* isolates from New South Wales, South Australia, Tasmania, Western Australia, Queensland, Northern Territory, New Zealand and China. These fatty acids were also tested against 10 *M. pluton* (the cause of European foulbrood) isolates from New South Wales, South Australia, Tasmania, Queensland and the United Kingdom. Fifteen fatty acids had activity against *P. l. larvae* in the following decreasing order of activity; undecanoic, homo-γ-linolenic, capric, 7, 10, 13, 16-docosatetraenoic, 13, 16, 19-docosatrienoic, linolenic, linoleic, myristoleic, lauric, 11, 14-eicosadienoic, ricinoleic, 13, 16-docosadienoic, arachidonic, γ-linolenic and ricinelaidic acid. Eight fatty acids showed activity against *M. pluton* in the following decreasing order of activity; undecanoic, lauric, myristoleic, 13, 16, 19-docosatrienoic, homo-γ-linolenic, ricinoleic, ricinelaidic and myristic. There was generally little variation in the sensitivity of the various strains of either *P. l. larvae* or *M. pluton* to any specific fatty acid.

The use of fatty acids as an alternative means for the control of honeybee bacterial diseases seems feasible based on microbiological assays. Further testing of the activity of these fatty acids in artificially raised larvae and honeybee colonies is required before the application of such an alternative control strategy can be implemented.

1. Introduction

There are two major bacterial diseases of honeybees in Australia. European foulbrood (EFB) caused by *Melissococcus pluton* and American foulbrood (AFB) caused by *Paenibacillus larvae* subsp. *larvae*.

EFB causes significant economic loss to the beekeeping industry in Australia and around the world. In Australia the impact of EFB on the bee farming industry necessitated the introduction of hive treatment in Australia in 1977 with the antibiotic oxytetracyline hydrochloride (OTC). In a recent RIRDC funded study [DAN136A; "Oxytetracycline sensitivity, diversity and study of *M. pluton* (European foulbrood)"] 104-*M. pluton* isolates from New South Wales, Victoria, Queensland, South Australia and Victoria were all demonstrated to be sensitive to low concentrations of OTC (Hornitzky and Smith, 1999) . However, recent advances in the detection of very low concentrations of OTC using high performance liquid chromatography have resulted in the detection of OTC in honey following hive treatment with this antibiotic, even after testing beyond the currently recommended withholding period for honey. These findings may have a serious impact on the marketing of honey as it is considered to be a natural product, and as such, an unadulterated food. Alternative methods for the control of EFB are needed if problems with residues are to be eliminated.

AFB is considered by many to be the most serious disease of honeybees in Australia. In many countries OTC is used in the treatment of this infection. However, in recent years OTC resistant *P. l. larvae* have been reported from the USA, Canada and Argentina (Miyagi, 2000). On mainland Australia OTC is not recommended for the treatment of AFB which is controlled by the destruction of infected hives or by the irradiation of hive material with Cobalt 60 once the bees have been killed.

Fatty acids are important in the development, nutrition and reproduction of honeybees. The main fatty acids found in 6 day old larvae and adult bees are oleic, palmitic and stearic but others such as linolenic, myristic, lauric and linoleic are also found in lesser concentrations (Manning, 2001). Some of these fatty acids are also found in varying amounts in Australian eucalypts (Manning and Harvey, 2002). Interestingly, many of the fatty acids also have bactericidal properties (Kabara, 1978). Fatty acids such as oleic, palmitic, stearic, myristic, linoleic, linolenic and others have also been demonstrated to have activity against *Clostridium perfringens* and *Streptococcus pyogenes* (Kabara, 1978). As control agents, fatty acids would be safe and environmentally-sound (Feldlaufer *et al* (1993b). Not only are these agents non-toxic to man but are actual foods and in the case of unsaturated fatty acids are essential for growth, development and health (Kabara, 1978). However, little work has been carried out on their effect on honeybee bacterial pathogens and nearly all this work has focused on the effects of fatty acids on *P. l. larvae*.

In 1992 Shimanuki *et al* (1992) reported that chalkbrood mummies contained an antimicrobial substance which in laboratory tests inhibited the growth of *P. l. larvae* and *M. pluton*. Feldlaufer *et al* (1993a) subsequently identified this substance to be the fatty acid linoleic acid. They also demonstrated that linoleic acid was active against *P. l. larvae* but did not test its effect on *M. pluton*. Feldlaufer *et al* (1993b) also reported the effects of 38 fatty acids against a spore suspension of *P. l. larvae* and found that 24 fatty acids had some activity against this organism of which the most active were undecanoic, palmitoleic, linoleic and ricinoleic acids.

It would be useful to determine the *in vitro* effects of fatty acids on a range of Australian isolates of both the major honey bee pathogens if further consideration is to be given for their use in honey bee disease control in Australia

2. Objective

The objective of this proposal was to determine the sensitivity of a range of geographically diverse isolates of *M. pluton* and *P. l. larvae* to a range of fatty acids including linoleic acid.

3. Materials and Methods

3.1 Fatty acids

Twenty-eight saturated and unsaturated fatty acids obtained from Sigma-Aldrich were used is this study (Table 1). These were selected from the range of fatty acids tested by Feldlaufer *et al* (1993b). The fatty acids chosen for this study were those acids that had previously been shown to have high levels of activity and some that had shown little or no activity against *P. l. larvae*. Eighteen were chosen that showed an inhibition zone on bacteriological culture plates of 40 mm diameter or greater in sensitivity tests by Feldlaufer *et al* 1993b). The remainder (10) were chosen with zones less than 40 mm, or those that were inactive. Fatty acids with little or no activity against *P. l. larvae* were also tested to determine whether they had any activity against *M. pluton.* The storage and properties of each fatty acid are also provided (Table 1).

3.2 *P. l. larvae* and *M. pluton* and isolates

Fifteen *P. l. larvae* isolates from all Australian states [New South Wales (MN 00/B062), New South Wales (FD 366), South Australia (FD 374), South Australia (MN 01/6683/120),Tasmania (MN 89/5002/7),Tasmania (MN 89/5002/11), Western Australia (SO.1/2233/1) Western Australia (SO. 1/2310Q15/1/1), Queensland (MN89/0736), Queensland (194460/2), Victoria (89/2302/1), Victoria (89/2302/4)], the Northern Territory (MN 94/4560/1) and one isolate each from New Zealand (MN 88/1541) and China (MN 92/3092) were also used in this study

Ten *M. pluton* isolates from all Australian states excluding Western Australia, where EFB has not been reported were used in this study [New South Wales (MN 96/2986/10), New South Wales (MN 96/2152/A), South Australia (MN 96/A964/L7463/L8-2), South Australia (MN 00/D578/51), Queensland (MN 96/A345/1), Queensland (MN 96/A345/3), Victoria (MN 00/8938/11), Victoria (MN 01/6215), Tasmania (MN 97/0331/D2)]. An isolate from the United Kingdom was also used.

3.3 Bioassays

3.3.1 Preparation of fatty acid stock solutions

Stock solutions of 10 mg/mL and serial dilutions (1:10, 1:100 and 1:1000) of each of the fatty acids listed in Table 1 were prepared in methanol.

3.3.2 Initial screen for fatty acid antibacterial activity

Preparation of discs containing fatty acids
Blank anti-microbial susceptibility test discs (Oxoid) (0.60 cm diameter) were placed into 0.658 cm diameter flat-bottomed ELISA plates. Twenty five µl of the test solutions were applied with an electronic pipette in final concentrations of 250, 25, 2.5 and 0.25 µg of fatty acid per disc. The ELISA plates facilitated the absorption of the fatty acid solution by the paper discs after which the discs were air-dried in the ELISA plates

TABLE 1: List of fatty acids tested

Fatty acid carbon and double bond number [a]	Fatty acid	Position of double bond [b]	Storage and properties
6:0	n-caproic		room temperature, corrosive
8:0	caprylic		room temperature, corrosive
9:0	nonanoic		0-8°
10:0	capric		room temperature
11:0	undecanoic		room temperature
12:0	lauric		room temperature
13:0	tridecanoic		<0°, desiccate
14:0	myristic		<0°, powder
14:1	myristoleic	9	<0°, light sensitive
16:0	palmitic		room temperature
16:1	palmitoleic	9	<0°
18:0	stearic		0-8°
18:1	petroselinic	6	<0°
18:1	oleic	9	<0°, air/light sensitive
18:1	ricinoleic	9 (12-hydroxy)	<0°, air/light sensitive
18:1	ricinelaidic	9t (12hydroxy)	<0°
18:2	linoleic	9,12	<0°, air/light sensitive
18:2	linolelaidic	9t,12t	<0°,air/light sensitive, not fully tested
18:3	linolenic	9,12,15	<0°
18:3	y-linolenic	6,9,12	<0°, air/light sensitive
20:2	11,14-eicosadienoic		<0°, air/light sensitive, combustible, not fully tested
20:3	homo-y-linolenic	8,11,14	<0°, air/light sensitive, combustible.
20:4	arachidonic	5,8,11,14	<0°,air/moisture sensitive, reactive unstable compound
22:1	brassidic	13t	<0°, air/light sensitive
22:2	13,16-docosadienoic		<0°, air/light sensitive, not fully tested
22:3	13,16,19-docosatrienoic		<0°, air/light sensitive, not fully tested
22:4	7,10,13,16-docosatetraenoic		<0°, air/light sensitive, not fully tested
22:6	4,7,10,13,16,19-docosahexenoic		<0°, air/light sensitive, combustible

[a] The first number is the number of carbons followed by the number of double bonds
[b] All configurations are *cis* unless noted as *trans (t)*

Seeding of plates with test isolates
For *P. l. larvae*, isolates grown on 7% sheep blood agar (SBA) (Hornitzky and Clark, 1991) (except that nalidixic acid was not incorporated into the agar) and incubated at 37^{o} C for 2 to 3 days in an atmosphere containing 5% CO_2 were used as inocula. For *M. pluton* isolates were grown on Bailey's medium (Bailey, 1957) and incubated at 37^{o} C for 4 to 6 days under anaerobic conditions containing 10% CO_2 were used as inocula. Three single lines of inoculum of each isolate were streaked across an agar plate of the prescribed medium. Paper discs containing each of the fatty acids were placed at intervals on this line. Only rates of 250 µg per disc (10 mg/ml) were used in the initial screen. To determine whether fatty acids had activity against *M. pluton* seeded plates were incubated as described above for 4 days and the *P. l. larvae* seeded plates were incubated for 3 days as described above. Those fatty acids, which produced a zone of inhibition with *M. pluton* and/or *P. l. larvae*, were used in the quantitative bioassay described below. Discs inoculated with methanol were used as a negative control.

3.3.3 Quantitative bioassay

This bioassay was essentially that described by Feldlaufer *et al* (1993a). For *P. l. larvae* fresh two to three day old cultures and four to six day old cultures of *M. pluton* prepared as described above were used for the assays. For *P. l. larvae* three to six colonies 1.5 to 3.0 millimetres in diameter were swiped with a sterile bacteriological culture loop into 4 mL of sterile saline to give a suspension of 3 x 10^{8} organisms/mL (McFarland 1). Six to ten *M. pluton* colonies, one to two millimetres in diameter were used to achieve the same suspension concentration. These suspensions were then poured onto the agar plates by the standard bacteriological CDS sensitivity test method (Bell *et al*, 1999). Following the preparation of these plates discs containing 250, 25, 2.5, 0.5 µg of each fatty acid were placed on the plates. Disc inoculated with methanol only were used as a negative control. *M. pluton* cultures were grown on Bailey's medium (Bailey 1957) to which 7% sheep blood had been added and *P. l. larvae* was cultured on SBA and these plates were incubated for 4 and 3 days respected as described above. Four replicates were carried out with each fatty acid with each isolate used in this study. Discs inoculated with methanol were used as a negative control.

After the appropriate incubation time measurements of the inhibition zone were carried out with Toledo Vita calipers. Some plates had zones of reduced growth of the size of the bacterial colony, i.e. the colony was smaller, and/or they had a reduced amount of bacterial growth (less dense). However, these were not included in the zone of inhibition. Only the area with zero growth was measured. If there was any contamination or doubtful results the tests were repeated. All serial dilution rates were tested with the first replication, however on subsequent replications only the dilutions that gave a positive result and the dilution thereafter were tested and recorded.

4. Results

4.1 Screening test results

P. l. larvae
The following fatty acids were demonstrated to have significant antibacterial activity against *P. l. larvae*: Undecanoic, lauric, capric, homo-y-linolenic, ricinoleic, linoleic, myristoleic, y-linolenic, arachidonic, linolenic, ricinelaidic, 4,7,10,13,16,19 docosahexanoic, 11,14 eicosadienoic, 13,16,19 docosatrienoic, 13,16 docosadienoic, 7,10,13,16 docosatetraenoic, myristic.

M. pluton
The following fatty acids were demonstrated to have significant antibacterial activity against *M. pluton*: Undecanoic, lauric, homo-y-linolenic, ricinoleic, myristoleic, ricinelaidic, 13,16,19 docosatrienoic, myristic.

All discs inoculated with methanol did not produce any inhibitory effects on both bacterial species.

4.2 Quantitative assay results

P. l. larvae
Fifteen fatty acids were shown to have antibacterial activity against *P. l. larvae*. The fatty acids and the zone diameters (average of four replicates) for each of the strains is presented in Tables 2a and 2b. All these fatty acids only showed activity at the highest concentration (250 µg) except for myristoleic and lauric acids which also showed reduced activity with discs containing 25 µg of the acid.

The mean inhibition zone of all the isolates for each fatty acid is presented in Table 3 along with the results reported using the Feldlaufer method.

M. pluton
Eight fatty acids were demonstrated to have antibacterial activity against *M. pluton* (Table 4). The fatty acids and the zone diameters (average of four replicates) for each of the strains is presented in Table 4. All eight fatty acids only showed activity at the highest concentration used (250 µg).

The mean inhibition zone of all the isolates for each fatty acid is presented in Table 3.

Comparative sensitivity of different strains of the same bacterial pathogen species
The sensitivity of the 15 *P. l. larvae* and 10 *M. pluton* isolates to each of the fatty acids was generally quite consistent for each species. There were no cases where any strain of either species was resistant or sensitive when others of the same species were sensitive or resistant.

The *P. l. larvae* isolates of both Chinese and New Zealand origin were shown to have similar sensitivity patterns to the isolates of Australian origin. This was also the case for *M. pluton* from the United Kingdom which had similar sensitivity patterns to the *M. pluton* isolates of Australian origin.

TABLE 2a. Sensitivity (diameter of zone in mm) of *P. l. larvae* strains to specific fatty acids (250 ug discs)

Isolate	capric	undecanoic	lauric	myristoleic	ricinoleic	ricinelaidic	linoleic	y-linolenic
NSW (MN 00/B062)	16	17	12	13	12	8	14	9
NSW (FD 366)	15	18	12	9	11	8	14	10
SA (FD 374)	18	17	13	13	12	9	14	10
SA (MN01/6683/120)	15	19	12	13	11	9	14	9
TAS (MN89/5002/7)	19	19	12	12	11	8	13	9
TAS (MN89/5002/11)	13	17	10	13	11	8	13	8
WA (SO.1/2233/1)	16	17	13	13	11	8	14	9
WA (SO.1/2310Q15/1/1)	17	19	13	15	11	9	13	10
QLD (MN89/0736)	15	16	11	13	11	8	13	10
QLD 194460/2	14	17	13	16	11	9	15	9
NT (MN 94/4560/1)	16	16	12	12	11	8	13	9
NZ (MN 88/1541)	12	17	13	13	12	8	14	9
VICTORIA (89/2302/1)	24	19	13	12	12	9	13	9
VICTORIA (89/2302/4)	17	21	13	15	12	9	18	10
CHINA (MN 92/3092)	14	17	12	12	11	8	13	9

TABLE 2b. Sensitivity (diameter of zone in mm) of *P. l. larvae* strains to specific fatty acids (250 ug discs)

Isolate	11,14 – eicosadienoic	linolenic	homo-y-linolenic	arachidonic	13,16-docosadienoic	13,16,19-docosatrienoic	7, 10, 13, 16 docosatetraenoic
NSW (MN 00/B062)	11	17	18	9	11	NA[a]	18
NSW (FD 366)	11	15	18	8	10	15	17
SA (FD 374)	11	15	16	9	10	15	16
SA (MN01/6683/120)	12	15	14	8	10	15	16
TAS (MN89/5002/7)	11	15	17	11	10	16	16
TAS (MN89/5002/11)	10	16	16	8	10	14	16
WA (SO.1/2233/1)	11	15	17	9	10	16	16
WA (SO.1/2310Q15/1/1)	12	12	17	8	10	16	14
QLD (MN89/0736)	12	13	17	8	10	15	15
QLD 194460/2	12	17	19	9	10	16	17
NT (MN 94/4560/1)	12	15	16	8	10	16	16
NZ (MN 88/1541)	12	14	18	8	10	16	16
VICTORIA (89/2302/1)	13	14	18	8	11	NA	16
VICTORIA (89/2302/4)	13	15	18	9	12	NA	16
CHINA (MN 92/3092)	11	14	17	8	10	15	16

NA[a] = Fatty acid no longer available

TABLE 3: Fatty acids and their activity against *P. l. larvae* and *M. pluton*

Fatty acid carbon and Double bond number	Fatty acid	Inhibition zones (mm)		
		Feldlaufer *et al* (1993b) ***P. l. larvae***	**This study** ***P. l. larvae***	**This study** ***M. pluton***
6:0	n-caproic	Inactive	Inactive	Inactive
8:0	caprylic	18	Inactive	Inactive
9:0	nonanoic	40	Inactive	Inactive
10:0	capric	54	16	Inactive
11:0	undecanoic	60	187	12
12:0	lauric	80	12	12
13:0	tridecanoic	40	Inactive	Inactive
14:0	myristic	10	Inactive	8
14:1	myristoleic	80	13	10
16:0	palmitic	Inactive	Inactive	Inactive
16:1	palmitoleic	72	Inactive	Inactive
18:0	stearic	Inactive	Inactive	Inactive
18:1	petroselinic	Inactive	Inactive	Inactive
18:1	oleic	Inactive	Inactive	Inactive
18:1	ricinoleic	60	11	7
18:1	ricinelaidic,	45	8	7
18:2	linoleic	68	14	Inactive
18:2	linolelaidic	40	Inactive	Inactive
18:3	linolenic	52	15	Inactive
18:3	y-linolenic	52	9	Inactive
20:2	11,14-eicosadienoic	Inactive	12	Inactive
20:3	homo-y-linolenic	40	17	7
20:4	arachidonic	50	9	Inactive
22:1	brassidic	Inactive	Inactive	Inactive
22:2	13,16-docosadienoic	28	10	Inactive
22:3	13,16,19-docosatrienoic	40	15	8
22:4	7,10,13,16-docosatetraenoic	50	16	Inactive
22:6	4,7,10,13,16,19-docosahexenoic	52	Inactive	Inactive

TABLE 4. Sensitivity (diameter of zone in mm) of *M. pluton* to specific fatty acids (250 ug discs)

	undecanoic	lauric	myristic	myristoleic	ricinoleic	ricinelaidic	13,16,19-docosatrienoic	homo-y-linolenic
NSW (MN 96/2986/10)	11	12	8	9	7	7	8	t
NSW (MN96/2152/A)	12	12	8	10	7	7	8	t
SA(MN96/A964	13	11	7	10	7	7	8	7
SA(MN00/D578/51)	12	13	8	11	7	7	8	t
TAS (MN97/0331/D2)	12	15	7	10	7	7	8	t
QLD (MN96/A345/1)	13	11	9	10	T	T	8	7
QLD (MN96/A345/3)	13	15	7	10	7	7	8	t
VIC (MN00/8938/11)	12	11	7	9	7	7	8	t
VIC(MN01/6215)	12	12	8	10	7	7	8	t
UK slide	12	12	7	11	7	7	8	t

5. Discussion

AFB and EFB cause significant economic loss to the beekeeping industry not only in Australia but in most major beekeeping areas worldwide. EFB infections are usually treated with OTC and AFB infections in many countries are also treated with OTC although on mainland Australia AFB is controlled by the destruction of diseased colonies or the irradiation of infected equipment after the bees have been destroyed. The development of OTC resistant strains of *P. l. larvae* in the USA, Canada and Argentina (Miyagi, 2000) and the detection of OTC residues in honey indicates that alternative control methods for these diseases would be useful.

Fatty acids are not only important in honeybee development, nutrition and reproduction but also have antifungal and bactericidal properties. Fatty acids and derivates can effect microorganisms by affecting their lipid membranes (envelopes). These effects lead to pertubations of the lipid phase and subsequent changes in the organisms permeability (Kabara, 1978). As control agents, fatty acids would be safe and environmentally-sound (Feldlaufer *et al* (1993b). Not only are these agents non-toxic to man but are actual foods and in the case of unsaturated fatty acids are essential for growth, development and health (Kabara, 1978). A broad range of fatty have been tested against a range of bacteria; however, little work has been carried out their effect on honeybee bacterial pathogens and most of this work has been carried out on *P. l. larvae* (Feldflaufer *et al*, 1993b).

The aim of this study was to test a range of fatty acids against a diverse range of Australian honeybee bacterial pathogens. Some overseas isolates were also included in this study for comparative purposes. This study has demonstrated that both *P. l. larvae* and *M. pluton* are sensitive to a range of fatty acids. Of the 28 fatty acids tested in this study 15 showed activity against *P. l. larvae*. On average, in decreasing order of activity the six most active were undecanoic, homo-y-linolenic, capric, 7,10,13,16-docosatetraenoic, linolenic and 13,16,19-docosatrienoic (Table 3). Of the 28 tested in this study 21 showed activity against *P. l. larvae* in Feldlaufer's study. The six most active in that study were lauric, myristoleic, palmitoleic, linoleic, ricinoleic and undecanoic.

The activity of the fatty acids against *P. l. larvae* was usually less than that reported by Feldlaufer *et al* (1993b). This was noted for capric, undecanoic, lauric, myristoleic, ricinoleic, ricinelaidic, linoleic, linolenic, y-linolenic, homo-y-linolenic, arachidonic, 13, 16-docosadienoic and 13, 16, 19-docosatrienoic (Table 2). There were six fatty acids which were active against *P. l. larvae* using the Feldlaufer *et al* (1993b) assay which did not show any activity in the assay used in this study (caprylic, nonanoic, tridecanoic, mystiric, palmitoleic and linolelaidic). However, there was one acid (11, 14 eicosadienoic) which did not show activity using the Feldlaufer method but did using the method used in this study.

In this study using *P. l. larvae* we modified the culture conditions used by Feldlaufer *et al* (1993b) to enhance the growth of *P. l. larvae* with the following changes; brain heart infusion agar was replaced with sheep blood agar and the incubation conditions had been changed to include CO_2 . Hornitzky and Nicholls (1993) had previously demonstrated that the addition of CO_2 to the culture conditions improves the growth of *P. l. larvae.* These changes were introduced to more closely mimic the conditions of the larval gut. To ensure that our method did not reduce the efficacy of the test compared with the Felduafer method we carried out tests on the fatty acids using the identical methodology used by Feldlaufer *et al* (1993b). Even when their methodology was used the zone of inhibition sizes were 33% of that obtained by them for undecanoic acid, 14% for lauric acid and 20% for that obtained for linoelic acid.

It should also be noted that serum albumin, cholesterol, lecithin, and Ca and Mg ions antagonise the inhibitory action of various fatty acids (Galbraith *et al* 1971). Kolmer, Rule and Madden (1934) reported that a 20% solution of sodium ricinoleate was completely bactericidal for *Staphylococcus aureus* in an exposure of 5 minutes, yet the higher concentration of a 10% solution was not completely bactericidal in exposures as long as 1 hour when tested. Many of the fatty acids including

linoelic acid are sensitive to air and light (Table 1). The sensitivity of fatty acids to decomposition should be taken into consideration when their use is considered for the treatment of honeybee diseases.

The only report that fatty acids may have activity against *M. pluton* was produced by Shimanuki *et al* (1992). They demonstrated that an extract from chalkbrood "mummies" inhibited *M. pluton*. Feldlaufer *et al* (1993a) subsequently demonstrated that the predominant antimicrobial in the fungus *Ascosphaera apis* (the cause of chalkbrood) was 9,12-octadecadienoic acid (linoleic acid). They demonstrated that this fatty acid inhibited the growth of cultures of *P. l. larvae* but did not test this fatty acid against *M. pluton*. The work reported here is the first study where a range of pure fatty acids has been tested against *M. pluton*. Of the 28 fatty acids, 8 showed activity against *M. pluton* (undecanoic, lauric, myristic, myristoleic, ricinoleic, ricinelaidic, homo-y-linolenic and 13, 16, 19-docsatrienoic) (Table 4). All these fatty acids except myristic also showed activity against *P. l. larvae.*

The sensitivity of the 15 *P. l. larvae* and 10 *M. pluton* isolates to each of the fatty acids was generally quite consistent for each species. There were no cases where any strain of either species was resistant or sensitive when others of the same species were sensitive or resistant. The *P. l. larvae* isolates of both Chinese and New Zealand origin were shown to have similar sensitivity patterns to the isolates of Australian origin. This was also the case for *M. pluton* from the United Kingdom which had similar sensitivity patterns to the *M. pluton* isolates of Australian origin. This general uniformity of sensitivity has been also been demonstrated in the sensitivity of *M. pluton* to OTC (Hornitzky and Smith, 1999). This observation indicates that if a fatty acid is considered to be appropriate in the treatment of honeybee colonies that all strains of the pathogen are likely to be sensitive to its use.

There are a number of options which could be used to determine what effects fatty acids have on EFB and AFB.

1. Treatment of diseased hives with candidate fatty acids. This method would require the use of a suitable carrier for the fatty acid.
2. Use of candidate fatty acids in larval assays where the larvae are also feed *P. l. larvae* or *M. pluton.*
3. Move diseased hives onto flora which is rich in fatty acids which have been demonstrated to have significant antibacterial activity against one or both of the pathogens.
4. Feed infected hives with pollens containing high concentration of fatty acids which have been demonstrated to have antibacterial activity against the honeybee pathogen under test.

6. Implications

This study has identified candidate fatty acids that, based on microbiologial assays, could be used in the control of AFB and EFB. These fatty acids have been listed in Table 5 in decreasing order of activity for each honeybee pathogen.

TABLE 5. Sensitivity of honeybee bacterial pathogens to fatty acids in decreasing order of activity

Fatty acids inhibitory to *P. l. larvae*	Fatty acids inhibitory to *M. pluton*
Undecanoic homo-γ-linolenic capric 7, 10, 13, 16-docosatetraenoic 13, 16, 19-docosatrienoic linolenic linoleic myristoleic lauric 11, 14-eicosadienoic ricinoleic 13, 16-docosadienoic arachidonic γ-linolenic ricinelaidic acid	undecanoic lauric myristoleic 13, 16, 19-docosatrienoic myristic homo-γ-linolenic ricinoleic ricinelaidic

The use of a fatty acid treatment would be best suited for the control of EFB. However, before fatty acids can be used in the treatment of honeybees, factors such as a carrier for the fatty acid, the stability of the fatty acid to be tested and appropriate doses must be determined.

7. References

Bailey, L (1957) The cause of European foulbrood. *Bee World* 38: 89-95.

Bell, SM; Gatus, BJ; Pham, JN (1999) Antibiotic susceptibility testing by the CDS method. *In* A Concise Laboratory Manual 1999. Arthur Productions Pty Ltd, Sydney p6

Feldlaufer, MF; Lusby, WR, Knox, DA; Shimanuki (1993a) Isolation and identification of linoleic acid as an antimicrobial agent from the chalkbrood fungus, *Ascosphaera apis*. *Apidologie* 24: 89-94.

Feldlaufer, MF; Knox, DA; Lusby, WR,; Shimanuki (1993b) Antimicrobial activity of fatty acids against *Bacillus larvae*, the causative agent of American foulbrood disease. *Apidologie* 24: 95-99.

Galbraith, H; Miller, TB; Paton, AM; Thompson, JK (1971) Antibacterial activity if long chain fatty acids and the reversal with calcium, magnesium, ergocaliferol and cholesterol. *Journal of Applied Bacteriology* 34(4): 803-813.

Hornitzky, MAZ; Clark, S. (1991) Culture of *Bacillus larvae* from bulk honey samples for the detection of American foulbrood. *Journal of Apicultural Research* 30(1) 13-16.

Hornitzky, MAZ; Nicholls, PJ. (1993) J medium is superior to sheep blood agar and brain heart infusion agar for the isolation of *Bacillus larvae* from honey samples. *Journal of Apicultural Research* 32(1) 51-52.

Hornitzky, MAZ; Smith L (1999) The sensitivity of Australian *Melissococcus pluton* isolates to oxytetracycline hydrochloride. *Australian Journal of Experimental Agriculture* 39(7): 881-883.

Kabara, JJ (1978) Fatty acids and derivatives as antimicrobial agents – a review. *In: The Pharmacological Effects of Lipids* (Kabara JJ ed) *Am Oil Chem Soc. Champaign, IL*

Kolmer, JA; Rule; AM; Madden (1934) Chemotherapeutic studies with sodium ricinoleate. *Journal of Laboratory and Clinical Medicine* 19: 972.

Manning, R (2001) Fatty acids in pollen: a review of their importance for honey bees. *Bee World* 82(2): 60-75.

Manning, R; Harvey, M (2002) Fatty acids in honeybee-collected pollens from six endemic Western Australian eucalypts and the possible significance to the Western Australian beekeeping industry. *Australian Journal of Experimental Agriculture* 42 (217-223).

Miyagi, T (2000) Verification of oxytetracycline-resistant American foulbrood pathogen *Paenibacillus larvae* in the United States. *Journal of Invertebrate Pathology* 75: 95-96.

Shimanuki, H; Knox, DA, Feldlaufer, MF (1992) Honey bee disease interactions: The impact of chalkbrood on other honey bee brood diseases *American Bee Journal.* 132: 735-736.