# EFFICACY OF CHLORINE DIOXIDE FUMIGATION ON THE SPORES OF *PAENIBACILLUS LARVAE*, THE CAUSATIVE AGENT OF AMERICAN FOULBROOD DISEASE IN HONEYBEES

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Osama Salih Mahdi

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**Title**

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

**MASTER OF SCIENCE**

SUPERVISORY COMMITTEE:

Dr. John McEvoy
Chair

Dr. Teresa Bergholz

Dr. Birgit Pruess

Dr. Kendra Greenlee

Approved:

October 2, 2019
Date

Dr. John McEvoy
Department Chair

## ABSTRACT

Honeybees (*Apis mellifera*) play a critical role in agricultural pollination. However, their health and numbers are in decline. A major cause of this decline is bacterial diseases, of which American foulbrood disease (AFB) is particularly important and troubling. Since the causative agent, *Paenibacillus larvae*, is spore forming, it can resist antibiotics, many disinfectants, and environmental stresses.

We provide protocols and methods for the growth, maintenance, sporulation, and germination of *P. larvae*. Also, this study investigates the sporicidal activity of $ClO_2$ on *P. larvae* spores. The gas efficacy depends on treatment time and gas level. The effective level was 645-811 ng/ml $ClO_2$ for 30 min, 191-198 ng/ml for 1 hour, 21-18 ng/ml for 2 h and 7-16 ng/ml $ClO_2$ for 4 h. For decontamination of contaminated surfaces, 214- 245 ng/ml $ClO_2$ for 1 h and 191-200 ng/ml $ClO_2$ for 2 h completely inactivate the spores.

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# 1. LITERATURE REVIEW

## 1.1. Introduction

Honeybees (*Apis mellifera*) are very important productive livestock as they play an essential role in pollination and they are often the only solution for farmers to ensure pollination. Nowadays, 90% of commercial pollination is performed by managed honeybees (Perez, 2003). Hence, honeybee health is important for agriculture and the environment. The added value for honeybee pollination services to agriculture in the United States is about 15 billion dollars annually with 130 commercial crops dependent on that service (Morse & Calderone, 2000). Modern farming practices use a high density of plants-per-acre which requires more pollination. Consequently, the demand for honeybees is growing faster than the global stock. Therefore, honeybee health is of crucial importance, not only for apiculture, but also for agriculture and human food security.

The number of honeybees is in decline in the United States and globally. A major cause of this decline is bacterial diseases (Genersch, 2010a). One of the most destructive bacterial infections in honeybees is American Foulbrood (AFB), which occurs all over the world. In 1906, the American microbiologist George F. White first isolated the etiological agent of AFB, the gram positive spore-forming bacteria, *Paenibacillus larvae* (White, 1906). The endospore (spores) is the infectious form of the disease. The infection leads to the death of infected honeybee larvae which can lead to the collapse of an entire colony. Antibiotics, which are effective against germinated *P. larvae* but not spores, can delay the collapse of an infected colony but they cannot prevent it, and infection spreads rapidly to other hives. Furthermore, the use of antibiotics for AFB treatment has been banned in most of countries due to drug resistance

and the potential for antibiotic residues to contaminate the honey. Currently, the only effective method to prevent AFB spread is to burn contaminated hives and all associated equipment. AFB is transmitted by adult honeybees and contaminated apiarist equipment. Currently, there is no effective method to disinfect surfaces contaminated with *P. larvae* spore. Chlorine dioxide gas ($ClO_2$), an oxidative agent that has proven effective against spores of *Bacillus anthracis* (Canter et al., 2005), *B. subtilis* (Wang et al., 2016), and *B. cereus* (Nam et al., 2014), is a promising treatment option. However, the efficacy of $ClO_2$ against *P. larvae* spores has yet to be determined.

This thesis examined the efficacy of $ClO_2$ gas against *P. larvae* spores. We determined the effect of gas concentration and treatment time on spores in liquid at different concentrations and on spores on stainless steel, wax, and wood surfaces.

The study showed that $ClO_2$ can be used as a sporicidal and as a disinfectant on surfaces contaminated with *P. larvae* spores. This finding is relevant to apiculture and agriculture. More broadly, these data on $ClO_2$ sporicidal activity will be useful in the prevention of other diseases caused by bacterial spores.

### 1.2. Honeybee Biology

In the hive, the queen can lay up to 2,000 eggs per day and the hive may contain 50,000 honeybees. The bee life cycle comprises the following stages: egg, larvae, pupa and adult, as shown in Figure 1 (Wilson 2007). Larvae take about 3 days to hatch, and they last for 5 days, during which time they are fed by adult workers. Development from an egg to an adult worker bee takes 21 days. Worker bees emerge from the hexagonal cell to perform different tasks. The bee worker is responsible for cleaning and building the hive, nursing developing bee larvae, guarding the hive entrance, and foraging (Winston, 1987). The life expectancy of workers is

about 4-8 weeks. The tasks performed by honeybees depend on their age. The oldest bees in the hive forage, collecting nectar, water and pollen for the hive. The foraging necessitates a high metabolism and flying for long distances, and bees die while doing it (Winston, 1987).

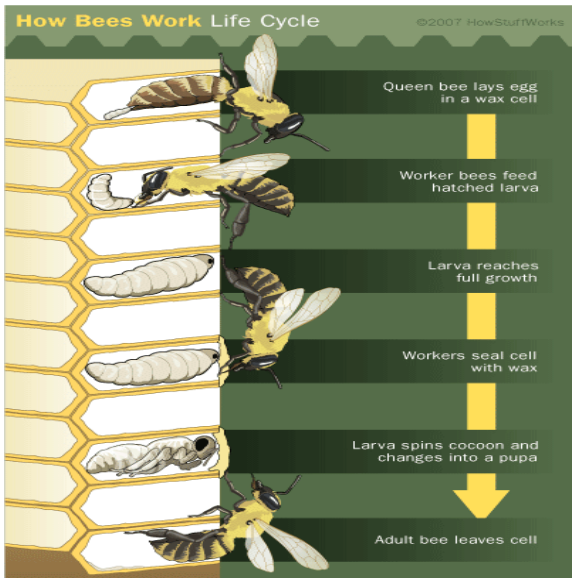


Figure 1. Honeybee life cycle.

Replenishing numbers is critical for hive health. AFB interferes with the honeybee life cycle, decreasing numbers and leading to colony collapse.

### 1.3. Spore Resistance

Spore formation is triggered by adverse conditions such as nutrient limitation. A spore is a metabolically dormant structure that can resist harsh environmental stress like heat, drought, toxic chemicals, and extreme pH levels. Spores can also resist disinfectant and cleaning solutions like formaldehyde and ethanol, and they are commonly used as biodosimeters to assess the efficiency of sterilization methods (McDonnell & Russell, 1999). Studies have shown that *B. subtilis* spores resist UV radiation and remain active without signs of DNA damage ( Setlow et al., 2002;  Setlow, 2006).

The spore structure includes a dehydrated core containing DNA, small acid soluble proteins (SASPs), enzymes, and large amounts of $Ca^{2+}$ and pyridine; 6-dicarboxylic acid (dipicolinic acid [DPA]); the spore cortex which is made primarily from peptidoglycan; and the spore coat (Paredes-Sabja et al., 2011; Setlow, 2006).

SASPs bind to DNA to provide resistance to enzymatic and chemical cleavage, dehydration, and temperature extremes (Fairhead et al., 1994).

The dehydrated core resists moist heat and inhibits enzyme activity, leading to spore metabolic dormancy and long term survival (Setlow, 1994). The inner membrane acts as a permeability barrier against small hydrophilic molecules, peroxides, and alkylating agents (Stelow 2006), while the coat protects the spore from mechanical damage. Large molecules, like lysozyme, cannot pass through the coat. Furthermore, the coat plays an important role in resistance to hydrogen peroxide, iodine, and glutaraldeyde (Bloomfield & Arthur, 1994; Tennen et al., 2000).

Spores monitor the environment for conditions that favor vegetative growth and the presence of specific nutrients called germinants. Following the binding of germinants to germinant receptors, spores commit to germination and outgrow to a vegetative cell. Spore commitment to germination will continue even after removing the germinant, but the mechanism of this commitment is still unknown (Setlow, 2003). The processes of germination and outgrowth are key steps in establishment of AFB, and germination is considered a weak link that can be targeted to control AFB.

*B. subtilis* has been extensively studied as a model organism for understanding the processes of germination and outgrowth in spore forming bacteria. The germination process is divided into two stages: Stage I comprises three steps. In the first step, the nutrient germinant

binds to a germinant receptor, forming one or more channels in the inner membrane of the spore, releasing cations like H+ and Zn2+ from the spore core, and leading to a change in the pH of the spore. This change increases spore metabolism and enzyme activity (Setlow, 2003). In the second step, dipicolinic acid (DPA) and $Ca^{2+}$ are released from the spore core, creating a small space, which allows the third step, the entry of a small amount of water and the partial hydration of the spore cortex. In stage II the partial hydration of the spore core leads to the activation of cortex-lytic enzymes (CLEs), which degrade the peptidoglycan cortex that is responsible for maintaining the dehydrated status of the spore and preventing the expansion of the core. More water intake and expansion of the germ cell wall and outgrowth follows germination, which is characterized by active metabolism, macromolecular synthesis, and an escape from the spore coat (Setlow et al., 2001; Setlow, 2003). In contrast to *B. subtilus*, *P. larvae* has not been well studied, and little is known about spore germination in this species. A gap in knowledge that limits studies on *P. larvae* is the absence of an easy to apply protocols for growth, maintenance, and preparation of viable spores.

### 1.4. *Paenibacillus larvae* and American Foulbrood Disease

The term foulbrood was first used to describe a honeybee disease characterized by a foul smell in the 18$^{th}$ century (Schirach,1769). In 1882, Dzierzon described two different types of foulbrood: the mild, unsealed foulbrood (most likely the current European foulbrood) and the malignant and uncurable sealed foulbrood, a description that applies to AFB. In 1906, White isolated a rod-shaped spore-forming bacteria from infected honeybee larvae and named it *Bacillus larvae* (White, 1906). Before 1906, AFB was not differentiated from European foulbrood disease (EFB). EFB is another honeybee bacterial disease caused by *Melissococcus plutonius*. In 1993, Ash et al. used 16S rRNA sequence analysis to reclassify the causative agent

of AFB to a new genus, *Paenibacillus*. By using repetitive element polymerase chain reaction (rep-PCR) and enterobacterial repetitive intergenic consensus primers (ERIC). *P. larvae* is differentiated into ERIC I, II, III, and IV genotypes (Peters et al., 2006). The former subspecies of *P. larvae* currently correspond to ERIC I and II. While the former subspecies *P. pulvifaciens* corresponds to ERIC III and IV (Genersch, 2010b).

*P. larvae* specifically attacks the honeybee larval stage 12-36 hours after hatching. During this time, the infectious *P. larvae* spores are transmitted to larvae via food and pass through the foregut into the midgut (Yue et al., 2008), where spores germinate and bacteria colonize and proliferate. Vegetative bacteria breach the epithelium of the honeybee midgut after secretion of extracellular protease and spread throughout the hemocoel (Dancer and Chantawannakul, 1997; Hrabak and Martinek,2007).

The main clinical signs of AFB are death of larvae and bad odor. Infected honeybee larvae die and turn into a brownish, semifluid glue-like mass called the ropy stage (Genersch, 2010b) . The ropy stage dries and turns into hard scales adhered to the cell wall in the hive and are called foulbrood scale.

*P. larvae* is highly adapted to honeybees as a host, and the vegetative form can be found in nature only inside bee larvae (Genersch, 2010b); although they can also form in complex media in a laboratory setting.

AFB can be transmitted among larvae in the brood nest by the activity of nurse bees, which inspect and feed many larvae. In this way, *P. larvae* is transmitted within the brood nest by larval food contaminated with *P. larvae* spores (Tarr, 1937).

The bacteria produce toxins and enzymes like proteases, collagenases, and chitinase enabling the bacteria to breach the midgut of honeybee larvae (Djukic et al., 2014). Eventually,

the honeybee larvae die. After consuming all the nutrients in honeybee larvae bodies, the bacteria sporulate (Davidson, 1973; Genersch et al., 2005; Peters et al., 2006). The new spores then contaminate the hive and spread the infection.

Honeybee workers act as passive vectors for AFB. The workers are resistant to the infection, yet they are considered the main source of infection. *P. larvae* spores have been recovered from wax debris (de Graaf et al., 2013) and commercial pollen (Gochnauer & Corner, 1974) . Also, honey is considered a major source of *P. larvae* spores (Fries & Camazine, 2001). Beekeepers routinely share frames of honey and move resources between hives. Such behavior spreads the infection (Korpela et al. 2008).

The four genotypes of *P. larvae*: ERIC I–IV co-segregate with phenotypic differences including differences in virulence (Alippi, 1999; Genersch et al., 2005). ERIC I is the most virulent and most abundant globally (Loncaric et al., 2009). Epidemiological studies have shown that ERIC I is frequently isolated from colonies infected with foulbrood disease in Europe and on the American continent, whereas ERIC II seems to be restricted to Europe (Ebeling et al., 2016). Relative to ERIC I, ERIC II–IV kill larvae quickly (7 days versus 12 days). More larvae are removed by nurse bees as part of the social immune defense before cell capping, prior to the production of infectious spores. The less ropy mass and spore containing foulbrood scales that are produced, the more slowly the disease will develop and spread within the colony (Genersch, 2010b).

If honeybee larvae die quickly and before cell capping, the adult bees will remove the dead larvae before sporulation of *P. larvae*, as is case of ERIC II-IV. This means that only a minor proportion of the larvae die after cell capping, resulting in the described clinical symptoms of AFB (ropy stage, foulbrood scale) in the case of ERIC II–IV (Ebeling et al., 2016). ERIC I, on

in contrast, generally kills honeybee larvae after cell capping, providing sufficient time for the bacteria to consume the nutrients from the larvae and subsequently sporulate. Virulence at the larval stage is negatively correlated with virulence at the colony level due to the hygienic behavior of nurse bees ( Morse & Calderone, 2000). The removal rate of infected larvae depends on the timeframe of disease progression. Larvae infected by a representative of the fast killing (at the larval stage) ERIC II genotype were removed more efficiently (up to 90% removal rate) than larvae infected by a representative of the slower killing (at the larval stage) genotype ERIC I genotype (around 60% removal rate).

### 1.5. AFB Management

Infection with *P. larvae* can lead to the collapse of entire colonies. The current management strategies set in place do not totally eliminate the disease. Antibiotics like tylosin, terramycin/oxytetracycline, and sodium sulfathiazole have been used to control AFB (Alippi, 1999), but infectious spores are not affected by antibiotics (Alippi, 1999; Shimanuki & Knox, 2000). Furthermore, antibiotic residues are spread throughout the hive and lead to the appearance of resistant *P. larvae* strains (Alippi, 1999; Genersch, 2010b).

Natural products have been used as an alternative treatment and control regime. Products like probiotics, essential oils, plant extracts and propolis have been used (Antunez et al., 2008; Fernández et al., 2014 ; Fuselli et al., 2010; Yoshiyama et al., 2013). Using disks with 1% natural propolis extract, the diameter zone of inhibition ranged between 20 mm and 30 mm. This effect was on vegetative bacteria (Antunez et al., 2008). In vitro, low concentrations of bee venom inhibit vegetative *P. larvae*, with minimal inhibitory concentrations (MIC) of 3.12 to 8.33 μg/ml (Fernández et al., 2014 ).Yoshiyama et al. (2013) tested 9 different lactic acid bacteria strain against *P. larvae* vegetative bacteria, and reported inhibition zones ranging from 9-25 mm. All

the natural products act against vegetative *P. larvae* bacteria but are ineffective against *P. larvae* spores.

The selection of certain honeybee breeds based on their hygienic behavior has been used to fight AFB. To select for hygienic behavior, a freeze-killed brood is placed inside the hive and the time taken to remove the dead brood is recorded (Wagoner et al., 2018).

The current method to control AFB infection is by the destruction of the infective hives and all contaminated apiarist equipment by burning followed by burial (Alippi, 1999), resulting in heavy economic loses.

Diagnosis and identification of *P. larvae* in suspected hives is very important to prevent the spread of AFB. Furthermore, routine sampling is equally important for AFB monitoring and prevention programs. Currently, there is no regional or national AFB detection program. It is important to establish such programs since many AFB cases have no obvious clinical signs. *P. larvae* isolation is the best approach to conduct epidemiological studies and diagnosis of AFB (de Graaf et al., 2013). AFB reduces the number of bees available for pollination, affecting crop production and the global food supply. The overarching objective of this thesis is to develop effective treatments to inactivate *P. larvae* spores, which will reduce the losses due to AFB, and reverse the decline in honeybee numbers. We proposed to develop and use a new AFB disinfectant method by using $ClO_2$ gas to inactivate *P. larvae* spores.

### 1.6. Chlorine Dioxide as a Sporicidal

Chlorine compounds have been used widely for their antimicrobial activity since the nineteenth century (Khanna & Naidu, 2000). For example, Robert Koch used it to treat water after a cholera outbreak in London in 1850.

$ClO_2$ is highly oxidative and has been used as a sporicidal agent against *B. subtilis* (Young & Setlow, 2003), *B. cereus* (Nam et al., 2014), *B. thuringiensis* (Kreske et al., 2006), and *B. anthracis* (Canter et al., 2005). $ClO_2$ is effective over a wide pH range (pH 3.0-10.0) and can be easily generated in the lab by mixing the two media parts—part A (sodium chlorite) and part B (ferric chloride) —in a gas generation sachet. Formaldehyde and ethylene oxide also have been used as antimicrobial gases. However, they are flammable and carcinogenic (Cheney & Collins, 1995; Natarajan et al., 1995).

The aim of this study is to develop a method to disinfect contaminated materials and beehives with *P. larvae* spore by using highly oxidative $ClO_2$, since gas treatment can reach to all treated surfaces.

Although the spores are highly resistant to many bactericidal treatments, they can be killed by induced damage to their DNA (Tennen et al., 2000) or inactivation of components of the germination apparatus ( Setlow et al., 2002).

In this study, we tested decontamination on stainless steel, wood, and bee wax, as most beehives are made from these components. An advantage of $ClO_2$ is that it does not leave significant residues (Smith & Herges, 2015).

There is a direct link between honeybees and global food security. Apiculture, agriculture, and the environment will benefit from the outcomes of this study. Damage to the spore's germinant proteins by using oxidative gaseous treatment will most likely lead to a new control regime for AFB. Honey consumers and all the industries that use honey as a raw material will directly benefit from a successful outcome of this study.

## 1.7. Study Aims

More research on AFB is needed and this requires the establishment protocols for growth, maintenance, and spore production. In this thesis we provide selected protocols in a recipe-like format that are easy to apply in the laboratory. In the same way, to apply effective treatment and control measures for AFB, we need to know about the environmental cues required to promote germination in *P. larvae* spore and the germination pathway.

It is important to develop a method to disinfect contaminated materials and beehive with *P. larvae* spore and a strong oxidative agent in a gaseous form like $ClO_2$ is a good candidate since the gas treatment can reach to all the treated surface.

The 3 aims of this thesis are:

- Provide protocols for the growth and maintenance of *P. larvae*
- Acquire a reliable method for sporulation and germination of *P. larvae* spores.
- Evaluate the sporicidal activity of $ClO_2$ gas to disinfect contaminated materials and beehive with *P. larvae* spore.

## 2. GROWTH AND LABORATORY MAINTENANCE OF *PAENIBACILLUS LARVAE*[1]

### 2.1. Abstract

*Paenibacillus larvae* is a Gram-positive, spore-forming bacterium and the causative agent of American Foulbrood disease (AFB), a highly contagious, fatal disease of managed honeybee colonies (*Apis mellifera*). This chapter describes protocols for the *in vitro* growth and laboratory maintenance of *P. larvae* (1).

### 2.2. Introduction

Managed honeybee colonies are crucial for pollination of many food crops and wild plants. Unfortunately, honeybee populations are currently declining due to myriad threats including overuse of pesticides, environmental change, and disease. American Foulbrood disease (AFB) is considered the most important bacterial disease of honeybees because it is highly contagious, fatal, and spread worldwide. *Paenibacillus larvae*, a Gram-positive, endospore-forming bacterium, first isolated by George F. White in 1906, is the etiological agent of AFB (White, 1906). *P. larvae* endospores are most often introduced into a honeybee colony by the actions of robber bees, which steal honey from other colonies that are too weak to defend themselves. The contaminated honey is then fed to young larvae. After ingestion, the *P. larvae* endospore undergoes the biophysical and biochemical processes of germination and outgrowth, which collectively allow the cell to return to the vegetative state. While initial stages of *P. larvae* pathogenesis are not well understood, it is clear that the bacterium eventually gains access to the honeybee hemolymph where it can evade host immune functions, rapidly multiply, and cause

[1]The material in this chapter was co-authored by Osama S. Mahdi and Nathan A. Fisher. Osama S. Mahdi had primary responsibility for the preparing and testing the protocols in this chapter. Osama S. Mahdi and Nathan A. Fisher drafted and revised all versions of this chapter. Nathan A. Fisher served as proofreader and checked the protocols results conducted by Osama S. Mahdi. This article has been published as (Mahdi, O. S., & Fisher, N. A. (2018). Growth and laboratory maintenance of *Paenibacillus larvae*. *Current protocols in microbiology*, *48*(1), 9E-1).

death (Djukic et al., 2014). Upon death of the infected honeybee larvae, *P. larvae* cells return to the endospore state and are spread throughout the colony by nurse bees. As the newly formed endospores are ingested by additional honeybee larvae, the disease cycle repeats. As more and more brood is lost to AFB, the colony grows progressively weaker until it can no longer sustain itself (Genersch, 2010b).

There are very few options for treatment of honeybee colonies with AFB. Antibiotic treatment is problematic because of an increase in antibiotic resistant strains and because government regulations often require apiarists to discontinue antibiotic treatment for some period of time (usually six weeks) before harvest. Due to the continual presence of endospores, AFB can recur during this period, threatening the infected colony and risking the spread of disease to other colonies in the area. Thus, most apiarists chose to destroy infected colonies by burning them along with any equipment that came into contact with the colony. As a result, the economic impact of AFB can be substantial. Additional research is needed to fully characterize the pathogenesis of *P. larvae* and develop more effective means to diagnose and successfully treat infected colonies. To that end, the protocols described in this chapter are designed to assist investigators in the growth and laboratory maintenance of *P. larvae.*

## 2.3. Strategic Planning

### 2.3.1. Laboratory Containment

All work should be performed inside a certified biological safety cabinet (BSC) in a BSL-2 containment laboratory in order to reduce the risk of environmental release. For general biosafety information, see Burnett et al., (2009).

### 2.3.2. Strain Selection

There are a number of *P. larvae* strains available from the American Type Tissue Collection (ATCC; www.atcc.org) in Rockville, MD. Phenotypes such as virulence, motility and sporulation efficiency can vary widely across strains. For example, ATCC 9545 belongs to the ERIC I clade, which is considered the most virulent, while ATCC 49843 belongs to the less virulent ERIC IV clade. Investigators should consider strain selection carefully depending on the goals of the study.

### 2.3.3. Growth Conditions

*P. larvae* isolates are generally slow growing, and some isolates are fastidious. Thus, growth under standard laboratory conditions is not guaranteed. Media supplemented with 5% sheep blood and an atmosphere of 5% $CO_2$ enhances the growth of most isolates and is absolutely required for some. Under these conditions, growth is usually visible after 48 hours of incubation at 37°C but extended incubation up to 5 days may be required in some cases. Investigators should evaluate several media to empirically determine which is best suited for each isolate of interest.

## 2.4. Basic Protocol 1. Growth of *P. larvae* From a Frozen Stock

Like most bacteria, *P. larvae* vegetative cells can be stored for long periods of time at -80°C or below. See Basic Protocol 3 for guidance in preparing frozen stocks. Experiments typically begin by reviving cells from a long-term seed stock or a working stock. In order to ensure the culture is pure of contaminating bacteria, we recommend streaking onto a fresh agar plate instead of reviving frozen cells in liquid media.

### 2.4.1. Materials

*P. larvae* frozen stock (see Basic Protocol 3)

MYPGP or CSA agar plates (see Reagents and Solutions)

Inoculating loop or needle, sterile

37°C incubator (an atmosphere of 5% $CO_2$ is recommended)

1. Remove the stock vial from long-term storage and, without allowing it to thaw, use an inoculating loop or needle to dislodge a small clump of frozen cells.
2. Gently streak the clump of bacteria cells onto an agar plate in order to isolate single colonies.
3. Invert the plate and place it in a 37°C incubator. We recommend using an incubator capable of generating an atmosphere of 5% CO2.
4. Incubate up to 5 days at 37°C.

## 2.5. Basic Protocol 2. Growth of *P. larvae* in Liquid Media

Trypicase soy broth (Stevenson, 2006) supports broth culture of most *P. larvae* isolates but MYPGP broth (Reagents and Solutions) can be used with more fastidious isolates (Dingman & Stahly, 1983).

### 2.5.1. Materials

*P. larvae*, grown on MYPGP or CSA agar (see Basic Protocol 1)

MYPGP or trypticase soy (TS) broth, sterile.

Inoculating loop or needle, sterile

Capped test tube or flask, sterile

37°C shaking incubator

1. Using aseptic technique, add sterile MYPGP or TS broth to a sterile test tube or flask (e.g., 3 ml to a 15 ml screw-cap conical tube or glass test tube, or one tenth volume to a flask). Culture vessels should have tight fitting lids that allow for gas exchange but not escape of aerosolized *P. larvae* into the environment. In order to minimize aerosolization, baffled flasks should not be used.
2. Using a sterile inoculating loop or needle, transfer a single colony from the agar plate to the sterile broth media. If a large volume of culture is needed (e.g., above 10 ml), it is preferable to first expand the culture in a small volume of broth (e.g., overnight in 1-3 ml) and then transfer this starter culture to the larger volume.
3. Tightly cap the test tube or flask and transfer it to an orbital shaking incubator.
4. Incubate the test tube or flask while shaking at 200-250 rpm for 24-48 hours at 37°C. 48 hours is usually sufficient for cultures to become visibly turbid, but some isolates may require extended incubation.

### 2.6. Basic Protocol 3. Preparation of *P. larvae* Frozen Stocks

Cryopreservation at -80°C or below is recommended for long-term storage of *P. larvae* stocks. Due to the risk of spontaneous germination, the authors do not recommend relying on endospore stocks as the sole source of critical strains. MYPGP broth supplemented with 30% glycerol works well for this purpose.

#### 2.6.1. Materials

*P. larvae*, grown on MYPGP or CSA agar (see Basic Protocol 1)

MYPGP broth, sterile (see Reagents and Solutions)

50% (v/v) glycerol, sterile

15-ml conical tubes, sterile

Inoculating loop or needle, sterile

1.5-ml freezer vials, sterile

Vortex

37°C shaking incubator

-80°C freezer

1. Aseptically transfer a single colony of *P. larvae* into 3 ml of sterile MYPGP broth in a 15 ml screw-cap conical tube.
2. Place in an orbital shaking incubator set to 37°C, 200 rpm.
3. Incubate 16 – 24 hours or until the culture is turbid. Some isolates may require extended incubation before turbidity is obvious. Transfer 0.5 ml of the turbid culture to a 1.5 ml freezer vial containing 0.75 ml 50% glycerol solution.
4. Secure the vial cap and vortex for 20 seconds to ensure the cells are well distributed and the solution is well mixed.
5. Immediately place the vial at -80°C for storage.

## 2.7. Reagents and Solutions

### 2.7.1. MYPGP Broth (Dingman and Stahly, 1983)

Mueller-Hinton broth base

Yeast extract

Potassium phosphate (dibasic)

10% (v/v) L- or D-Glucose solution, sterile filtered

Sodium Pyruvate

Graduated cylinder

Autoclave

1. Add 10 g Mueller-Hinton broth base, 15 g yeast extract, 3 g potassium phosphate, and 1 g sodium pyruvate to 900 ml distilled, deionized water.
2. Mix well, transfer to a graduated cylinder and bring the total volume to 980 ml by adding additional distilled, deionized water.
3. Autoclave at 121°C for 20 minutes, remove from autoclave and allow to cool.
4. Add 20 ml 10% glucose solution and mix well. Broth can be stored at room temperature for 1-2 weeks or at 4°C for 2-4 weeks.

### 2.7.2. MYPGP Agar

Bacteriological agar

Petri plates, sterile

1. Add 20 g per liter of agar to MYPGP broth before autoclaving. Add glucose solution after agar has cooled to approximately 55°C, but before it has begun to solidify.
2. Add approximately 20 ml of media per petri plate, cover, and allow plates to solidify at room temperature before using. Plates can be stored at 4°C for 1-2 weeks. In order to avoid excess condensation, ensure plates are cool before transferring to 4°C.

### 2.7.3. CSA Agar (Hornitzky & Karlovskis, 1989)

Columbia sheep blood agar base

Defibrinated sheep blood, room temperature

Petri plates, sterile

Graduated cylinder

Autoclave

1. Suspend 28 g of Colombia sheep blood agar base in 800 ml of distilled, deionized water.
2. Transfer suspension to a graduated cylinder and bring to a total volume of 950 ml by the addition of distilled, deionized water.

3. Autoclave the suspension at 121°C for 20 minutes.
4. Remove from the autoclave and allow to cool to approximately 45-50°C.
5. Aseptically add 50 ml (5% v/v) sterile defibrinated sheep blood and mix gently. The media should be red in color due to the presence of red blood cells. A brown color indicates that the agar was too hot, which caused lysis of the red blood cells and oxidation of released hemoglobin.
6. Dispense approximately 20 ml of media per petri plate, cover, and allow to solidify at room temperature before using. Plates can be stored at 4°C for 1-2 weeks. In order to avoid excess condensation, ensure plates are cool before transferring to 4°C.

## 2.8. Commentary

### 2.8.1. Background Information

Techniques for the laboratory growth and maintenance of *P. larvae* are generally similar to those used for other bacteria, with subtle modifications for the increased fastidiousness of this organism such as incubation in an atmosphere of 5% $CO_2$ and the addition of supplements to standard microbiological media. In addition to those recommended in this chapter, a number of alternative media optimized for the growth of *P. larvae* have been described (de Graaf et al., 2013). Investigators should compare the performance of various media with their isolates of interest if optimization of growth is a concern.

### 2.8.2. Critical Parameters

The authors have observed that an atmosphere of 5% $CO_2$ enhances growth of nearly all isolates of *P. larvae* on solid media and is absolutely required for growth of some environmental isolates.

The temperature of the agar at the time of sheep blood addition is a critical parameter during formulation of CSA agar. If the agar is above ~50°C, the red blood cells may lyse. If the temperature is below ~45°C, the agar will begin to solidify without proper mixing. It is also

important that the red blood cells are equilibrated to room temperature before addition in order to avoid uneven cooling and solidification.

### 2.8.3. Troubleshooting

*P. larvae* cells maintain viability for extended periods of time when stored at -80°C. If viable cells cannot be recovered on solid media (Basic Protocol 1), it is possible that an insufficient number of cells were transferred or that the inoculation loop was too hot during transfer. Start another culture with a larger ice chip and ensure that the inoculation loop or needle cools completely if using a Bunsen burner to sterilize it before transfer. Do not store glycerol stocks at -20°C because incomplete freezing results in settling of the cells to the bottom of the tube.

### 2.8.4. Anticipated Results

Basic Protocol 1 describes how to culture *P. larvae* cells on solid agar plates starting from a frozen stock. The culture should be pure (contain only one type of colony) and colonies will appear small, flat, with regular margins, and white to beige in color.

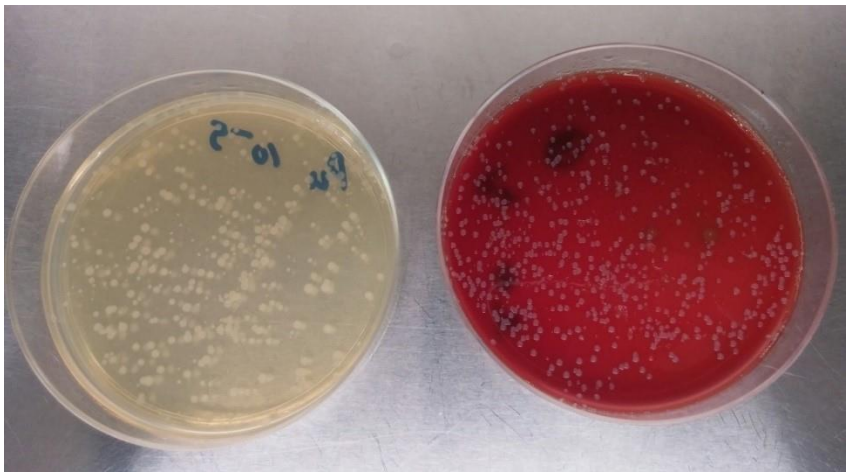

Figure 2. *P. larvae* strain ATCC 9545 on MYPGP (left) and CSA (right) agar plates after 72-hour incubation at 37°C in an atmosphere of 5% $CO_2$. Colonies appear small, flat and regular in shape and white to beige in color.

Basic Protocol 2 describes how to culture *P. larvae* in liquid media. After 24-48 hours, cultures should appear turbid. Basic Protocol 3 describes preparation of frozen stock suspensions of *P. larvae* cells. Transfer of a small volume from the frozen cell suspension should result in a large number of colonies on agar media.

### 2.8.5. Time Considerations

Many *P. larvae* isolates are slow growing and require extended incubation in order to reach turbidity in liquid culture or for colonies to be visible on agar plates. Investigators should consider this factor when scheduling experiments with *P. larvae*.

## 2.9. Acknowledgement

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# 3. SPORULATION AND GERMINATION OF *PAENIBACILLUS LARVAE* CELLS[2]

## 3.1. Abstract

Endospores are metabolically dormant cells formed by a variety of Gram-positive bacteria within the phylum Firmicutes in response to nutrient limit or otherwise unfavorable growth conditions. American foulbrood disease (AFB) is a serious disease of honeybees that is caused by the introduction of *P. larvae* endospores into a honeybee colony. Progression to fulminant disease and eventual collapse of the colony requires multiple rounds of endospore germination, vegetative replication, endospore formation, and subsequent spread within the colony. This chapter includes protocols for the in vitro sporulation and germination of *P. larvae* to assist investigators in the study of these processes (2).

## 3.2. Significance Statement

*P. larvae* is the etiological agent of American foulbrood disease (AFB), the most significant bacterial pathogen of honeybees. The metabolically dormant endospore is the infectious particle, but the cell must convert to the vegetative morphology in order to replicate and cause disease. Therefore, the conversion between vegetative cell and endospore (sporulation and germination) is critically linked to virulence.

## 3.3. Introduction

Managed honeybee colonies are responsible for pollination of 90% of all commercial crops. Therefore, honeybee health is critically linked to the availability of food and ultimately to human health. In the twelve-month period between April 2015 and March 2016, 44.1% of all

[2] The material in this chapter was co-authored by Osama S. Mahdi and Nathan A. Fisher. Osama S. Mahdi had primary responsibility for the preparing and testing the protocols in this chapter. Osama S. Mahdi and Nathan A. Fisher drafted and revised all versions of this chapter. Nathan A. Fisher served as proofreader and checked the protocols results conducted by Osama S. Mahdi. This article has been published as (Mahdi, O. S., & Fisher, N. A. (2018). Sporulation and germination of *Paenibacillus larvae* cells. *Current protocols in microbiology*, *48*(1), 9E-2.).

managed honeybee colonies in the United States were lost, many of these to viral, fungal, parasitic, and bacterial diseases (Apiary Inspectors of America., 2016).

The Gram-positive bacterium *Paenibacillus larvae* is the causative agent of American foulbrood disease (AFB). It is an endospore-forming bacteria and the endospore is the only infectious cell type; vegetative cells are non-pathogenic. Briefly, dormant endospores from the environment or from previous cycles of amplification within the brood are ingested by honeybee larvae. Once in the honeybee digestive track, the endospores germinate and outgrow to form vegetative cells that produce a number of toxins. The cells breach the intestinal barrier and replicate within the hemolymph until the death of the larvae, at which time the *P. larvae* cells undergo sporulation. The next round of amplification then takes place when these endospores contaminate the food of an additional brood (Djukic et al., 2014).

The protocols within this chapter describe procedures for the in vitro sporulation and germination of *P. larvae* cells in order to assist investigators in the study of these important cellular processes.

## 3.4. Strategic Planning

### 3.4.1. Laboratory Containment

All work should be performed inside a certified biological safety cabinet (BSC) in a BSL-2 containment laboratory in order to reduce the risk of environmental release. For general biosafety information, see (Burnett et al., 2009).

### 3.4.2. Variation Between Stocks

For most endospore forming bacterial species, phenotypic differences between endospore lots can be a major contributor to experimental variation. For this reason, it is common to

perform experiments using a minimum of three different endospore stocks, each prepared on a different day.

### 3.5. Basic Protocol 1. Sporulation of *P. larvae* on Solid Media

While it is recognized that sporulation and subsequent germination of *P. larvae* cells is critical to the lifecycle of this bacterium, most isolates exhibit poor sporulation efficiency in vitro. Most isolates will sporulate to some degree on solid MYPGP media. However, yields are typically lower than for other endospore forming species.

#### 3.5.1. Materials

*P. larvae*, turbid broth culture (see previous chapter, Basic Protocol 2)

MYPGP agar plates (see previous, Reagents and Solutions)

1 ml pipette with sterile tips

L-spreader, sterile

37°C incubator (an atmosphere of 5% CO2 is recommended)

1. Using aseptic technique, transfer 250 µl of turbid *P. larvae* broth culture to each MYPGP agar plate and spread using a sterile L-spreader.

   The broth culture should be in logarithmic growth phase, if possible. Cultures that have been in stationary phase for an extended period of time should not be used.
2. Allow the liquid to absorb into the agar plates, invert them, and transfer to a 37°C incubator with an atmosphere of 5% CO2.
3. Incubate the plates at 37°C, 5% CO2 for 4-7 days.

## 3.6. Alternate Protocol 1. Sporulation of *P. larvae* in Liquid Media

Sporulation in liquid media can yield higher numbers of endospores as compared with growth on solid media. However, not all isolates will sporulate in liquid media. Low levels of aeration and nutrient exhaustion seem critical to endospore formation in this species.

### 3.6.1. Materials

*P. larvae,* turbid broth culture (see chapter 2, Basic Protocol 2)

TMYGP broth (see Reagents and Solutions)

1 ml pipette with sterile tips

1-liter plastic flask with screw cap lid

37°C shaking incubator

1. Using aseptic technique, add 400 ml of TMYGP broth to a 1-liter plastic flask and allow it to equilibrate to room temperature.

   Aeration inhibits *P. larvae* sporulation. Thus, a larger volume of liquid should be used relative to the total capacity of the culture vessel as compared to standard practices with other spore forming bacterium.
2. Transfer 1 ml of stationary phase *P. larvae* cells from a turbid liquid culture to the flask.
3. Securely close the cap and transfer the flask to a 37°C shaking incubator set to 190 rpm.
4. Incubate for at least 4 days at 37°C, 190 rpm. Slow shaking is used to minimize aeration.

## 3.7. Basic Protocol 2. Purification and Concentration of *P. larvae* Endospores

Endospores from *P. larvae* grown on solid media or in liquid broth should be separated from culture detritus for storage and future experimental manipulation. The following protocol results in highly purified suspensions of endospores.

### 3.7.1. Materials

*P. larvae*, grown on MYPGP agar plates or in TMYPG broth (see Basic Protocol 1 or Alternate Protocol 1, respectively)

Deionized distilled water, sterile

HistoDenz gradient (see recipe)

15- and 50-ml conical centrifuge tubes

1.5-ml screw cap tubes

1.5-ml snap-cap tube

Serological pipets

Water bath set to 65°

top centrifuge

Microcentrifuge

1. If following Basic Protocol 1, flood each MYPGP plate with 5 ml sterile deionized distilled water. Tip plate gently to the side and rinse plate surface several times using a 10-ml serological pipet in order to dislodge the bacterial cells from the agar surface. Combine the yield from multiple plates into 40 ml aliquots in sterile 50-ml conical tubes. CAUTION: In order to prevent the spread of *P. larvae*, avoid splashing.
2. If using Alternate Protocol 1, transfer aliquots of 40 ml TMYPG broth culture to sterile 50-ml conical tubes.
3. Tightly cap conical tubes and transfer to a 65°C water bath.
4. Incubate at 65°C, 20 min.
5. Transfer tubes to a top centrifuge and spin at 4000 × g, 20 min.

CAUTION: Ensure the centrifuge is properly balanced and follow all operating instructions.

Dry the outside of the tubes with a paper towel before placing in the centrifuge.

6. Carefully pipet or decant supernatant from the 50-ml tube without disrupting the cell pellet.
7. Suspend cell pellet in 40 ml sterile deionized distilled water and repeat steps 5 and 6 for a total of two washes.
8. After the second wash, suspend pellet in 5 ml sterile deionized distilled water.
9. Carefully transfer 5 ml aliquots of suspension and slowly add them to 15-ml conical centrifuge tubes containing 5 ml 20% to 50% HistoDenz gradient (see recipe). Ensure that the cell suspension is layered on top of the HistoDenz gradient slowly and carefully, making sure not to disrupt the gradient.
10. Secure tube lids and transfer them to a microcentrifuge.
11. Pass cell suspension through the HistoDenz gradient by centrifugation at 7393 × g (11,500 rpm), 35 min.

    CAUTION: Ensure the centrifuge is properly balanced and follow all operating instructions.
12. Optional: Carefully remove upper layers of the tube with a pipet and suspend the pellet in 5 ml sterile deionized distilled water. Transfer suspension to a fresh 15-ml conical centrifuge tube containing a 20% to 50% HistoDenz gradient for a second round of purification.
13. Carefully remove upper layers of the tube with a pipet and suspend pellet in 1 ml sterile deionized distilled water.

14. Secure tube lids and centrifuge at 10,956 × g (14,000 rpm), 3 min.

    CAUTION: Ensure the centrifuge is properly balanced and follow all operating instructions.

15. Carefully remove supernatant and be careful to avoid disruption of the spore pellet. Suspend pellet in 1 ml sterile deionized distilled water and transfer to 1.5-ml snap cap tube and wash twice more to remove residual HistoDenz. Depending on the efficiency of the HistoDenz purification step, at this point the pellet may have two distinct phases: A dense phase on the bottom of the tube and a lighter, fluffyappearing phase right above it. In this case, the fluffy phase can be removed by gently swirling the liquid above the pellet and slowly pipetting up and down. The material in this phase is primarily composed of cell debris that should be removed prior to endosporestorage or subsequent experiments. During these washes, the pellets from multiple tubes can be combined to increase the concentration of endospores in the final suspension.
16. Carefully remove supernatant and be careful to avoid disruption of the spore pellet. Suspend pellet in 1 ml sterile deionized distilled water and transfer to a 1.5-ml screw-cap tube.
17. Tightly secure cap and transfer tube to a 4°C refrigerator for storage. Endospores are typically stable at 4°C for months or years. However, they should be periodically checked for spontaneous germination. Due to the possibility of spontaneous germination and subsequent cell death, investigators should not rely on endospore stocks as the sole means of long-term storage.

### 3.8. Support Protocol 1. Quantification of *P. larvae* Endospore Stocks

Efficiency of sporulation and germination varies widely among *P. larvae* isolates. The following protocol describes a technique for quantification of the concentration of viable, colony-forming endospores within a given suspension (usually an endospore stock).

#### 3.8.1. Materials

*P. larvae*, endospore stock suspension

MYPGP agar plates

Deionized distilled water, sterile

1.5-ml screw-cap tubes

1.5-ml snap-cap tubes

Pipet with sterile tips

L-shaped spreaders

Vortex

65°C water bath

37°C incubator with 5% CO2 atmosphere

1. Prepare a series of dilution blanks by adding sterile deionized distilled water to sterile 1.5-ml snap-cap tubes. We recommend that the first dilution tube contain 990 µl deionized distilled water in a screw-cap tube and that all subsequent dilution tubes contain 900 µl deionized distilled water in snap-cap tubes. If other volumes are used, the volumes of subsequent steps in this protocol should be altered accordingly. We recommend a total of seven dilution tubes including the first dilution tube.
2. Remove endospore stock suspension from storage and vortex briefly to mix.

3. Aseptically transfer 10 µl stock suspension to the first dilution blank containing 990 µl sterile deionized distilled water in a screw-cap tube.
4. Tighten cap, vortex briefly to mix, and place tube in a 65°C water bath 15 min to kill any germinated bacteria.
5. Remove tube from the water bath, dry outside using a paper towel, and transfer 100 µl to the first snap-cap tube containing 900 µl sterile deionized distilled water.
6. Cap tube, vortex briefly to mix, and transfer 100 µl to the next snap-cap tube containing 900 µl sterile deionized distilled water. Repeat until the end of the dilution series. Pipet tips should be changed between each transfer to avoid accidental carryover of endospores from higher concentration suspensions to lower concentration suspensions.
7. Transfer triplicate, 100 µl aliquots from each dilution tube to MYPGP agar plates and spread with a sterile L-shaped spreader.
8. Allow excess liquid to absorb into the agar, invert, and transfer plates to a 37°C incubator with 5% CO2 atmosphere.
9. Incubate plates 3 to 4 days or until colonies are visible.
10. Count number of colonies on each plate that contains between 30 and 300 total colonies.
11. Determine average number of colonies across all three plates from the same dilution and convert to colony forming units (CFUs) per ml of stock suspension by multiplying the CFU by the dilution factor and volume factor.

If 100 µl was spread on each plate, the volume factor is 10. The dilution factor is the inverse of the fraction of the original solution still present in each tube. For example, the first tube is a 1/100 dilution for a dilution factor of 100 while the seventh tube is a $1/10^8$ dilution for a dilution factor of $10^8$.

## 3.9. Basic Protocol 3. Measuring Germination of *P. larvae* Endospores by Change in Optical Density

Dormant endospores are more optically dense than vegetative cells due to the formation of a crystalline lattice of calcium and dipicolinic acid in the interior of the cell. As endospores undergo germination, extracellular water rushes into the cell causing it to swell. Hydration of the core breaks down the crystalline lattice and results in a loss of optical density. This change in optical density can be used to quantify the percentage of the endospore population that germinates in response to a given germinant molecule and the speed with which that germination takes place.

### 3.9.1. Materials

*P. larvae*, endospore stock suspension (see Basic Protocol 2)

PBS, pH 7.0 to 7.4

3 mM uric acid solution or test solution

1.5-ml screw-cap vials

0.22-μm sterile filter

96-well clear polystyrene microtiter plate with lid

Spectrophotometer

Pipet with sterile tips

65°C water bath

1. Prepare 3 mM solution of uric acid in PBS (pH 7.0 to 7.4) and pass solution through a 0.22-μm sterile filter. Uric acid serves as a positive germinant control for *P. larvae*. Prepare similar solutions of test germinants as required by the experiment. Typical

evaluation will begin by testing for induction of germination in the low micromolar range.

2. Dilute endospore stock solution to an optical density at 580 nm (OD580) of 0.7 ± 0.02 in PBS (pH 7.0 to 7.4). Prepare a large enough volume so that each germinant can be tested in triplicate against at least two different endospore stocks.
3. Transfer 1.0 ml aliquots to 1.5-ml screw-cap vials and incubate in a 65°C water bath 15 min. Use of a screw-cap vial is important to prevent pressure from building inside the vial and forcing a snap-cap tube open.
4. Add 0.2 ml PBS (pH 7.0 to 7.4) to three wells of a clear polystyrene microtiter plate to serve as a blank.
5. Transfer 0.1 ml aliquots endospore suspension to the remaining wells of the microtiter plate.
6. Transfer 0.1 ml aliquots of each test solution (see step 1) to individual wells and mix quickly by pipetting up and down. This step should be completed quickly for all test solutions. The drop in optical density will begin in 30 to 90 sec after addition of germinant. Use of a multichannel pipet is recommended if more than a couple of germinants are to be tested on the same plate.
7. Place microtiter plate in a microtiter plate reader and use blank wells to set the baseline absorbance of the buffer. Refer to the microtiter plate reader operator's manual.
8. Read absorbance of each well at 580 nm at 30 to 60 sec intervals for 1 h. If automated reading is not possible, read the absorbance manually at 0, 1, 2, 5, 10, 20, 40, and 60 min.

9. Determine percentage decrease in optical density at each time point using the following equation:

   (OD580 at T0) − (OD580 at Tn) / (OD580 at T0) ×100%

## 3.10. Alternative Protocol 2. Measuring Germination of *P. larvae* endospores by Loss of Heat Resistance

Hydration of the endospore core is concomitant with a loss of heat resistance. Therefore, resistance to heat (typically 65° or 70°C) can be used to measure the percentage of endospores in a given population that have undergone germination. This assay is simpler to execute compared to the loss in optical density assay, but it requires significantly more materials (e.g., agar plates, tubes) and time.

### 3.10.1. Materials

*P. larvae*, endospore stock suspension (see Basic Protocol 2)

MYPGP agar plates (see previous chapter)

PBS, pH 7.0 to 7.4

3 mM uric acid solution or test solution

1.5-ml screw-cap vials

1.5-ml snap-cap tubes

0.22-μm sterile filter

L-shaped spreaders

Pipet with sterile tips

37°C and 65°C water bath

37°C static incubator

1. Prepare 3 mM solution uric acid in PBS (pH 7.0 to 7.4) and pass solution through a 0.22-μm sterile filter. Uric acid serves as a positive germinant control for *P. larvae*. Prepare similar solutions of test germinants as required by the experiment. Typical evaluation will begin by testing for induction of germination in the low micromolar range.
2. Determine number of time points to be sampled (e.g., 5, 10, 20, 60 min). For each time point, aliquot 250 μl PBS (negative control), uric acid (positive control), or test solution into a sterile 1.5 ml-screw-cap vial and set aside. It is not practical to collect a zero time point because some germination will take place during the subsequent heating step.
3. Dilute endospore stock solution to a concentration of _5 × $10^4$ CFU/ml in PBS (pH 7.0 to 7.4). Prepare a large enough volume so that 250 μl aliquots can be added to each tube prepared in step 2.
4. Transfer 1.0 ml aliquots to 1.5-ml screw-cap vials and incubate in a 65°C water bath15 min. Use of a screw-cap vial is important to prevent pressure from building inside the vial and forcing a snap-cap tube open.
5. Remove vials from the water bath and allow them to equilibrate to room temperature.
6. Aliquot 250 μl heat activated endospores and mix with each vial prepared in step 2. Transfer to 37°C water bath.
7. At the prescribed time points, remove one vial PBS control, uric acid control, and test compound from the water bath.
8. From each vial, remove a 50 μl aliquot and dilute it 1/10 by adding to a 1.5-ml snap-cap tube containing 450 μl PBS. Spread three 100 μl aliquots from each tube on MYPGP

agar plates. Colony counts from these plates serve to determine the total count of bacterial cells present in each vial. These plates should have ~250 colonies each.

9. Recap each germination vial, transfer to a 65°C water bath, and incubate 20 min.
10. Remove vials from the 65°C water bath.
11. From each vial, spread three 100 µl aliquots on MYPGP agar plates. Then remove a 50 µl aliquot and dilute it 1/10 by adding to a 1.5-ml snap-cap tube containing 450 µl PBS. Spread three 100 µl aliquots from each dilution tube on MYPGP agar plates.
12. Incubate plates from steps 7 and 11 at 37°C up to 72 h and observe for colonies at 24 h increments.

    Colony counts from these plates serve to determine the count of heat-resistant endospores present in each vial.
13. Calculate viable colony forming units present in each suspension before and after heat treatment by multiplying the average number of colonies by the appropriate volume and dilution factors.
14. Determine percentage germination according to the formula below:

    (CFU no heat) − (CFU heated) / (CFU no heat) ×100%

## 3.11. Reagents and Solutions

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions.

### 3.11.1. HistoDenz Gradient

HistoDenz (Sigma-Aldrich)

Deionized distilled water

Make a 20% w/v solution of HistoDenz in deionized distilled water. Mix until thoroughly dissolved. Make a 50% w/v solution of HistoDenz in deionized distilled water. Mix until thoroughly dissolved. Pass both solutions through a 0.22-µm sterile filter and collect the filtrates in separate sterile vessels. Transfer 2.5 ml of the 50% w/v solution to the bottom of a 15-ml conical tube. Carefully layer 2.5 ml of the 20% w/v solution on top of the 50% w/v solution without disturbing the lower layer. Store at 4°C for 48 h.

### 3.11.2. TMYPG Broth

Yeast extract

10% (v/v) L- or D-glucose solution, sterile filtered

Sodium pyruvate

0.3 M Tris·maleic acid buffer (pH 7.0)

Add 15 g yeast extract, 3 g potassium phosphate, and 1 g sodium pyruvate to 800 ml distilled deionized water. Mix well, transfer to a graduated cylinder and add 100 ml 0.3 M Tris·maleic acid buffer. Bring the total volume to 960 ml by adding additional distilled deionized water. Autoclave at 121°C for 20 min, remove from autoclave, and allow to cool. Add 40 ml 10% glucose solution and mix well. Broth can be stored at room temperature for 1 to 2 weeks or at 4°C for 2 to 4 weeks. Pipet slowly to avoid mixing of the gradient. Gradients should be prepared for immediate use. See (Dingman & Stahly, 1983).

## 3.12. Commentary

### 3.12.1. Background Information

The procedures described here are adapted from those developed for other endospore forming species within the genera Bacillus. Despite the importance of morphotypic changes in the lifecycle of *P. larvae*, in vitro sporulation and germination of *P. larvae* can be inefficient in

comparison to most well-studied species. In some cases, both sporulation and germination efficiency can be <10%. This makes HistoDenz purification (Basic Protocol 2) extremely important to subsequent studies of germination. Investigators should consider the impact low efficiency sporulation and germination may have when planning experiments.

### 3.12.2. Critical Parameters

The nutrient requirements for *P. larvae* sporulation are not well understood. However, production of highly pure stocks of endospores (Basic Protocol 2) is critical to downstream analysis of germination, especially by optical density (Basic Protocol 3). Care should be taken to avoid mixing of the HistoDenz layers during column preparation and endospore addition. Removal of all residual HistoDenz during the subsequent wash steps is also critical as it may interfere with endospore stability and germination. There are four recognized genetic subtypes of *P. larvae* (Genersch, 2010b) denoted as ERIC I through IV. ERIC types I and II are associated with American foulbrood disease, with ERIC I being the more virulent of the two. All of the protocols described here, including the use of uric acid as the positive control germinant, have been developed for ERIC I. Investigators using other ERIC types may need to adjust parameters to optimize each protocol for the isolate of interest.

### 3.12.3. Troubleshooting

Over-aeration seems to inhibit *P. larvae* sporulation but some amount of mixing is required. Investigators should adjust the rate of shaking during liquid endospore production (Alternate Protocol 1) if spores are not evident within 72 h. If titration plates (Support Protocol) show a smeared lawn instead of distinct colonies, allow the plates to dry in a biological safety cabinet for up to 1 h before use.

### 3.12.4. Anticipated Results

Endospore concentrations before and after HistoDenz purification will vary greatly for different *P. larvae* isolates but they are typically between $10^6$ and $10^9$ CFU/ml. Exposure to uric acid should trigger germination of >60% of the endospore population within 30 min at 37°C.

### 3.12.5. Time Considerations

*P. larvae* is relatively slow growing in comparison to more commonly studied endospore forming species. Therefore, endospore production requires several days. Generation of a purified endospore stock solution (Basic Protocol 2) requires several hours and should be completed on the same day it is initiated. Germination occurs rapidly, usually within seconds of exposure to germinant signals, and can be tracked in real time using the optical density assay (Basic Protocol 3).

### 3.12.6. Acknowledgement

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# 4. EFFICACY OF CHLORINE DIOXIDE FUMIGATION ON THE SPORES OF PAENIBACILLUS LARVAE, THE CAUSATIVE AGENT OF AMERICAN FOULBROOD DISEASE IN HONEYBEES

## 4.1. Introduction

Honeybees (*Apis mellifera*) play a critical role in the pollination of crops, fruits, and vegetables, with 90-130 commercial crops depending on or supported by honeybee pollination services like almonds, apples, and cherries. Honeybee pollination added 15 billion dollars in value to agriculture production in 2000 in the United States (Morse and Calderone, 2000). Current crop planting and management schemes require the movement of millions of hives around the country, but the number of honeybees available for pollination is in decline as the beekeepers are unable to compensate for all the losses (Genersch, 2010a). On the other hand, commercial crop production places a high and continue growing. The demand on honeybees for pollination services has never been higher and is continuing to grow, but declining numbers of honeybees will eventually lead to shortage in pollination services, a decrease in lands devoted to commercial crop production, a decrease in food production, and higher food prices.

Infectious diseases such as American Foulbrood disease (AFB) are a major cause of honeybee decline (Genersch, 2010a). AFB is a bacterial disease with a global distribution that can lead to the collapse of entire colonies (White, 1906). The causative agent, *Paenibacillus larvae*, is a Gram-positive bacteria that forms highly infectious spores. The *P. larvae* spore is transmitted within the brood nest by larval food (Tarr, 1937). A survey of honeybee hives used to pollinate almonds in California showed that 4% of colonies contained significant AFB load (Cox et al., 2005). Another survey in Australia indicated 50% of bee keepers have encountered AFB during their beekeeping (Rhodes, 2007).

Spores monitor the environment for favorable conditions and the presence of germinants such as L-alanine and L- valine in the case of *Bacillus subtilis* (Stlow 2014). Germinants bind to germinant receptors located in the spore inner membrane, leading to spore germination and outgrowth to a vegetative cell ( Setlow, 2003). The environmentally-resistant spores ( Setlow et al., 2002) are transmitted within and between colonies by the activity of nurse bees and the behavior of the apiarist. Bee larvae are susceptible to infection 12-36 hours after hatching, while the adult honeybees are resistant to infection (Genersch, 2010b). In contrast to adults, the midgut and hindgut of honeybee larvae are disconnected, causing an accumulation of waste products and nutrients in the midgut (Winston, 1987). Therefore, *P. larvae* spores accumulate and are exposed to the germinant. After germination, *P. larvae* proliferate and invade the honeybee larval tissue.

AFB management strategies include antibiotics, natural products, selective breeding for hygienic behavior in honeybees, and the burning of infected hives (Genersch, 2010b). These methods do not effectively eliminate AFB and come with high economic cost. Antibiotics and natural products are aimed at the vegetative form of *P. larvae* and are ineffective against spores. Antibiotics are not permitted in honey production in most of Europe due to concerns about the spread of antibiotic resistance and the presence of antibiotic residues in honey (Alippi, 1999; Genersch, 2010b). Given the poor efficacy of current decontamination treatments, new approaches to control AFB are required.

Liquid disinfectants have been used to decrease or eliminate pathogenic bacteria and the diseases they cause (Rutala et al., 2000). However, bacterial spores have a structure that makes them resistant to most chemical disinfectants. The spore core that contains the DNA is protected by several layers: inner membrane, germ cell wall, an outer cell membrane, spore coat, and an outer layer called the exosprium ( Setlow 2003). These barriers provide protection and prevent

disinfectant agents from penetrating and killing the spore (Henriques and Moran 2000). Liquid disinfectants have been used to inactivate bacterial spores with limited success. Some disinfectants inactivate the spore if they are used with high concentration, high temperature, and longer treatment time (Sagripanti and Bonifacino 1996; Whitney et al., 2003).

Contaminated apiary tools are considered as a major source of AFB infection (Genersch 2010b). However, no previous study assessed the used of liquid disinfectants on *P. larvae* spores. In this study, we evaluate the efficacy of some common commercial disinfectants (10% Bleach, 70% Isopropyl Alcohol, Benzarid Hospital Grade Cleaner, Citrus II Hospital Germicidal Cleaner, Dettol, Rescue™ Ready to Use and Spor-Klenz® Ready-To-Use). The oxidizing agent $ClO_2$ gas is an EPA approved disinfectant (EPA, 2006) that represents a promising alternative treatment for hives contaminated with spores of *P. larvae*. The gas has been used to disinfect laboratory duct work (Lowe et al., 2012), hospital rooms, ambulances (Lowe et al., 2013), cafeterias (Hsu et al., 2014), and entire buildings (Wood & Blair Martin, 2009). Several studies have shown that it is sporicidal, with activity against *Bacillus anthracis* (Canter et al., 2005), *B. thuringiensis* (Han et al., 2003), *B. cereus* (Nam et al., 2014), *B. atrophaeus* (Shirasaki et al., 2016), and *B. subtilis* (Wang et al., 2016). Furthermore, $ClO_2$ can decontaminate large spaces such as buildings, a quality that was used to good effect to decontaminate *B. anthracis* spores during the bioterrorism attack that took place in the fall of 2001, when letters contaminated with *B. anthracis* were sent through the U.S. postal system. The building volumes that were successfully decontaminated following the 2001 anthrax attacks ranged from 45,312 to 410,640 $m^3$ (Wood & Blair Martin, 2009) making ClO2 an attractive putative disinfectant for *P. larvae.*

Although $ClO_2$ shows promise as a treatment for spore contamination, its effectiveness against spores of *P. larvae* is not yet known. Factors to be considered when assessing

effectiveness include the type of surface being decontaminated, $ClO_2$ concentration, humidity, and the presence of organic material (Dychdala, 1991); (Young & Setlow, 2003). This study was aimed at assessing $ClO_2$ effectiveness against spores of different *P. larvae* strains (two reference strains and four environmental isolates) on stainless steel, wood, and beewax as most of the beehives are made from these components.

## 4.2. Materials and Methods

### 4.2.1. *Paenibacillus larvae* Strains and Enumeration of Spores

Typed strains of *P. larvae*, belonging to the ERIC I (ATCC 9545) and ERIC IV (ATCC 49843) clades, were obtained from the American Type Culture Collection (www.atcc.org). ERIC I is the most virulent clade and it is the only clade associated with foulbrood disease in the US. Four environmental isolates of *P. larvae* (E1, E2, E3, and E4) were obtained from naturally infected honeybee hives in North Dakota.

Spores were enumerated as described previously (Mahdi and Fisher, 2018a and 2018b). Briefly, spores were serially diluted in sterile water, 100 µl was aliquoted onto an MYPGP( Mueller-Hinton broth, yeast extract, potassium phosphate, glucose, and Sodium pyruvate) agar plate, which was incubated for 4 days at 37 °C, and colony forming units were counted.

### 4.2.2. Treatment of Spores with Commercially Available Liquid Disinfectants

Spores of *P. larvae* suspended in 1 ml of sterile water were incubated with 80 µl of 10% Bleach, 70% Isopropyl Alcohol, Spor-Klenz® Ready-To-Use, Dettol, Rescue™ Ready to Use (Hydrogen Peroxide), Benzarid Hospital Grade Cleaner, and Citrus II Hospital Germicidal Cleaner for 20 min at room temperature. The final concentrations for the above disinfectants were 0.008%, 0.056%, 0.08%, 0.384%, 0.04%, 0.0028%, and 0.008% respectively. Spores of *P. larvae* suspended in 1 ml of sterile water and incubated for 20 minutes at room temperature

served as a negative control. Treatment and control samples were washed three times in sterile water, at 1398 × g for 10 minutes before spore enumeration. Disinfectant efficacy was calculated by subtracting the treated-sample spore count from the control-sample spore count.

### 4.2.3. Treatment of Spores in Solution with $ClO_2$ Gas

4.2.3.1. Treatment chambers

Spores were treated using either a one- or two-chamber treatment system. In the one-chamber system, a single chamber was used for gas generation and spore treatment. The chamber comprised an air-tight 6 L polypropylene container (STX-1000-CE Rotating marinator; Chef's Elite, Mercantile Station 2; Lincoln, NE) that was wrapped in duct tape to protect the gas from light. The top of the container had a hole through which gas could be purged and it was sealed using a 13 mm butyl rubber stopper (Kimble Kontes). In the two-chamber system (Figure 3), one chamber (chamber A) was used for gas generation and a second chamber (chamber B in Figure 3) was used for spore treatment. The two chambers (6 L polypropylene tanks) were connected by 1-inch diameter brass gas tubes, each fitted with a gas valve to control flow. Both tanks were wrapped with duct tape to protect the gas from light. A fan in the gas generation chamber was used to move gas to the treatment chamber.

4.2.3.2. Generation of $ClO_2$ gas

$ClO_2$ gas was generated and used on site by mixing a two-part dry Fruitgard media system (ICA TriNova; Newnan, GA) consisting of a zeolite carrier impregnated with sodium chlorite (Dry Media A) and an acid activator (FeCl3; Part B). The $ClO_2$ gas concentration was proportional to the amount of dry media. Dry matrix reagents were sequentially and separately weighed in a Tyvek sachet (10 x 12 cm), mixed by hand agitation, and placed inside the gas generation chamber. In the one-chamber system the gas generation chamber was also the

treatment chamber. In the two-chamber system, the valves were closed between the gas generation chamber and treatment chamber for 2 h, after which time the treatment was started by placing the spore sample inside Chamber B, opening the valves, and turning on the fan to move the gas into Chamber B. To end the treatment, the fan was turned off and the spore samples were removed from Chamber B.

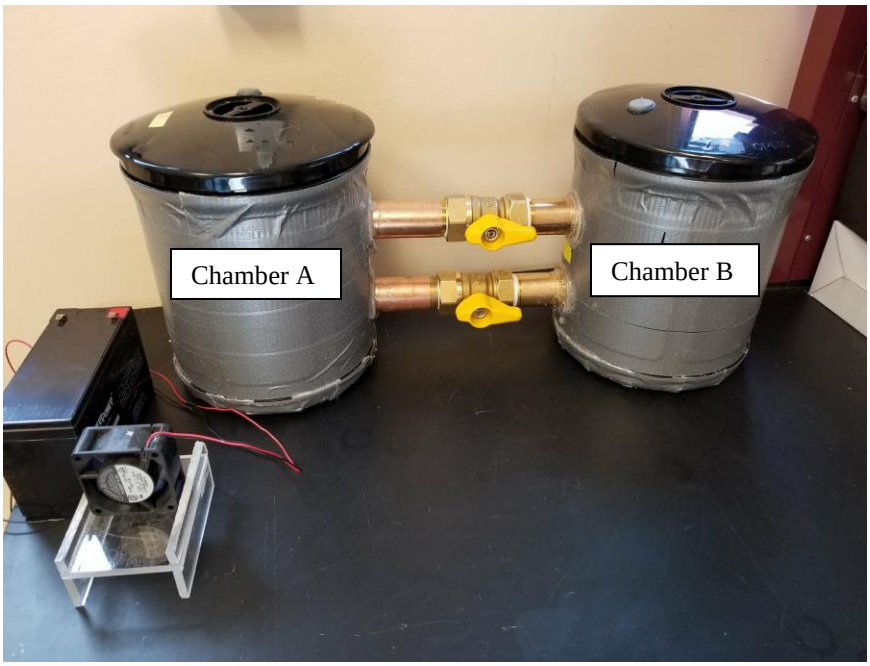


Figure 3. Closed two-chamber system

### 4.2.4. Measurement of $ClO_2$ Gas Concentration

The $ClO_2$ concentration in the treatment chamber was measured using the method described in (Smith & Herges, 2018). A 1 ml gas sample was collected using a gastight syringe (SGE Analytical Science; Melbourne, Australia) equipped with a 22-gauge syringe needle (Becton Dickinson; Franklin Lakes, NJ). Sampling of gas concentration was carried out at 0, 15, 30, 45, and 60 min (in the one-chamber system only), and after 2, 3, 4, 5 and 6 h (in both the one- and two-chamber systems). The gas samples were bubbled through 8 ml of 0.02% potassium iodide (KI) in buffer (1.5 mM sodium bicarbonate and 1.5 mM sodium carbonate, to

trap $ClO_2$ as chlorite ion ($ClO_2^-$). A gas sample was collected from five different treatment chambers at each time point.

Chlorite was quantified by injecting 100 µL aliquots of KI trapping buffer onto a Dionex AS2100 ion chromatograph equipped with Dionex AG9 and AS9 guard and analytical columns, respectively. Chlorite was eluted with an isocratic mobile phase of 9 mM potassium carbonate originating from an in-line eluent generator. Chlorite was detected as suppressed conductivity and quantified using least squares regression of peak area against a standard curve. The standard curve was generated with 0, 50,75, 150, 225, 750, 1500, 3000, and 5000 ng/ml of sodium chlorite dissolved in KI buffer. The software Chromeleon version 7.1.1 was used to control the chromatograph and analyze the data.

**4.2.5. Effect of Exposure Time on Viability of Spores**

We tested the susceptibility of *P. larvae* spores (from the two typed strains and four environmental isolates) to $ClO_2$ gas at different exposure times. The treated group was exposed to the gas, while the control group was without treatment. Experiments were conducted in both the one-chamber and two-chamber system. In the one-chamber system, spore suspension in distilled water was used, and the exposure times were 0.5, 1, and 5 h. The 0.5 and 1 h treatments were carried out in triplicate, and the 5 h exposure treatment was carried out one time.

In the two-chamber system, spore suspension in distilled water was used and the exposure times were 5, 10, 15, 30, and 60 min. Each treatment was carried out in triplicate.

To show the effect of longer treatment periods and low gas concentration, we treated the spore suspension with average gas concentration of 12.5 ng/ml $ClO_2$ for treatment periods of 1, 2, 3 and 4 h. After each experiment, 100 µl of spore sample was inoculated on MYPGP agar in triplicate and the CFU/ml was determined for the treated and control groups.

### 4.2.6. Effect of $ClO_2$ Gas Concentration on Viability of Spores

The two reference stains and one environmental isolate spore suspension in distilled water were used in this study. Similarly, in each experiment treated group with the gas and control group without treatment were used. The first treatment time was 1 h. We started with gas concentration of an average range of 645.3±150 -811.8± 105 ng/ml $ClO_2$ and reduced the gas concentration by using different media weight, that is 20, 10, 7, 5,2.5,1.25, 0.75, 0375,and 0.187 g.

The second treatment time was 2 h and we started with gas average range 48.2±10 -53± 13ng/ml $ClO_2$ and continued reducing $ClO_2$ concentrations. Similarly, after each treatment the CFU/ml for each group was determined by inoculating 100 µl of spore sample on MYPGP agar in triplicate.

### 4.2.7. Treatment of Spores on Surfaces with $ClO_2$ Gas

4.2.7.1. Preparation of Coupon Material

Three materials were used: stainless steel, wood, and bee wax, which represent surfaces commonly used or found in beehives. The materials were cut to form 1x1 cm coupons, sterilized by autoclaving at 121°C for 30 minutes, and maintained aseptically until required for testing. The tested coupons were divided into four groups placed in sterile petri dish in a class II biological safety cabinet, inoculated with 50 µl of *P. larvae* ATCC 9545 spores (group 1 and 2), and allowed to dry for 30-45 min. Non-inoculated coupons were included in the treatment as a control (group 3 and 4). The coupon group 1 (inoculated with spores and act as the treated group) and group 3 (non- inoculated) were transferred to the $ClO_2$ gas treatment chamber. While the inoculated coupon in group 2 remained outside the treatment chamber as an untreated control. Group 4 a non-inoculated group was also kept outside the treatment chamber as additional

control to check for any contamination in the coupons. For the $ClO_2$ gas, we tested various gas levels and specific treatment time figures 9 and 10. The specific treatment time was either 1 or 2 hrs.

After the gas treatment, each coupon was aseptically placed in a 50 ml conical tube containing 10 ml MYPGP broth. All the tubes were incubated in shaking incubator at 37°C for 48-72 h and examined for turbidity.

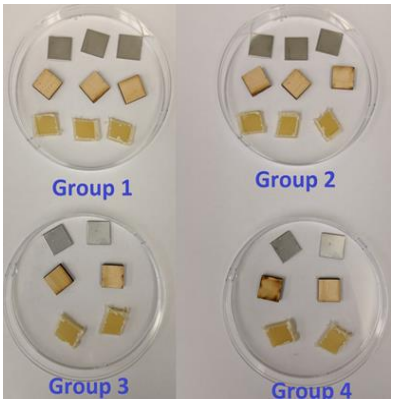


Figure 4. Coupon groups. Group1was inoculated with spores and treated with the gas. Group 2 was inoculated with the spores and without treatment. Group 3 was sterile and treated with the gas. Group 4 was sterile and without treatment.

### 4.2.8. Statistical Analyses

Each experiment was done in 3 biological replicates with 3 technical replicates in each biological replicate except for the 5 hours treatment time, that time point was done only once with 3 technical replicates. For the effect of liquid disinfectant and the effect of $ClO_2$ gas on *P. larvae* spores in water, the data were presented as a $\log_{10}$ CFU/ml. While for the effect of the $ClO_2$ gas on the bacterial spores attached to the coupons, data were presented as growth or no growth. Log reduction was determined by subtracting the log of the treated group from the log of the control group. The detection limits for *P. larvae* spores, using the plating method, was 10 CFU/ml when no colonies were detected in the treated group which resulted in 1 $\log_{10}$ CFU/ml.

Statistical analyses for the data from the effect of liquid disinfectants and the effect of $ClO_2$ gas on the spores in water were performed using multi-factor analysis of variance (ANOVA) to detect significant differences ($p < 0.05$) between treatment conditions and strains response using the SAS program, version 9.3 (SAS Institute, Inc., Cary, N.C.). Tukey test was used as *post hoc* test in case the comparisons resulted in statistically significant differences. A general association test was used for the analysis of the coupon growth data using above software. The test examined the general association between coupons and growth status, while controlling for the gas concentration. Likewise, we tested for a general association between concentration and growth status, while controlling for the type of coupon material in order to identify the effect of the coupons material on spores survival.

## 4.3. Results

### 4.3.1. Effect of Liquid Disinfectants on *P. larvae* Spores

Several commercially available liquid disinfectants were tested for their efficacy against spores of *P. larvae* ATCC 9545 (Figure 5). With the exception of 10% bleach, spore reductions following all treatments were less than one $\log_{10}$ CFU/ml. None of the treatments caused statistically significant reductions in spore numbers, relative to the control.

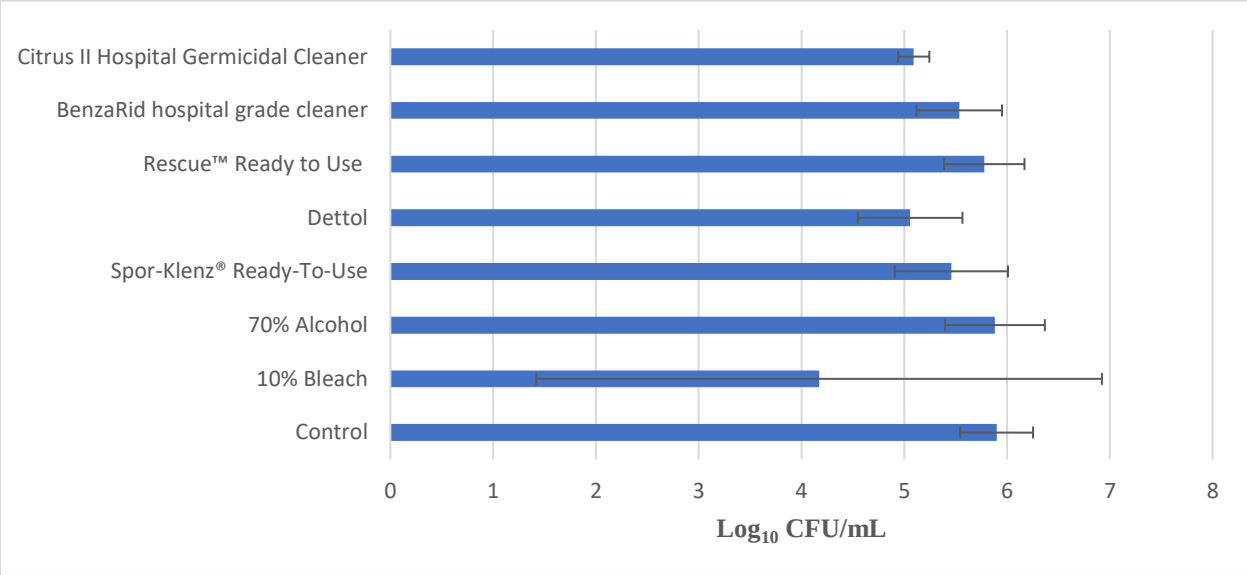


Figure 5. Mean numbers of P. larvae ATCC 9545 spores (log10 CFU/ml) ± SD in an untreated control and in samples treated with different commercial disinfectants for 20 minutes at room temperature.

### 4.3.2. Effect of $ClO_2$ Gas on *P. larvae* Spores in a One Chamber Treatment System

$ClO_2$ gas was generated by mixing 10 g of each media part in a sachet at room temperature. The theoretical gas levels at different time points were calculated using information provided by the manufacturer (each 1 g of media A and media B should release 8.9 mg $ClO_2$ in 6 h) and compared to actual $ClO_2$ gas levels, determined by ion chromatography (Figure 6). The results show good agreement between theoretical and actual gas levels, with the $ClO_2$ gas concentration increasing from 532 ±58 ng/ml after 1 h to 805±104 ng/ml.

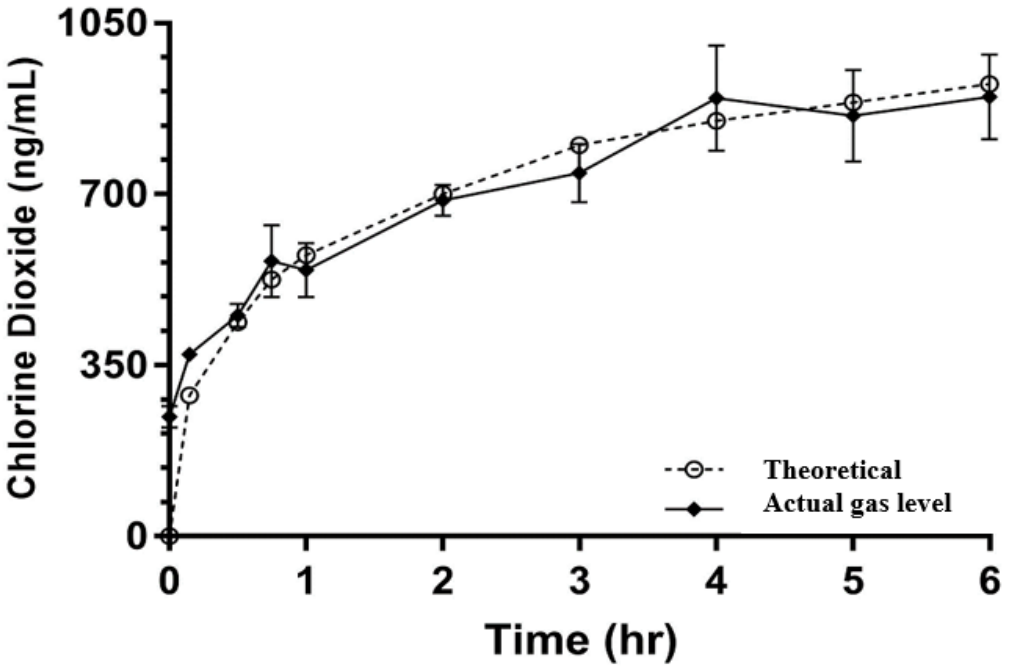


Figure 6. Comparison of predicted and actual ClO2 gas levels in the one-chamber treatment system over time. Data represent mean ± SD.

The spores of *P. larvae* strains ATCC 9545 and ATCC 49834 and four environmental isolates – E1, E2, E3, and E4 – were used in this experiment. Based on data reported in figure 6, average $ClO_2$ gas concentrations for 30 min, 1 h, and 5 h treatments were 451±24, 532 ±58, and 805±114 ng/ml, respectively. The 1 h and 5 h treatments resulted in approximately 5-8 and 5.2-8 log CFU/ml before treatment to below the detection limit. The 30 min treatment reduced spore numbers by 1.7-4.4 log CFU/ml (Figure 7).

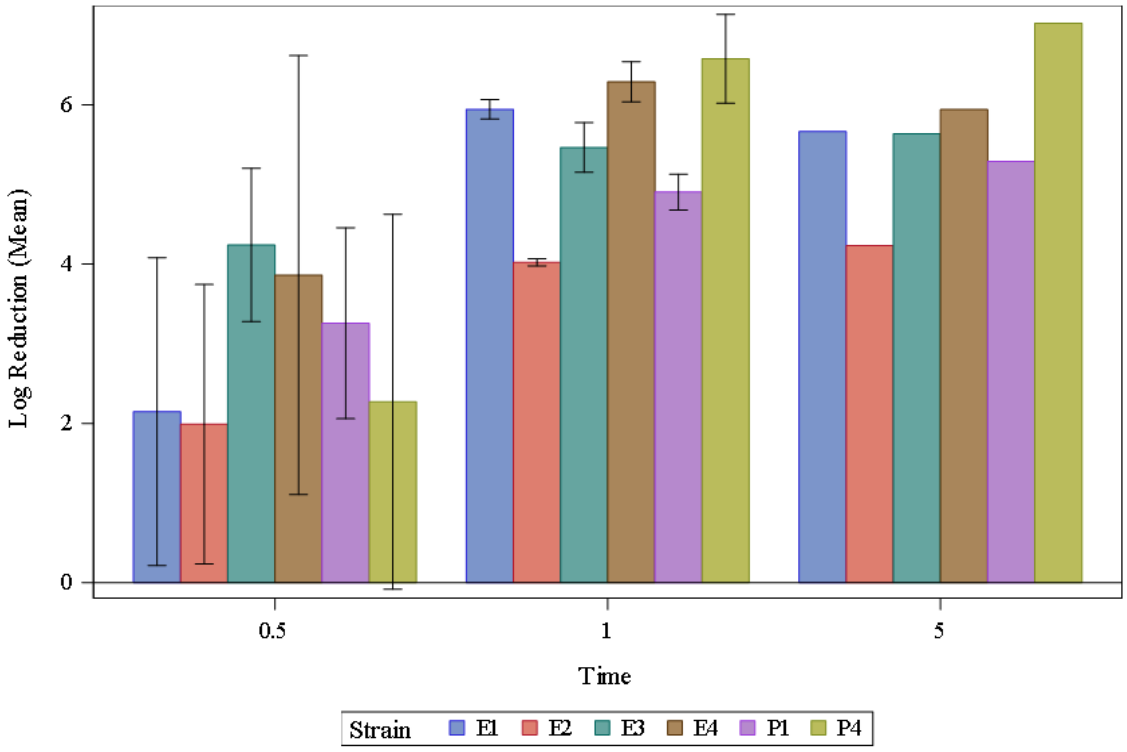


Figure 7. Effect of $ClO_2$ gas on *P. larvae* spores. Samples were exposed to $ClO_2$ gas for 5, 1, and 0.5 hours in single chamber fumigation system. P1= reference strain ATCC 9545, P4= reference strain ATCC 49834 and E1-4= environmental isolates. Time in hours. Data represent $log_{10}$ reduction mean ± SD.

### 4.3.3. Effect of ClO2 Gas on *P. larvae* Spores in a Two Chamber Treatment System

A limitation of the one-chamber treatment system was that gas concentration varied considerably, particularly during shorter treatment times when gas was being generated. To overcome this problem, we used a two-chamber system, whereby gas was allowed to generate for 2 h in a gas generation chamber before beginning the treatment. The volume of the two-chamber system (12 L) is double that of the one-chamber system so 20 g of each media part was used to achieve the same gas concentration as 10 g of media in the one-chamber system. Two hours after mixing the media parts in the gas generation chamber, a valve was opened to release the gas into the treatment chamber. Gas levels were measured in the treatment chamber 0, 1, 2, 3, and 4 h after opening the valve (Table 1).

To test the effect of exposure time on spore inactivation, spores were treated with $ClO_2$ gas, generated from 20 g of each media part, for 5, 10, 15, 30, and 60 min. During that time, the gas levels ranged from 645.3 ± 150 ng/ml (immediately after the valve was opened) to 811.8 ± 105 ng/ml (at 60 min).

For all strains tested, treatment with $ClO_2$ gas for 30 and 60 min reduced spore numbers from 4.5-7.2 and 4.9-7.1 $log_{10}$ CFU/ml to below the detection limit. All other time point treatments reduced spore numbers with greater reductions at longer treatment times (Figure 8).

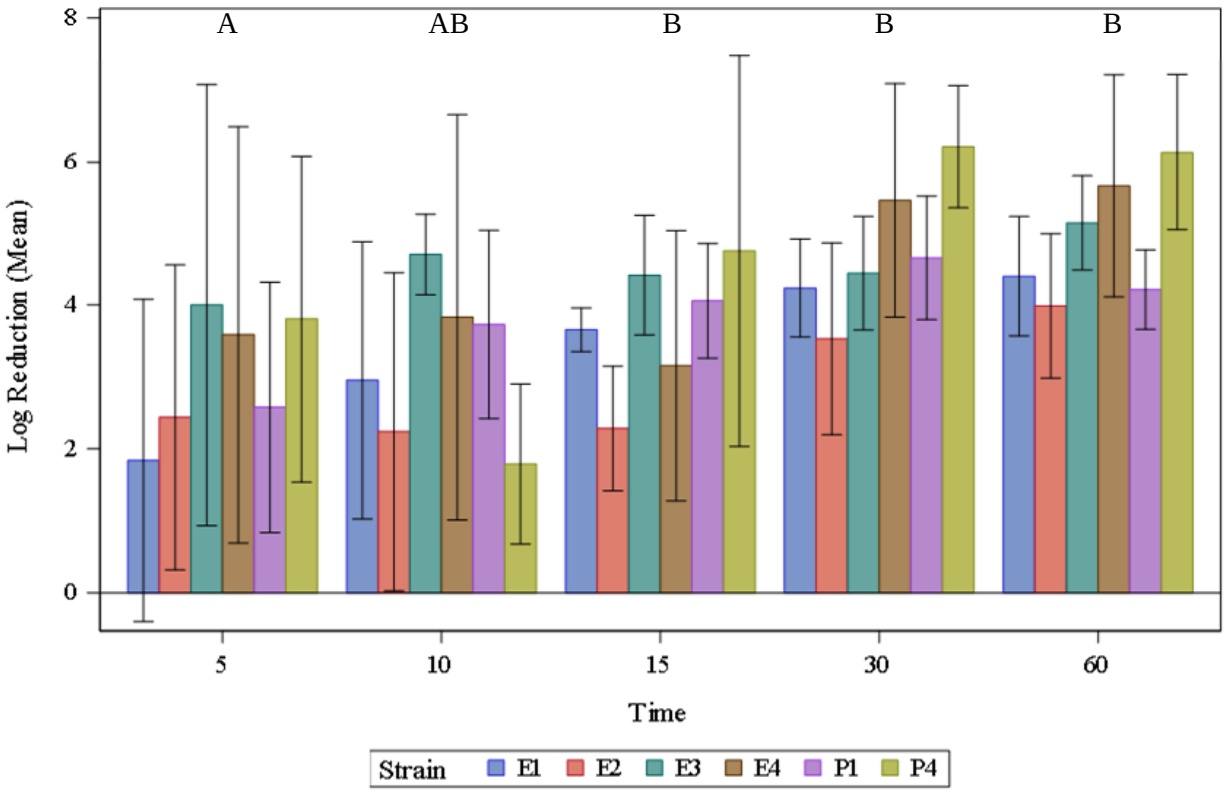


Figure 8. Effect of $ClO_2$ gas on *P. larvae* spores at various exposure times. P1= reference strain ATCC 9545, P4= reference strain ATCC 49834 and E1-4= environmental isolates. Means with the same or overlapping letters are not significantly different. Each letter represents the aggregate of all the strains. Time in minutes. Data represent $log_{10}$ reduction mean ± SD.

Although 15 min exposure to the gas did not achieve complete inactivation for all spores, it resulted in reduction range from 2.9 for E2 to 5.4 $log_{10}$ CFU/ml for P4. The 5, and10 min resulted in range reduction of 2.9 for E1 to 4.6 for E3, and 1.9 for P4 to 5.7 for E3 $log_{10}$ CFU/ml,

respectively. The mean log reductions following the 15 min treatment were significantly greater than those following the 5 min treatment (ANOVA, $F_{4,14}$=3.47, $p$=0.0095) using Tukey test. The 10 min treatment did not differ significantly from the 5and 15min. treatments. Also, no significant difference was observed among the strains during all treatment time.

Next, we varied the $ClO_2$ gas levels during a 1 h treatment. The different $ClO_2$ gas levels were achieved by mixing 20, 10, 7, 5, 2.5, 1.25, 0.75, 0.375 and 0.018 g of media parts A and B (Table 1). $ClO_2$ gas was below the detection limit when using 0.375, and 0.18 g of media. For the 1 h treatment time, the $ClO_2$ gas level was ranging. At the beginning of the treatment the gas level was as shown under 0 hour, and at the end of the treatment the gas level was as shown under 1 h in Table 1. The other set of experiments were carried out to test a 2 h exposure time. We used identical media weights and extend the treatment time to 2 h. So, the bacterial spores were exposed at the beginning of the treatment to the gas level as shown at 0 hour, and at the end of the treatment the gas level was as shown at 2 h in Table 1. In addition to 1 and 2 h treatments, the bacterial spores from three strains – the reference strains ATCC 9545, ATCC 49834, and one environmental isolate – were subjected to the gas for 3 and 4 h.

Table 1. Chlorine dioxide gas level (ng/ml) release from control media weight. Gas samples were taken at 0,1,2,3, and 4 hours after opening the valves. Data represent mean ± SD.

| Time(h) / Weight of each media part (g) | 0 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| 20 | 645.3 ± 150 | 811.8 ± 105 | 866 ± 92 | 824 ± 89 | 816 ± 129 |
| 10 | 435.4 ± 87 | 456.1 ± 130 | 449.4 ± 157.5 | 437.7 ±152 | 411.4 ± 155 |
| 7.5 | 214 ± 110 | 245.1 ± 145 | 258 ± 168 | 252 ± 188 | 231.8 ± 185 |
| 5 | 191.5 ± 64 | 198 ± 48 | 200.6 ± 61 | 219.3 ± 59 | 217.3 ± 54 |
| 2.5 | 48.2 ± 10 | 53.3 ± 15 | 53 ± 13 | 60 ± 20 | 59 ± 21 |
| 1.25 | 21 ± 6 | 20 ± 3 | 18 ± 3 | 19 ± 5 | 22 ± 6 |
| 0.75 | 7 ± 1.6 | 15 ± 4.3 | 13 ± 8 | 15 ± 7 | 16 ± 6.6 |

The result showed that the treated spores were below the detection limit of all *P. larvae* strains when using 191.5± 64 - 198 ± 48ng/ml and higher for the 1 h treatment (Figure 9). Reducing $ClO_2$ gas concentration to 48.2± 11- 53.3 ± 15 ng/ml result in $log_{10}$ reduction by 0.7-2.9 with a significant difference (ANOVA, $F_{5,23}$=3.16 $p$=0.0116) observed between that group and other groups. The $log_{10}$ reduction decreased with lower gas concentration.

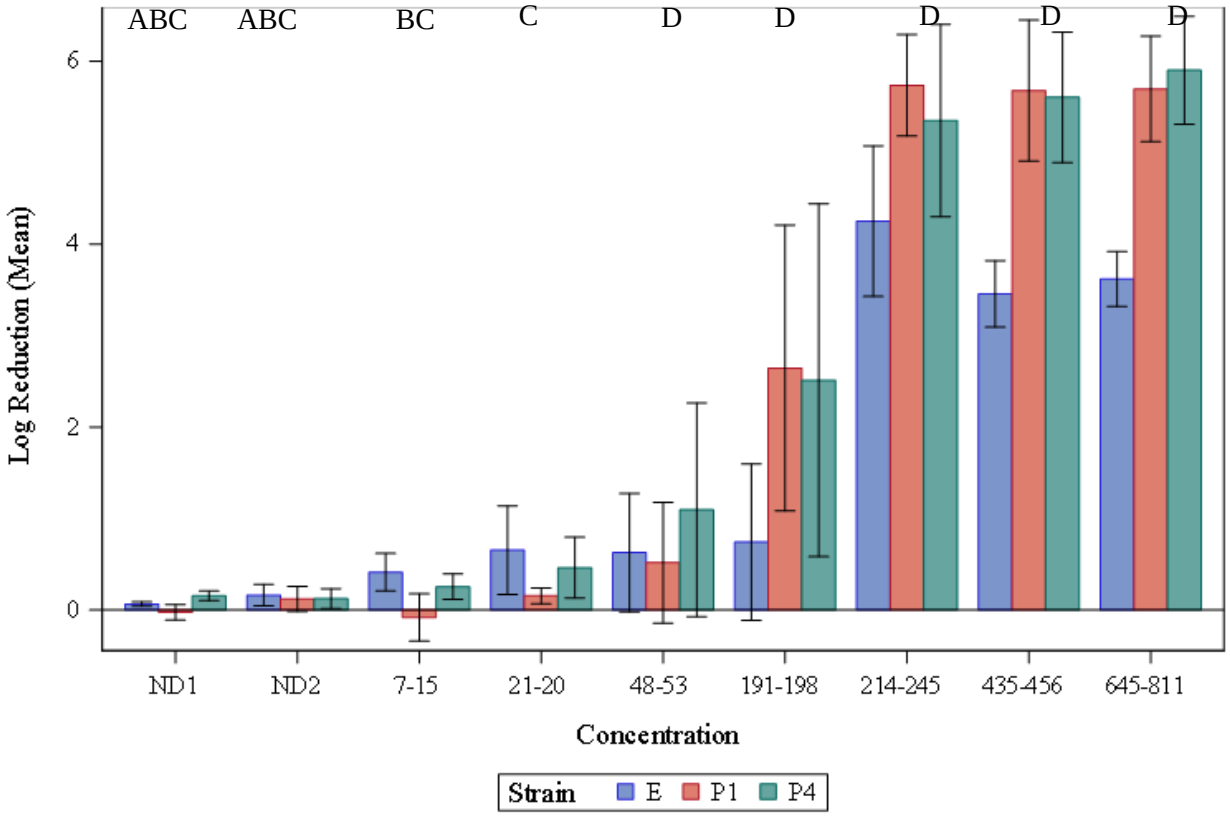


Figure 9. Effect of $ClO_2$ gas on *P. larvae* spores at various concentrations and 1 h exposure time. P1: reference strain ATCC 9545, P4: reference strain ATCC 49834 and E: environmental isolates. Means with the same or overlapping letters are not significantly different. ND1 and ND2 means gas below detection limit. Gas concentration ng/ml. Data represent $log_{10}$ reduction mean ± SD.

When extending the treatment time to 2 h, a lower gas concentration 21 ± 6 -18 ± 3 ng/ml achieved inhibition of the spores to below detection limit in all the bacterial spore samples with a significant difference, (ANOVA, $F_{1,1}$=4.84 $p$=0.0347) observed between ND1 and ND2 group and other groups (Figure 10).

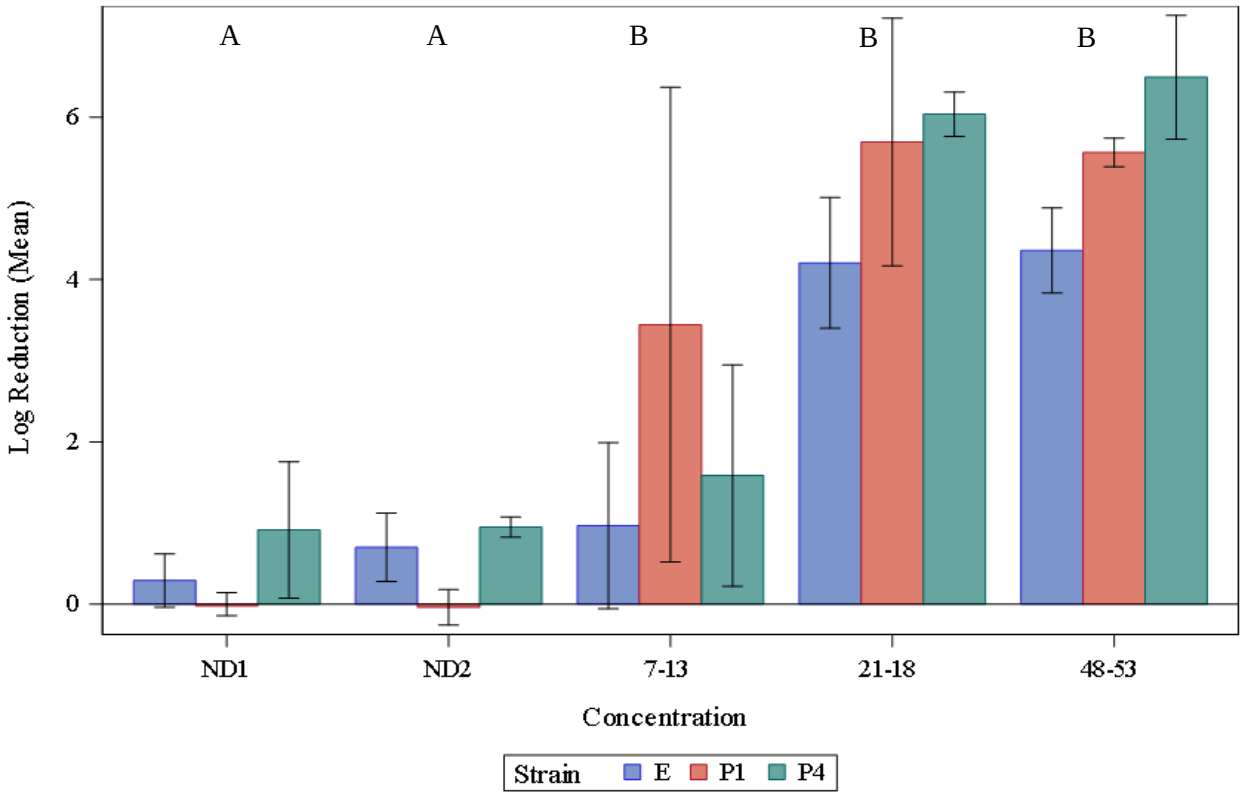


Figure 10. Effect of $ClO_2$ gas on *P. larvae* spores at various concentrations and 2 h exposure time. P1: reference strain ATCC 9545, P4: reference strain ATCC 49834 and E: environmental isolates. Means with the same or overlapping letters are not significantly different. ND1 and ND2 means gas below detection limit. Gas concentration ng/ml. Data represent $log_{10}$ reduction mean ± SD.

Spores were reduced by 1-2.1 $log_{10}$ with exposure to 7 ± 1.6 -13 ± 8 ng/ml for 2 h. The reduction was decreased with the lower gas concentrations. While a 1 h treatment with 21 ± 6 - 20 ± 3 ng/ml $ClO_2$ did not totally inhibit the spores, a 2 h treatment with the same $ClO_2$ concentration inhibit the treated samples to below detection limit in all spore samples. To evaluate the sporicidal activity of the $ClO_2$ gas for longer treatment periods, we used $ClO_2$ gas level generated from mixing 0.75 g each media part as shown in Table 1 for 1,2 ,3 and 4 h. The result showed 0.51, 1.23, and 0.62 $log_{10}$ CFU/ml reduction for the three strains when exposed to 1 h treatment as shown in Figure 11. Extending the treatment for 2 h increased the $log_{10}$ reduction to 2.15, 1.78 and 1.03 $log_{10}$ CFU/ml for ATCC 9545, ATCC 49834, and the

environmental isolate respectively. Furthermore, 3 h treatment resulted in 3 and 3.04 $log_{10}$ reduction for *P. larvae* ATCC 9545 and ATCC 49834, while for the environmental strain it was 3.71 $log_{10}$ reduction.

Spores were below detection limit when treating for 4 h with 5.62, 6.32, and 4.43$log_{10}$ reduction (Figure 11).

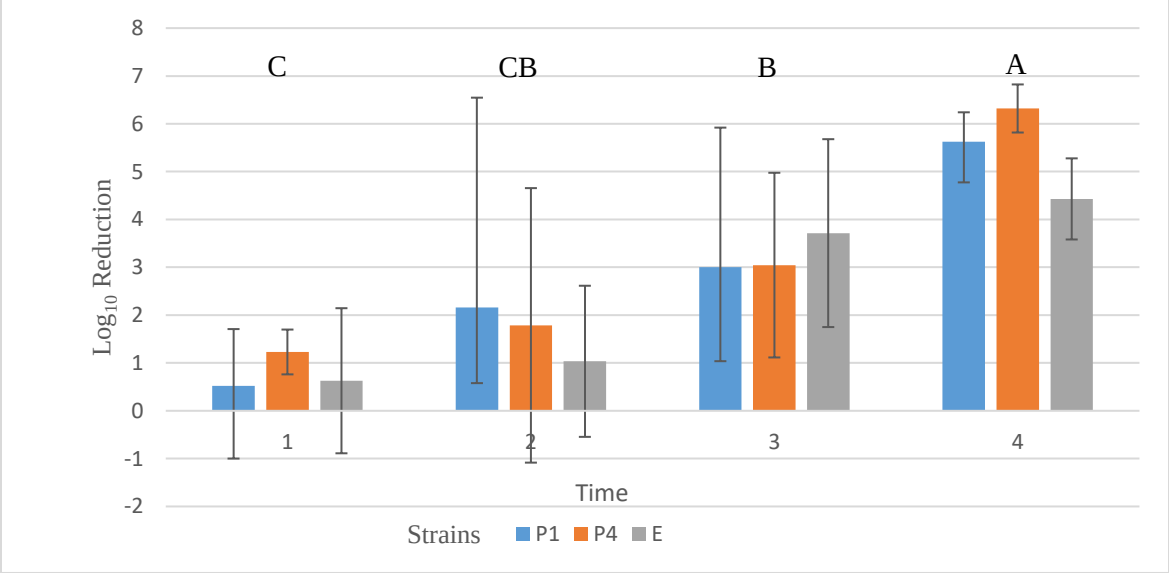


Figure 11. Effect of $ClO_2$ gas on *P. larvae* spores at various exposure times(hrs). P1= reference strain ATCC 9545, P4= reference strain ATCC 49834 and E=environmental isolate. Treatment time in hours. Log reduction with the same or overlapping letters are not significantly different. Data represent $log_{10}$ reduction ± SD.

### 4.3.4. Inactivation of *P. larvae* Spores Using Various $ClO_2$ Levels and Specific Exposure Time Attached to the Coupons

We tested the sporicidal activity of $ClO_2$ on *P. larvae* spores attached to coupons. For the coupons, we used surfaces: stainless steel, wood, and bee wax as most of the beehives are made from these components. After the gas treatment each coupon was aseptically placed using sterile forceps into 50 ml tube containing 8-10 ml MYPGP broth. All the tubes were incubated in shacking incubator at 37°C for 48-72 h. Any viable spores will result in germination and growth in the MYPGP broth. $ClO_2$ levels used during the 1 h treatment are shown in Table 1.

The 1 h treatment time showed no growth in the treated groups with (214± 110 - 245.1± 145) ng/ml $ClO_2$ and higher, some coupons showed growth when treated with (191.5± 64 -198 ± 48) ng/ml, (48.2± 11 -53.3 ± 15) ng/ml $ClO_2$ did not inhibit the spores attached to all coupons (Figure 12).

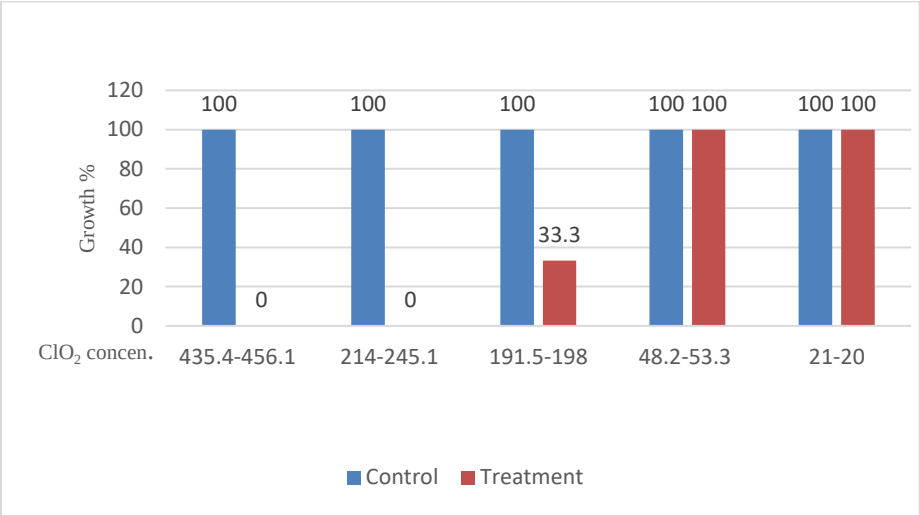


Figure 12. Effect of ClO2 gas on *P. larvae* reference strains ATCC 9545 spores attached to coupons for 1hour treatment time. Total number of coupons was 9. Data represent percentage of coupons with growth.

By extending the treatment to 2 h, (191.5± 64 - 200.6 ± 61) ng/ml $ClO_2$ achieved complete spore inhibition in all coupon types. While (48.2± 11 -53 ± 13) ng/ml $ClO_2$ only 11.11% of the coupons showed growth. Treatment with (21 ± 6 -18 ± 3) ng/ml $ClO_2$ for 2 h resulted in growth in 66.67% of the coupons. In contrast, (7 ± 1.6 -13 ± 8) ng/ml $ClO_2$ failed to inactivate spores in all coupons (Figure 13). A significant difference (ANOVA, $F_{2,3}$=6.81 $p$=0.0111) was observed between 2 h and 1 h treatment time.

There was no significant difference in spore survival among the types of coupon materials during both treatment times.

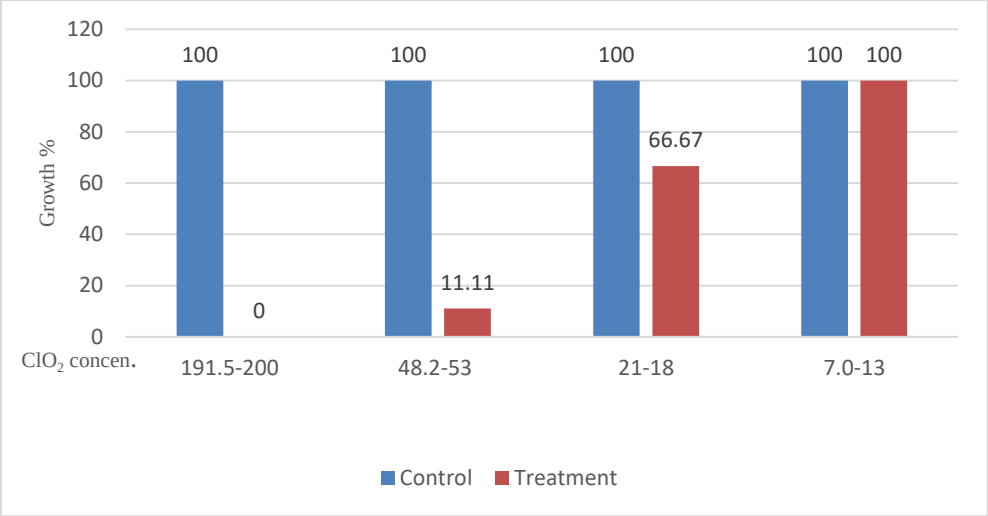


Figure 13. Effect of $ClO_2$ gas on *P. larvae* reference strains ATCC 9545 spores attached to coupons for 2hour treatment time Total number of coupons was 9. Data represent percentage of coupons with growth.

## 4.5. Discussion

### 4.5.1. Effect of Liquid Disinfectants on *P. larvae* Spores

We evaluated the sporicidal activity of seven commercial disinfectants against *P. larvae* ATCC 9545 spores. This was the first study to examine the effects of liquid disinfectants against spores of *P. larvae*. None of the tested disinfectants significantly reduced spore numbers. Studies examining the effectiveness of liquid disinfectants against other spore forming bacteria have found that spore reductions depend on disinfectant type, concentration, exposure time, temperature, and bacterial strain (Andre et al., 2012; Blakistone et al., 1999; Hilgren et al., 2007; Peng et al., 2002; Sagripanti & Bonifacino, 1996; Sudhaus et al., 2012). A possible explanation for the ineffectiveness of liquid disinfectants in the present study was the relatively short treatment time (20 min in this study). Andre et al. (2001) found that peracetic acid-based disinfectants, such as Spor-Klenz® Ready-To-Use, were ineffective against *Clostridium*, *Geobacillus*, and *Moorella* spores when the treatment time was 5 min, but their efficacy was significantly higher after 30 min treatments. Similarly, peracetic acid was only effective against

*B. cereus* spores when treatment duration was longer, with 0.16-0.97 $\log_{10}$ CFU/ml reductions following a 30 min treatment and 3.56-3.99 $\log_{10}$ CFU/ml reductions following a 60 min treatment (Sudhaus et al., 2014).

We found that disinfectants with alcohol, hydrogen peroxide (Rescue™ Ready to Use), and benzalkonium chloride (Citrus II Hospital Germicidal Cleaner) as the active ingredients were ineffective against spores of *P. larvae*, which is consistent with findings of previous studies on spores of other bacterial species. Alcohol is a broad-spectrum antimicrobial for vegetative bacterial cells but it is not sporicidal (Trujillo & Laible, 1970), and because of that it is not recommended to be used for sterilization of spores. Blakistone et al., 1999 found that *B. cereus, B subtilis,* and *G. stearothermophilus* were highly resistant to hydrogen peroxide-based sanitizer. This chemical has biocidal activity against vegetative bacteria, fungi, and viruses, but not bacterial spores. The bacterial spore permeability barrier blocks alkylating agents such as Benzalkonium chloride (García & Cabo, 2018).

Some liquid disinfectants can damage germination proteins resulting in spore inhibition. We expect that was the case when using the bleach, in which the active ingredient is hypochlorous acid. This compound causes unfolding of bacterial proteins in an irreversible manner (Winter et al., 2008). Similarly, chloroxylenol, the active ingredient in Dettol, disrupts cell membrane proteins (WHO, 2008), which may explain the 0.84 $\log_{10}$ CFU/ml reduction we saw in the present study. Part of the high resistance of *P. larvae* spores might be due to the use of single liquid disinfectant in this study. For example, use of peracetic acid and chlorine in combination exhibited synergism in sporicidal effect on *Bacillus* spores with 5 log CFU/ml reduction when treating the spores for 5 min (Alasri et al., 1993). Few *P. larvae* spores can trigger AFB infection and subsequently the collapse of a colony (Genersch et al., 2005). So, the

use of commercial liquid disinfectants used in this study to decontaminate hives and apiary tools is not recommended.

### 4.5.2. The Sporicidal Activity of $ClO_2$ Gas

In contrast to liquid disinfectants, $ClO_2$ inactivated *P. larvae* spores in a concentration- and time-dependent manner. Susceptibility to $ClO_2$ did not vary significantly among the *P. larvae* strains tested. In experiments carried out in a one-chamber system, treatments for 1 and 5 h reduced spore numbers to below the detection limit. However, a treatment for 30 min reduced spore likely because the gas continued to be generated over time in the treatment chamber. For example, the gas concentration increased from 451±24 ng/ml at 30 min to 805±104 ng/ml after 5 h. Therefore, the greater inactivation from the 5 h treatment relative to the 30 min treatment was probably due to a higher gas concentration in addition to the longer treatment time. To minimize the effect of gas concentration as a variable, we created a 2-chamber treatment system, where gas was generated in one chamber for 2 h before being added to the treatment chamber. In contrast to the one-chamber system, a 30 min treatment in the two-chamber system completely inactivated spores of *P. larvae*. Treatments for 5, 10, and 15 min also reduced spore numbers, but viable spores remained. Previous studies have shown that $ClO_2$ is more effective against vegetative cells than spores (Lee et al., 2006; Nam et al., 2014; Spotts Whitney et al., 2003; Wang et al., 2016; Young & Setlow, 2003).

$ClO_2$ gas concentration, relative humidity (HR) and exposure time are the main factors affecting its biocidal activity. Also, longer contact time and/or higher concentration are needed to inactivate bacterial spores compared to vegetative bacterial forms.

We next examined spore inactivation using lower gas concentrations for longer treatment time. A treatment with (7± 1.6 - 16± 6.6) ng/ml $ClO_2$ for 4 h completely inactivated spores, while the last treatment did not completely inactivate *P. larvae* spores after 1,2, and 3 h.

Our result agrees with previous findings that a longer treatment time results in greater spore inactivation (Foegeding et al., 1986; Lee et al. 2006; Caiet et al., 2015; Wang et al., 2016). The 2 h treatment time using 21± 6- 18± 3 ng/ml reduced spore numbers to below detection limit, while the 1 h treatment required a higher gas concentration 191.5± 64- 198± 48. Our results agree with these of Wang et al. (2016), who reported that higher concentrations result in greater inactivation of *B. subtilis* and *B. thuringiensis* spores.

$ClO_2$ gas release was consistent with the theoretical levels. This is an indication that the tank, which is made from plastic, did not absorb the gas or leak during treatments of up to 6 h. Our result regarding the gas release was consistent with the findings of Smith and Herges (2018), who used similar method of gas generation.

We evaluated the efficacy of chlorine dioxide gas in inactivating *P. larvae* spores attached to stainless steel, wood and bee wax surfaces. It was difficult to retrieve *P. larvae* spores from the contaminated surfaces. Therefore, we incubated the materials in liquid media and used the presence or absence of growth as an indicator of sporicidal activity. We tested different gas concentrations during 1 h and 2 h treatments. For the 1 h treatment, a gas level of 214± 127 - 245±145 ng/ml $ClO_2$ or higher reduced spore numbers to below the detection limit. 2 h treatment at 191.5±64 -200± 61 ng/ml $ClO_2$ also reduced spores below the detection limit. Alvarado et al. (2017) used indole analogs to inhibit *P. larvae* spore germination, as germination is an important first step toward the development of AFB. They showed that honeybee larval survival was 36-75%, compared to 22% in the control.

Earlier studies showed that treatment time, decontaminant concentration, RH and pH are factors to consider when cleaning surfaces contaminated with bacterial spores (Lee et al., 2006; Nam et al., 2014; Spotts Whitney et al., 2003; Wang et al., 2016). Similarly, our result indicated that higher $ClO_2$ gas concentration is required to inactivate *P. larvae* spores attached to surfaces versus spores in water. However, we did not evaluate the effect of RH, and pH in this study. The spores may penetrate the coupon and embed in the cavities. Also, $ClO_2$ gas attracts more to water and more humid surfaces. To our knowledge, no previous study evaluated the sporicidal activity of $ClO_2$ on surfaces contaminated with *P. larvae* spores. Nam et al. (2014) used *B. cereus* spores attached to stainless steel and 115± 5 ng/ml gas peak for 0, 10, 20, and 30 min, and 1, 2 and 6 h. Their result indicates $ClO_2$ effectively killed the spores within 1h. The spores of *B. thuringiensis* attached on paper, wool, epoxy and plastic surfaces were inactivated with $ClO_2$ gas at 20, 25 and 30 ng/ml for 12 h (Han et al., 2003). Shirasaki et al. (2016) reported that exposing filter paper contaminated with *B. atrophaeus* to 10.6 ng/ml peak concentration result in compete inactivation. The filter papers were placed inside an 87 $m^3$ room and the treatment was carried out overnight.

$ClO_2$ gas has a high oxidation capacity, even at low concentrations and over wide pH range (Winniczuk & Parish, 1997). Unlike chlorinated water, $ClO_2$ cannot react with ammonia, and humic acid to from harmful chloramine and chlorophenols (Sadiq & Rodriguez, 2004).

The oxidizing properties of $ClO_2$ causes the antimicrobial effect of the gas since $ClO_2$ can react with many organic component (Fukayama et al., 1986), particularly cell surface membrane proteins (Jeng & Woodworth, 1990). $ClO_2$ reacts with the amino acid cysteine which contains sulphur (Ison et al., 2006). Furthermore, it reacts with aromatic amino acids like tyrosine and tryptophan (Ogata, 2007). $ClO_2$ treatment cause changes in the proteins. In aromatic amino acids,

it transforms the sulfhydryl SH- group into -S-S- group (Kiokias et al., 2007). Such a change inactivates enzymes (Huang et al., 1997).

Spore coat play an important role in the resistant of the spores. Young and Setlow (2003) described the role of spore's coat and inner membrane in the resistance of *B. subtilis*. The *cotE* gene is necessary for *B. subtilis* spore coat assembly. The mutant showed more susceptibility to $ClO_2$ treatment. Furthermore, $ClO_2$ caused damage to the inner membrane, resulting in a change in permeability and a failure of spore germination. A similar effect of the gas on cell membrane permeability has been reported and cause cell death in *Escherichia coli* (Berg et al., 1986). Moreover, $ClO_2$ gas causes enzymatic damage to bacterial spores. These enzymes are required to initiate the germination process, and damage occurs when $ClO_2$ oxidizes the fatty acids and proteins which results in severe damage to the spore inner membrane and enzymes (Young & Setlow, 2003).

For future studies, measuring RH is needed as we did not include its effect in this study. RH is considered as an important factor affecting $ClO_2$ biocidal activity (Wang et al., 2010; Nam et al., 2014). Similarly, the effect of different temperatures should also be considered to identify any synergistic or additive effect to optimize $ClO_2$ treatment.

The U.S. Environmental Protection Agency [EPA] has approved the use of $ClO_2$ gas, and it is currently used to decontaminate enclosed spaces or building like laboratory duct work (Lowe et al., 2012), hospital rooms and ambulances (Lowe et al., 2013), cafeterias (Hsu et al., 2014) and entire buildings (Wood & Blair Martin, 2009). None of the tested disinfectants significantly reduced spore numbers. Its performance in sterilizing materials contaminated with P. larvae make it attractive for putative commercial use.

In summary, $ClO_2$ gas showed high sporicidal activity against *P. larvae* spores at higher concentrations and longer treatment times. The result was expected and in line with the previous findings for other bacterial spores. However, more studies are needed to evaluate the gas for indoor fumigation on a larger scale. For that purpose, a model with hive boxes in an enclosed space should be used. This would allow the influence and interaction of the hive boxes on the $ClO_2$ gas concentration to be determined (i.e. determine how much gas will be consumed by the boxes). Next, testing should be performed to optimize gas concentration, RH, treatment time, and temperature to disinfect contaminated hive boxes with *P. larvae* spores. The mechanism of gaseous chlorine dioxide in killing *P. larvae* spores also should be investigated.

# 5. CONCLUSIONS

*P. larvae* is a highly adaptive pathogen with only one known host: the honeybee larvae. The environment inside the honeybee's midgut provides the bacteria with all the requirements for spore germination and subsequent bacterial growth and proliferation.

The causative agent of AFB has been known since 1906. However, little is known about the pathogenic mechanisms, sporulation, and germination pathways in *P. larvae*. Part of this gap in our knowledge is due to the fastidious nature of *P. larvae*, which makes it difficult to culture and study. Chapter 2 describes protocols to grow and maintain *P. larvae*, which will be useful in the diagnosis of AFB, the rapid confirmation of suspected cases, epidemiological investigations, and scientific research.

There is a wealth of knowledge on bacterial spores, including spore structure and the processes of sporulation and germination. However, this knowledge is generally restricted to *Bacillus spp*. and *Clostridia spp*. and comparatively little is known about spores of *P. larvae*. *Bacillus* and *Clostridium* spores recognize nutrient germinants through proteins called germinant receptors (GRs) located in the intermembrane of the spore (Setlow, 2014). The binding of specific nutrient germinants to GRs triggers unidirectional biophysical and biochemical processes (beginning with germination and ending with outgrowth) that allow the cell to break quiescence and return to vegetative growth. Nutrient triggers of germination are species-specific (Setlow, 2014). In all spore forming bacteria, spore germination is important to establish the disease (such as anthrax, tetanus, botulism and food poisoning). AFB is not an exception as *P. larvae* spore germination is the first important step toward the development of AFB, and it is considered an Achilles heel that can be exploited to kill the pathogen.

The germination pathway in *P. larvae* remains unknown, and genes encoding germinant receptors have not been reported. Such knowledge will be necessary to develop control measures that inhibit germination. Chapter 3 describes protocols for sporulation and measurements of germination in *P. larvae*.

Chapter 4 examined treatments to inactivate spores of *P. larvae.* Although there have been several studies on treatments to inactivate spores of *Bacillus* and *Clostridia* species, we are not aware of studies on the inactivation of *P. larvae* spores.

Commercial liquid disinfectants were largely ineffective, but $ClO_2$ gas showed excellent efficacy against spores of *P. larvae* in water and on various surfaces. While the current method to control AFB requires burning the hive and any other equipment that may come in contact with the disease, causing heavy economical losses, gas treatment will help to reduce such losses. Moreover, $ClO_2$ can be used in routine decontamination of apiarist tools, which are considered one of the main sources for AFB infections. It is possible that $ClO_2$ targets amino acids, causing oxidative damage to proteins involved in germination, however this would need to be verified by biochemical analyses of protein structure and electron microscopy to examine ultrastructural changes in spore structure. Other oxidants such as ozone, decon, and hypochlorite might also be effective at inhibiting germination and are worthy of further investigation.

Bacterial spores are widely distributed in the environment (both indoor and outdoor). However, $ClO_2$ works effectively in enclosed areas where treatment parameters can be carefully controlled. Therefore, an effective field treatment will comprise an enclosed, sealable treatment container.

One of the future approaches to be taken is to treat and control AFB by interrupting the intestinal infection process and toxin inhibition. In order for the *P. larvae* to proliferate and

induce the intestinal infection of honeybee larvae, it has to compete with the microbiome of the bee larvae midgut. Genome sequences of *P. larvae* ERIC I and II have been published by (Djukic et al., 2014). The genome sequence revealed a potential peptide antibiotic.

In addition to the microbiome, the digestive tract in insects contain a peritrophic matrix (PM) rich with chitin as a further line of defense against pathogens (Terra, 2001). Invasive pathogens like *P. larvae* must have the ability to breach the chitin-rich PM and epithelial cells (Langer & Vinetz, 2001).

Recently, the chitin-degrading enzyme, PICBP49, had been identified in *P. larvae* ERIC I, and its pathogenesis role in AFB was verified (Garcia-Gonzalez et al., 2014). Furthermore, several other toxins have been characterized as well (Ebeling et al., 2016). These toxins allow the bacteria to attack the midgut epithelial cells.

So, for interrupting the intestinal infection process, different methods may achieve toxin inhibition like the inhibition of toxin gene transcription (Cegelski et al, 2008), the blockage of toxins with inhibitor molecules (Benghezal et al, 2007) and cell entry inhibition (Tautzenberger et al, 2013).

This approach will not only aid in the development of a new regime for AFB, but also have a potential implication for other diseases and microbiology in general.

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## APPENDIX. LIST OF PUBLICATIONS

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