# SURVEILLANCE AND IMPROVED CONTROL OF AMERICAN FOULBROOD IN SASKATCHEWAN HONEY BEES THROUGH THE DETECTION OF *PAENIBACILLUS LARVAE* SPORES IN POOLED, EXTRACTED HONEY

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**ABSTRACT**

The North American beekeeping industry is heavily reliant on antimicrobial metaphylaxis to prevent and control outbreaks of the destructive bacterial disease, American foulbrood (AFB). The infectious endospores (spores) of the causative agent, *Paenibacillus larvae*, are incredibly resilient to environmental extremes and are impervious to antimicrobials licensed for use against AFB (1–3). Consequently, non-curative antibiotic use fosters dependency and sustained, indiscriminate use to ensure industry profitability. In the face of growing international concern regarding antimicrobial resistance (AMR) and the emergence of strains of *P. larvae* with AMR, North American beekeepers are inadequately prepared to reduce their reliance on antimicrobials without risk of significant economic losses to AFB (2,4–11). Current methodology to guide evidence-based antimicrobial use through AFB risk assessment relies on the sampling and testing of individual hives and is logistically impossible for large-scale, commercial beekeeping operations to implement (12–21).

To address this issue, we evaluated the use of spore detection in conveniently collected, pooled, extracted honey to determine AFB risk at a yard or operation level within antibiotic-reliant apiculture. Large-scale, commercial honey bee operations in Saskatchewan, Canada, with a history of antimicrobial use and recent outbreaks of AFB were clinically characterized and opportunistically sampled to compare the detection of spores and predictive ability of pooled, extracted honey to the current standard of samples collected from individual hives. We demonstrated that pooled honey was predictive of the spore contamination identified through individual hive testing and appeared to have prognostic value in assessing the risk of AFB at the yard or operation level.

Accordingly, we expanded our testing of pooled honey to 116 Saskatchewan beekeepers representing approximately 75% of the province's 110,00 registered colonies during the study period. By correlating spore concentrations to the incidence of AFB, we established reliable prognostic thresholds at 2 and 100 spores per gram of honey to determine a beekeeper's future risk of disease. Additionally, we highlighted management practices related to a higher risk of AFB that could represent key targets for improved biosecurity to mitigate risk. Given their expertise in herd-health management and biosecurity, veterinarians should play an integral role in a beekeeper's health management team in the prevention and control of AFB. Together, beekeepers who are at

low risk of AFB through the testing of their pooled honey and are simultaneously incorporating low-risk management practices through the help of their health management team may temporarily cease antimicrobial metaphylaxis. Improved, judicious antimicrobial use in apiculture will reduce the threat of AMR and will help to ensure the sustainability of the North American industry.

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## DEDICATION

This work is dedicated to the beekeepers of Saskatchewan, whose devotion and passion for their industry made this research possible. May our findings help ensure the continued growth and prosperity of apiculture in Saskatchewan.

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| | |
|---|---|
| AFB | American foulbrood |
| AMR | antimicrobial resistance |
| BC | brood chamber |
| CFU | colony forming unit |
| CI | confidence interval |
| CLSI | Canadian Laboratory Standards Institute |
| CSA | Columbia sheep blood agar |
| ERIC | enterobacterial repetitive intergenic |
| HS | honey super |
| IPM | integrated pest management |
| L | large-scale |
| MALDI-TOF MS | matrix-assisted laser desorption/ionization time-of-flight mass spectrometry |
| MYPGP | complex medium consisting of Mueller Hinton broth, yeast extract, potassium phosphate, glucose, and sodium pyruvate |
| OIE | World Organisation for Animal Health |
| PCR | polymerase chain reaction |
| PLA | *Paenibacillus larvae* agar |
| qPCR | quantitative polymerase chain reaction |
| S | small-scale |
| SK | Saskatchewan |

# CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

## 1.1 American Foulbrood

American foulbrood (AFB) is a long-known and well-described disease of honey bee (*Apis mellifera*) larvae that continues to prove itself destructive and economically significant despite decades of intensive research (2,4,5,22–24). AFB is considered the most devastating brood disease of honey bees and is widespread across the planet (4,25). If left unrecognized, a clinically-affected colony will usually die as a result of natural disease progression (4,25). Upon recognition of clinical signs, however, beekeepers will often destroy affected colonies and either burn or irradiate contaminated equipment in an effort to minimize the spread of disease to surrounding hives (2,25–27). Due to these adverse outcomes, AFB has the potential to cause substantial economic losses for both beekeepers and agricultural producers who rely on honey bee pollination services (4,5).

The destructive capability of this disease warrants its inclusion as a notifiable disease in many countries (2). In Saskatchewan, AFB was added to the provincial notifiable animal disease list in the fall of 2019 (28). Federally, AFB has been reportable since 1924 (as per the Apiaries Act of 1930) and the government of Canada includes AFB on its list of annually notifiable diseases for the purpose of annual reporting to the World Organisation for Animal Health (OIE) (29).

### 1.1.1 Etiology and Pathogenesis

AFB is caused by the Gram-positive, facultatively anaerobic, endospore-forming bacterium, *Paenibacillus larvae*, and disease occurs when infectious spores are inadvertently fed to newly hatched larvae in contaminated larval diet (30–33). Only newly hatched larvae less than 48 hours of age are considered susceptible to infection (33–37). Although the exact explanation for this age-related resistance remains unknown, it has been suggested that the appearance of inhibiting substance(s) within the larvae older than 48 hours may be responsible (38). Regardless, the number of spores required to establish lethal infection in a susceptible, individual larva can be incredibly low ($LD_{50}$ of 8.49 ± 1.49 spores) (4,6,32), but may result in the production of over a billion new spores per infected larva (25,39).

Spores germinate into motile, vegetative rods in the midgut of larvae within 12 hours of ingestion that feed off of incoming larval diet and rapidly proliferate (2,4,22,40). Proteases released by *P. larvae* throughout vegetative multiplication penetrate the protective peritrophic

membrane of the midgut and allow bacterial translocation into the hemocoel, where the bacteria continue to multiply, leading to a fatal septicemia (2,40,41). As larval remains break down they visually undergo a color change, becoming tan to brown (42). The mass of decaying tissue itself, along with the tremendous number of accompanying vegetative bacteria, also adopt a characteristic glue-like consistency, or ropiness, which can be demonstrated through a useful field-side diagnostic test (26). Vegetative bacterial cells undergo sporulation as the larval remains undergo desiccation to form a hard, flattened scale that adheres relatively tightly to the dependent surface of the honey comb cell (4,6,26,42). It is these larval scales that contain the highest density of infectious spores throughout the entirety of the bacterium's life cycle (25,39). Importantly, spores remain infectious for decades after production, as experiments have shown that spores recovered from infected cells within comb over 35 years of age are still capable of germinating and establishing fatal infection within susceptible larvae (1).

### 1.1.2 Gross Pathology

#### *1.1.2.1 Larval Clinical Signs*

Visual evidence of AFB within an individual larva only occurs following larval death, which is the result of septicemia due to *P. larvae* invasion into the hemocoel (26). Death generally occurs when larvae are in their prepupal stage during cell capping, although this timing is dependent upon the strain of *P. larvae* (26,43). Following death, infected larval remains become moist and slumped within the cell and undergo a characteristic color change, becoming a diffuse coffee-brown (26,42). Moist larval remains of a recently deceased larva can be macerated within a cell and drawn out using a probe, and only those macerated cell contents that may be drawn out to a length of at least 2 cm are considered compatible with AFB (25,26). This technique, known as the "ropiness test" or "matchstick test", is key for differentiating AFB from other brood diseases, and the long length (i.e., > 2 cm) of the glue-like rope is nearly pathognomonic for the disease (10). Co-infection of larvae with European foulbrood and *Paenibacillus alvei* may also produce glue-like larval remains, but only have "pseudo-ropiness", as macerated cell contents will not draw out as far as with AFB infection (10).

If larval remains are left unadulterated by nurse bees, they will eventually undergo desiccation and become scale, which may be brown to black in color (25,26,44). Scale adheres tightly along the length of the gravitationally dependent wall (i.e., lower wall) of the cell, reflecting

the stretched out position of the prepupal larva when it died (25,44). Ultraviolet light with a wavelength of 360 nm has been shown to fluoresce scale, although care must be taken to distinguish fluorescent scale from fluorescent pollen or moulds (26,45).

In rare situations, death may not occur until larvae reach the pupae stage (26). With pupal death, a thin thread will occasionally be observed stretching across the face of the cell. This thread, which represents the tongue of the pupa, is considered a pathognomonic finding for AFB (26).

#### *1.1.2.2 Colony-level Clinical Signs*

Suspicion of AFB within a colony occurs when a beekeeper recognizes compatible clinical signs within frames of brood comb during hive inspection (26). The visual inspection of brood frames involves an assessment of the capped brood, open cells, and overall laying pattern (26). The first visual indication of AFB is often recognition of a weak colony with a reduced bee population (due to disease progression) and a "spotty" or "shotgun" brood pattern, so-called due to the scattershot and non-uniform appearance of capped and uncapped cells (26,44). This finding is relatively non-specific, and may signal a wide range of both infectious and non-infectious differential diagnoses in addition to AFB (26,44). Other most common potential causes of a "spotty" brood pattern include, but are not limited to, European foulbrood, varroosis, nosemosis, a failing queen, or the presence of a pseudo-queen (i.e., laying worker bee) (26). In the case of AFB, this brood pattern results from larval mortality, the irregular uncapping and cleaning out of affected cells by worker bees, as well as the reluctance of the queen to lay in cells containing firmly adhered scale (44).

Changes to the color and texture of brood cappings are another suggestive – albeit non-specific – sign of AFB (26). Whereas healthy brood cappings are slightly raised and light to dark brown in color, the cappings of cells affected by AFB are noticeably darker and may be nearly black in color (26). Affected cappings will also become sunken and take on a "greasy" appearance (26). In addition to these changes, the capping of an affected cell may be marred by the presence of an irregular, off-center hole that has been chewed by a worker bee upon identification of a problem within the cell (26). The marking of cell cappings in such a way acts as a flag that the underlying contents require removal. Importantly, such holes must be differentiated from other holes that occur in healthy cells during the normal capping process and the emergence of new bees (26).

Finally, a characteristic odor reminiscent of dead fish may be present in hives affected by AFB (25,26,44). Indeed, the term "foulbrood" comes from the foul smell associated with this disease (26). Although suggestive of AFB when present, the detection of this odor is an insensitive method to reliably detect disease, as it is dependent on both the severity of disease and external factors such as ambient temperature (26).

Recognition of any of the above colony-level signs should signal the need for closer examination, particularly for the presence (or absence) of the more specific, larval clinical signs of AFB, including larval "ropiness" (reflective of active infection) and/or larval scale (reflective of chronic infection) (see 1.1.2.1). Any suspicious cell may be evaluated for larval ropiness so long as there is access to a linear instrument that may be used as a probe (i.e., matchstick, small stick, applicator stick, stem of grass or thin branch) (10,10,26,44). Inspection of brood frames for scale is best performed by holding the frame at its top and orienting it in such a way as to highlight the gravitationally dependent portion of the cell wall with direct light from the sun or other handheld light source (25,26)

### 1.1.3 Transmission

Transmission of *Paenibacillus larvae* occurs predominantly through the movement of infectious spores (4,6,46). Both horizontal and vertical transmission occur (46,47). With regard to horizontal transmission, spores are transmitted within a colony, thereby promoting the progression and propagation of disease within a single hive (intra-colony transmission) (46). In addition, spores are also horizontally transmitted between colonies, so-called inter-colony transmission (46).

#### *1.1.3.1 Horizontal Transmission Within a Colony*

Transmission within a colony (intra-colony transmission) occurs when nurse bees identify an infected brood cell, unwittingly contaminate their mouthparts and gastrointestinal tract with spores as they clean the cell out, and transfer these spores to larval diet that is subsequently fed to naïve larvae (4). In addition to the contamination of larval food, worker-caste honey bees carrying spores further contribute to intra-colony transmission by transferring spores to other adults of the hive through routine trophallaxis, as well as interaction with food and honey stores (20,48). The artificial introduction of spores into a colony by means of comb containing larval remains affected by AFB results in comparable spore loads among adult bees within the colony to spores introduced within contaminated honeycomb (20). Spores of *Paenibacillus larvae* fail to germinate within the

gut of adult bees, and spores that maintain their infectivity have been recovered from the guts of adult bees up to four weeks after initial ingestion (49). Adult bees are therefore considered an "insect vector" for AFB, and play a vital role not only in the dissemination of spores within a colony, but between colonies as well (inter-colony transmission) (32,46–48,50,51).

#### *1.1.3.2 Horizontal Transmission Between Colonies*

Natural, inter-colony transmission is achieved primarily through robbing behaviour, although spread through drift has been investigated as well (50–52). Robbing behaviour occurs in colonies any time when naturally-occurring, environmental sources of nectar are scarce (51). Foraging honey bees resort to targeting weaker colonies and attempt to steal their honey to bolster their own food stores (51). If the robbed colony is weakened by AFB, then honey stores contaminated with spores of *P. larvae* are returned to the assaulting colony through this behaviour and, if a large enough spore-load is fed to larvae within this colony, can initiate a new, colony-level infection (50,51). It has been demonstrated that the shorter the distance between the interacting honey bee colonies – especially those within 0.5 – 1km of one another – the higher the likelihood of sufficient spore transmission to result in clinical disease in the robbing colony (51). AFB is a unique infectious disease of honey bees due to its high colony-level virulence (46). This trait is strongly selected for because it allows for efficient inter-colony transmission of spores through robbing (46). Drifting bees, in contrast, are likely of relatively little importance in terms of inter-colony transmission (26,50,52). One study attempted to incite clinical disease in healthy colonies through the random drifting of bees between colonies by placing them in close proximity to a clinically affected hive, but was unable to cause clinical disease in over 90% of the healthy hives despite prolonged exposure to the diseased hive over a period of several months (52).

The role of the beekeeper, and routine management practices within the apiculture industry as a whole, have been widely recognized as another important source of inter-colony transmission of *P. larvae* spores (4,26,51). In New Zealand, Goodwin reported that two of the most effective, beekeeper-driven means of transmitting spores of AFB are through the indiscriminate placement and swapping of contaminated, wet honey supers on colonies following honey extraction and the transfer of frames containing brood between colonies (practices encountered frequently in North American apiculture) (26). Contaminated honey supers placed on spore-free colonies that were in turn placed within spore-free bee yards resulted in all colonies within the yard testing positive for

*P. larvae* spores two days following the introduction of the contaminated supers (26). Other aspects of poor management, such as failing to properly dispose of contaminated honey or weakened to dead colonies, serves as a means of unintentionally attracting bees and encouraging robbing behaviour, which can effectively contribute to AFB transmission as described above (26,50,51). The intentional transfer of bees between colonies may also contribute to disease transmission, as the long-distance transport of packaged bees taken from AFB infected colonies caused clinical disease when established in new colonies with sterile equipment and clean frames (53). Given their propensity to disseminate throughout a hive, spores may be recovered from the surfaces of hive equipment, including the wooden surfaces of the hive boxes, beekeeping gloves, and hive tools, although the importance of contaminated equipment with regard to disease transmission is questionable, particularly if it has undergone decontamination (26,54). Further work in New Zealand has suggested that empty drawn comb, foundation, and the soil and flowers around a colony are not considered important factors with regard to the spread of AFB disease (26).

#### *1.1.3.3 Vertical Transmission*

Vertical transmission of AFB has been demonstrated to occur through regular colony fission (swarming) (47). Whereas horizontal transmission is associated with significant virulence, vertical transmission of *Paenibacillus larvae* spores has been shown to be relatively benign in comparison and rarely results in clinical disease in daughter colonies, which may help to explain why the disease may be maintained in feral populations (46,47). Importantly, feral populations of honey bees in New Zealand were found to have lower numbers of spores than those of managed bees, and were concluded to not be a major source of *P. larvae* transmission (26,55).

### 1.1.4 Determinants of Disease

Despite the low dose required to initiate infection within an individual larva (4,6,32), the presence of spores within a colony does not signal imminent clinical disease at the level of the colony, which has been demonstrated multiple times as referenced by Hansen and Brødsgaard (1999) (4,54). The number of spores that a colony must be inoculated with in order to generate colony-level clinical disease, as well as the time from inoculation to the first appearance of observable clinical signs within the colony, has varied within the literature (4,26,39,50,52). Early studies determined that a minimum of approximately 50,000,000 spores of *P. larvae* fed as a single

dose in 1L of sugar syrup was necessary to cause clinical disease in a hive (39). More recent studies have shown that 5,000,000 spores fed in 100mL of sugar syrup, is sufficient (52).

Rather than a straightforward correlation, the development of disease at the colony-level is instead dependent on a number of host and pathogen-related factors (2,4,22,39,43,50,52,56–58,58–63). From a host perspective, consideration of factors contributing to disease development are most clinically relevant at the level of the entire colony rather than the individual larvae within it, and research has focused on colony strength, the proportion of adult bees to brood, and the overall hygienic behaviour of a colony (4,62,63). Early experiments investigating colony-level mechanisms of resistance against AFB found that relatively resistant colonies had worker nurse bees that exhibited strong hygienic behaviours and removed larvae inoculated with *P. larvae* before the infected larvae could advance to the point of bacterial sporulation (63). Earlier work had established that this hygienic behaviour was a heritable trait that could be selectively bred for (59,60). More recent work has shown that bees selected for strong hygienic behaviour inoculated with sections of comb containing multiple larval scales are much more effective at preventing or recovering from the development of clinical disease compared to non-hygienic colonies (62).

From the perspective of the pathogen, certain strains of *P. larvae* are associated with different levels of virulence (2,22,58). The application of repetitive element PCR fingerprinting to subtype *P. larvae* resulted in the identification of four distinct genotypes of epidemiological importance (31,58,64,65). These genotypes, identified using enterobacterial repetitive intergenic (ERIC) primers, are appropriately named ERIC I-IV (31,58,65). ERIC I and ERIC II (which correspond to the former *P. larvae* subspecies, *P. larvae larvae*) are commonly isolated from field samples obtained during epidemiological studies, whereas ERIC III and ERIC IV (which correspond to the former *P. larvae* subspecies, *P. larvae pulvifaciens*) have not been isolated from field samples for decades and currently only exist as laboratory stocks (2,6,22,66). Exposure bioassays, through which *in vitro* reared honey bee larvae were fed larval diet inoculated with varying concentrations of *P. larvae* from different ERIC strains, demonstrated differences in lethality, particularly between ERIC I and ERIC II-IV (58). ERIC I manages to kill all infected larvae 12 to 14 days after inoculation, while ERIC II-IV kill all larvae by 7 days (2,22,43,58). The degree of virulence at the level of individual larvae has a negative correlation with colony-level virulence (43,58). ERIC II, a strain associated with a high degree of virulence at the larval level, results in lower colony-level virulence relative to ERIC I (43,58). A larger proportion of larvae

infected by ERIC II *P. larvae* die before capping and are more readily recognized by nurse bees and removed before a significant amount of sporulation can occur (43). The lower larval virulence of ERIC I results in a greater proportion of infected larvae dying after capping, which are less easily recognized by attending nurse bees (43). Dead, capped larvae go longer before removal and therefore have longer to build up large numbers of spores, which potentiates intra-colony transmission and results in a more rapid collapse of the colony (43). Recent work has identified a fifth genotype, ERIC V, from a Spanish honey sample, which has a level of individual virulence comparable to that of ERIC II-IV (57).

### 1.1.5 Diagnosis of Clinical Disease

A confirmatory diagnosis of AFB disease requires both the presence of compatible clinical signs within a colony, as well as laboratory identification of the pathogenic agent, *P. larvae* (6).

#### *1.1.5.1 Recognition of Supportive Clinical Signs*

Clinical signs that are supportive of American foulbrood are discussed in section 1.1.2. For laboratory evaluation and isolation of *P. larvae* from a clinically affected hive, laboratory submissions may include either entire brood frames or excised sections of comb with larval remains exhibiting ropiness and/or scale formation (6).

#### *1.1.5.2 Laboratory Isolation of Causative Agent*

Appropriate diagnostic techniques to isolate *P. larvae* include cultivation using microbiological culture, molecular identification through the use of multiple forms of Polymerase Chain Reaction (PCR), biochemical profiling, bacteriophage sensitivity, immunological assays with antibodies, direct microscopy, and mass spectrometry including matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (6,10,42,66,67).

Direct microscopy may be performed using slides prepared from either cultured, vegetative bacteria or direct larval smears (6,10,42,66). A Gram-stain is most often used to identify preparations of vegetative rods, but if spores are present, as with larval remains (depending on the advancement of disease), carbol fuschin or Schaeffer and Fulton stains may be used to highlight spore morphology (6,10,68). Alternatively, non-stained suspensions of larval remains may be examined for Brownian Movement of spores, which helps to differentiate spores of *P. larvae* from

those of other species that tend to have fixed spores, including *P. alvei* and *Brevibacillus laterosporus* (42).

Microbiological cultivation of *P. larvae* remains a cornerstone of many diagnostic testing protocols (6,10,15,42,66). Some evidence exists that the use of microbiological culture techniques may actually be more accurate in detecting clinical disease within a colony than real-time PCR, although this depends highly on the sampling and DNA extraction techniques used (69). Briefly, samples containing bacterial spores require some form of heated pasteurization to eliminate contaminant vegetative microorganisms, followed by plating and incubation on a semi-selective agar media prepared with antibiotics and/or antifungals, such as Nalidixic acid, Pipemidic acid, and amphotericin B (6,10,66). Pasteurization protocols must take into account the variability in sensitivity to heat between different genotypes of *P. larvae* (70). Specifically, bacteria of the ERIC II genotype exhibit reduced germination to a wide range of commonly used pasteurization temperatures, whereas ERIC I bacteria show enhanced germination following higher pasteurization temperatures (70). Ideal parameters for incubation are 37°C +/- 1°C in air kept at 5 – 10% $CO_2$ (6). Inoculated plates are generally incubated for 3 to 6 days to evaluate for colony growth, although incubation up to 15 days may be necessary for cultivation in some situations (6).

Many formulations of nutrient-enriched agar have been used and developed to encourage the efficient germination of bacterial spores and selective growth of *P. larvae* (6,10,66,71–76). Basic nutrient agar is not sufficient to support the growth of *P. larvae*.(42) Other, nutrient-rich agars including Columbia sheep-blood agar (CSA) and J-agar, are effective for growth, and CSA is still regularly used by some diagnostic centers today (73,74,77). Early developments of specialized culture media included the supplementation of liquid brain-heart infusion with thiamine to enhance bacterial growth (42,72). A commonly used agar, referred to by its acronym, MYPGP, is comprised of Mueller-Hinton broth, yeast extract, pyruvate, glucose, and potassium phosphate, and was initially developed as a means to promote sporulation of vegetative bacteria (6,66,71). Development of this media revealed that the addition of both glucose and pyruvate were necessary requirements for good sporulation (71). A modification of MYPGP using tris-HCl buffer and called T-HCl-YGP was developed and found to relatively outcompete J-agar and brain-heart infusion supplemented with thiamine in the germination and cultivation of spores recovered from honey samples (76). Another experiment tested the sensitivity of different solid media in detecting

spores of *P. larvae* from samples of honey (78). J-agar, MYPGP, brain-heart infusion with thiamine, CSA, and horse blood agar were compared under varying incubation conditions, and MYPGP was found to be superior by having the highest percentage of germinating spores (78). One of the most recently developed, *P. larvae*-specific solid culture medias was developed using a combination of molten *Bacillus cereus* agar, trypticase soy agar, supplemented nutrient agar, and egg yolk supplementation (75). This medium, aptly referred to as *Paenibacillus larvae* agar (PLA), was found to be more sensitive than brain-heart infusion with thiamine, J-agar, and Bailey & Lee agar (75). Research continues into fully establishing the nutrient requirements of *P. larvae* for maximal spore germination (79). An *in vitro* investigation of the determinants for spore germination used a variety of compounds expected to be present within the larval midgut in combination with varying temperatures and pH levels (79). Both L-tyrosine and uric acid were found to be potent activators of spore germination *in vitro*, whereas indole and phenol were identified as strong inhibitors of germination (79).

As an alternative to traditional microbiological cultivation, increasing numbers of studies have developed PCR protocols as a means to not only isolate, but also quantify and/or characterize *P. larvae* (31,64,67,69,80–83). Some of these protocols rely on the amplification of DNA from vegetative bacteria, a straightforward and sensitive method to confirm the presence of *P. larvae* in samples of affected brood from hives with suspected AFB (69,80–82,84). In contrast, the use of PCR for the detection of bacteria within the endospore form can prove challenging, likely due to the thickened wall of the endospore itself that inhibits efficient DNA extraction (69).

### 1.1.6 Control

The marked resiliency of spores, combined with their high degree of infectivity and prolific replicative potential, are the main factors that make this disease extremely difficult for beekeepers to control (2,56). As a result, there are numerous strategies currently employed to deal with AFB, with as many or more prospective therapies under research and development for future employment (56).

#### *1.1.6.1 Integrated Pest Management*

Integrated Pest Management (IPM) is a term commonly used in the beekeeping industry. It refers to the implementation of a combination of varied management strategies to prevent and control a specific disease, such as AFB (25). IPM techniques include a range of cultural, physical,

and chemical strategies (25). Cultural strategies refer to those practices that minimize transmission of the etiologic agent, emphasizing concepts of hygienic behaviour and biosecurity as means to reduce intra- and inter-colony transmission, respectively (see section 1.1.6.1.1). Physical control refers to more reactionary strategies, focusing on effective elimination of the source of disease when identified (see section 1.1.6.1.2). Finally, chemical control refers to the use of antimicrobials to prevent the expression of clinical disease (25). Preventative antimicrobial use and its limitations are discussed in section 1.1.6.2.

*1.1.6.1.1 Cultural Control*

The heritable trait of colony-level hygienic behaviour is described in section 1.1.4. When re-queening their colonies, beekeepers are encouraged to include the expression of hygienic behaviour in their selection criteria for identifying prospective breeder colonies (25). By consistently selecting for colony-level hygiene in their hives, beekeepers may increase the natural resistance of their hives against AFB by reducing intra-colony transmission, as worker bees would rapidly remove diseased larvae before substantial bacterial sporulation could occur (25,62,63).

Consideration for – and implementation of – biosecurity measures throughout all aspects of apiary management is critical for minimizing and preventing inter-colony transmission of *P. larvae* spores (4,25,26). The most significant mechanisms of inter-colony transmission of *P. larvae* spores, including those that are the direct result of beekeeper management practices, are detailed in section 1.1.3.2. Generally, biosecurity practices should strive to minimize the movement and interchange of frames, colonies, and other beekeeping equipment (such as supers) between hives, apiaries, and different beekeeping operations whenever possible (25,26). Colonies should be adequately supplemented with feed during seasonal periods of nectar scarcity to discourage robbing behaviour, and all hive equipment should be well maintained and free of cracks or other unintentional entrances that could be exploited by robbing bees (4,25,26,44). Finally, colonies should be subject to frequent and thorough brood chamber inspection to ensure early detection of clinical disease and prompt remedial action, as described in section 1.1.6.1.2.

*1.1.6.1.2 Physical Control*

In the event that clinical signs consistent with AFB are recognized within a hive, government regulations often mandate destruction of the hive itself, usually through burning, as a means to limit the spread of disease (2,4,26). Destruction generally encompasses the entirety of a

hive and includes the bees, frames, and boxes of the colony, as it is often the most practical course of action for beekeepers (2,26,85). If available, some equipment, including wooden frames, may be recoverable by subjecting them to either gamma irradiation or high velocity electron-beam irradiation, if allowed by governmental laws regulating AFB control (27,86). Other methods of wooden equipment decontamination including, but not limited to, chemical disinfectants with sporicidal properties, superficial "scorching" with a open flame, dry heat exposure in ovens, autoclaving, and hot paraffin dips have variable efficacy (4,54,85,87). Even decontamination methods that demonstrate effective destruction of spores (heat paraffin dipping, dry heat exposure, some chemical disinfectants) are accompanied by considerable limitations including safety to the beekeeper, especially with the prolonged use of highly concentrated chemical disinfectants, or low throughput, as with high heat exposure in ovens (85,87).

As an alternative to complete destruction of the entire colony, some countries may allow for the shaking of bees, also referred to as shook-swarming, as a means of control when clinical disease is recognized within a hive (4,88–91). This technique is not curative, but can be used as a means to salvage the bees from an affected colony, thereby lessening the economic loss imposed by the identification of clinical disease and subsequent colony destruction (4,88–91). Adult bees are shaken from the frames of the clinically diseased colony into boxes where they are temporarily deprived of food to encourage social grooming and ingestion of any remaining spores on the bodies of the bees (90). The bees are then transferred to a new, uncontaminated colony with new foundation and comb, which effectively acts to reduce the level of *P. larvae* spores to only those that are carried within the guts of the transferred honey bees (91). The majority of spores within the guts of these bees are then eliminated outside of the hive with the passage of feces (90). Despite its effectiveness in reducing spore counts, low levels of spores can still be detected within newly established colonies, and the original, contaminated hive boxes and frames are generally subject to destruction (88,91).

Other than remedial action in the face of clinical disease, physical control of AFB includes the routine replacement of old brood comb, as it may act as a reservoir for spores of *P. larvae* (4,92,93). Recommendations regarding the frequency of brood comb replacement range from once every three years to as frequently as annual replacement (4).

#### *1.1.6.2 Preventative Antimicrobial Use and its Limitations*

Due to the radical control measures that are often required with the recognition of clinical disease, great effort is put into the prevention of clinical disease. Some countries permit the use of antimicrobials for the control of AFB, including the United States and Canada, but antibiotic use is fraught with many significant limitations (2,11,94). Foremost among these limitations, commonly relied-upon registered antimicrobials, the most prominent of these being the bacteriostatic compound, oxytetracycline hydrochloride, are effective only at preventing the replication of vegetative bacteria, and are incapable of penetrating the thickened spore wall (2,3). Metaphylactic and therapeutic antibiotic applications are therefore non-curative, and only capable of masking or suppressing clinical signs of disease without eliminating the causative agent from a colony (2–4,95). In one study, therapeutic oxytetracycline hydrochloride treatment of hives artificially inoculated with AFB saw a resolution of clinical signs following treatment, but a re-emergence of clinical signs between 5 and 10 months later (96). Another study demonstrated that colonies with a light infection of AFB – those with between 50 and 100 visible scales in two or more frames – saw a resolution of clinical signs 3 – 4 weeks following oxytetracycline hydrochloride treatment, but re-emergence of clinical disease by the next production season (95).

Another significant limitation of preventative antibiotic use is the variable withdrawal times associated with registered antimicrobials that can place restrictions on the amount of honey that can be marketed for human consumption, thereby limiting the economic productivity of treated hives (2,94,97). Countries that prohibit the use of antimicrobials in apiculture have zero tolerance for any residue levels in both honey and other hive products, whereas those countries that allow for the use of antimicrobials have zero tolerance for antimicrobials in honey only (3). Degradation of oxytetracycline hydrochloride within honey takes between 42 and 70 days to fall below 0.1ppm, depending on the temperature the honey is stored at (96). Tylosin takes approximately 60 days to fall below the 2ppm limit of detection (96). A 2000 survey of honey samples in Germany identified residues of chlortetracycline and oxytetracycline in 0.7% and 3.6% of samples, respectively (3). More recent work using combined residue identification techniques on honey samples from the United States, Asia, and Europe found that honey samples with detectable residues were often positive for more than one class of drug (98).

The chronic, non-curative use of oxytetracycline hydrochloride for the control of AFB for over 50 years is also thought to have contributed to the development of widespread antimicrobial resistance (AMR) (2,3,7–9,99,100). Specifically, oxytetracycline-resistant *P. larvae* have been identified in the United States and Argentina and, in Canada, resistance has been documented in the literature in Alberta and Manitoba (8,100), as well as in Saskatchewan (Wilson – personal communication). Instead of a single evolutionary origin of resistance within this bacteria and subsequent geographic dissemination, molecular work suggests that oxytetracycline resistance has developed independently, multiple times, in geographically distinct areas (3,7). A combination of both genomic and horizontal, epigenetic transfer mechanisms, including plasmids and conjugal transposons, have been implicated in the spread of resistance (3,7,99). Interestingly, AMR has emerged relatively recently given the decades-long use of oxytetracycline, and this has been suggested to be a result of the ever-increasing onslaught of newly emerging infectious disease in domestic honey bee populations (7). Increasing numbers of infectious colony stressors may be weakening colonies and necessitating the use of increased, prolonged antibiotic therapy to keep AFB in check, although this hypothesis has not been investigated (7). The emergence of oxytetracycline hydrochloride resistance led to the search for alternative antimicrobials, namely the macrocyclic lactone, tylosin (2,96,101,102). Despite on-label approval of this antibiotic for the control of AFB, it suffers similar to its predecessors due to an inability to penetrate and eliminate *P. larvae* spores, thereby maintaining the concern of emerging resistance (2,3,103). In response to concern of the development of AMR within multiple animal-based production systems, apiculture included, recent federally-invoked regulatory changes within Canada were passed to increase veterinary oversight of medically important animal antimicrobials and work to slow the further development of AMR (104).

A final major limitation of antibiotic use against AFB are the direct and indirect effects of antimicrobial compounds on honey bee larvae and adult bees within a treated colony (96,105,106). One study showed that feeding larvae doses of chlortetracycline higher than 0.0025% (greater than 0.025μg/mg of larval diet) can retard larval development, increase larval mortality, and delay adult emergence (96,105). Unfortunately, recommended field-realistic doses of tetracyclines in the United States equate to a 0.1% dose within larval diet (96,105). Adult bees have a very high tolerance for oral toxicity by oxytetracycline, and mortality can only be induced by feeding concentrations that greatly exceed those used within the field (96). Tylosin, by comparison, is

relatively safe for both larvae and adults at field-realistic concentrations, although there is still some evidence that use of this antibiotic may adversely impact colony strength (102,103). Adult bees may still be indirectly, adversely impacted through long-term exposure to antibiotics in feed, as research to evaluate the microbiome within the gut of chronically treated bees has identified a high incidence of oxytetracycline resistance within these naturally-occurring gut microbes (106). Concern exists that pathogens may be capable of accessing these resistance genes for their own benefit, thereby overcoming preventative antibiotic management practices performed by beekeepers (106).

#### *1.1.6.3 Future Alternative Treatments*

Due to the significant limitations of antibiotic use in the control of AFB, substantial research has been performed to identify efficacious, natural alternative strategies to prevent the development of clinical disease (56,60–62,103,107–110). Some areas of this research interest focus on compounds with demonstrated antimicrobial properties that include, but are not limited to, the use of essential oils, plant extracts, propolis, royal jelly, and fatty acids (56,110). The majority of studies investigating the potential use of these compounds are limited to *in vitro* assays against the replicative, vegetative state of *P. larvae* and the assessment of toxicity to adult honey bees (56,110). Although some of these compounds demonstrate some degree of efficacy against *P. larvae*, substantial work remains to evaluate the efficacy, pharmacodynamics, pharmacokinetics, and methods of delivery at the level of the colony within field conditions, as well as the sporicidal potential of these compounds to eliminate spores within a colony.

Other antibiotic alternatives include investigation into the use of prebiotics, probiotics, selective breeding for hygienic behaviour, and phage therapy (56,60–62,107–110). Multiple studies into the use of probiotics have successfully demonstrated that several natural bacterial constituents of the honey bee gut microflora, including lactobacilli and bifidobacteria, can exhibit an inhibitory effect on the replication of *P. larvae* when isolated and applied *in vitro* (110). Despite the degree of success achieved *in vitro*, however, translation to the colony level has been disappointing (103). A recent study evaluating the efficacy of probiotics against AFB at the colony level supplemented honey bee feed with a concoction of honeybee-specific lactic acid bacteria (lactobacilli and bifidobacteria), and evaluated AFB infected colonies for changes in clinical signs and spore counts (103). This supplement was unable to significantly reduce clinical signs of

disease, unlike hives treated with tylosin, and was unable to reduce the number of spores within a colony, which was similar to tylosin-treated colonies (103). It has been postulated that the complexity of the honey bee colony as a superorganism may override any positive effect that might be gained from the supplemental feeding of probiotic compounds, and suggest that alternative delivery methods or multi-modal therapies require further investigation (103). A great degree of colony-level complexity is attributed to overall hygienic behaviour of the worker bees within the colony (59–63,103). As discussed above, breeding for robust hygienic behaviour has been demonstrated as a viable option as a component of honey bee management for disease control, and experiments evaluating the ability of hygienic behaviour to help control AFB are promising (60–63).

The use of bacteriophages as an alternative to antibiotic use continues to garner growing interest across many aspects of therapeutic medicine (107–109,111,112). The advantages of bacteriophages over many forms of conventional antibiotic therapy include their ability to auto-regulate their own dosing, their relatively low degree of inherent toxicity, and their high degree of specificity toward their target bacterium (107–109,111). In apiculture, multiple bacteriophages have been identified against various strains of *P. larvae* (107–109). Recent experimental trials have also shown a high degree of efficacy in the use of bacteriophage cocktails as both a prophylactic and a therapeutic treatment against AFB when administered orally to adult bees in sugar syrup, while concurrently demonstrating no negative health effects to the treated colony itself (108,109). Furthermore, there is evidence that some bacteriophages are capable of binding to *P. larvae* spores and, although this binding may not directly kill the spore itself, there is hope that substantial binding may effectively neutralize spores, thereby acting as a curative treatment (109). Further investigation into the neutralization potential of bacteriophages is required (109). Regardless, significant evaluation of the safety profiles of phages needs to be undertaken as a pre-requisite before therapeutic applications can be considered (107,112,113). Specifically, bacteriophages may inadvertently contribute to the virulence of the pathogenic bacteria they target through several mechanisms including generalized bacterial transduction, acting as vessels for genomic rearrangement, possession of genes with a large degree of homology to bacterial virulence and AMR genes that may be exploited, and lysogenic conversion (107,113). Additionally, extensive confirmation of the specificity of bacteriophages is required to ensure that

phage activity does not detrimentally impact any other beneficial microbial ecosystems within a colony (107).

### 1.2 Detection of Spores for Risk Assessment

Diagnostic tools to isolate *P. larvae*, particularly through microbiological cultivation techniques, are commonly used in applications beyond the confirmatory diagnosis of clinical, active infection (66). Microbiological and molecular techniques may be carried out on samples collected from a hive as a means to identify sub-clinical contamination with *P. larvae* through the identification of spores (66). Accordingly, the identification of sub-clinically contaminated hives may allow for the determination of a colony's future risk of developing AFB disease (15–17,69,92,100,114–116).

Given the ability of *P. larvae* spores to effectively disseminate throughout the resident bee population and colony-derived products of a hive, there are a plethora of studies evaluating the ability to detect and quantify spores in different populations of bees, wax, pollen, debris, and honey as a means of identifying colonies at future risk of disease, colonies likely to be experiencing active clinical disease, and establishing thresholds for the importation and trade of hive products such as honey (4,6,10,15–21,39,42,66,74–76,92,93,100,114,116). Indeed, the development of many of the selective, solid, culture media described previously (see section 1.1.5.2) were largely driven by the desire to improve the sensitivity of spore detection in samples obtained from various sample types within a hive.

The majority of studies evaluating the applications of spore detection within a hive focus on bees and honey, which are explored in sections 1.2.1 and 1.2.2, respectively. Spores can also be successfully recovered from samples of colony debris and wax when these materials are homogenized using heated Tween 80, although these samples are prone to containing a large number of microbial contaminants that can hinder or overwhelm any growth of *P. larvae* (93).

#### *1.2.1 Use of Bees for AFB Risk Assessment*

Research investigating spore detection in bees generally favors their use as a measurement of a colony's current health status, rather than as a predictor of future disease (14,18–21,69,100). Extensive work in Sweden has been done to understand the relationship between spores recovered from bees within a colony and the severity of clinical AFB disease within that same colony (18,21).

Results show that there is a positive relationship between the proportion of spore-positive bees and the spore-load of individual bees, as well as between the proportion of spore-positive bees and the number of larval cells within the colony that are clinically affected with AFB (21). These positive relationships were in turn used to develop a formula that can be used to calculate the threshold of bees needed to be sampled from an individual hive in order to detect very mild, clinically active AFB disease with negligible risk for false negative results (21). Importantly, the results suggest that the most sensitive method of detection requires the testing of individual hives, whereas composite samples from an entire apiary may be more useful as a screening tool (21).

Multiple experiments have compared the recovery of spores from bees (and honey) collected from different colony chambers to determine if the localization of sample collection within a hive has an impact on spore recovery (18–21,114). Work comparing bees collected from brood chambers and bees collected from the overlying honey supers found that although some bees in the brood chamber may carry higher numbers of spores, the overall difference in the proportion of bees with detectable spores and the overall spore load in bees between these two location was not significantly different (18,21). The distribution of spores amongst bees within these different chambers, however, is not random, and only a relatively few bees in a chamber will carry the majority of spores (21). The comparison of spore recovery from brood bees and brood chamber honey from clinically affected colonies reveals that more spores are recovered from bee samples (19). This may be due to capped stores of honey in the brood chamber predating current infection (19).

In one study, the sampling of both bees collected from the brood chambers and bottom-board debris that had accumulated during the over-wintering period was tested as a way to predict the health status of 11 colonies clinically affected by AFB (69). Samples were evaluated by either cultivation on MYPGP media or real-time PCR, and the presence and quantity of detectable spores was related to the disease status of the colony (69). Samples of adult bees that were cultured proved to be superior to samples of hive debris when used to predict current colony health status, but debris was found to be a more useful tool to screen for sub-clinical colonies (69). This is likely because hive debris is representative of a relatively long period of time for the colony compared to the relative “snap shot” offered by the current adult bee population (69).

In Manitoba, Canada, Pernal and Melathopoulos (2006) analyzed the concentrations of *P. larvae* spores on PLA media from bees collected from brood chambers during government, spring health inspections, as well as honey sampled from settling tanks (100). Each of the 19 sampled beekeeping operations had an average of 790 colonies, and operations were stratified into different groups based on the average percentage of colonies within an operation exhibiting clinical signs of AFB over the previous 3-year period (100). Culture results were compared to the number of colonies within each beekeeping operation that exhibited clinical signs of active AFB (100). The number of colony forming units (CFUs) cultured per gram of honey and per adult bee were both positively correlated to the number of clinically affected colonies within an operation, but adult bee samples were ultimately determined to be a superior predictor of current, active disease status over honey (100).

Recently, work has been done to evaluate the efficacy of combining surveillance techniques with intensive management practices to reduce the prevalence of *P. larvae* spores within a modestly-sized, commercial beekeeping operation in Sweden (14). This operation, which had 56 bee yards with a minimum of 10 colonies per yard at the start of the study, had previously been coping with chronic, recurrent outbreaks of clinical AFB by reactively destroying affected colonies (14). Surveillance for yards with a high risk of developing clinical AFB was done through the screening of sampled bees in the fall before overwintering from each colony within a yard for *P. larvae* spores through bacterial culture on MYPGP agar (14). Individually identified colonies with high spore counts were quarantined by first shaking the bees onto new, previously unused hive frames (shook swarm method), destroying the old, contaminated frames, and then using dedicated equipment for the high-risk colony that was kept separate from any other colony within the apiary (14). This combined approach of the identification of sub-clinically contaminated hives and intensive quarantine management, performed over a 5 year period, was successful at reducing the proportion of yards positive for *P. larvae* spores from 74% to 4% (14). Importantly, this study identified that only a small number of colonies within a yard identified as positive for *P. larvae* spores were considered highly contaminated with spores, while the other colonies within the yard were either free of detectable spores, or had very low levels (14).

Instead of using bees as a means to identify the presence of current, active disease within a colony, Gende *et al.* (2011) evaluated the use of bees as a predictor of the risk of disease onset by determining the threshold of recoverable spores per adult bee that is linked to the future

appearance of clinical signs of disease (16). The spore loads of bees from both visibly affected and apparently healthy colonies were determined through culture on MYPGP media, and a threshold for clinical disease of approximately 3,000 spores per bee was identified (16). This threshold may be useful to identify sub-clinical colonies at a high risk for the development of clinical disease, but the external validity of this work is limited, as this threshold is defined within the context of the sampling and culture procedures and may not necessarily be reproducible by other laboratories (16).

More recent work has investigated *P. larvae* spores in brood chamber bee, honey, and bottom-board debris samples collected in the winter as a means of predicting the risk of AFB in the following spring on an individual hive basis (17). A total of 165 hives were sampled amongst ten apiaries. In this study, sensitivity was defined as the ability to correctly identify diseased colonies in the following spring, whereas specificity referred to the ability to correctly identify colonies free of disease (17). Overall, increasing numbers of spores detected in winter samples were positively correlated with increasing risk of AFB in the spring (17). Regardless of the cut-off threshold of spore concentration used for the calculation of sensitivity and specificity, bees were found to have the highest sensitivity of all sample types. When using the presence/absence of detectable spores as a cut-off, however, debris was ultimately considered the best predictor of future AFB disease due to a higher specificity and positive predictive value relative to bees. Honey still had some use as a predictive tool (sensitivity of 81.25%), but failed to detect *P. larvae* spores in three colonies that went on to develop clinical disease in the following spring (17).

Overall, applications for the use of bees for the detection of *P. larvae* spores appear to favor their role as a measurement of a colony's current health status, rather than as a predictor of future disease (14,18–21,69,100). This may be due to bee samples representing a "snap shot" of colony health as presented by the current (and transient) adult bee population (69) Although a few studies have established bees as a prognostic indicator of future AFB risk, the use of bees as a routine diagnostic sample is ultimately hampered by the need to intensively sample hives on an individual, hive-by-hive basis (14,16,17)

### *1.2.2 Use of Honey for AFB Risk Assessment*

Honey has long been used as an effective albeit underutilized tool to predict the risk of AFB disease (15,90,115,116). Studies that compare the spore content in honey to other sample

types, such as bottom-board debris and bees, tend to use samples of honey collected directly from individual hives (17,20). A number of other studies, however, have explored the use of pooled, extracted (i.e., harvested) honey as a matrix for spore detection (15,39,74–76,90,92,100,115–117). One of the earliest records of the detection of *P. larvae* spores in extracted honey is by Sturtevant (1932), who investigated the potential role of extracted, "commercial" honey collected from honey supers in the transmission of *P. larvae* (39). Through centrifugation and direct microscopy, Sturtevant concluded that the concentrations of spores were too small to be considered capable of producing disease, and were likely ineffective as a means of AFB transmission (39).

Many years later, Hansen and Rasmussen (1986) cultivated spores of *P. larvae* from 532 samples of extracted honey collected from 243 apiaries following regional inspections (115). This study was performed in Denmark, and there was no mention of antibiotic use by any of the participating beekeepers (115). The authors used J-agar media for cultivation. Most honey samples had no detectable spores, however, 11% of the samples had variable spore concentrations that allowed for the generation of different categories of spore contamination (115). Beekeepers were inspected the year following extraction, and the incidence of AFB was compared to the spore concentrations identified the previous year (115). Of the 476 samples with no detectable spores, only two samples came from apiaries that went on to develop clinical signs of AFB. In contrast, four of 32 samples (13%) from the lowest category of spore concentration (30,000 – 60,000 spores per 5 g of honey) and five of 21 samples (24%) from the highest category (>600,000 spores per 5 g of honey) were found to be from apiaries that developed AFB in the following year (115).

Further work using bulk honey samples cultivated on sheep blood agar has compared spore contamination to current or recent AFB disease, demonstrating that increasing numbers of *P. larvae* spores in pooled honey samples were positively correlated with either current AFB or a recent historical occurrence of disease (116). This study examined a total of 505 honey samples representing 315 beekeepers in Australia and antibiotic use amongst these beekeepers was not reported (116). Rather than approximating spore concentrations from counts of colonies on cultivation plates, the authors directly scored the number of colonies on a plate into 1+, 2+, and 3+ categories corresponding to increasing severity of contamination (116). Of the 315 beekeepers, 52 had detectable spores of *P. larvae* (16.5%). All beekeepers within the 3+ category were found to have active AFB within their hives, whereas 78.6% of 2+ beekeepers and 56.4% of 1+ beekeepers had either active AFB or a recent history of the disease (116).

Von der Ohe and Dustman (1997) tested over 3,500 samples representing a combination of honey collected directly from the brood chambers of individual hives and packaged, marketable honey (presumed to represent pooled, extracted honey) (15). For brood chamber honey, samples were specified to have been sealed and located in close proximity to the brood nest itself. Although the authors did not indicate the proportions of each sample type in the total sample count, nor report any inherent differences in cultivability, they still successfully generated prognostic reference ranges, or contamination classes, from the concentrations of *P. larvae* spores in honey (15). These classes not only designated the immediate risk of AFB infection, but provided important interventive measures to be followed by the beekeeper to help mitigate the current risk designated by the degree of spore contamination (15). Specifically, a contamination class of 0 equated to a sample with no detectable spores and represented between 90 and 97% of all samples analyzed. Contamination classes of "low" and "high" indicated some degree of future risk of disease and were delimited by a threshold of 4,500 spores per gram of honey (15). Beekeepers at a low risk of AFB (i.e., honey samples had detectable spores in low levels) were recommended to renew comb, only provide nutrition from known, disease-free sources, clean their tools and equipment, prevent robbing whenever possible, and maintain diligent, frequent inspection of colonies (15). In contrast, beekeepers at a high risk of AFB (i.e., honey samples had high concentrations of detectable spores) were found to be likely to already have evidence of clinical signs in their hives and were recommended to undertake more drastic remedial action. Recommendations included the removal and subsequent destruction of all comb and food supplies (except honey for human consumption), along with the use of shook-swarming and strict quarantine (15,90). The authors concluded that the analysis of honey sampled from comb provided a more efficient and effective way to identify and subsequently remove sources of AFB than treatment with antibiotics (15).

The previously described study by Pernal and Melathopoulos (2006) (see section 1.2.1), which included an analysis of *P. larvae* spore concentrations in pooled honey sampled from settling tanks, concluded that although spore concentrations in both honey and bees were positively correlated to the number of clinically affected colonies within an operation, adult bees were the better of the two sample types as an indicator of the severity of current, active disease (100). The authors reasoned that pooled honey may be more valuable as a prognostic screening tool for the risk of future disease outbreaks rather than as a predictor of current disease status, suggesting that

the establishment of thresholds for risk could potentially screen out operations with no or little apparent risk of disease at the operation level (100).

### 1.3 Rationale

For decades, the prevention and control of AFB in North American apiculture has been heavily dependent on the chronic and sustained use of antimicrobials in the form of metaphylaxis (2,4). Although generally able to control vegetative *P. larvae*, antibiotic use against AFB is fraught with many well-described limitations (see section 1.1.6.2), not least of which includes an inability to eliminate infectious *P. larvae* endospores (2,3). In recent years, however, continued reliance on antimicrobials has been further hindered by the widespread emergence of antimicrobial resistant strains of *P. larvae* across the Americas, resulting in situations where clinical signs of AFB may appear in hives treated with metaphylactic antibiotics in an otherwise appropriate and on-label manner (2,3,7–9,99,100).

This scenario is by no means unique to apiculture (11). Over the last two decades, AMR has been recognized as an international threat to public health and has even been designated as the top health challenge of the 21$^{st}$ century (11). Left unchecked, the spread of AMR is poised to dramatically reduce the effectiveness of modern medicine for future generations (11). There is excessive use of antimicrobials across production animal systems, including apiculture, where treatment of entire populations rather than individuals is considered the norm due to practicality (11). This overuse of antibiotics has serious ramifications for not only the management of animal diseases, but for those of humans, too, as animals may act as reservoirs of resistance for human infections (11). The industry's continued reliance on indiscriminate antimicrobial metaphylaxis is therefore unsustainable, both for the long-term control of AFB and for public health as a whole. In Canada, recent regulatory changes have been implemented that increase veterinary oversight of medically important antimicrobials in animals (104). Although an important step to improve the regulated use of antibiotics, it does little to alter the fundamental dependency of North American apiculture on their use. Rather, North American apiculture is in urgent need of alternative, evidence-based management strategies to control AFB in order to reduce its overall reliance on antibiotics, thereby ensuring the continued sustainability and profitability of the industry for years to come.

Many countries, including member countries of the European Union, New Zealand, and Australia, are prohibited by law from using antimicrobials in apiculture (2,15,26,90). Instead, control of AFB disease in these countries is the result of intensive inspection and surveillance programs that take advantage of the ability to detect *P. larvae* spores in a wide variety of samples from a colony (14,15,26,90). The effectiveness of these surveillance programs is derived from both a thorough understanding of the risk factors associated with AFB disease within their respective management systems and the ability to develop prognostic reference ranges for spore concentrations from a particular sample type to assess for either the current presence or the future risk of an outbreak of AFB disease (17,21,26,69,90,100,116).

Due to its long-time status as a standard of practice, it is likely unreasonable to expect a future where North American apiculture steps away from the use of antimicrobials entirely (25,44). That said, the adoption of strategies of surveillance and evidence-based management through reliable risk assessment, as seen in other jurisdictions, could help to ensure a more judicious use of antibiotics. Instead of performing indiscriminate annual metaphylaxis, beekeepers could instead only use them when absolutely necessary, as dictated by evidence gathered through established surveillance of their hives.

Diagnostic laboratories currently offer testing capacity for spores of *P. larvae*; however, samples are generally restricted to adult bees or honey collected directly from the brood chambers of individual hives (12,13). Similarly, many of the studies evaluating the quantification of spores in different sample types rely on the collection of samples on a hive-by-hive basis (14–19,21,51,90). Although an individual hive approach may be an acceptable foundation for the development of surveillance programs for beekeepers managing a small number of hives, North American apiculture is dominated by large-scale, commercial beekeeping operations that manage hundreds to thousands of hives at a time. Accordingly, it is logistically impossible for such operations to sample all of their hives as a means to determine AFB risk. Sampling a representative subset of individual hives may not be reasonable compromise either, as Locke *et al.* (2019), through the testing of bees collected from individual hives, found that *P. larvae* spores were not equally distributed amongst hives within a given yard (14). Instead, only 1 – 2 hives within each yard (containing 10 – 12 hives) were contaminated with significant numbers of spores (14). If similar spore distributions are present in commercial beekeeping operations in North America, then subsets of individual hives for sampling would still need to be of a very large number that

would preclude convenience in order to ensure the sampling of the relative few, contaminated hives at high risk of disease.

Instead of samples of bees or brood chamber honey collected from individual hives, pooled honey collected during end-of-season extraction may represent an ideal solution for the establishment of risk assessment in North American apiculture. Extracted honey is collected from frames located in honey supers, which are located above brood chambers and generally exclude queens through the use of an excluder screen. During extraction, honey super frames are collected in aggregate within a given yard and spun together on a honey extractor machine for storage in bulk tanks. We estimate, assuming the use of a 60-frame commercial extractor (most commonly used in Saskatchewan), that 60 frames of honey represent between three and six separate hives within the yard from which they were collected, which would allow more efficient coverage of a yard relative to an individual hive approach. Additionally, the collection of such samples could be easily incorporated into the extraction workflow, thereby representing a convenient and time-saving alternative to collecting samples from the brood chambers of individual hives.

In light of previous studies demonstrating a correlation between spore concentrations in pooled, extracted honey samples and either recent or future incidence of AFB disease, we believe that spore concentrations from pooled, extracted honey of Saskatchewan beekeepers can be used to establish reliable, prognostic reference ranges indicative of the risk of future outbreaks of AFB at the yard or operation level (100,115,116). Furthermore, correlation of spore concentrations with management practices of beekeepers submitting samples provides an opportunity to identify those practices associated with high risk of AFB in antibiotic-reliant management systems (92). Altogether, surveillance for spores of *P. larvae* in pooled, extracted honey will provide North American apiculture with the ability to make improved, evidence-based decisions in the prevention and control of AFB that will reduce the industry's reliance on antibiotic use and reduce the spread of AMR.

## 1.4 Hypothesis and Objectives

The overarching hypothesis of this thesis was that concentrations of *P. larvae* spores obtained from pooled honey samples collected during routine extraction would be predictive of the future risk of AFB at the yard or operation level. With this in mind, the specific objectives of this thesis were:

1. To clinically characterize outbreaks of American foulbrood disease in large-scale, commercial, honey bee operations in Saskatchewan with recent or ongoing histories of antimicrobial metaphylaxis.
2. To opportunistically and intensively sample these operations in order to:
    i. Assess *P. larvae* spore concentrations from different sample types from individual hives;
    ii. Assess the use of honey collected from honey supers of individual hives as a surrogate for brood chamber honey or adult bees;
    iii. Correlate the concentration of *P. larvae* spores in pooled honey collected during end-of-season extraction with honey super samples collected from individual hives.
3. Develop province-wide surveillance for *P. larvae* spores in pooled, extracted honey in order to:
    i. Establish reliable prognostic thresholds for *P. larvae* spore concentrations for the determination of future AFB risk at the yard or operation level;
    ii. Identify key management practices as targets for mitigation of AFB risk in antibiotic-reliant apiculture.

Taken together, the establishment of prognostic thresholds for *P. larvae* spore concentrations in pooled, extracted honey for future AFB risk, along with the identification of key management practices as targets for mitigation of AFB risk, will provide effective and alternative strategies for North American beekeepers to prevent and control AFB, thereby reducing the industry's reliance on antibiotic use.

## PREFACE TO CHAPTER 2

During the summer of 2019, we were made aware of a number of outbreaks of AFB in four large-scale, commercial, honey-producing, beekeeping operations within Saskatchewan. As these outbreaks coincided with the initiation of our AFB research, we sought to investigate and characterize them as a means to better understand AFB disease within antimicrobial-reliant management systems, exploring management practices that may be relevant in the development of AFB risk assessment. Additionally, we used these outbreaks as an opportunity to establish effective, working relationships with the beekeepers of these operations and the Provincial Specialist in Apiculture. These collaborative and inter-disciplinary relationships proved to be critical for both the development of our province-wide surveillance capacity for *P. larvae* spores and our study to demonstrate the use of pooled, extracted honey as a risk assessment tool.

In this chapter, we confirmed the occurrence of AFB in all operations through the combined demonstration of supportive clinical signs and isolation of the causative agent through laboratory testing. Beekeepers from each operation were subjected to a basic questionnaire that included information on the size of their respective operations, the use of antimicrobials for metaphylaxis, and the frequency and thoroughness of brood chamber inspections. Results from this study identified off-label metaphylactic use of antibiotics in three operations, and the recent cessation of antimicrobials in the fourth. These findings highlight the need for risk assessment tools to allow for evidence-based decision-making regarding antimicrobial use and the need for improved on-label adherence when they are deemed necessary. Accordingly, this study was published as educational material for practicing Canadian veterinarians while emphasizing their role in apiculture health management.

# CHAPTER 2: INVESTIGATION OF CLINICAL OUTBREAKS OF AMERICAN FOULBROOD IN HONEY-BEE OPERATIONS IN SASKATCHEWAN

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## 2.1 Abstract

Four outbreaks of American foulbrood were investigated in honey-bee operations in Saskatchewan during the summer of 2019. Clinical signs were confirmed by the Saskatchewan Provincial Specialist in Apiculture and the causative agent was cultured and identified through matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). Evaluation of management practices revealed off-label metaphylactic use of oxytetracycline in 3 of 4 operations and a discontinuation of antibiotic use in the fourth. Recent regulatory changes regarding access to medically important antimicrobials has provided an opportunity for veterinarians to promote evidence-based use of antimicrobials in apiculture while safe-guarding the health of commercial honeybee populations and the economic viability of their producers.

## 2.2 Introduction

American foulbrood (AFB), caused by *Paenibacillus larvae*, is a highly infectious, destructive bacterial disease that affects the young brood of honey-bee colonies (25,34). Newly hatched larvae are inadvertently fed bacterial spores, which germinate and replicate in the midgut causing an overwhelming septicemia and producing over 1 billion new spores per infected larva (25,39). As deceased larvae are cleaned from their cells by worker caste bees, spores are picked up and disseminated throughout the hive products including honey and wax, as well as hive equipment including frames and boxes (6,51,66,93). Spores are resilient to environmental conditions and chemical treatments and have been reported to maintain infectivity for decades (1). This resiliency poses significant challenges regarding the management and control of disease. If clinical signs consistent with AFB infection are observed, burning the bees and equipment of affected colonies or, if available, subjecting equipment to gamma irradiation or high velocity electron-beam irradiation are the only practical solutions to eliminate spores (26,27,56,85).

In Canada, prevention of clinical disease relies heavily on the use of biannual, metaphylactic, antibiotic treatments with oxytetracycline in the spring and fall. Treatments are administered as either a dusting of antibiotic mixed with powdered sugar, or as a direct feed within sugar syrup. If Oxysol-62.5 (DIN 00560189) is used, each dusting treatment consists of mixing 400 g of oxytetracycline HCl (62.5 mg/g) with 3.5 kg of powdered sugar, and dusting 32 g of this mixture into each hive 3 times at 4-to 5-day intervals. Syrup feed is made by dissolving 400 g of oxytetracycline HCl (62.5 mg/g) within 300 kg of a 1:1 mixture of sugar and water and feeding

2.5 kg of this solution to each hive 3 times at 4- to 5-day intervals. If Oxytet-25 (DIN 02231111) is used, then 454 g of oxytetracycline HCl (55 mg/g) is mixed with 3.5 kg of powdered sugar or 300 kg of 1:1 sugar syrup for dusting powder or syrup administration, respectively. Volumes can be scaled down as needed. Antibiotic use can prevent the replication of the vegetative state and suppress clinical manifestation, but it does not affect the spores present in treated colonies (3). This metaphylactic treatment is thought to have contributed to both the development and subsequent spread of antibiotic resistant strains of *P. larvae* and necessitated the use of alternative antibiotics such as tylosin for control (7,8,99,118).

A definitive diagnosis of AFB requires the identification of clinical signs consistent with disease (Figure 2.1) and isolation of the causative agent through laboratory testing (6). As of the fall of 2019, AFB was added to the list of provincially notifiable diseases in Saskatchewan, and laboratory-confirmed cases must be reported to the Chief Veterinary Officer within 24 h of confirmation. The disease is annually notifiable in Canada.

Following infection, larvae and pupae change from pearly white to tan to brown as they are invaded by large numbers of replicating vegetative bacilli (42). The large number of bacteria create a glue-like consistency to the pre-pupa/pupa that can be macerated and strung out using a probe to test for larval ropiness. If macerated pupal tissue can be drawn out and stretched more than 2 cm from a cell (Figure 2.1A), it is highly suggestive of AFB infection (26). When worker bees recognize an abnormal larval or pupal cell either before or after capping, they will clean and remove the diseased or dead larva or pupa, which can produce a scattered, “shotgun” appearance to the brood pattern (Figure 2.1B) (119). Remaining cell cappings of affected larvae may also appear sunken, greasy, or perforated (Figure 2.1C) (25). Scattered, “shotgun” brood appearance and abnormalities in cell cappings may be suggestive for AFB, but they are not as specific as the presence of ropey brood and firmly attached larval scale (26). As infection advances, dead larvae will desiccate and form dark brown scales that adhere firmly to the ventral lateral wall of the brood cell (Figure 2.1D) and a strong, unpleasant foul odor (hence the name “foulbrood”) may be noted from affected bee frames (42). Samples of comb that contain evidence of ropey larvae and/or scales can be cultured to isolate the bacterium (6,66,71,78), or tested using molecular techniques such as polymerase chain reaction (PCR) (66,81).

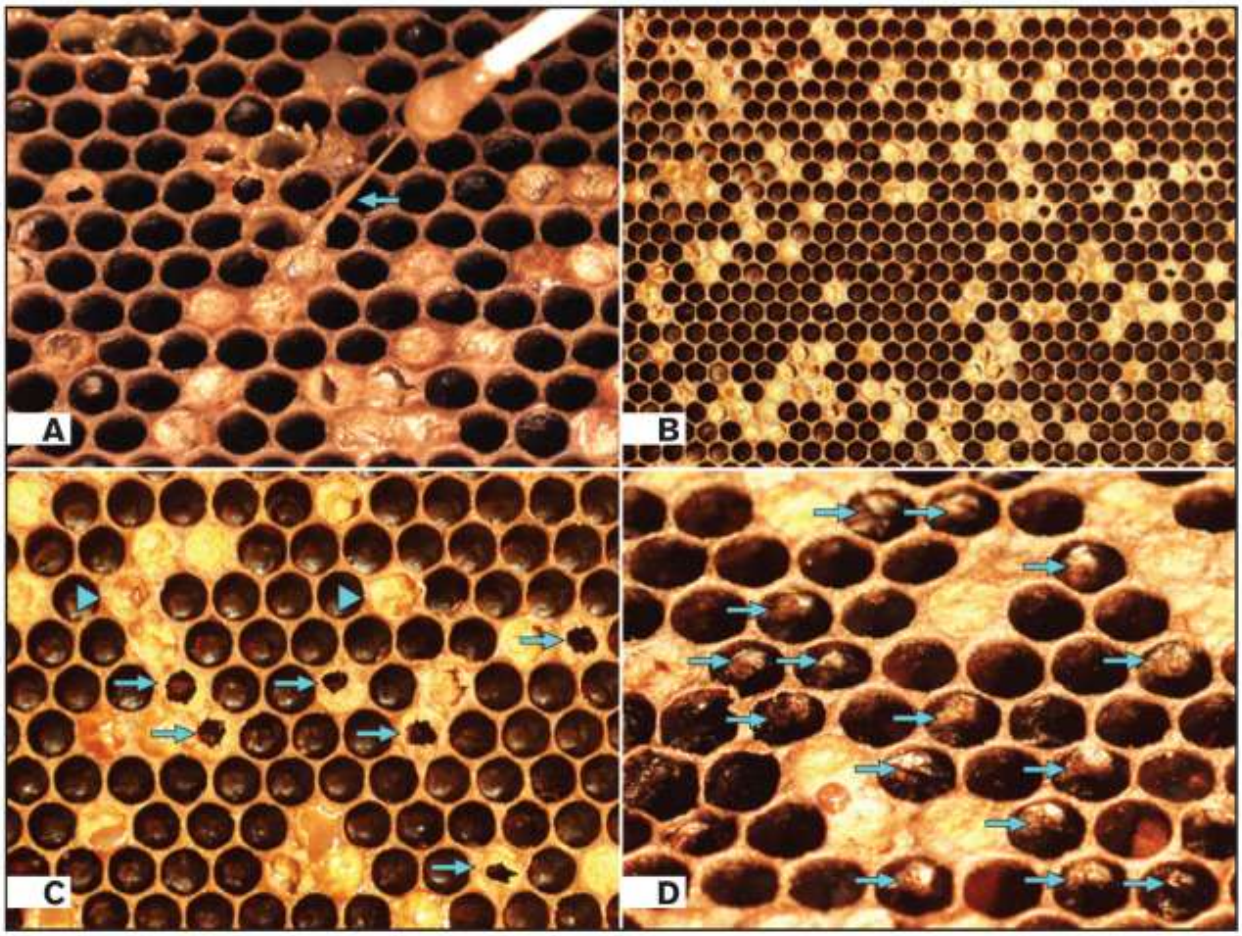


**Figure 2.1. Common clinical signs of American foulbrood.** A — larval ropiness, representing decaying and macerated pre-pupae (arrow); B — Scattered, 'shotgun' brood pattern as a consequence of larval/pupal mortality; C — Perforated cell cappings (arrows) and sunken cell cappings (arrowheads); D — Scale, representing dead, desiccated pre-pupae (arrows)

## 2.3 Clinical Description

Four suspected outbreaks of AFB were identified in commercial honey-bee operations across central and southeastern Saskatchewan in June, 2019. In this manuscript, outbreaks are defined as an incidence of clinical disease in hives in excess of what is expected. All 4 operations — identified here as Operations A, B, C, and D — varied in their management practices for control of AFB, which are summarized in Table 2.1. All 4 operations are large honey-producing operations with 2100 to 4000 honey-producing colonies. Given the use of metaphylactic antibiotics in 3 operations and the long duration of freedom from observable disease in the 4th, antibiotic-free operation, the expected incidence of clinical disease in each operation is zero.

### 2.3.1 Operation A

Operation A had ceased using metaphylactic antibiotics and had last treated its colonies with oxytetracycline in the fall of 2016. On June 10th, 2019, staff identified a single colony in 1 yard with a scattered, "shotgun" brood pattern, abnormal cell cappings, larval scale, and larval ropiness. The colony was treated with a single application of oxytetracycline administered over the frames in icing sugar, and the Provincial Specialist in Apiculture was contacted to inspect the affected hive. A clinical diagnosis of AFB was confirmed based on the described clinical signs, and the entire hive was destroyed through burning; the remainder of the yard was treated with oxytetracycline as per label directions. Several affected frames were submitted to the laboratory for bacteriological culture.

### 2.3.2 Operation B

Operation B treats its colonies twice annually with oxytetracycline mixed with powdered sugar in the spring and fall, but only performs a single application of antibiotics per feed treatment instead of the on-label instructions requiring 3 applications given 4 to 5 days apart. Brood frames are inspected frequently throughout the season and, on June 14th, 2019, a single colony within a large yard of nucleus colonies (n = 195) was marked with clinical signs similar to those described in Operation A. Frames and bees from the colony were destroyed by burning, and the remaining colony materials (i.e., super boxes, lids, and bottom) had their surfaces scorched with a torch before being re-used in circulation. The last suspected case of AFB in this operation was identified 3 y ago, and a clinical diagnosis was made by the Provincial Specialist in Apiculture at that time.

**Table 2.1. Summary of management variables from 4 commercial honey-bee operations in Saskatchewan diagnosed with a clinical outbreak of American foulbrood during the summer of 2019.**

[a] Estimated frame replacement proportion for new nucleus hives, from which the affected colony originated.

| Management Variable | Operation ID | | | |
|---|---|---|---|---|
| | A | B | C | D |
| Total Number of Honey-producing Colonies | 4000 | 2700 | 2800 | 2100 |
| Total Number of Bee Yards | 125 | 85 | 45 | 50 |
| Number of Affected Yards | 1 | 1 | 1 | N/A [b] |
| Total Number of Colonies Within Affected Yard | 40 | 195 | 44 | N/A [b] |
| Number of Affected Colonies Within Affected Yard | 1 | 1 | 1 | 7 |
| Affected Colony Status | Alive | Alive | Dead | Dead |
| Metaphylactic Antibiotic Use | No | Yes | Yes | Yes |
| Antibiotic Used | - | Oxytetracycline | Oxytetracycline | Oxytetracycline |
| On-Label Antibiotic Use? | - | No | No | No [c] |
| Frequency of Brood Inspection | Spring and Whenever Weak | Spring and Whenever Weak | Overwinter Loss, Spring, and Whenever Weak | Spring and Whenever Weak |
| Who Performs Brood Inspection | Owner and Staff | Owner and Staff | Owner and Staff | Owner |
| Proportion of Brood Frames Inspected | Few | Few | Few | All |
| Percentage of Brood Frames Renewed Annually | 10% | Over 50% [a] | 10% | Less than 5% |
| Last Suspected Case of American Foulbrood | 25 years ago | 3 years ago | 3 years ago | 1 year ago |

[b] All colonies were overwintered together indoors and individual yard assignments were not tracked for nucleus colonies.

[c] Fall treatment of oxytetracycline in 2018 was given as 2 applications, not 3.

N/A — Not applicable

### 2.3.3 Operation C

Unlike operations A and B, which had identified clinical signs in living colonies, Operation C identified a suspect colony during its annual inspection of dead colonies that failed to survive overwintering. Frames contained scattered, abnormal cappings and adhered scale along the ventral aspect of numerous cells. The Provincial Specialist in Apiculture was alerted, and the entire hive was burned. Similar to Operation B, this commercial outfit reported a 3-year gap between this case of AFB and its last suspected case. The previous case was identified by clinical signs observed by the operation owner alone without visual confirmation by the Provincial Specialist. The owner of this operation also noted that a nearby hobbyist had lost their colonies to AFB in 2013 but had left the hives out instead of having them destroyed. There was a high likelihood of these contaminated colonies being robbed out by bees belonging to Operation C, a behavior whereby foraging bees from strong hives will collect (steal) unprotected honey within weak and dead hives and bring it back into their hives. Operation C had therefore been using metaphylactic oxytetracycline, but only once annually in the spring since 2010 instead of the label-prescribed twice annual treatments.

### 2.3.4 Operation D

Operation D identified a total of 7 deceased nucleus colonies suspected to have succumbed to AFB during examination of its overwinter mortalities. This operation overwinters all of its colonies together indoors and performs rigorous inspection of all brood frames 2 to 3 times each spring. Suspect colonies were initially identified due to a noticeable malodor, and subsequent inspection of frames revealed capping abnormalities and the presence of scale. All identified colonies and frames were destroyed by burning. The operation reported administering oxytetracycline twice annually and adhering to label instructions but noted that the fall treatment in 2018 was only given as 2 applications instead of the label-prescribed 3.

From all operations, only those frames with either visible larval scale or cells identified as positive for ropiness were collected for bacterial culture. Scales were collected from affected frames, vortexed in sterile water, and subjected to heat treatment to eliminate other microbial contaminants. Samples were plated on both Columbia sheep blood agar (CSA) (BD Biosciences, San Jose, California, USA) and MYPGP [prepared according to published protocols (6,66)] and incubated at 37°C with 5% CO2 for 48 h. Plates were confirmed for lawns of colony growth consistent with the high concentration of spores expected from contaminated larval scale and with

colony morphology consistent with *P. larvae* (6). Two serial sub-cultures were performed from these lawns to generate a pure culture. Colony growth from the second sub-culture was submitted to Prairie Diagnostic Services, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, and tested using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) with a pre-existing library for *Paenibacillus larvae*. Bacterial isolates from all operations were positively identified as *Paenibacillus larvae*. An isolate from Operation A was confirmed as *Paenibacillus larvae* using both MALDI-TOF MS and PCR with primers designed by Dobbelaere et al (81). Independent samples collected by the Provincial Specialist in Apiculture were submitted to the Animal Health Laboratory, (University of Guelph, Guelph, Ontario) for antimicrobial susceptibility testing by Kirby Bauer disk diffusion following Canadian Laboratory Standards Institute (CLSI) VET06 guidelines. Isolates from all operations were confirmed to be susceptible to oxytetracycline.

Follow-up with all 4 operations was performed following routine fall inspections. All operations confirmed that they had detected no additional clinical evidence of AFB. Operation A returned to metaphylactic, on-label use of oxytetracycline and the 3 other operations had adjusted their metaphylactic antibiotic protocols to follow label instructions.

**2.4 Discussion**

The current landscape of AFB in Saskatchewan remains largely unknown due to the long-term masking of disease through antibiotic metaphylaxis; outbreaks are difficult to predict. Suppression of clinical disease can lead to a high build-up of infectious spores within contaminated colonies, unknown to both hobbyists and commercial beekeepers applying treatment (15,95,99). In Saskatchewan, cases of AFB are identified through a combination of random inspections and self-reporting by beekeepers. Four outbreaks of AFB were reported in 2018, primarily among small-scale beekeepers and hobbyists. In 2019 the number of cases increased to 8, but were mostly seen in commercial operations, 4 of which were investigated here. Many factors associated with the pathogen, host, and environment are thought to play a role in determining the development of clinical disease, including strain virulence, the hygienic behavior of worker bees within a colony (social immunity), and basic management practices, respectively (6,58,62,92). How these factors interplay in the presence of chronically treated colonies remains unknown. Investigations of potential risk factors for disease have helped to shed light on management practices critical to

American foulbrood management and control in countries such as New Zealand and Belgium, but because antibiotic use in these regions is prohibited, the applicability of the results of these investigations to Saskatchewan and Canadian beekeeping is uncertain (26,92).

With the recent regulatory changes on December 1, 2018, requiring all medically important antimicrobials for veterinary use to be sold by prescription only, the current use of antibiotics in the honey-bee industry must be re-evaluated, and Canadian veterinarians have an opportunity to establish themselves as leaders in the judicious use of antimicrobials in this industry (104). Common to all operations investigated here was the variability of approaches taken with regard to antibiotic use. Operation A had discontinued antibiotic metaphylaxis in their management program, and although operations B, C, and D were using regular antibiotic metaphylaxis, all were doing so with different, off-label protocols. It is possible that the cessation of antibiotics in operation A, or under-dosing of antibiotic in the other operations, may have contributed to the clinical appearance of AFB. Until there is a better understanding of the risk factors associated with clinical outbreaks of AFB in antibiotic-managed systems, Canadian veterinarians are uniquely poised to build effective, working veterinary-client-patient relationships with hobbyist and commercial beekeepers around communicating the importance of on-label use of antibiotic therapy to minimize the likelihood of clinical disease and slow the development of antimicrobial resistance within the apiculture industry.

Conversely, if beekeepers wish to cease the use of antibiotics for metaphylactic control of AFB, veterinarians can be relied upon to consider this decision in an evidence-based manner through risk assessment, the testing of hive products, and the implementation of frequent brood frame examination to minimize the likelihood of occurrence of clinical disease. Here, Operation A had discontinued the use of oxytetracycline due to concern about the development of antimicrobial resistance and saw an emergence of clinical disease less than 3 y after cessation of treatment in an operation that had had no clinical disease for the previous 25 y. It is suspected that the long-term viability of bacterial spores, combined with a silent accumulation of spores through clinical masking with chronic antimicrobial use, can create a false sense of contamination-free colony status (95). Removing the suppressive effects of antimicrobials in these hives without an understanding of underlying contamination of *P. larvae* spores and relevant risk factors could potentially be disastrous. If antibiotic use is discontinued, then a rigorous health management plan must be in place that emphasizes frequent and thorough examination of brood frames to improve

the early detection of disease and prompt destruction of affected colonies to reduce the spread of AFB. This approach for control of AFB has been implemented in New Zealand and many European countries and it is being adopted successfully by a few commercial beekeepers in Saskatchewan and Alberta (15,26). Veterinarians again have an opportunity to educate and aid in the implementation of such plans, and can advocate the testing of hive products such as honey and bees to detect the presence of spores of *P. larvae* and the presence of antimicrobial resistance that may influence the decision to use antibiotic metaphylaxis or not (15,100).

Practitioners are advised to follow the procedures listed in suspected AFB cases:

i. Contact the Provincial Specialist in Apiculture to establish a working relationship with their team. Provincial apiculture teams are a valuable resource in both knowledge and skills concerning beekeeping practices and the detection/diagnosis and management of honey-bee diseases. Strong collaboration is necessary for efficient prevention, early detection, and optimal management of AFB. Provincial apiculturists also perform a regulatory role to implement and enact provincial acts related to beekeeping.

ii. Inspect the colony frames for presence of pathognomonic signs: ropiness and/or scales.

iii. Submit suspect frame(s) to an appropriate microbiology laboratory to confirm the presence of *P. larvae*.

iv. If a diagnosis of AFB is confirmed and is a notifiable disease within your province, report to the Chief Veterinary Officer within 24 h of laboratory diagnosis.

v. Burn affected colonies and equipment or irradiate exposed equipment if this service is available. Treat the remaining colonies with antibiotics or implement frequent and rigorous inspections to identify and destroy bacteria in new cases at early stages to prevent spread of the disease.

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TOF MS identification. Finally, thank you to beekeepers who allowed us to use their data in this manuscript, but who shall remain anonymous to preserve confidentiality.

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## PREFACE TO CHAPTER 3

In Chapter 2, we characterized outbreaks of AFB in four separate commercial beekeeping operations in Saskatchewan, Canada. In Chapter 3, we returned to three of these operations within days of the reporting of these outbreaks, opportunistically and intensively sampling individual hives from multiple yards (hereafter referred to as apiaries throughout Chapter 3). This chapter focuses on establishing the use pooled, extracted honey as a means of risk assessment of AFB at the apiary or operation level. To do this, we first compared and correlated the spore content in samples of brood chamber honey and bees to those of honey from the overlying honey supers of individual hives, determining that honey super (HS) honey was an acceptable surrogate for more routinely submitted and researched brood chamber (BC) samples (12–19,21,51,90). We then evaluated the ability of pooled, extracted honey collected from each beekeeping operation at the end of the honey-producing season (i.e., pooled HS honey) to predict the degree of spore contamination identified through the testing of individual hives from earlier in the season.

Chapter 3 highlights several important findings that support the use of pooled, extracted honey as a convenient risk assessment tool for large-scale, commercial beekeeping operations. First, we demonstrated that concentrations of *P. larvae* spores in samples of pooled, extracted honey were reflective of the spore contamination identified from the testing of each operation's individual hives. Second, we showed that pooled, extracted honey may have utility as a risk assessment tool, as the most severely contaminated operation was the only one of the three operations to develop clinical AFB in the following spring. Finally, we found – similar to studies performed in Europe – that spores were not equally distributed amongst hives within any given apiary, and that most spores were isolated to a relatively small proportion of hives (14,120). Consequently, an individual hive approach for risk assessment would be logistically impossible for large-scale, commercial operations, as it would necessitate the sampling of a large number of individual hives to identify those few with heavy spore contamination.

# CHAPTER 3: COMPARISON OF INDIVIDUAL HIVE AND APIARY-LEVEL SAMPLE TYPES FOR SPORES OF *PAENIBACILLUS LARVAE* IN SASKATCHEWAN HONEY BEE OPERATIONS

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## 3.1 Abstract

Three commercial honey bee operations in Saskatchewan, Canada, with outbreaks of American foulbrood (AFB) and recent or ongoing metaphylactic antibiotic use were intensively sampled to detect spores of Paenibacillus larvae during the summer of 2019. Here, we compared spore concentrations in different sample types within individual hives, assessed the surrogacy potential of honey collected from honey supers in place of brood chamber honey or adult bees within hives, and evaluated the ability of pooled, extracted honey to predict the degree of spore contamination identified through individual hive testing. Samples of honey and bees from hives within apiaries with a recent, confirmed case of AFB in a single hive (index apiaries) and apiaries without clinical evidence of AFB (unaffected apiaries), as well as pooled, apiary-level honey samples from end-of-season extraction, were collected and cultured to detect and enumerate spores. Only a few hives were heavily contaminated by spores in any given apiary. All operations were different from one another with regard to both the overall degree of spore contamination across apiaries and the distribution of spores between index apiaries and unaffected apiaries. Within operations, individual hive spore concentrations in unaffected apiaries were significantly different from index apiaries in the brood chamber (BC) honey, honey super (HS) honey, and BC bees of one of three operations. Across all operations, BC honey was best for discriminating index apiaries from unaffected apiaries ($p = 0.001$), followed by HS honey ($p = 0.06$), and BC bees ($p = 0.398$). HS honey positively correlated with both BC honey ($rs = 0.76$, $p < 0.0001$) and bees ($rs = 0.50$, $p < 0.0001$) and may be useful as a surrogate for either. Spore concentrations in pooled, extracted honey seem to have predictive potential for overall spore contamination within each operation and may have prognostic value in assessing the risk of future AFB outbreaks at the apiary (or operation) level.

## 3.2 Introduction

American foulbrood (AFB) is a destructive disease of honey bee larvae caused by the gram-positive bacterium, *Paenibacillus larvae* (6). Depending on its surrounding environment, *P. larvae* may exist in one of two distinct forms: either a replicative, vegetative state, or a resilient, infectious endospore (hereafter referred to as spore) (4,6). Spores, once ingested by newly hatched and susceptible larvae, undergo germination within the larval midgut into the vegetative form, which replicate prolifically, eventually invading the haemocoel, killing the larva, and undergoing

sporulation to produce over a billion new, infectious spores (2,32,33,39,40). Bacterial spores of *P. larvae* are durable, capable of surviving and maintaining infectivity for decades, and able to withstand environmental extremes and common disinfection procedures (1,34). Prevention, eradication, and control of AFB are challenging due to the combination of spore resiliency and the dissemination of large numbers of spores throughout a hive by the normal caretaking actions of worker bees (56). These same characteristics, however, have been useful in the development of diagnostic testing for the presence of spores in different hive- and colony-associated matrices (66). These include bees, wax, pollen, bottom-board debris, and honey, all of which have been used as a diagnostic tool to determine the clinical status of a hive, or as a predictive tool to determine the risk of disease outbreak (4,6,10,15–21,26,39,42,66,69,75,78,92,93,95,100,114,116,121–123).

Many of these studies have been performed in regions such as Europe and New Zealand, where the use of antimicrobials in beekeeping is prohibited (10,15,17–21,26,69,78,92,93,114,115,123), and are focused predominantly on the collection and testing of matrices from individual hives. Conversely, there are relatively few studies evaluating the distribution of *P. larvae* spores within North America, where apiculture is heavily reliant on the sustained use of antimicrobials to prevent clinical outbreaks of AFB (39,76,100,121). Metaphylactic antimicrobial use against *P. larvae* is only successful at eliminating the vegetative state of the bacteria; spores are unaffected by antibiotics and remain infectious (2,3). Antimicrobial use is therefore only successful at controlling and preventing clinical signs of disease (2,3,95). As a result, many North American beekeeping operations that incorporate antimicrobial use in their routine management practices assume that there is widespread spore contamination within their hives, fostering continued, indiscriminate antimicrobial treatment to help ensure sustainability and profitability. This practice precludes a true understanding of the distribution and load of spores of *P. larvae* within antibiotic-reliant operations, information that could promote more judicious use of antimicrobials through evidence-based decision-making.

The documented emergence of antimicrobial resistance in *P. larvae*, along with recent regulatory changes in Canada to strengthen the veterinary oversight of medically important antimicrobial use in animals (104), may have important ramifications for the control of AFB in not only Saskatchewan, but all of North America, as Saskatchewan beekeeping practices are representative of those across much of the continent (7,8,99,100,104). Without a thorough

understanding of the distribution and concentration of *P. larvae* spores within its beekeeping operations, the North American beekeeping industry is inadequately prepared to safely reduce its reliance on the metaphylactic use of antibiotics and is at risk of significant economic losses. As such, more work is needed to determine the value of different individual and apiary-level sample types in describing the distribution and degree of contamination of *P. larvae* spores within antibiotic-reliant beekeeping operations.

We previously identified clinical outbreaks of American foulbrood in four large-scale, commercial, honey-producing, beekeeping operations across Saskatchewan in the summer of 2019 with recent or ongoing histories of metaphylactic antibiotic use (124). We reported a description of these outbreaks in the context of relevant management practices as a means of continuing education for Canadian veterinarians, who have only recently become responsible for the prescription of antimicrobials for apiculture (104). We returned to three of these operations within days of the recognition of clinical signs of AFB to intensively sample individual colonies from the apiaries with a confirmed case of AFB and apiaries without clinical evidence of AFB to quantify contamination by spores of *P. larvae*. In addition, pooled honey samples across multiple apiaries were collected from each beekeeper during routine extraction at the end of the honey-producing season for comparative testing. The objectives of this study were: i) to assess *P. larvae* spore concentrations from different sample types within individual hives across apiaries of beekeeping operations that had experienced recent outbreaks of AFB; ii) to assess whether honey collected from honey supers could be used as a surrogate for brood chamber honey or adult bees when assessing spore concentrations within a hive; and iii) to assess the ability of pooled honey samples collected during end-of-season extraction to predict the degree of spore contamination identified through individual hive sampling. Through a descriptive and comparative evaluation of different approaches for detecting spores of *P. larvae* from these beekeeping operations, we sought to improve our understanding of AFB outbreaks within antibiotic-reliant management systems. The detection of spores in this study was performed with the intent of identifying techniques with potential use as prognostic indicators of future AFB risk, rather than as a means of evaluating the current health status of the investigated hives. The ultimate goal of this study was to evaluate if pooled, extracted honey (i.e., pooled honey from honey supers) could be used for risk assessment of AFB at the apiary or operation level.

## 3.3 Materials and Methods

### 3.3.1 Beekeeping Operations

In this study, samples were collected from three large-scale, honey-producing, commercial beekeeping operations across Saskatchewan whose management practices are described in greater detail in Zabrodski *et al.* (2020) (Figure 3.1) (124). Both here and in this previous study, these commercial beekeeping operations are referred to as operations A, B, and C (124). All three beekeeping operations are located in central Saskatchewan, Canada (as defined by latitude). In North America, a commercial beekeeping operation refers to beekeeping performed at a very large scale with large numbers of hives across multiple apiaries. The owner(s) of a commercial beekeeping operation rely on hired staff to assist with routine apiary management, and operations may include honey production and/or pollination services. The beekeeping operations sampled in this study ranged in size between 2,700 and 4,000 honey-producing colonies across 45 to 125 apiaries during the summer of 2019 (124). Here, the term apiary is synonymous with bee yard (a term predominantly used in North America) and is defined as a collection of hives at a single geographical location. Apiaries of commercial beekeeping operations in Saskatchewan each typically contain between 36 and 56 bee colonies.

One operation (operation A) had recently ceased metaphylactic antibiotic use with oxytetracycline, having last treated its colonies approximately three years prior to sample collection described in this study. The remaining two operations (operation B and operation C) had been treating their apiaries with metaphylactic oxytetracycline in an off-label manner (124). Operation B had been treating with oxytetracycline twice annually (on-label), but only performing a single application per treatment (off-label) instead of the instructed 3 applications per treatment (124). Operation C, on the other hand, had only been treating with oxytetracycline once annually in the spring (off-label) (124).

**Case Report (Zabrodski *et al.* 2020)**

- Identification of AFB in four beekeeping operations (A, B, C, D)
- Comparison of management practices
- Identification of off-label antimicrobial use in all operations

**Current Study**

- Return to three beekeeping operations (A, B, C)
- Sampling of individual hive/colony matrices and pooled, extracted honey
- Cultivation and quantification of *P. larvae* spores
- Correlation of results from different matrices

**Figure 3.1. Schematic comparing previous case report (Zabrodski *et al.* 2020) and current study.**

As previously described, each outbreak of AFB was considered to be limited to a single colony (hereafter referred to as the index case) within a single apiary (124). A confirmed diagnosis of AFB within a colony required both the presence of compatible clinical signs (a combination of larval ropiness and/or larval scale) and laboratory isolation of the causative agent from samples of diseased larval tissue taken from the suspect hive (6,124). Samples collected by the Provincial Specialist in Apiculture from the index cases in each beekeeping operation were submitted for antimicrobial susceptibility testing using Kirby Bauer disk diffusion at the Animal Health Laboratory (University of Guelph, Guelph, Ontario), and all isolates were confirmed to be susceptible to oxytetracycline (124). All three operations in this study were contacted throughout 2020 to determine the incidence of AFB disease over the 2019-2020 winter and 2020 honey-producing season.

### 3.3.2 Sample Collection from Individual Hives

Following initial reporting of a suspected AFB outbreak (operation A: June 10, 2019; operation B: June 14, 2019; operation C: early June 2019) and subsequent inspection and confirmation by the Provincial Specialist in Apiculture, all three beekeeping operations were subjected to intensive sampling within several days after confirmation (Figure 3.2). Two apiaries were selected for sampling from each beekeeping operation: the apiary with a recently confirmed case of AFB disease in a single colony (index case), hereafter referred to as index apiary, and a second apiary with no detected clinical evidence of AFB disease, hereafter referred to as unaffected apiary. The second apiary was chosen at the discretion of the beekeeper, with instructions to select a location that was geographically distant from (i.e., not within flying distance of) the index apiary and that they believed to be free of AFB. It should be noted that all sampled colonies in both the index and unaffected apiaries were confirmed to be negative for clinical AFB disease based on the absence of the following clinical signs: a scattered brood appearance, sunken and/or perforated cappings, larval scale against the dependent wall of uncapped cells, and larval ropiness within capped cells (25). Permission to access each apiary was provided directly by the owner of each beekeeping operation, who was also present during each sampling event.

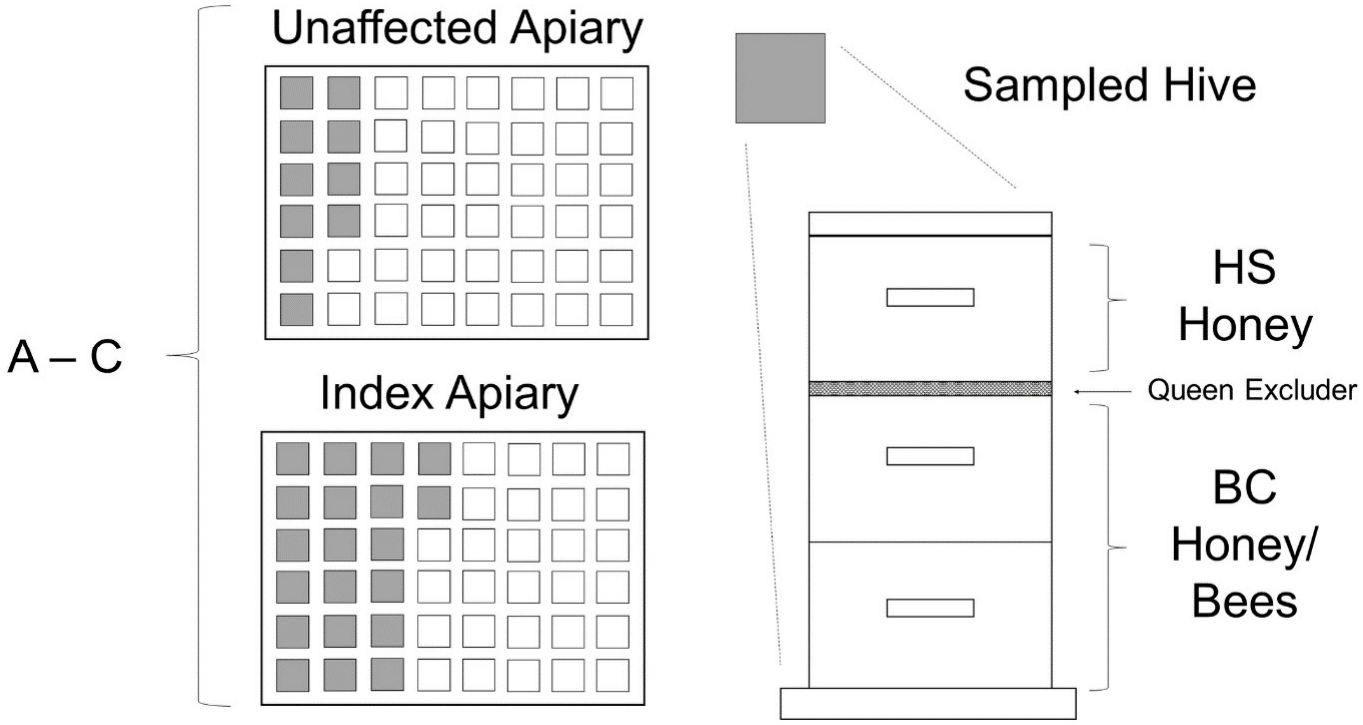


**Figure 3.2. Visual schematic of sampling collection from individual hives.** A total of 2 apiaries were sampled for each beekeeping operation (A, B, and C; letters denote operation ID): an apiary with an index case of clinically and laboratory confirmed AFB disease (index apiary) and a second apiary with no detected clinical evidence of AFB disease (unaffected apiary). Twenty randomly selected hives were sampled from the index apiary in each operation (represented by grey-shaded boxes), and ten randomly selected hives were sampled from each unaffected apiary. Samples of honey super honey (HS honey), brood chamber honey (BC honey), and brood chamber bees (BC bees) were collected from each sampled hive, when available.

A total of 30 hives were sampled from each beekeeping operation: 20 hives randomly selected from the index apiary and ten hives randomly selected from the unaffected apiary. The following samples were collected from each hive: i) live, adult bees from a brood chamber frame with uncapped brood (brood chamber bees; hereafter, BC bees); ii) unsealed honey from a brood chamber frame with uncapped brood (brood chamber honey; hereafter, BC honey); and iii) if available, unsealed honey from a frame within the overlying honey super closest to the brood chambers (honey super honey; hereafter, HS honey). If there were no frames of uncapped brood, samples of BC bees and BC honey were collected from frames with or adjacent to capped brood. Samples were collected in individual plastic bags, transported on ice from apiary to laboratory within a few hours after collection, and stored at -20 °C until analysis. The Canadian Council on Animal Care (CCAC) does not require permission for research on insects; however, all sampling and animal handling procedures were performed in accordance with the Saskatchewan Apiaries Act.

### 3.3.3 Sample Collection During End-of-Season Extraction

To determine if spore concentrations in pooled honey at end-of-season extraction could predict the degree of spore contamination identified through individual hive sampling, beekeepers were instructed to collect a total of 18 honey samples during routine, end-of-season extraction using an adapted, two-stage sampling protocol (125). For each operation, six apiaries or lots were chosen at the discretion of the beekeeper, and three different samples of honey were collected from each apiary or lot. Here, a lot refers to a collection of several apiaries in close geographic proximity that are collected and extracted together as a single unit during routine honey extraction. Beekeepers selected six different lots or apiaries that were geographically distant from one another. Correlation of these apiaries or lots to the index and unaffected apiaries sampled in June of 2019 was not attempted. Each sample was collected from a different extractor load to ensure that the same frames/hives were not sampled multiple times. Assuming the use of an at least 60-frame commercial extractor and that 60 frames represent between three and six hives within an apiary, the collection of three unique samples from three different extractor loads would represent between nine and 18 hives from a single apiary or lot. Based on this sampling approach, it is assumed that 18 honey samples represented 54 to 108 hives from six geographically distant locations for each

operation. Samples were collected and sealed in 500 g plastic tubs and stored at room temperature until processing.

### 3.3.4 Preparation of Culture Media

All samples were cultured on a complex MYPGP medium adapted from previously published protocols (66,71) with added agonists of *P. larvae* spore germination (i.e., L-tyrosine and uric acid (79)). For 1 L of media, 10 g of Difco™ Mueller Hinton Broth (BD, 275730), 15 g of Bacto™ Yeast Extract (BD, 212750), 3 g of potassium phosphate dibasic (Fisher BioReagents, BP363-1), 1 g of sodium pyruvate (Fisher BioReagents, BP356-100), and 15 g of agar (Fisher BioReagents, BP1423-2) were autoclaved in 880 mL of distilled water and combined with 20 mL of separately autoclaved, 10% glucose (Sigma, G-5767). To attain a final volume of 1 L, 50 mL of 60 mM L-tyrosine (Alfa Aesar, A11141) dissolved in 1 M hydrochloric acid (Fisher Chemical, A144-500), 50 mL of 60 mM uric acid (Alfa Aesar, A13346) dissolved in 1 M sodium hydroxide (Fisher Chemical, S318-500), and 1 mL of 20 mg/mL Nalidixic acid (Alfa Aesar, J63550), each sterilized through separate, 0.22 micron filters, were immediately added to the molten media. The resulting MYPGP medium enhanced by germination agonists is hereafter referred to as enhanced MYPGP medium. Plates were refrigerated at 4 °C until use.

### 3.3.5 Cultivation of *P. larvae* From BC Bees

A total of 100 worker bees were counted into a plastic bag, mixed with 25 mL of sterile water, and manually crushed according to previously published protocols (18,19,114). A portion of the resulting fluid was heat-treated at 85 °C for 15 min (126), allowed to cool to room temperature, and spread onto three plates of enhanced MYPGP media (200 µL per plate, 600 µL total). An additional 200 µL of unpasteurized sample was spread onto a separate plate of enhanced MYPGP media as a control. Samples were briefly vortexed immediately prior to plating. Plates were incubated at 37 °C with 5 % $CO_2$ for seven days (6,66). After incubation, the number of bacterial colonies with a morphology consistent with *P. larvae* were averaged across the three technical replicates and the number of spores per bee was calculated using the assumption that a single colony forming unit (CFU) is equivalent to a single spore (6,16). Serial, ten-fold dilutions were prepared and re-cultured for any samples yielding one or more plates with greater than 100 colonies consistent with *P. larvae* morphology to avoid underestimating colony counts due to

crowded, confluent colonies (19). Suspect colonies were submitted to Prairie Diagnostic Services of the Western College of Veterinary Medicine in Saskatoon, Saskatchewan, and were identified as *P. larvae* using diagnostic matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS).

### 3.3.6 Cultivation of *P. larvae* from Honey

For BC honey and HS honey collected from individual hives, a mixture ~20 g of honey with accompanying comb wax was mixed with 20 mL of sterile water and shaken overnight at 37 °C to ensure complete dissolution of honey (6,66). The following morning, samples were filtered through two sheets of loose, autoclaved cheesecloth to remove comb wax and other debris, and the final weight of honey in the suspension was calculated by subtracting the weight of water that had been previously added. Honey suspensions were balanced with additional sterile water and centrifuged at 6,000 *g* for 40 min at room temperature (6,66) to pellet the spores. The supernatant was poured off and centrifuge tubes left upside-down on paper towel to drain for approximately five min. Pellets were re-suspended in 2 mL of sterile water and the samples vortexed for 20 seconds. Steps for heat treatment, plating, and incubation were identical to those for bee samples.

Samples collected from the extracted, pooled honey contained very little to no wax and did not require straining to remove wax and debris. Accordingly, 20 g from each extracted honey sample was weighed out, mixed with 20 mL of sterile water, and shaken overnight at 37 °C. The following morning samples were centrifuged, heat-treated, plated, and incubated as per the steps for honey samples from individual hives. Data for all honey samples were presented as spores per gram of honey.

### 3.3.7 ERIC Genotyping of *P. larvae* Isolates

To characterize *P. larvae* genotypes isolated from these beekeeping operations, five isolates from each operation (a total of 15 isolates) were subjected to repetitive element PCR fingerprinting (rep-PCR) using ERIC primers (64,65). Spore suspensions obtained from both larval scale of the index case and the four BC honey samples with the highest spore concentrations from each operation's index apiary were revived from -80 °C on enhanced MYPGP medium incubated at 37 °C with 5 % $CO_2$ for 72 hours. The resulting populations of *P. larvae* colony

growth were confirmed as uniform which, as per Bassi *et al* (2015), allows for a single representative colony from each sample to be sub-cultured for rep-PCR (127).

DNA was extracted from sub-cultured isolates using a DNeasy Blood & Tissue kit (Qiagen) following the protocol for gram positive bacteria with minor changes. First, centrifugation of the second buffer was performed at 15,000 rpm rather than 20,000 rpm. Second, 150 µL of elution buffer was used for elution instead of 200 µL.

The DNA sequences used for ERIC primers were as described by Versalovic *et al.* (1994) (65), and the ingredients and reaction cycle parameters for rep-PCR reactions were carried out according to a previously established protocol by Genersch and Otten (2003) (64), but with 40 amplification cycles instead of 35 (Mastercycler™ Pro, manufactured by Eppendorf™, Germany). PCR products were visualized with UV light (302 nm) (AlphaImager HP Imaging System, manufactured by ProteinSimple bio-techne®, San Jose, CA, USA) following gel electrophoresis (Bio-Rad) with ethidium bromide run in 1.0% agarose gel at 90 V for 50 min. Differentiation between ERIC I and ERIC II genotypes was determined by the presence or absence of a migrating band between 2500 and 2800 bp that is characteristic of ERIC II (31).

### 3.3.8 Data Analysis

Statistical analyses were carried out using STATA software (Version 16.1; StataCorp LLC). Descriptive statistics were provided by apiary within beekeeping operation to assess the parameter estimate and its variability (i.e., median and interquartile range) for each individual hive sample type (BC honey, HS honey, BC bees). Normality of data were tested using a Shapiro-Wilk test, and equality of variances assessed with a Levene's test. Within each operation, the difference between the unaffected and index apiary was compared using a Wilcoxon rank sum test for each sample type. To assess the overall ability of each sample type to discriminate between index and unaffected apiaries, a Poisson regression was used that accounted for operation ID. The robust option was used to estimate the variance–covariance matrix (VCE) corresponding to the parameter estimates, which is robust to some types of misspecification so long as the observations are independent. Correlation between HS honey and BC honey, as well as HS honey and BC bees, was determined using the Spearman rank correlation. A Kruskal-Wallis test was used to compare pooled, extracted honey samples between beekeeping operations. To evaluate pooled, extracted honey samples as a predictor of results of individual hive sampling, all pooled samples within each

beekeeping operations were compared to an arbitrary threshold set at 1 spore per gram of honey. An α cut-off of 0.05 was used for all statistical analyses with the exception of post-hoc pairwise comparisons following Kruskal-Wallis assessment of pooled, extracted honey samples between operations, where an α cut-off of 0.017 was used following Bonferroni correction.

## 3.4 Results

### 3.4.1 Descriptive Evaluation of *P. larvae* Spore Concentrations in Individual Hives Within Apiaries

In all operations, a small number of hives within apiaries accounted for the majority of spore contamination. Variability in spore concentrations between hives within individual apiaries was dependent on the beekeeping operation (Table 3.1, Figure 3.3). In operation A, spore concentrations within the unaffected apiary were low and tightly clustered regardless of sample type. Within the index apiary, a relatively small number of samples within each sample type accounted for the majority of spore contamination. A single BC honey sample (1/20) and a single bee sample (1/20) from the index apiary had spore concentrations greater than 100 spores/g or spores/bee, respectively.

For operation B, variability of spore concentrations between hives across all sample types in the unaffected apiary was relatively comparable to that of the index apiary. One to three samples from each sample type were responsible for the majority of spore contamination within each apiary. One BC honey sample (1/10) and one HS honey sample (1/10) from the unaffected apiary had spore concentrations greater than 100 spores/g. Three BC honey samples (3/20) and two HS honey samples (2/14) from the index apiary had spore concentrations greater than 100 spores/g.

In operation C, the distribution of spore concentrations between hives across all sample types in the unaffected and index apiary were similar, and overall had a greater number of highly contaminated samples in each sample type relative to other operations. Three samples of BC honey (3/9), three HS honey samples (3/9), and one bee sample (1/10) had spore concentrations greater than 100 spores/g or spores/bee in the unaffected apiary. In the index apiary, five BC honey samples (5/18), three HS honey samples (3/18), and two bee samples (2/20) had spore concentrations greater than 100 spores/g or spores/bee.

**Table 3.1. Summary statistics of concentrations of *Paenibacillus larvae* spores from individual hives.** Summary statistics of concentrations of *Paenibacillus larvae* spores from individual hives for honey collected from brood chambers (BC Honey) and honey supers (HS Honey) and bees collected from brood chambers (BC Bees) within apiaries with a recent, confirmed case of American foulbrood (AFB) in a single hive (index apiaries) and apiaries unaffected by AFB (unaffected apiaries). Capital letters denote operation ID (A, B, or C).

| | | Unaffected apiary | | | Index apiary | | |
|---|---|---|---|---|---|---|---|
| Operation ID | | BC honey (spores/g) | HS honey (spores/g) | BC bees (spores/bee) | BC honey (spores/g) | HS honey (spores/g) | BC bees (spores/bee) |
| A | Sample size | 10 | 9 | 10 | 20 | 10 | 20 |
| | Median | 0.0 | 0.0 | 0.0 | 1.8 | 0.7 | 1.3 |
| | Minimum | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | Maximum | 2.7 | 0.3 | 1.3 | 10,703.2 | 56.0 | 1,237,500.0 |
| | Interquartile range | 0.0 | 0.0 | 0.0 | 13.9 | 1.4 | 1.9 |
| B | Sample size | 10 | 10 | 10 | 20 | 14 | 20 |
| | Median | 7.9 | 15.8 | 0.4 | 8.1 | 16.6 | 0.4 |
| | Minimum | 1.8 | 3.0 | 0.0 | 0.2 | 1.4 | 0.0 |
| | Maximum | 243.8 | 747.6 | 8.3 | 670.0 | 61,666.7 | 5.4 |
| | Interquartile range | 8.2 | 24.4 | 5.4 | 14.6 | 37.6 | 1.2 |
| C | Sample size | 9 | 9 | 10 | 18 | 18 | 20 |
| | Median | 2.9 | 6.9 | 0.5 | 25.8 | 14.9 | 3.0 |
| | Minimum | 0.0 | 0.4 | 0.0 | 0.0 | 0.4 | 0.0 |
| | Maximum | 1,163.6 | 2,684.9 | 262,500.0 | 94,589.5 | 3,574.1 | 245,833.3 |
| | Interquartile range | 185.2 | 130.4 | 36.2 | 143.2 | 50.8 | 6.2 |

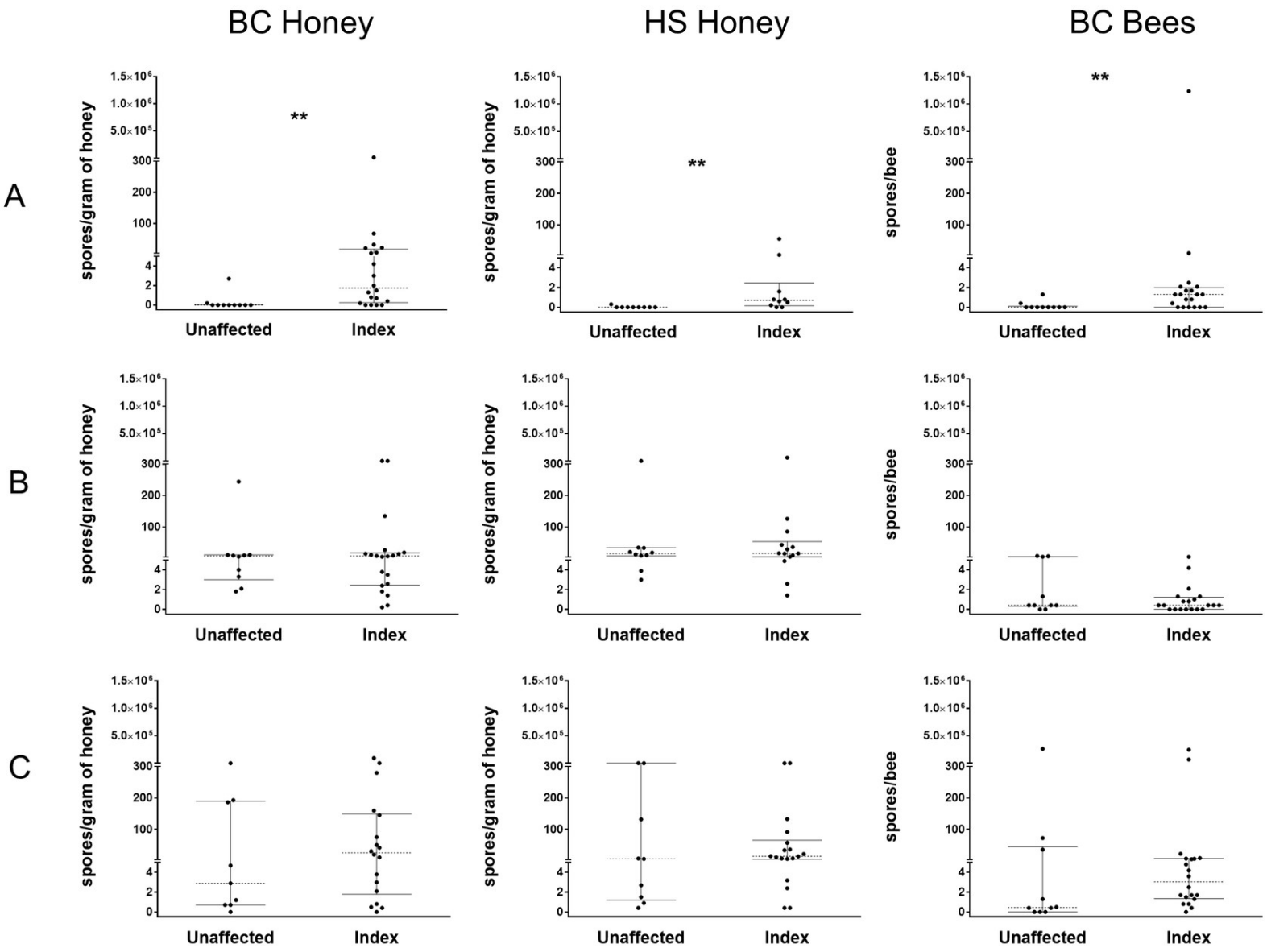


**Figure 3.3. Concentrations of *Paenibacillus larvae* spores for individual hives from index and unaffected apiaries.** A single index apiary and single unaffected apiary were sampled from each operation (six apiaries total). Apiaries are grouped vertically by sample type (BC honey, HS honey, and BC bees) and horizontally by beekeeping operation (A, B, and C; letters denote operation ID). Dotted lines represent median values and bars represent interquartile ranges. ** denotes statistical significance where $p < 0.01$ (Wilcoxon rank sum test).

### 3.4.2 Comparison of *P. larvae* Spore Concentrations in Individual Hives Between Index and Unaffected Apiaries

With regard to the overall discriminating ability of each individual hive sample type, only BC honey was able to detect a difference between index and unaffected apiaries (after accounting for operation ID) (Robust Coefficient [RC] = 3.39, 95% Confidence Interval [CI] = 1.36 to 5.41, p = 0.001; Poisson regression). No statistically significant difference was detected between index and unaffected apiaries (after accounting for operation ID) when measuring spore concentrations from either HS honey (RC = 2.14, 95% CI = -0.091 to 4.36, p = 0.06; Poisson regression) or BC bees (RC = 1.08, 95% CI = -1.43 to 3.59, p = 0.398; Poisson regression).

Within each operation, the difference between index and unaffected apiaries was dependent on the operation ID and the sample type used for assessment (Fig 2). Within operation A, statistical differences in spore concentrations between index and unaffected apiaries were observed for BC honey (z = 3.115, p = 0.0018; Wilcoxon rank sum test), HS honey (z = 3.003, p = 0.0027; Wilcoxon rank sum test), and BC bees (z = 2.723, p = 0.0065; Wilcoxon rank sum test). No significant differences were found between the index and unaffected apiaries within operations B and C by any of the individual hive sample types.

### 3.4.3 Comparison of *P. larvae* Spore Concentrations in Individual Hive HS Honey to BC Honey and BC Bees

HS honey was assessed as a surrogate for BC honey and BC bees in measuring spores of *P. larvae* by correlating these values in individual hives. HS honey was positively correlated with BC honey ($r_s = 0.76$, $p < 0.0001$, Figure 3.4a). Similarly, HS honey was positively correlated with BC bees, albeit less strongly ($r_s = 0.50$, $p < 0.0001$, Figure 3.4b). Hives with only one of the two samples under comparison were excluded from analysis, resulting in 68 pairwise observations for HS honey and BC honey, and 70 observations for HS honey and BC bees.

In operation A, 19 out of possible 30 hives from across both apiaries had honey samples available from both the honey super and brood chamber at the time of sampling. Of these, ten hives (10/19) had detectable spores in at least one of either HS honey or BC honey. Spore concentrations from BC honey samples were higher than those from honey supers in five of these hives (5/10). Twenty-four of 30 hives across both apiaries in operation B had available honey from both the

honey super and brood chamber. All 24 of these hives had detectable spores in at least one of their honey samples, and spore concentrations from BC honey were higher than those from honey supers in three hives (3/24). In operation C, 25 out of a possible 30 hives from across both apiaries had honey samples available from both the honey super and brood chamber. All 25 of these hives had detectable spores in at least one of their honey samples, and spore concentrations from BC honey samples were higher than or equal to those from honey supers in 12 hives (12/25).

The majority of bee samples across all apiaries of all beekeeping operation (82/90) had low levels of detectable spores with fewer than 10 spores per bee (Range = 0 – 9.3 spores/bee). Five of the eight hives with bee samples greater than 10 spores per bee had concurrently high concentrations of detectable spores in HS honey (Range = 131.9 – 2,684.9 spores/g). Of the remaining three hives with relatively high spore concentrations in BC bees, two had no available HS honey, whereas the third (10.8 spores/bee) detected a maximum of 5.1 spores per gram of honey in its super honey sample.

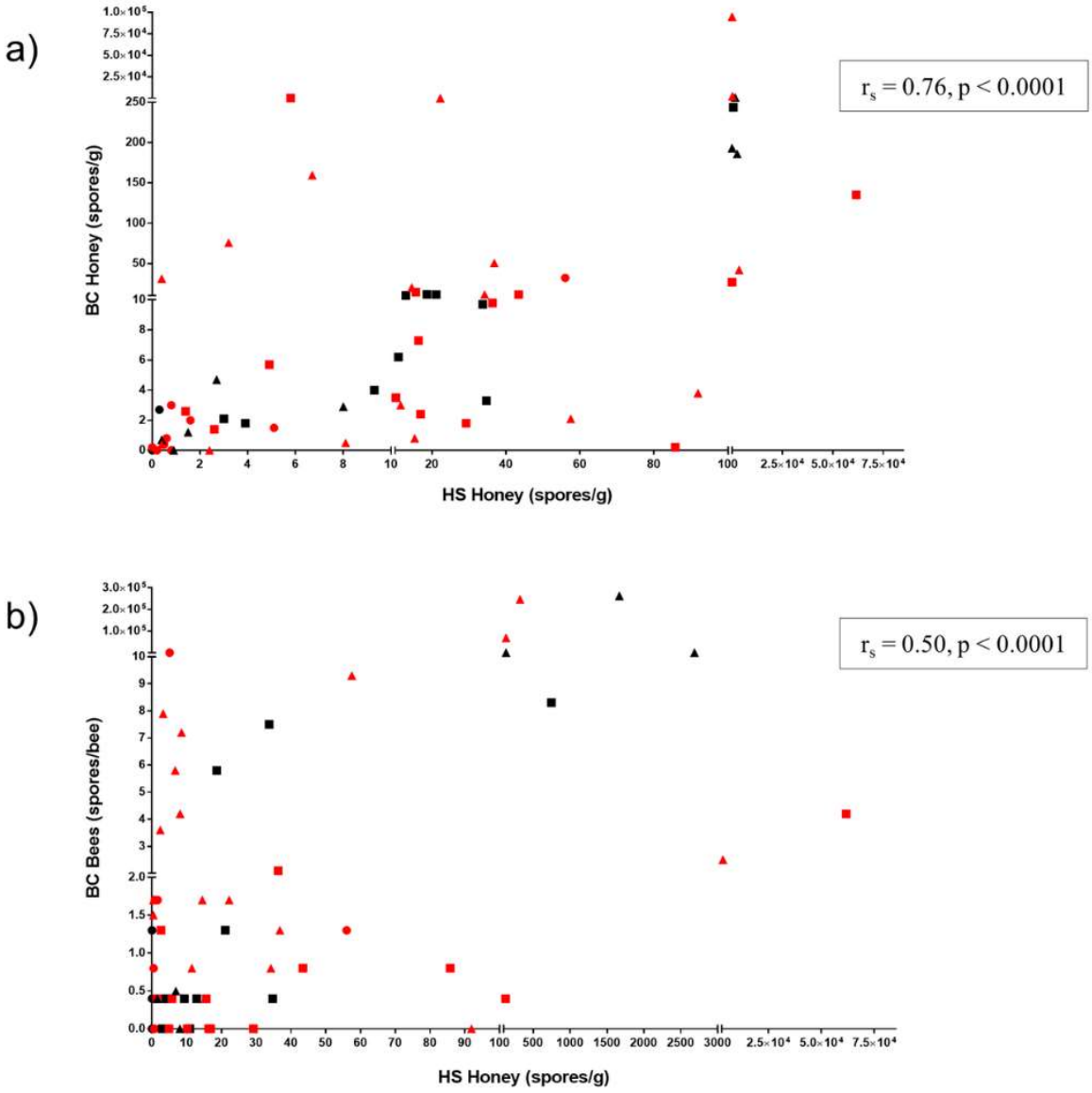


**Figure 3.4. Comparison of concentrations of *Paenibacillus larvae* spores between HS honey and BC honey and BC bees within individual hives.** Data include all apiaries of all beekeeping operations. a) HS honey is positively correlated with BC honey ($r_s = 0.76$, $p < 0.0001$); b) HS honey is positively correlated with BC bees, albeit less strongly ($r_s = 0.50$, $p < 0.0001$). Hives with only one of the two samples under comparison are excluded from each plot. Circles = operation A, squares = operation B, triangles = operation C; black = hive from apiary unaffected by American foulbrood (AFB), red = hive from index apiary with a recent, confirmed case of AFB in a single hive.

### 3.4.4 Apiary-Level Sampling for Spores of *P. larvae* in Pooled, Extracted Honey and Subsequent Incidence of AFB

Spore concentrations of extracted honey samples from all operations were different from one another (Figure 3.5). Operation A had the lowest concentration of detectable spores per gram of honey across its pooled samples (M = 0, Range = 0 to 0.8, IQR = 0.2), followed by increasing concentrations of detectable spores in operation B (M = 0.7, Range = 0 to 3.6, IQR = 1.3), and operation C (M = 17.1, Range = 2.0 to 280.3, IQR = 28.8). When compared to an arbitrary threshold of 1 spore per gram of honey, 0% of operation A's 18 pooled samples (0/18), 39% of operation B's pooled samples (7/18), and 100% of operation C's samples (18/18) fell above this threshold line.

Median spore concentrations were assessed as a rough assessment of the ability for pooled honey samples to reflect the degree of contamination detected by individual HS honey samples across apiaries within an operation. The median spore concentration of all individual hive HS honey samples from Operation A was 0 spores/g, which was comparable to the median spore concentration from pooled honey samples (0 spores/g). The median spore concentration of all HS honey samples from Operation B was 16.6 spores/g, whereas the median spore concentration from the pooled samples was 0.7 spores/g. The median spore concentration of all HS honey samples from Operation C was 11.5 spores/g and the median concentration from the pooled samples was 17.1 spores/g.

Both operation A and operation B reported no further cases of AFB throughout 2020. Operation C reported clinical signs consistent with AFB within several colonies that died overwinter (2019/20), which were confirmed by the provincial specialist in apiculture. These colonies belonged to apiaries that had not been included in either individual or apiary-level sampling during 2019.

### 3.4.5 ERIC Genotyping of *P. larvae* Isolates

The banding patterns of the ERIC-PCR products of all 15 isolates across all beekeeping operations were uniform and consistent with ERIC I (Figure 3.6). These patterns consisted of a 970 bp migrating band and the absence of a migrating band between 2500 and 2800 bp (31). No isolates were compatible with ERIC II.

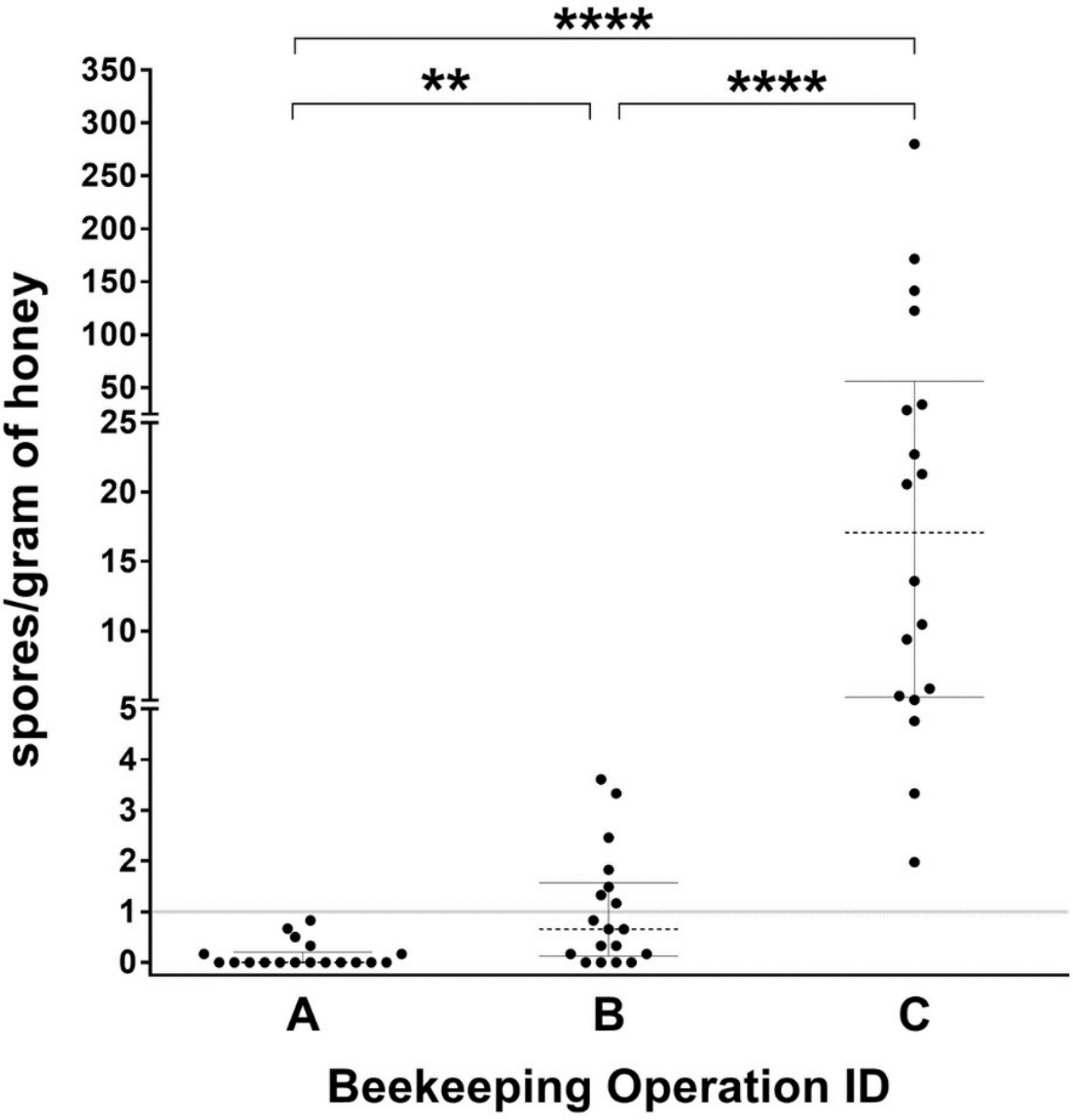


**Figure 3.5. Spores of *Paenibacillus larvae* per gram of honey from pooled honey samples collected during routine extraction at the end of the honey-producing season for each beekeeping operation.** Each operation submitted 18 samples representing six randomly selected apiaries or lots; three unique samples were collected from each apiary or lot. Dotted lines represent median values and bars represent interquartile ranges. The solid grey line represents an arbitrary threshold value of one spore per gram of honey. Capital letters denote operation ID. ** denotes statistical significance where $p < 0.01$; **** denotes statistical significance where $p < 0.0001$ (Kruskal-Wallis test).

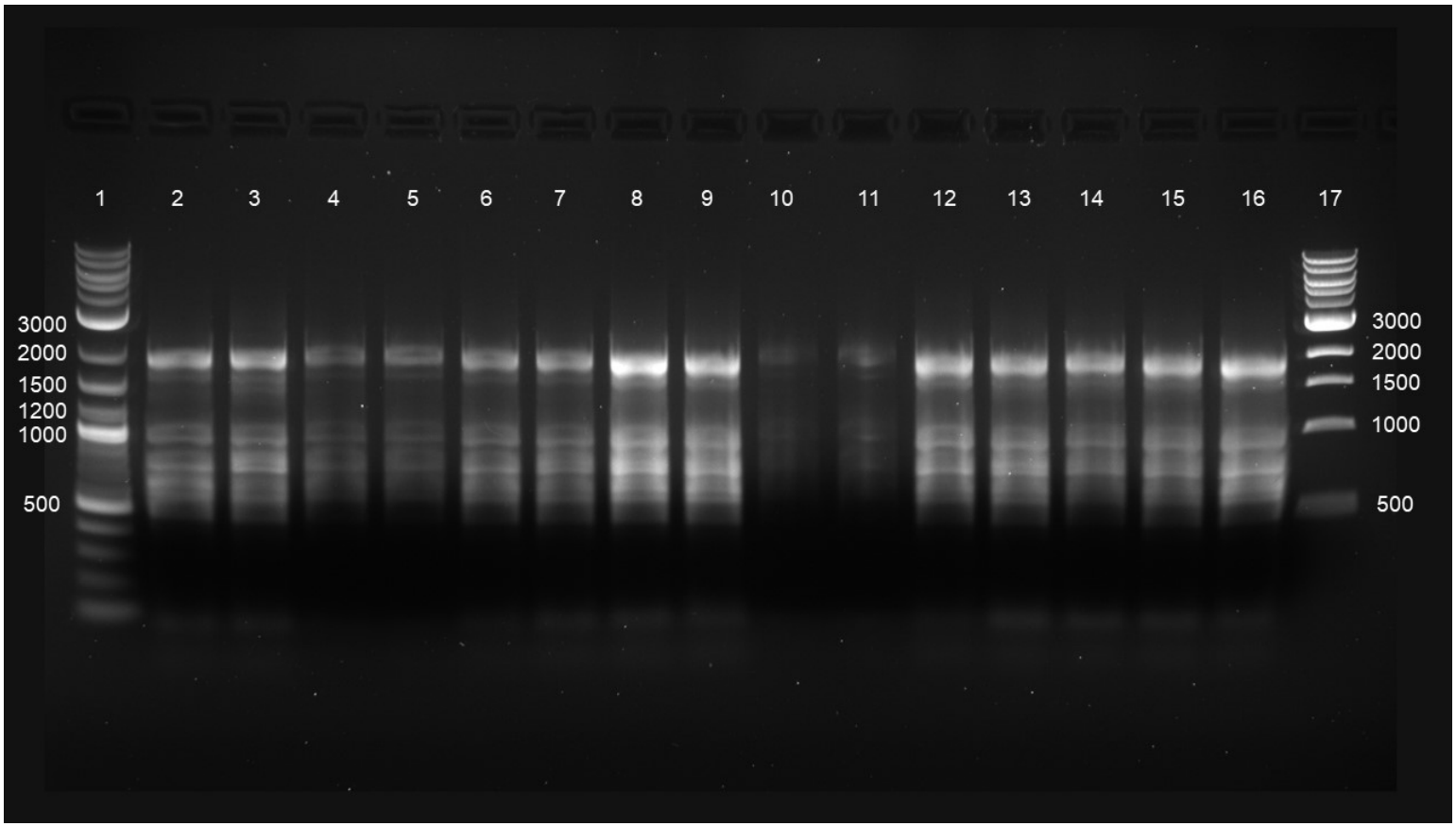


**Figure 3.6. Gel electrophoresis patterns for *Paenibacillus larvae* genotyping using rep-PCR with ERIC primers.** Lanes 1 and 17 contain N0550A and N0468S Quick-Load® DNA ladders, respectively. Lanes 2, 4, 5, 6, and 7 contain isolates from operation A; lanes 8 through 12 contain isolates from operation B; lanes 3 and 13 through 16 contains isolates from operation C. All patterns include a 970 bp migrating band and the absence of a migrating band between 2500 and 2800 bp. Differentiation between ERIC I and ERIC II genotypes was determined by the presence or absence of a migrating band between 2500 and 2800 bp that is characteristic of ERIC II.

### 3.5 Discussion

Through the opportunistic and intensive sampling of beekeeping operations with recent clinical outbreaks of AFB, we found that pooled samples of HS honey collected during end of season extraction were reflective of the overall severity of contamination by AFB spores within an operation and may have potential utility as a prognostic indicator of AFB risk. In addition, we demonstrated that only a few hives were heavily contaminated with spores and most hives had few to no detectable spores in any given apiary. Index apiaries tended to have higher concentrations of spores than unaffected apiaries, but this was largely dependent on sample type and the beekeeping operation under examination. In the context of overall discriminatory ability, BC honey was best for differentiating between index and unaffected apiaries, although HS honey was strongly correlated with BC honey and may be used as a surrogate in place of brood chamber samples.

Similar to studies performed in Europe, where antibiotic use in apiculture is prohibited (14,120), we found that spores are not homogenously distributed amongst hives within apiaries treated with antibiotics, and apiary contamination with spores appears to be driven by only a few heavily contaminated hives. This is contrary to our initial expectation that hives within an apiary would have comparable levels of detectable spores, as previous work has shown that the introduction of spore-laden, recently extracted (wet) honey supers onto some AFB-free hives within an AFB-free apiary results in rapid dissemination of spores to bees from all hives (26). Similarly, research evaluating the impact of robbing behaviour on the horizontal transmission of *P. larvae* spores found that hives within close proximity of clinically diseased hives were at a high risk of contracting high levels of spores (51).

Although BC honey had a better overall ability to discriminate between index and unaffected apiaries relative to HS honey based on the detection of spores, we found that HS honey was positively correlated with spore concentrations in both BC honey and BC bees. Furthermore, the overall burden of spores identified within each operation through pooled, extracted honey from honey supers was comparable to the severity of contamination identified in individually sampled hives (Figure 3.7). We therefore suggest that HS honey may be used as a surrogate for brood chamber sample types as a means of identifying spore concentrations within hives and that the use of pooled HS represented in extracted samples may have a couple of distinct advantages over the use of BC bees and BC honey in large, antibiotic-reliant beekeeping operations.


**Figure 3.7. Conceptual representation of the detection of a spore "signal" in pooled, apiary-level honey samples.** The corresponding apiaries the pooled samples are derived from are indicated by letters for operations A, B, and C. Small boxes within an apiary represent individual bee hives, and red boxes represent those hives heavily contaminated with spores of *P. larvae*. A lighter "signal" (light pink/red) in the pooled samples reflects operations with very little contamination in individual hives, whereas higher concentrations of spores (stronger "signal" – red) in pooled samples correspond to more widespread and/or chronic contamination. Index and unaffected apiaries are used in this theoretical example.

First, HS honey can be easily scaled-up into pooled samples representing multiple hives within an apiary by collecting during routine honey extraction at the end of a honey-producing season. The main advantage of this sampling approach is in its relative convenience to the beekeeping operation, as honey samples may be rapidly collected during the spinning of frames on an extractor with minimal disturbance to normal workflow (100,116). Due to the skewed distribution of spore contamination within an apiary, a large sample size is required on a per apiary basis to identify the relatively few, heavily contaminated, and presumably high-risk hives. The sampling of individual hives is therefore time consuming, laborious, and logistically impossible for large-scale North American beekeeping operations. Collecting several samples of honey from separate extractor loads incorporates multiple hives at a time with a higher chance of "capturing" the few, heavily contaminated hives that may be present within an apiary.

Second, the detection of a spore "signal" through apiary-level testing may reflect the overall chronicity and severity of *P. larvae* spore contamination across an entire operation, despite use of antibiotic metaphylaxis (Figure 3.7) (100). At the very least, this approach has merit in its ability to screen apiaries within a beekeeping operation for contamination level by *P. larvae* spores that may signal the need for closer investigation on a per hive basis, similar to work done in other studies (15,76,100,115,116). In this study, where a limited number of operations are examined, AFB re-occurred in the most contaminated operation (operation C) within 12 months, suggesting that spore concentrations in pooled, extracted honey may be predictive of the risk of clinical AFB, as suggested in other studies performed in regions where antibiotic use in apiculture is prohibited (15,115). Accordingly, we are expanding the scope of this investigation to test the predictive value of pooled honey across a large number of commercial beekeeping operations reliant on chronic antibiotic use in the control of AFB.

Finally, honey samples in this study had a more gradated distribution of spore concentrations relative to those in bees, which were either very low or very high. Honey samples, within the context of these operations, may therefore have greater utility as a predictor of AFB risk due to an increased ability to discern low, medium, and high concentrations, which may in turn correspond to low, moderate, and high categories of AFB risk. This idea is supported by Von der Ohe and Dustmann (1997), who used spore concentrations in honey to establish contamination classes that corresponded to the risk of developing AFB disease (15). Similarly, Hansen and

Rasmussen (1986) demonstrated that spore concentrations in pooled honey samples had predictive potential for the development of clinical signs of AFB in the following year (115). Verification of such risk categories, however, would require longitudinal observations for changes in spore concentrations within these hives, as well as the reporting of any emergence of AFB, which we are currently investigating.

In contrast to the gradated distribution of results in honey, the either very low or very high results for bee samples in this study suggest that bees may be better suited as an indicator of current colony health status, as has been demonstrated by previous studies (16,18,19,21,69,114,120). There are several factors that, taken together with antimicrobial use, may account for the either very low or very high results observed. First, spores of *P. larvae* are not uniformly distributed amongst bees, and only a small proportion of bees within a contaminated hive will carry the majority of spores (21). Second, the proportion of spore-positive bees within a hive is positively correlated to the severity of active infection (21). Third, spore abundance within bees is also positively correlated to the severity of active infection (16,20,100,120). Erban *et al.* (2017), when comparing the abundance of *P. larvae* spores within the microbiome of honey bees sampled from colonies with clinical signs of AFB to those from adjacent, asymptomatic colonies and distant, asymptomatic colonies, found increased abundance of *P. larvae* in clinically affected colonies only (120). Unaffected colonies in close proximity to those with clinical signs of AFB were found to be no different from those outside of the designated AFB zone (120). In beekeeping operations with a chronic reliance on metaphylactic antibiotic use to prevent and control clinical signs of AFB, there is expected to be very little evidence of active disease. This may, in turn, reduce the overall number of bees with high spore burdens, thereby reducing the likelihood of sampling these relatively few individuals. This idea may be indirectly supported by a study assessing the use of bees and bulk honey samples as a means to detect *P. larvae* spores in Manitoba beekeeping operations, whose management practices are comparable to those in Saskatchewan (100). Pernal and Melathopoulos (2006) observed that bee samples yielded no *P. larvae* in some operations with histories of chronic, recurrent, and current AFB infection (100).

This study has several important limitations. First, as a cross-sectional, opportunistic survey, this study lacks longitudinal assessment of the individually sampled hives. Second, the sampled operations ranged in size between approximately 2,700 and 4,000 honey-producing hives

distributed amongst anywhere between 45 and 125 separate bee apiaries. Sampling in this study was limited to 30 individual hives (0.8 – 1.1% of total honey-producing hives) and two apiaries (1.6% – 4.4% of total apiaries) per operation. This small selection may not reflect the entire operation as a whole, however, the results from individually sampled hives positively correlate with the corresponding degree of spore contamination identified within the pooled, apiary-level honey samples, which we estimate are representative of between 54 and 108 hives from across each operation. Third, there are inherent limitations to the plate culturing technique used in this study. It is recognized that our collective, overall ability to cultivate vegetative *P. larvae* from spores on different artificial media is relatively poor (66). Not all spores will readily germinate, and previous studies have determined that fewer than 10 percent of *P. larvae* spores within a sample will germinate and produce visible colony growth (42,128). The spore concentrations reported in this study may therefore underestimate the true number of spores present within the sampled hives and pooled honey samples. While this may be the case, all operations would be equally affected by suboptimal germination rates and would therefore not affect the comparative AFB risk assessment. Finally, in addition to poor overall germination, protocols for the cultivation of *P. larvae* spores are biased toward the detection of ERIC I strains over ERIC II strains (70). Heat treatment, which is a necessary step to eliminate contaminate overgrowth, stimulates the germination of ERIC I strains at temperatures over 90 °C while inhibiting the germination of ERIC II (70). The protocols used in this study were designed to be permissive to the growth of any ERIC II strains through the use of a heat treatment that did not exceed 85 °C and through the simultaneous culture of suspension not subjected to heat. Despite this, all isolates tested across all beekeeping operations, including those obtained from each index case, were identified as ERIC I. The exclusive recognition of ERIC I strains in these three beekeeping operations is consistent with our limited understanding of the prevalence of ERIC genotypes in the Americas (22).

Surprisingly, of the three operations asked to select an unaffected apiary free of AFB, only operation A was successful in identifying an apiary that concurrently had a relatively low degree of spore contamination. Although the unaffected apiaries in both operation B and operation C were free of clinical signs of disease, they still had individual hives with levels of spore contamination comparable to those in the index apiaries. Based upon spore thresholds for clinical AFB in other studies (14,16), it is reasonable to expect that very high concentrations of spores in these seemingly healthy hives would likely be associated with the presence of clinical/subclinical AFB if not for

the recent and/or continual application of antibiotic therapy. This raises concern that beekeeping operations, through the chronic use of antibiotic metaphylaxis, may underestimate the severity of contamination within their operations and could potentially benefit from convenient, apiary-level testing of pooled honey, as identified in this study. This would allow beekeeping operations to identify those apiaries at risk of AFB outbreaks, allowing them to implement targeted interventions to mitigate risk and make evidence-based decisions regarding the use of antibiotics.

In conclusion, by comparing BC bees, BC honey, and HS honey within individual hives, we have demonstrated the usefulness of HS honey as a surrogate for both BC bees and BC honey. In addition, we have shown that pooled samples of HS honey collected during routine extraction are reflective of the overall degree of spore contamination within a given operation and may potentially be used as a prognostic indicator of the risk of future AFB outbreak. These findings improve our understanding of AFB outbreaks within antibiotic-reliant management systems and provide a potential avenue for the development of prognostic testing for AFB risk through pooled, apiary-level honey that will help to establish meaningful surveillance data for commercial beekeepers in North America.

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## PREFACE TO CHAPTER 4

In Chapter 4, we expanded the sampling of pooled, extracted honey to a large number of Saskatchewan beekeepers from a wide demographic range, including those practicing beekeeping on a relatively small-scale (i.e., one to fewer than 100 hives) and those operating on a large-scale (i.e., greater than 100 hives). Together, these beekeepers owned over 82,000 of the province's 110,000 honey bee colonies during the study period, which spanned 2019 and 2020. In addition to the annual submission of honey samples, participating beekeepers received follow-up to determine the subsequent incidence of AFB amongst their hives and were asked to complete a questionnaire regarding their management practices and experience with AFB. This chapter focuses on the establishment of prognostic reference ranges for *P. larvae* spore concentrations in pooled, extracted honey to determine the risk of AFB at the apiary or operation level, as well as identifying management practices in Saskatchewan apiculture that are correlated with a high risk of disease.

Beekeepers were assigned to categories of risk based on their highest (maximum) spore concentration in a given year. By correlating spore concentrations with the incidence of AFB the following year, final prognostic thresholds were set at 2 spores per gram of honey and 100 spores per gram of honey, delimiting low from moderate risk and moderate from high risk, respectively. At these cut-offs, no large-scale (L) or small-scale (S) beekeepers assigned a low risk of disease reported the subsequent development of AFB, whereas the highest proportion of beekeepers reporting disease were those assigned a high risk of disease.

Predictive modelling of AFB risk using questionnaire data identified several management practices related to biosecurity as important targets for intervention to mitigate risk identified through the testing of pooled, extracted honey. Chapter 4 highlights that the combined testing of pooled, extracted honey and the avoidance of practices correlated with high AFB risk provides beekeepers with the ability to make evidence-based decisions regarding metaphylactic antibiotic use. We suggest that beekeepers with less than 2 spores per gram of honey that do not practice any high-risk activities may temporarily discontinue antimicrobial metaphylaxis while maintaining other components of their IPM strategies.

# CHAPTER 4: ESTABLISHMENT OF APIARY-LEVEL RISK OF AMERICAN FOULBROOD THROUGH THE DETECTION OF *PAENIBACILLUS LARVAE* SPORES IN POOLED, EXTRACTED HONEY IN SASKATCHEWAN

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## 4.1 Abstract

*Paenibacillus larvae*, the causative agent of American foulbrood (AFB), produces spores that may be detectable within honey. We analyzed the spore content of pooled, extracted honey from 52 large-scale (L) and 64 small-scale (S) Saskatchewan beekeepers over a two-year period (2019-2020). Our objectives were: i) establish reliable prognostic reference ranges for spore concentrations in extracted honey to determine future AFB risk at the apiary level; ii) identify management practices as targets for mitigation of risk. *P. larvae* spores were detected in 753 of 1476 samples (51%). Beekeepers were stratified into low (<2 spores/gram), moderate (2-<100 spores/gram), and high (≥100 spores/gram) risk categories. Of forty-nine L beekeepers sampled in 2019, those that reported AFB in 2020 included 0/26 low, 3/18 moderate, and 3/5 high risk. Of twenty-seven L beekeepers sampled in 2020, those that reported AFB in 2021 included 0/11 low, 2/14 moderate, and 1/2 high risk. Predictive modelling included indoor overwintering of hives, purchase of used equipment, movement of honey-producing colonies between apiaries, beekeeper demographic, and antimicrobial use as risk category predictors. Saskatchewan beekeepers with fewer than 2 spores/gram in extracted honey that avoid high risk activities may be considered at low risk of AFB the following year.

## 4.2 Introduction

*Paenibacillus larvae*, an endospore-forming bacterium, is the causative agent of the honey bee disease, American foulbrood (AFB) (6). *P. larvae* endospores (hereafter referred to as spores) are infectious to newly hatched larvae (32–34), within which they germinate, undergo massive vegetative replication, and produce hundreds of millions of new spores (39). These spores subsequently spread throughout the colony and hive products by the caretaking actions of nurse bees (4). Spores are exceptionally resilient, capable of maintaining infectivity for decades (1) and able to withstand treatment by common disinfectant solutions (85,87) and antibiotics (3). North American apiculture is heavily reliant on the chronic and sustained use of antibiotics in the form of metaphylaxis to prevent and control outbreaks of AFB, as on-label antibiotics are generally effective at controlling the vegetative state of *P. larvae* and preventing the expression of clinical disease (95,96,118). In recent years, however, several studies have documented and characterized the emergence of antibiotic resistant strains of *P. larvae* in North America (7–9,99). Consequently, beekeepers may be faced with the development of clinical disease despite seemingly-appropriate

and on-label antibiotic use if these resistant strains are present within their apiaries without their knowledge.

In addition to the appearance of antimicrobial resistance, the routine use of antimicrobials in apiculture is generally undesirable due to a potential persistence of residues in honey that limit marketability for human consumption, as well as the direct adverse effects they may have on larval development and survival (2,105). It is reasonable to suggest that reducing reliance on antibiotics in North American apiculture, if done in a safe and evidence-based manner, would improve the overall sustainability and profitability of the industry as a whole. Indeed, there is an urgent need to strive toward this goal given the growing global concern regarding antimicrobial resistance in both humans and animals (11) and the resulting, necessary regulatory changes that have directly affected beekeepers' access to antimicrobials (104,129).

Reducing antimicrobial reliance and ensuring more judicious use of antibiotics in apiculture requires beekeepers to have access to efficacious alternative treatments to control AFB (2) and/or have an improved ability to confidently make evidence-based management decisions through effective risk assessment tools. If a beekeeper or beekeeping operation could incorporate reliable and logistically feasible risk assessment into their integrated pest management strategies against AFB, the recognition of a low risk of future AFB disease may signal that the use of antibiotics is temporarily unnecessary. Conversely, identification of a high risk of disease would justify the judicious use of antimicrobial metaphylaxis while simultaneously providing an opportunity to evaluate for ways to mitigate said risk.

Multiple studies have evaluated the quantification of *P. larvae* spores in bees, bottom board debris, and honey as a means of either predicting future AFB risk or detecting early clinical disease (14–18,69,92,100,130); however, many of these studies rely on the collection of samples on an individual hive (i.e., hive-by-hive) basis. Although an individual hive approach may be useful for beekeepers managing a small number of honey bee colonies, we previously demonstrated that spores are heterogeneously distributed amongst hives within the apiaries of large-scale, commercial beekeeping operations (14,120,131). Consequently, the ability to identify high-risk colonies using a hive-by-hive approach in these operations would require very large numbers of individually sampled hives, a practice that is logistically impossible for many large-scale, commercial beekeeping operations in North America.

It has long been known that extracted, commercial honey may be a source from which to cultivate spores of *P. larvae* (39). Several studies have demonstrated that spore concentrations obtained from extracted, pooled (bulk) honey may be useful in AFB risk assessment; however, these studies originate from countries where the use of antimicrobials in apiculture is prohibited, test samples from beekeepers managing a relatively small number of hives, or use sampling strategies that preclude traceability to original apiaries (15,92,100,115,116). For large-scale, commercial beekeeping operations, the collection of samples of extracted honey traceable to a specific apiary would be substantially more convenient than individual hive sampling, as sample collection could be easily integrated into the routine honey extraction workflow while spinning frames on an extractor machine (131).

We previously showed that pooled honey collected from honey supers during routine extraction could be used as an apiary- or operation-level surrogate for more conventional, individual hive sampling strategies (i.e., adult bees or honey from brood chambers of individual colonies) for the detection of *P. larvae* spores (131). We also demonstrated that, similar to historical studies, such samples may have prognostic value in assessing the future risk of AFB at the apiary or operation level (131). Accordingly, we have expanded the sampling of pooled, extracted honey to a large number of both commercial and small-scale beekeepers across the province of Saskatchewan, Canada, to i) establish prognostic reference ranges for *P. larvae* spore concentrations for the determination of AFB risk at the apiary or operation level; and ii) use predictive modelling to identify management practices that may represent key targets for intervention and mitigation of AFB risk. The results of this study will enable enhanced risk assessment for future AFB at the apiary or operation level, along with potential intervention strategies to mitigate AFB risk; accordingly, beekeepers and beekeeping operations in North America will be better equipped to safely reduce their reliance on antimicrobials in the prevention and control of AFB. This evidence-based and logistically feasible approach will help to ensure not only the continued sustainability of the industry, but will also serve to reduce antimicrobial resistance within North American apiculture.

## 4.3 Materials and Methods

### 4.3.1 Sample Collection and Questionnaires

To ensure province-wide representation across Saskatchewan, beekeepers approached for study enrollment belonged to one of five subjectively bordered regions: Saskatoon region (defined by a 250 $km^2$ area around the municipality of Saskatoon), Regina region (defined by a 250 $km^2$ area around the municipality of Regina), Northeast (longitudinally defined from Highway 2 and the Eastern provincial border, latitudinally defined from Highway 5 to the town of La Ronge – approximately $1.06 \times 10^5$ $km^2$), Northwest (longitudinally defined from the Western provincial border to Highway 2, latitudinally defined from Highway 14 to the town of Beauval – approximately $9.68 \times 10^4$ $km^2$), and South (south of Highways 14 and 5, excluding the area defining the Regina region – approximately $2.04 \times 10^5$ $km^2$). Enrolled beekeepers and beekeeping operations were stratified into two demographic categories: small-scale beekeepers, defined as those with fewer than 100 hives (hereafter referred to as S beekeepers; range 1 to 98, average = 16, standard deviation = 24, median = 8), and large-scale beekeepers and beekeeping operations, defined as those managing greater than or equal to 100 hives (hereafter referred to as L beekeepers range 100 to 6,300, average = 1,578, standard deviation = 1,584, median = 935). Demographic variables used to describe these two categories included total number of hives, number of apiaries, full-time/part-time beekeeping status, percentage of income derived from beekeeping, and employment of staff. S beekeepers typically refer to those managing honey bees part-time or as a special interest (i.e., hobby), whereas L beekeepers tend to refer to commercial beekeeping operations performing beekeeping on a large scale and employing staff.

Sample collection spanned the honey-producing seasons of 2019 and 2020. All S beekeepers were solicited for honey samples from each of the sampling years, whereas L beekeepers were solicited once in 2019, and a selected subset solicited again in 2020. This selected subset included beekeepers with 2019 maximum spore concentrations greater than or equal to five spores per gram of honey, beekeepers that had experienced at least one case of AFB within the previous 10 years (determined based on questionnaire – see below), select beekeepers (n = 3) with low 2019 spore concentrations (i.e., less than five spores per gram of honey) not currently using antimicrobial metaphylaxis, select beekeepers (n = 3) with low 2019 spore concentrations currently using antimicrobial metaphylaxis, and additional select beekeepers (n = 2) with low 2019 spore

concentrations managing very large commercial beekeeping operations (approximately 5,000 hives).

Samples were collected from the last honey harvest (i.e., late August to early September) to ensure as wide a gap in time as possible from spring treatment with antibiotics. S beekeepers were asked to submit a single sample of extracted honey per year that was collected from their largest apiary. L beekeepers were asked to submit a total of 18 extracted honey samples per year using an adaptation of a two-stage sampling protocol (125). Specifically, L beekeepers randomly selected six geographically separate apiaries or lots and collected three honey samples from each. For any given apiary or lot, each sample was collected from a separate extractor load to avoid repeated sampling of frames and hives. With this strategy, we estimated that these three unique honey samples represent between nine and 18 hives within an apiary or lot (131). Here, the use of the term 'apiary' is synonymous with the North American term 'bee yard', whereas a lot refers to geographically clustered apiaries that are extracted together as a single unit (131). Upon receipt, all samples were stored at room temperature until quantitative bacterial cultures of *P. larvae* spores were performed.

In addition to the submission of honey samples, beekeepers were asked to complete an accompanying questionnaire regarding their beekeeping experience, current management practices, and history with AFB (92) (S1 Fig). Questionnaires were distributed in May of 2020, the majority of which were completed throughout the remainder of the year. Accordingly, collected data reflected routine beekeeper practices during 2019 and 2020. Collection and storage of personal information within these questionnaires was done in accordance with the requirements set by the Behavioural Research Ethics Board (Beh-REB) of the University of Saskatchewan (Ethics Approval ID 1868). In 2020, participating S and L beekeepers that developed clinical signs consistent with AFB were identified by one or more of the following: direct verbal communication, reporting to and/or inspection by the provincial specialist in apiculture, and/or recording of occurrence of AFB in the study questionnaire. In 2021, L beekeepers that developed clinical signs consistent with AFB were identified as those that had reported to and/or been inspected by the provincial specialist in apiculture. Determination of the 2021 AFB status of S beekeepers was not attempted. L beekeepers with subjectively large discrepancies in spore concentrations between 2019 and 2020 were contacted in an attempt to identify any changes in management practices

(including antibiotic use), overall colony strength, and/or prevalence of other diseases that could potentially explain these differences.

### 4.3.2 Media Preparation and *P. larvae* Cultivation

A complex MYPGP medium was used for the cultivation of *P. larvae* spores, combining conventional MYPGP medium (66,71) with germination agonists (i.e., L-tyrosine and uric acid) reported by Alvarado *et al.* (2013) (79). For a complete description of medium preparation, hereafter referred to as enhanced MYPGP medium, please refer to our previous study (131). Poured plates were stored at 4 °C until use. Any unused plates older than 2 months of age were discarded.

To cultivate *P. larvae* spores from honey, samples were processed as previously described (131). Briefly, approximately 20 g of honey was weighed out in a falcon tube and mixed with 20 mL of sterile water. The exact weight of honey was recorded and used for the subsequent calculations. This mixture was shaken overnight at 37 °C to allow for honey dissolution, and the resulting suspension was centrifuged at 6000 g for 40 minutes at 21 °C (6,66). Supernatant was discarded, the pellet re-suspended in 2 mL of sterile water, and the final suspension vortexed for 20 seconds. A portion of this suspension was heat-treated at 85 °C for 15 minutes and cooled to room temperature (126). Heat-treated suspension was spread across three technical replicate plates of enhanced MYPGP medium (200 µL per plate, 600 µL total), and 200 µL of unheated suspension was spread onto a fourth plate as a control (131). Plates were incubated at 37 °C with 5% $CO_2$ for seven days (6,66). For unheated control plates, bacterial colonies morphologically consistent with *P. larvae* were counted after 72 hours of incubation before overgrowth by contaminant bacteria and fungi could occur, then counted again after seven days on plates that were not overgrown by contaminant microorganisms. Bacterial colonies consistent with *P. larvae* were counted on all plates after seven days of incubation, and the number of colonies were averaged across the three technical replicates. Spore concentrations were calculated under the assumption that each bacterial colony was representative of a single *P. larvae* spore (6,16). If one or more replicate plates of heat-treated suspension for a given sample cultivated greater than 100 colonies of *P. larvae*, the suspension was re-plated and incubated following serial, ten-fold dilutions (19). Any bacterial colonies with an equivocal *P. larvae* morphology were sub-cultured to thoroughly assess morphological characteristics. If colony identification remained ambiguous following sub-culture,

suspect colonies were identified using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) by Prairie Diagnostic Services Inc., Western College of Veterinary Medicine, Saskatoon, Saskatchewan.

### 4.3.3 Establishment of Prognostic Reference Ranges for Honey

To assess the ability of pooled, extracted honey to predict the risk of an outbreak of AFB at the apiary or operation level, spore concentrations were stratified into low, moderate, and high categories using natural breakpoints (thresholds) within the combined dataset of S and L beekeeper samples. Tentative categories for future AFB risk (i.e., low, moderate, and high risk) corresponded to low, moderate, and high spore concentrations. Beekeepers were assigned to a risk category for a given year based on their highest (maximum) spore concentration honey sample from that year. Final thresholds for risk categories were determined using data on future occurrences of AFB, the historical occurrence of AFB for each beekeeper, and prediction graphs generated from the final predictive model to evaluate its ability to differentiate between the different risk categories.

### 4.3.4 Identification of Risk Factors and Predictive Modelling

Questionnaire responses were transcribed and coded using Microsoft Excel and analyzed using STATA software (Version 15.1; Statacorp. LLC). Coding differentiated between responses that were inapplicable for a particular respondent (based on their demographic or previous questionnaire answers), and those responses that were truly missing (not answered when they should have been). For ambiguous or incomplete responses, clarification was obtained directly from the responding beekeeper whenever possible. If beekeepers were unavailable for follow-up, ambiguous or incomplete responses were encoded with the lowest or most conservative value possible to ensure as conservative an interpretation as possible for analysis. For responses provided as a range of numerical values, the average of these values was used for analysis.

To identify management practices that may be important in predicting a beekeeper's risk of AFB disease, individual management variables were analyzed using ordered logistic regression comparing both combined moderate and high risk (i.e., moderate/high) to low risk, and high risk to combined moderate and low risk (i.e., moderate/low). A single odds ratio was reported for each individual variable following verification of proportionality using the Wolfe Gould proportional odds test. Variables with odds ratios with greater than 65 observations and a p-value of less than 0.3 were used as candidate explanatory variables to generate a predictive model using a

beekeeper's highest category of risk of AFB across 2019 and 2020 as an outcome. Antibiotic use was included as a model variable regardless of its univariable p-value due to its possible role as an important confounder. Models were differentiated from one another by pseudo R-squared values, number of observations, and overall model stability. Within each proposed model, attainment of the highest pseudo R-squared value possible was prioritized over an individual variable's retainment of statistical significance once added. Explanatory variables within the final model (i.e., highest pseudo R-squared model with model stability) were considered to be management factors relevant for the prediction of risk for future AFB disease.

In order for beekeepers to utilize identified risk factors as points of intervention to mitigate AFB risk, management practices were categorized into one of the following: prevention of AFB, control of AFB, and general (non-specific). Variables under prevention of AFB included frequency of inspection of brood frames, number of brood frames evaluated during inspection, investigation of winter dead-outs, training of staff in disease recognition and diagnosis, current antimicrobial metaphylaxis use, and questions relating to the handling and movement of brood frames, hives, and extracted honey supers within and between apiaries. Variables under control of AFB included destruction of suspect and/or confirmed AFB colonies and hives, reactionary use of antimicrobials following identification of suspect and/or confirmed AFB colonies and hives, use of a quarantine or isolation apiary, and reporting of suspect cases of AFB to the provincial specialist in apiculture. General management practices included mobility of hives and apiaries (i.e., for pollination services), indoor overwintering, barrel feeding, and the purchase/selling of used equipment and colonies.

## 4.4 Results

### 4.4.1 Summarization of Honey Samples over Study Period

A total of 116 Saskatchewan beekeepers were represented in at least one of the honey-producing seasons within the study period (2019 and/or 2020), consisting of 64 S and 52 L beekeepers. Sixty-one S beekeepers were represented in 2019, and 45 were represented in 2020. Forty-three S beekeepers provided samples from both sampling years, 18 provided samples from 2019 only, and the remaining three S beekeepers provided a sample from 2020 only. Fifty-two L beekeepers were sampled in 2019, and a subset of these (27 L beekeepers) were sampled again in 2020. Together, these 116 S and L beekeepers owned approximately 75% (over 82000 out of ~110000) of Saskatchewan's registered honey bee hives during the study period, consisting of over 56000 honey-producing hives, approximately 21000 nucleus hives, and 5000 hives of unspecified designation.

Over the study period of 2019 and 2020, a total of 1476 honey samples were tested for spores of *P. larvae*, consisting of 111 honey samples submitted by S beekeepers and 1365 samples submitted by L beekeepers. Sixty-five S beekeeper samples from 2019 and 46 samples from 2020 were tested. For L beekeepers, 888 samples were tested from 2019 and 477 samples were tested from 2020.

### 4.4.2 Establishment of Prognostic Thresholds for *P. larvae* Spore Concentrations in Honey

Spores of *Paenibacillus larvae* were detected in 753 of 1476 honey samples (51%) across the entire study period. Of those samples with detectable spores, 718 were from L beekeepers (52.6% of all L beekeeper samples) and 35 were from S beekeepers (31.5% of all S beekeeper samples). The visual distribution of spore concentrations of all samples with detectable spores is presented in Figure 4.1. The visual threshold delimiting low spore concentrations from moderate spore concentrations was established intuitively at two spores per gram of honey, and the threshold delimiting moderate from high was established intuitively at 100 spores per gram of honey. Across 2019 and 2020, 28 of 35 (80.0%) of S beekeeper samples with detectable spores had <2 spores per gram of honey, 6 of 35 samples (17.1%) had 2 – <100 spores per gram of honey, and 1 of 35 (2.9%) had a concentration of spores ≥100 spores per gram of honey (maximum of 156 spores per gram). Across 2019 and 2020, 396 of 718 (55.2%) of L beekeeper samples with detectable spores had <2 spores per gram of honey, 274 of 718 samples (38.2%) had 2 – <100 spores per gram of

honey, and 48 of 718 samples (6.7%) had ≥100 spores per gram of honey (maximum of 4983 spores per gram).

Potential categories of future AFB risk were chosen using the intuitive thresholds of two and 100 spores per gram of honey delimiting low, moderate, and high spore concentrations. Beekeepers were assigned to low, moderate, and high categories of risk each year based on their single, highest spore concentration in that given year (Figure 4.2). In 2019, 27 of 52 L beekeepers (51.9%) were assigned to low risk of AFB, 19 of 52 (36.5%) were assigned to moderate risk, and six of 52 (11.5%) were assigned to high risk of AFB. For the selected subset of L beekeepers in 2020, 11 of 27 (40.7%) were assigned to low risk, 14 of 27 (51.8%) were assigned to moderate risk, and two of 27 (7.4%) were assigned to high risk of AFB. For S beekeepers in 2019, 58 of 61 (95.1%) were assigned to low risk of AFB, two of 61 (3.3%) were assigned to moderate risk, and one S beekeeper was assigned to high risk of disease. In 2020, 41 of 45 (91.1%) of S beekeepers had a low risk of AFB, while the remaining 4 of 45 (8.9%) were assigned moderate risk.

To determine if categories of low, moderate, and high spore concentrations corresponded with low, moderate, and high risk of AFB disease, respectively, L beekeepers received follow-up throughout 2020 and 2021 and S beekeepers received follow-up in 2020 to identify occurrences of AFB. Six out of 49 L beekeepers sampled in 2019 (that provided follow-up) reported AFB occurrence in 2020; out of these six, zero of 26 (0%) were assigned to low risk, three of 18 (17%) to moderate risk, and three of five (60%) to high risk (Figure 4.2a). Out of the 27 L beekeepers sampled in 2020, three reported occurrences of AFB directly to the provincial specialist in apiculture in 2021. Out of these three, zero of 11 (0%) were assigned to low risk, two of 14 (14.2%) to moderate risk, and one of two (50%) to high risk (Figure 4.2b). The three L beekeepers that reported AFB in 2021 also reported AFB in 2020. One out of 52 S beekeepers sampled in 2019 (that provided follow-up) reported AFB occurrence in 2019; this S beekeeper had 156 spores per gram of honey and was the only S beekeeper assigned to high risk in 2020 (Figure 4.2c). Overall, the seven occurrences of AFB identified in 2020 and three occurrences of AFB identified in 2021 included all official cases of AFB across the entirety of Saskatchewan that were reported to the provincial specialist in apiculture in each of these years.

### 4.4.3 Relation of *P. larvae* Spore Concentrations to Historical Occurrence of American Foulbrood

Through available questionnaire data, spore concentrations in pooled, extracted honey were compared to any historical occurrence of AFB during the preceding four years of the date of sample collection (Figure 4.2). These four preceding years included the sampling year itself, as samples were collected at the very end of the honey-producing season and would reflect changes earlier in the year. Nine out of 45 L beekeepers sampled in 2019 (that provided historical information) reported AFB occurrence from 2016 to 2019 (inclusive); out of these nine, one of 25 (4%) was assigned to low risk, five of 16 (31.3%) to moderate risk, and three of four (75%) to high risk (Figure 4.2a). Eleven out of 26 L beekeepers sampled in 2020 (that provided historical information) reported AFB occurrence from 2017 to 2020 (inclusive); out of these 11, four of 11 (36.4%) were assigned to low risk, five of 14 (35.7%) to moderate risk, and two of two (100%) to high risk (Figure 4.2b). None of 48 S beekeepers sampled in 2019 that provided information regarding historical AFB, including the single S beekeeper with AFB in 2020, had ever previously detected a case of AFB within their hives.

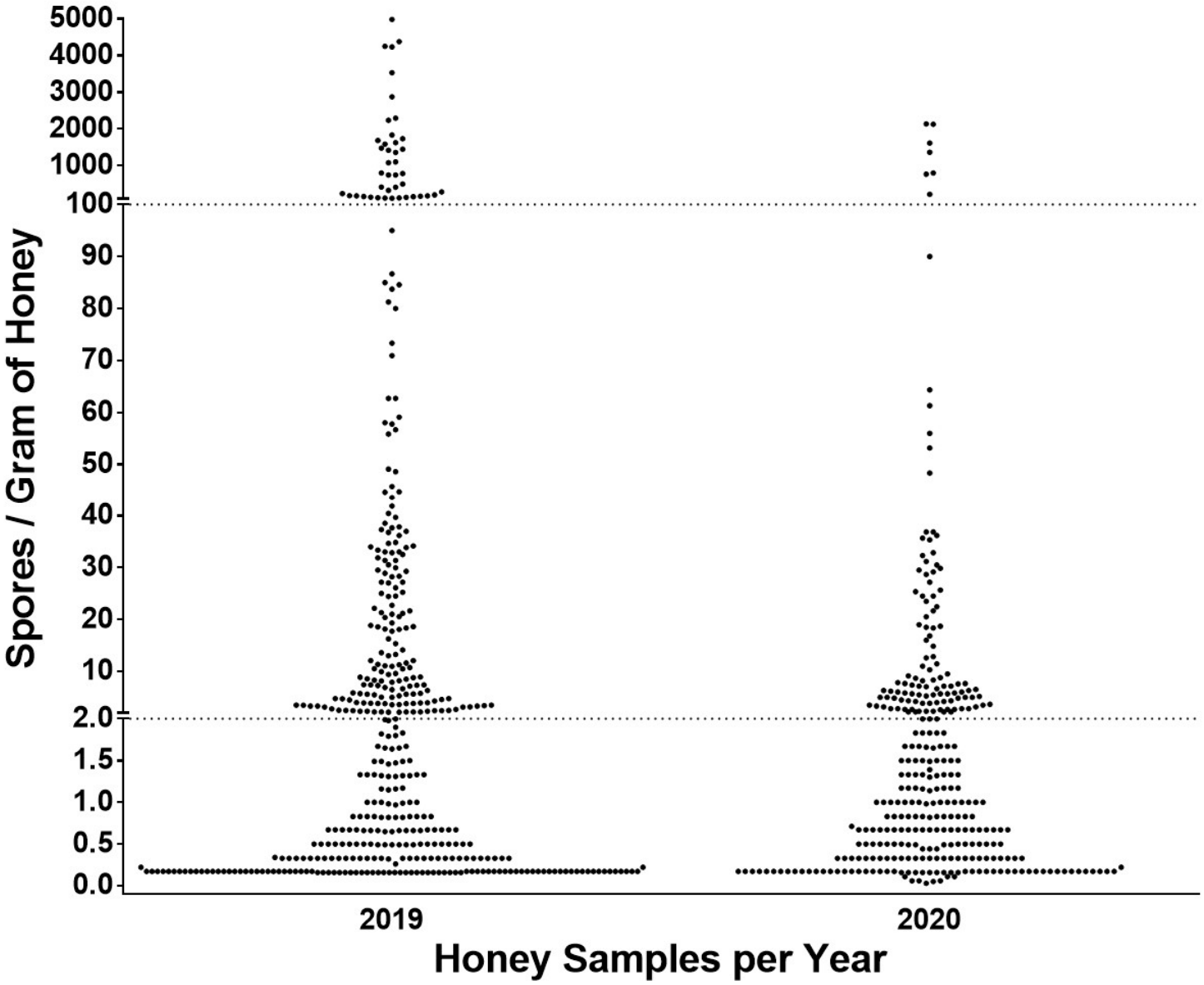


**Figure 4.1. Samples of pooled, extracted honey with detectable spores of *Paenibacillus larvae*.** Plotted values represent 753 honey samples (out of 1476) collected from both small-scale (S) and large-scale (L) beekeepers across two honey-producing seasons (2019 – 2020) that had detectable spores of *P. larvae* (range = 0.1 – 4983 spores per gram of honey). Samples with no detectable spores are not plotted. Dotted lines represent intuitive visual breakpoints (thresholds) with cut-off values delimiting categories of spore contamination where two spores per gram of honey differentiates low from moderate concentrations and 100 spores per gram of honey differentiates moderate from high concentrations.

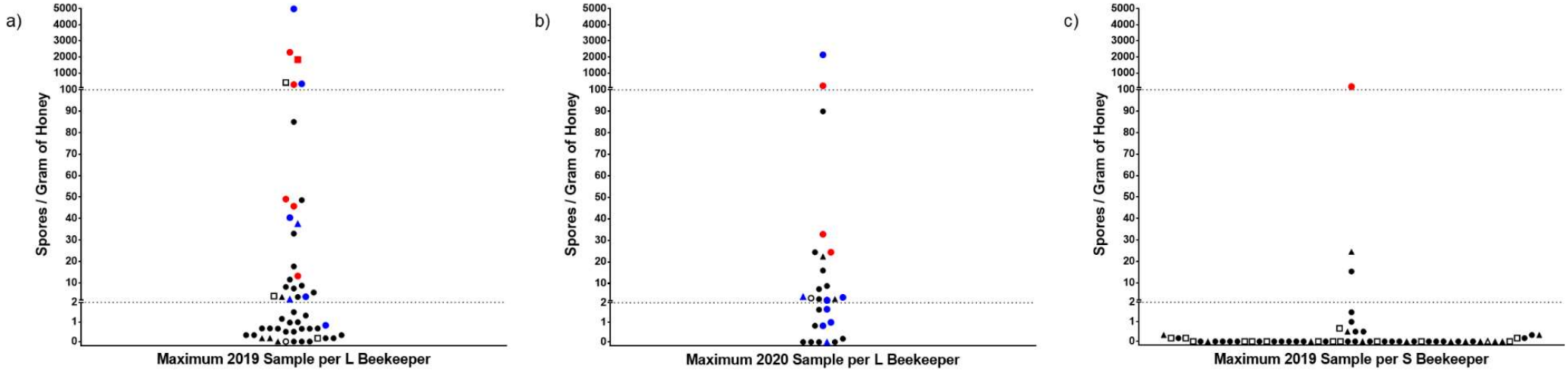


**Figure 4.2. Maximum spore concentrations of *Paenibacillus larvae* in pooled, extracted honey relative to future and historical AFB occurrence.** a) Maximum 2019 spore concentrations of large-scale (L) beekeepers; b) maximum 2020 spore concentrations of L beekeepers; c) maximum 2019 spore concentrations of small-scale (S) beekeepers. Samples highlighted in red indicate occurrence of AFB in the subsequent year from the year the sample was collected in. Samples highlighted in blue indicate historical occurrence of AFB within the preceding four years. Samples with open centers are those missing information on future and/or historical AFB occurrence. Dotted lines represent cut-off values delimiting categories of future AFB risk where two spores per gram of honey differentiates low from moderate risk and 100 spores per gram of honey differentiates moderate from high risk. Circles represent current antibiotic use or cessation of antibiotic use after 2017. Triangles represent those beekeepers who have never used antibiotics or cessation during or prior to 2017. Squares represent missing information on antibiotic use.

### 4.4.4 Comparison of 2019 and 2020 *P. larvae* Spore Concentrations in Individual L Beekeepers

The distributions of spore concentrations for the selected subset of L beekeepers that were sampled in both 2019 and 2020 are presented in Figure 4.3. Seventeen of 27 (63.0%) L beekeepers did not change risk categories between 2019 and 2020 based on the maximum spore concentrations of *P. larvae* detected in pooled, extracted honey. Seven of 27 (25.9%) were in a lower risk category in 2020 relative to 2019. Of these, six of seven decreased from moderate risk to low risk, and one L beekeeper decreased from high risk to low risk. Three of 27 (11.1%) were in a higher risk category in 2020 relative to 2019, all of which increased from low risk to moderate risk.

Six L beekeepers with relatively large decreases in maximum spore concentrations were contacted for follow-up. One or more apiaries were sampled during both years for four of six of these L beekeepers. Three of six L beekeepers implemented one or more of the following management changes in an active and concerted attempt to reduce their degree of spore contamination: discarding most to all of brood comb and equipment from colonies that died overwinter (winter dead-outs), performing more thorough inspections of winter dead-outs, replacing as much old brood comb as possible across the entirety of the beekeeping operation, increasing the frequency and thoroughness of brood chamber inspections in live colonies, implementing operation-wide use of antimicrobial metaphylaxis, and/or improving on-label adherence of antimicrobial use. The remaining three L beekeepers did not actively implement any changes specific to AFB management, although one of these three reported an increased use of queens with improved hygienic behavior between 2019 and 2020.

The apiaries of one of the three L beekeepers whose spore concentrations increased between 2019 and 2020 were found to be within close proximity to a confirmed case of AFB that occurred between these sampling events. This case of AFB belonged to a beekeeper not included in this study. Clinical disease was confirmed by the provincial specialist in apiculture.

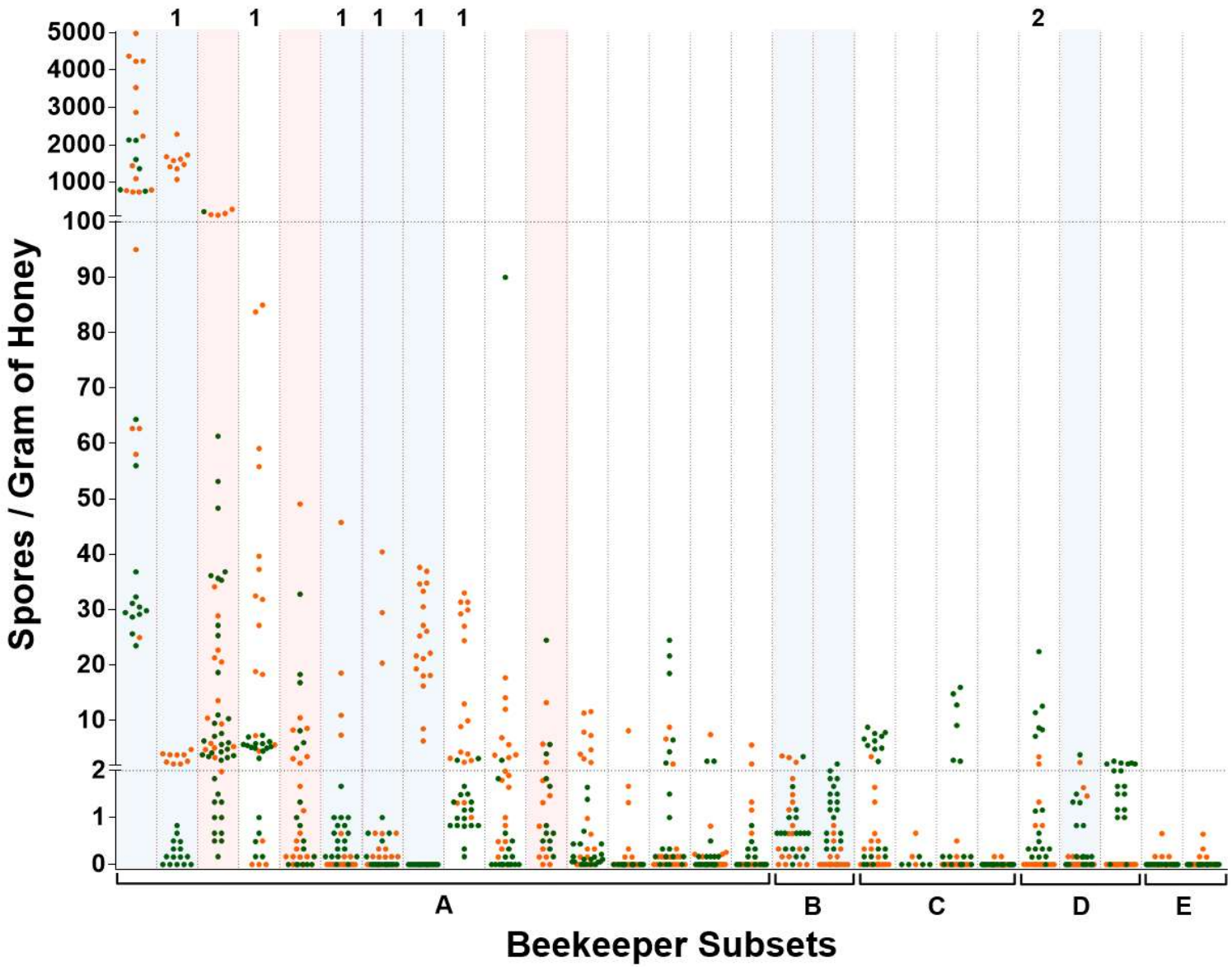
1
1
1
1
1
1
2
5000
4000
3000
2000
1000
100
90
80
70
60
50
40
30
20
10
2
1
0
Spores / Gram of Honey
A
B
C
D
E
Beekeeper Subsets

**Figure 4.3. *Paenibacillus larvae* spore concentrations in extracted honey of large-scale (L) beekeepers sampled in both 2019 and 2020.** Orange dots indicate spore concentrations in honey samples collected in 2019 and green dots for 2020. L beekeepers are separated from one another by vertical dashed lines. Beekeepers highlighted in light red are those that reported AFB in 2021. Beekeepers highlighted in light blue are those that reported a historical occurrence of AFB within the previous four years (2017 – 2020). L beekeepers were selected for repeat (2020) sampling by meeting one of the following subset criteria: A = maximum 2019 spore concentration greater than or equal to five spores per gram of honey; B = at least one case of AFB within the previous 10 years; C = select beekeepers with a maximum 2019 spore concentration less than five spores per gram of honey currently using antibiotics as a part of AFB management; D = select beekeepers with a maximum 2019 spore concentration less than five spores per gram of honey not currently using antibiotics; E = select beekeepers with a maximum 2019 spore concentration less than five spores per gram managing very large commercial beekeeping operations (approximately 5000 hives). 1 = L beekeepers that provided follow-up regarding decrease in spore concentrations between sampling years. 2 = L beekeeper with apiaries within flying distance of reported case of AFB between 2019 and 2020 sampling years.

### 4.4.5 Identification of Risk Factors and Predictive Modelling

Evaluation of the relationship between individual management practices and the three risk categories was performed using ordered logistic regression, where combined moderate and high (moderate/high) risk was compared to low risk, and high risk was compared to combined low and moderate (low/moderate) risk. These outcome risk categories were based on a beekeeper's highest category of risk of AFB across both 2019 and 2020. Odds ratios for individual management practices are summarized in Table 4.1. For L beekeepers, the odds of being at or in a higher risk category(ies) than being in a lower risk category(ies) (i.e., higher risk categorization) is 9.3 times that of S beekeepers (N = 97, 95% confidence interval [CI] = 3.1 – 27.3, p <0.001). Additionally, for full-time beekeepers, the odds of being at or in a higher risk category(ies) than being in a lower risk category(ies) (i.e., higher risk categorization) is 8.4 times that of part-time beekeepers (N = 96, 95% CI = 3.0 – 23.4, p <0.001). Due to the disparity between S and L beekeepers, we chose to use beekeeper size in all models to account for these differences as a possible confounder.

Following univariate analysis, candidates for explanatory variables in a predictive model included beekeeper size, current/recent antimicrobial use, percentage of hives overwintered indoors, movement of brood frames between apiaries, movement of honey-producing colonies between apiaries, mixing of extracted honey supers between apiaries, confidence in recognition of AFB clinical signs, historical purchase of used equipment, and selling of equipment to other beekeepers. Despite a univariate p-value of 0.36, the variable of current/recent antimicrobial use was included in all models due to its potential role as a confounder. Proposed predictive models using a combination of these variables are detailed in Table 4.2. Of the two models, model 1 was selected as the final predictive model due to a higher pseudo R-squared value relative to model 2. This final model (model 1) included a single demographic variable (beekeeper size), three management practices related to the prevention of AFB (current/recent antibiotic use, confidence in AFB recognition, and movement of colonies between apiaries), and two general management variables (overwintering of hives indoors and the historical purchase of used equipment).

**Table 4.1. Odds ratios for individual management practices with moderate/high and high future AFB risk categories as an outcome.** Only variables with >65 observations and a p-value of <0.3 are included, with the exception of Current/Recent Antibiotic Use. For each variable, the categories analyzed are described with the reference category displayed in brackets.

| Management Variable | Categories (Reference in Brackets) | Observations (N) | Odds Ratio (OR)* | 95% Confidence Interval | p-value |
|---|---|---|---|---|---|
| Occurrence of AFB Within Previous Five Years | Yes (no) | 93 | 11.5 | 3.3, 40.6 | <0.001 |
| Beekeeper Size Code | Large-scale (Small-scale) | 97 | 9.3 | 3.1, 27.3 | <0.001 |
| Inspection Personnel | Both Owner and Staff (Either Owner or Staff) | 96 | 8.6 | 3.3, 22.9 | <0.001 |
| Full-time/ Part-time Status | Full-time (Part-time) | 96 | 8.4 | 3.0, 23.4 | <0.001 |
| Highest Confidence Score with AFB Recognition between Owner and Staff | Very Confident (Somewhat or Not Confident) | 97 | 7.5 | 2.7, 20.8 | <0.001 |
| Confidence of Owner with AFB Recognition | Very Confident (Somewhat or Not Confident) | 97 | 6.2 | 2.3, 16.5 | <0.001 |
| Percentage of Hives Overwintered Indoors | Continuous - each increment equal to 10% | 94 | 1.2 | 1.1, 1.4 | 0.001 |
| Movement of Colonies between Apiaries | Sometimes or Often (No) | 97 | 4.3 | 1.7, 11.1 | 0.002 |
| Handling of Extracted Honey Supers | No Regard (Try to Retrun Supers to the Same Apiary or Colony) | 95 | 3.4 | 1.2, 9.3 | 0.02 |
| Historical Purchase of Used Equipment | Yes (No) | 92 | 8.6 | 1.1, 68.2 | 0.04 |
| Selling of Equipment to Other Beekeepers | Yes (No) | 96 | 2.6 | 0.99, 6.6 | 0.05 |
| Mobility of Hives | Mobile (Stationary) | 97 | 0.45 | 0.16, 1.2 | 0.11 |
| Current/Recent Antimicrobial Use | Current Use/Stopped Use After 2017 (Never used/Stopped Use in 2017 or Before) | 96 | 1.6 | 0.55, 5.02 | 0.36 |

* All odds ratios meet the assumption of proportional hazards

**Table 4.2. Proposed ordered logistic regression models for prediction of a beekeeper's risk of AFB.** Combined medium and high risk categories were compared to the low risk category (less than two spores per gram of honey), and the high risk category (greater than or equal to 100 spores per gram of honey) was compared to combined low and medium risk. Models were differentiated from one another by pseudo R-squared values, number of observations, and overall model stability.

| Proposed model | Variables included | Observations (N) | Pseudo R-squared | Model stability |
|---|---|---|---|---|
| 1 | Beekeeper Size Code<br>Current/Recent Antimicrobial Use<br>Percentage of Hives Overwintered Indoors<br>Purchase of Used Equipment<br>Movement of Colonies between Apiaries<br>Highest Confidence Score for AFB Recognition<br>Recent Occurrence of AFB | 87 | 0.2580 | Stable |
| 2 | Beekeeper Size Code<br>Current/Recent Antimicrobial Use<br>Percentage of Hives Overwintered Indoors<br>Movement of Colonies between Apiaries<br>Highest Confidence Score for AFB Recognition<br>Recent Occurrence of AFB | 91 | 0.2351 | Stable |

The ability of the final model (model 1) to predict a beekeeper's risk category for AFB was evaluated by comparing the model's predictions to each beekeeper's designated, maximum risk category over the two-year sampling period (2019 and 2020). Model 1 correctly predicted the low risk category (i.e., <2 spores/gram of honey) for 56 of 61 beekeepers (92%) assigned to this category by maximum spore concentration. This model correctly predicted the moderate risk category for 11 of 22 beekeepers (50%), and correctly predicted the high risk category for zero of four beekeepers (0%). When moderate and high risk beekeepers were combined into a single grouping (i.e., moderate/high risk), the predictive ability of the model improved to 14 of 26 beekeepers (54%). Due to the model's ability to differentiate between low risk and higher risk categories, the lower prognostic threshold was maintained at two spores per gram of honey. There was insufficient differentiation between moderate and high risk to reliably alter the upper prognostic threshold of 100 spores per gram of honey.

**4.5 Discussion**

In this study, we have provided evidence that *P. larvae* spore concentrations in pooled, extracted honey may predict a beekeeper's risk of clinical AFB disease in the following year. By establishing prognostic thresholds at two spores per gram of honey and 100 spores per gram of honey, we have identified three demonstrably different categories of low, moderate, and high risk for future AFB at the apiary or operation level. In addition, we created a predictive model that identified several management practices that may represent key targets for intervention and mitigation of AFB risk within the context of antibiotic-reliant apiculture. Through an understanding of both *P. larvae* spore concentrations within their apiaries and management practices that may be contributing to the risk of AFB, beekeepers will be better equipped to make evidence-based decisions to prevent and control AFB, and will improve their ability to use antimicrobials in a more judicious manner.

The use of pooled, extracted honey to correlate spore concentrations of *P. larvae* with the occurrence of AFB has been documented as early as 1978, when Hansen and Rasmussen (1986) requested beekeepers to submit samples of honey from their annual harvest (115). In this study, the authors used the number of *P. larvae* colonies cultivated on J-agar plates following heat treatment and direct inoculation to generate categories of spore concentrations ranging from no detectable spores to over 600000 spores per 5 grams of honey. There were very few occurrences

of AFB the following year in those apiaries with no detectable spores (two out of 476 apiaries), whereas the highest proportion of apiaries that went on to develop AFB the next year were in the highest spore concentration category (five of 21 apiaries) (115). In our present study, the range of spore concentrations was much smaller than Hansen and Rasmussen (1986), although this could potentially reflect differences in methodology of sample collection, culture medium, antibiotic use amongst the sampled beekeepers (no antibiotic use was mentioned by Hansen and Rasmussen), and/or the amount of honey used for cultivation (approximately 20 grams in this study, 0.08 grams in Hansen and Rasmussen's). Regardless, our results corroborate the use of pooled, extracted honey as a tool to determine future AFB risk. In addition, we established useful prognostic thresholds to guide evidence-based decisions regarding AFB management. In comparison to Hansen and Rasmussen's study, where spores were detected in 11% of samples and two of 11 cases of AFB were from apiaries with no detectable spores, we detected spores in 51% of samples and all reports of AFB were from beekeepers with detectable spores, which may reflect a higher sensitivity for spore detection with our methodology.

In addition to future risk, spore concentrations in pooled, extracted honey may also reflect the overall severity and chronicity of AFB within a beekeeping operation. Hornitzky and Clark (1991) used bulk honey samples to cultivate *P. larvae* colonies on sheep blood agar following heat treatment and centrifugation (116). Colony counts on plates were scored into 1+, 2+, and 3+ categories corresponding to severity of contamination, where 1+ corresponded to 1 to 20 colonies on a plate, 2+ to 21 to 50 colonies, and 3+ to over 50 colonies. The authors found that 56.4% of honey samples assigned a 1+ score, 78.6% of 2+ honey samples, and 100% of 3+ honey samples were from beekeepers with either active or historical AFB disease (116). Although it was not possible to correlate *P. larvae* colony counts on culture plates to actual spore concentrations in honey samples in Hornitzky's study, in our present study, we also found that 100% of beekeepers assigned to our highest risk category (i.e., those with a maximum spore concentration greater than or equal to 100 spores per gram of honey) had reported either the subsequent development of AFB or a recent historical occurrence of the disease. These findings suggest that beekeepers assigned a high risk of future AFB are also likely to be dealing with chronic contamination within their hives or operations.

The findings of this current study will be relevant to beekeepers across western Canada, not just those within the borders of Saskatchewan. Pernal and Melathopoulos (2006) previously

analyzed *P. larvae* spore content in pooled, extracted honey from commercial beekeeping operations in the province of Manitoba, Canada (100). Located immediately adjacent to the province of Saskatchewan, management practices of beekeepers in Manitoba are considered comparable to those of the beekeepers in this study. In fact, spore concentrations detected in honey by Pernal and Melathopoulos were within the same range of magnitude as in our study. The authors evaluated the use of adult bees from brood chambers and honey collected from settling tanks as an indicator of the severity of active disease within a beekeeping operation. Although they successfully demonstrated that the concentration of spores in honey samples was positively correlated with the number of colonies exhibiting clinical signs of AFB, they ultimately found that bees were a stronger predictor of current, active disease status (100). Pernal and Melathopoulos (2006) reasoned that pooled honey may be more valuable as a tool to identify operations at high risk of developing AFB, suggesting that threshold levels may be able to screen out operations with no apparent risk. In our study, we have established these prognostic thresholds for honey samples coming from hives managed very similarly to those in Manitoba, although there are important differences between our work and that of Pernal and Melathopoulos (2006). We analyzed unique samples of pooled, extracted honey collected directly from extractor loads in a two-stage sampling strategy (125), as opposed to collecting samples in 3000 kg increments from settling tanks (100). We also used an enhanced MYPGP culture medium instead of modified PLA medium (100). Nevertheless, we believe that our prognostic thresholds from pooled, extracted honey are applicable to beekeepers across western Canada, given the uniformity of management practices across the country.

Many strategies and interventions to prevent and control AFB are universally accepted as standard of practice, regardless of whether or not the region in question uses antimicrobials in beekeeping (15,25,26,44). These strategies include frequent and thorough inspection of brood chambers, evaluation of deceased colonies, annual replacement of brood comb, training of staff in disease recognition and diagnosis to improve early detection, and the practice of biosecurity and quarantine in the handling and movement of equipment within and between apiaries. Several of the L beekeepers whose spore concentrations dramatically decreased between 2019 and 2020 reported implementation of one or more of these strategies upon learning of high spore concentrations within their operations in 2019. In particular, these L beekeepers emphasized the replacement of large amounts of old brood comb, an increased frequency and thoroughness of

inspection of winter dead-outs and/or live colonies, and implementation or improved adherence to on-label antimicrobial metaphylaxis as major changes made in management. Further investigation of the potential ability of these mitigative interventions to rapidly decrease spore concentrations in antibiotic-reliant apiculture are warranted, as they may represent key intervention strategies for those beekeepers assigned a high risk of AFB. Recent work by Locke *et al.* (2019) is promising in this regard. They demonstrated that the consistent quarantine of individual colonies with high levels of spores, a process involving the replacement of all old hive materials including comb, successfully reduced the percentage of apiaries with detectable *P. larvae* spores from 74% to 4% over a period of 5 years within a modestly-sized beekeeping operation (between 560 and 670 hives amongst 56 apiaries) in Sweden (14).

In addition to the above mentioned strategies, our predictive modelling supports the value of biosecurity between the apiaries of different beekeepers, as well as between the apiaries of a single beekeeper, as we identified the historical purchase of used equipment and the movement of honey-producing colonies between apiaries, respectively, as management practices correlated with a moderate to high risk category (Table 4.3). Beekeepers should therefore make efforts to minimize both the purchase of used equipment and the movement of equipment and colonies between apiaries whenever possible (25,44). If used equipment must be purchased, then professional inspection by a specialist in apiculture should be performed prior to purchase. Newly introduced, used equipment should also be established in separate (quarantine) apiaries in order to minimize any risk of spore transmission to other apiaries or hives. At the very least, all introduced equipment and hives with colonies should be appropriately labeled or otherwise identified to improve traceability in the event of an outbreak.

Our final model identified the indoor overwintering of bees as a general management variable predictive of moderate to high risk of future AFB disease. In western Canada and other regions of similar climate, some beekeepers elect to move colonies indoors during the cold months of winter (44). In our univariable analysis, the odds of being in a higher risk category increased for every 10% increase in the proportion of hives overwintered indoors. To the best of our knowledge, this is the first report of an association between indoor overwintering and future AFB risk. Importantly, we do not believe the act of indoor overwintering itself to be a direct cause of AFB. Rather, we suspect this finding may reflect a tendency to bring relatively weaker colonies indoors that would otherwise perish if left outdoors overwinter. These weaker colonies may, in

turn, be predisposed to AFB if considerable numbers of spores are present within the hive and could subsequently act as a source of infection. This risk could be avoided if weak colonies were left outdoors or quarantined elsewhere. Alternatively, the overwintering of colonies indoors may further reflect issues with biosecurity, as beekeepers do not necessarily return individual hives to the same apiary that they were collected from in the fall. We hypothesize that improving biosecurity in indoor overwintering protocols, including identifiers that ensure consistent return of hives to their apiary of origin, may help to mitigate this source of risk.

We found that there were increased odds of a beekeeper being identified as large-scale relative to small-scale in higher AFB risk categories. This may partially be the result of selection bias. Candidate L beekeepers were identified primarily through publicly available listings of mandatory registration information for their operations. Conversely, S beekeeper candidates were identified largely through solicitation in municipal beekeeping clubs, which reflects those S beekeepers with a high level of interest and motivation in apiculture and honey bee health. Consequently, we were unable to sample the demographic of S beekeepers uninterested in attending such meetings. Alternatively, the association of L beekeepers with higher risk categories of AFB may relate to the sheer size of some of these beekeeping operations, each of which manage large numbers of apiaries spread over a wide geographic area. De Graaf *et al.* (2001) demonstrated that honey samples from apiaries in close proximity to apiaries with recent AFB were at a higher risk of being contaminated by spores (92). This is probably the result of robbing behavior by nearby bees (51). We speculate that the sprawling distribution of some of these operations may increase their likelihood of being in close proximity of other beekeepers with recent AFB or, at the very least, high spore contamination. Indeed, we identified one L beekeeper whose spore concentrations increased from low risk to moderate risk between 2019 and 2020, an increase that was attributed to the presence of a confirmed case of AFB within a nearby beekeeper's hive.

The remaining variables represented in our final model (use of current antimicrobial metaphylaxis, confidence in AFB recognition, and historical occurrence of AFB), although essential to explain variation in our dataset, may be reflective of the consequences of AFB rather than true predictive risk factors (Table 4.3). We suspect that any beekeeper with a recent history of AFB would consequently be more likely to recognize its clinical signs as opposed to a beekeeper who has never had experience with the disease. Similarly, such beekeepers would also be more likely to be using antimicrobials in response to recognition of a problem with AFB.

**Table 4.3. Proposed significance of variables in final model of prediction of future AFB risk.**

| Variable Category | Variable | Explanation of Odds Ratio | Proposed Significance for Beekeepers |
|---|---|---|---|
| Demographic | Beekeeper Size | The odds of being in a higher AFB risk category are greater for large-scale beekeepers than for small-scale beekeepers | -Potential selection bias during beekeeper enrolment<br>-Large size of operations may increase likelihood of close proximity to sources of spores |
| General Management | Indoor Overwintering of Hives | The odds of being in a higher AFB risk category are greater for beekeepers overwintering a large proportion of hives indoors than beekeepers overwintering fewer hives indoors | -Biosecuirty must be considered with indoor overwintering;<br>-Return colonies to apiaries they are collected from; identify hives for traceability<br>-Reduce any tendency toward the indoor overwintering of weak colonies |
| | Purchase of Used Equipment | The odds of being in a higher AFB risk category are greater for beekeepers that have purchased used equipment than for those beekeepers who have not | -Minimize the purchase of used equipment whenever possible<br>-If used equipment must be purchased, ensure professional inspection and/or testing<br>-Ensure appropriate quarantine and identification to improve traceability |
| Prevention of AFB | Current Antibiotic Use | The odds of being in a higher AFB risk category are greater for those beekeepers that are currently using or have recently used antimicrobials than for those that have never used antimicrobials or have not used them recently | -Current or recent use likely reflective of recent experience with or known risk of AFB |
| | Confidence in AFB Recognition | The odds of being in a higher AFB risk category are greater for beekeepers that are very confident at recognizing AFB than those that are somewhat or not confident at recognizing AFB | -Ability to recognize AFB likely reflective of previous experience with AFB |
| | Movement of Colonies between Apiaries | The odds of being in a higher AFB risk category are greater for beekeepers that move honey-producing colonies between apiaries than those who do not | -Minimize the movement of honey-producing colonies between apiaries whenever possible<br>-If colonies must be moved, ensure appropriate identification to improve traceability |

Overall, our final predictive model was capable of explaining 25.8% of variation, as approximated by pseudo R-squared. Although the ability of the final model to discriminate between low risk and higher risk (i.e., moderate to high risk) categories was excellent, the model was relatively poor at predicting beekeepers in moderate and high risk categories and could not differentiate between moderate and high risk based on our threshold of 100 spores per gram of honey. Nevertheless, we advocate the inclusion of this moderate to high risk threshold, as it stands to reason that a beekeeper with a maximum spore concentration of two spores per gram of honey would require less intensive intervention than one with hundreds to thousands of spores per gram of honey. Taken together, the low pseudo R-squared value of the model and poor differentiation of higher risk categories is likely reflective of the absence of important variables that could further explain variation in our dataset. One such variable may be apiary proximity to areas of recent or active AFB, as multiple other studies have identified this as an important risk factor (23,26,51,92). We asked beekeepers to enumerate nearby beekeepers to determine beekeeper density as a proxy of nearby AFB, however, poor response rates in our questionnaire precluded evaluation. Additionally, de Graaf *et al.* (2001) identified brood comb replacement as an important risk factor for the contamination of honey by *P. larvae* spores (92). We assessed comb replacement and found no statistical significance ($N = 85$, $p = 0.97$); however, we measured comb replacement as a continuous variable (i.e., 0 to 100%), whereas de Graaf *et al.* (2001) assessed comb replacement as a binary 'yes' or 'no'. The near uniform incorporation of some amount of brood comb replacement amongst participant beekeepers in our study, especially amongst L beekeepers, precludes evaluation of this variable in a comparable way to de Graaf *et al.* (2001).

In conclusion, we have developed a logistically feasible means for Saskatchewan beekeepers of all demographics, and particularly those that manage large-scale commercial beekeeping operations, to assess their risk of future AFB disease at the apiary or operation level. We suspect that these findings are also relevant for beekeepers in other western Canadian provinces with similar beekeeping management practices. Since the great majority of commercial beekeeping operations in this study were stationary, it is not clear if our thresholds are applicable to commercial beekeeping operations in the USA, as approximately 75% of their commercial honey bee colonies are subject to migratory management. In addition, through predictive modelling, we have identified management practices that potentially increase the risk of AFB in antibiotic-reliant apiculture, thereby providing beekeepers and their health management teams (i.e., provincial

specialists in apiculture and veterinarians) targets for intervention to mitigate risk. Together, an understanding of i) AFB risk through spore concentrations in pooled, extracted honey, and ii) management practices that contribute to risk in antibiotic-reliant management systems, will improve evidence-based decision making in western Canadian apiculture in relation to the prevention and management of AFB. In turn, this will promote a shift toward more judicious use of antimicrobials, helping to ensure the future sustainability of this sector by reducing antimicrobial resistance.

### 4.6 Recommendations for Beekeepers and their Health Management Teams in Western Canada

1. Identify one or more apiaries (honey bee yards) for sampling during the final honey pull of the season. Sampling late into the honey-producing season is recommended to ensure sampling is as far removed from the last application of antibiotics as possible. If sampling more than one apiary, ensure selected apiaries are geographically separate from one another and representative of the entire beekeeping operation.
2. During extraction of honey from frames, collect a total of three honey samples from each selected apiary. Each honey sample must come from a separate spin cycle/extractor load to ensure that the same frames are not repeatedly tested. Each sample should fill a 50 g container.
3. Submit samples to the appropriate diagnostic laboratory for detection and quantification of *P. larvae* spores and assignment of risk category for future AFB disease
4. If a beekeeper tests as moderate to high risk for future AFB disease, they should consult and collaborate with their health management team (i.e., provincial specialist in apiculture and veterinarian) to perform thorough inspections of affected apiaries, apply antimicrobials as needed, and identify management practices requiring intervention (see Table 4.1). Special attention should be paid to the following:
    - Biosecurity measures associated with the labeling and movement of hives during indoor overwintering
    - Selection criteria used for indoor overwintering candidates (for example, avoiding overwintering weak colonies)

- Biosecurity measures associated with the labeling and movement of honey-producing colonies between apiaries
- Biosecurity measures associated with the purchase and integration of used equipment from other beekeepers

5. If a beekeeper tests as low risk for future AFB disease and does not practice any high-risk activities (see list in point 4), they may temporarily discontinue antimicrobial metaphylaxis while maintaining other components of their integrated pest management strategies.
6. Testing of pooled, extracted honey should continue on an annual basis to allow for ongoing, evidence-based decision-making between the beekeeper and their health management team. Adjustments to preventative management practices may be made as indicated by test results.

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# CHAPTER 5: GENERAL DISCUSSION

## 5.1 The Need for Judicious Antimicrobial Use in Beekeeping

Despite more than a century of intensive research, *Paenibacillus larvae*, the causative agent of AFB, remains a significant and destructive pathogen of honey bees to the present day (2,4,5,22–24). AFB is capable of causing significant economic losses to beekeepers through both natural disease progression and the remedial actions necessary to eradicate disease outbreaks (5). Left undetected, the emergence and development of clinical disease within a colony often leads to its death through the progressive destruction of its brood (4,25). Similarly, recognition of clinical signs by a beekeeper often necessitates destruction of the colony and associated hive equipment in order to minimize the spread of infectious spores (2,25,26).

The continued challenges surrounding prevention and control of AFB stem from the incredible resiliency and high infectivity of bacterial spores combined with the prolific replication of its vegetative state (1,4,6,25,32,39). The definitive eradication of spores can only be achieved through the burning of bees and equipment or the use of gamma or high velocity electron-beam irradiation (26,27). Considerable research has been undertaken to find alternative and less severe means to eliminate disease, but these prospective therapeutics require further development to confirm their efficacy in both laboratory and field trials (56,103,107–110). Consequently, current preventative programs often rely on IPM strategies emphasizing a combination of cultural, physical, and chemical control to minimize spore contamination in hives and prohibit the replication of vegetative bacteria should spores infect susceptible larvae (25,26,44,90,92).

In North America, IPM strategies rely heavily on chemical control in the form of antimicrobial metaphylaxis with oxytetracycline (2,4). Although capable of preventing vegetative bacterial replication, and thereby preventing clinical signs of disease, antibiotics are incapable of affecting the infectious spores and treatment is non-curative (2,3). As a result of this caveat, the majority of North American beekeeping operations using antibiotics assume some degree of spore contamination within their hives, a mentality that fosters continual reliance on indiscriminate metaphylactic treatment in order to maintain profitability. In addition to being non-curative, antibiotic use limits marketability of honey fit for human consumption and has direct adverse effects on the development and survival of the larvae they are administered to protect (2,94,96,97,105,106).

In more recent years, the emergence of antimicrobial-resistant strains of *P. larvae* have added yet another significant limitation to chemical control strategies for the prevention and control of AFB (2,3,7–9,99,100). If a beekeeper unknowingly harbors a resistant strain of *P. larvae* within their hives, then clinical disease may occur regardless of on-label adherence to antibiotic metaphylaxis. In such a case, beekeepers must necessarily switch to one of the two other approved antibiotics for control of AFB in Canada, tylosin or lincomycin (2,96,101,102). This change is ultimately a delaying tactic, however, as sustained and chronic antibiotic use fosters the continued selection and development of AMR (11). Without an immediate shift away from a reliance on chemical control, North American apiculture may one day be faced with widespread resistance amongst *P. larvae* and be ill-equipped to maintain the sustainability of the industry.

The growing concern regarding AMR extends far beyond the realm of apiculture and is considered to be one of most significant health challenges faced in the 21$^{st}$ century (11). Left unaddressed, the progressive spread of AMR threatens public health systems across the world (11). There is growing evidence that overuse of antimicrobials in the practice of veterinary medicine, particularly in the preventative treatment of herds or large populations of animals instead of tailored, individual prescription, may be significantly contributing to AMR in human medicine (11). In Canada, the federal government has taken important steps to help address this problem by increasing veterinary oversight of the prescription and use of medically important antimicrobials in animal production systems, apiculture included (104). Although a necessary step, restricting access to antimicrobials fails to address the apiculture industry's underlying reliance on their use.

Taken together, the continued and predominantly indiscriminate application of antibiotics in North American apiculture is unsustainable, both from the perspective of AFB management and the wider scope of the impact of AMR on modern medicine and public health (2,11,15,104). In order to reduce the spread of AMR, and thereby ensure the continued sustainability of the industry in the management of AFB, beekeepers in North America must endeavour to use antibiotics in a more judicious manner, only relying on their use when absolutely necessary (104). The ability to safely reduce antimicrobial use, however, relies upon evidence-based decision-making through the implementation of proven and effective risk assessment strategies. Such programs are currently lacking or logistically unfeasible for the great majority of commercial beekeeping operations in North American. Indeed, there is limited information available on the fundamental distribution and concentration of *P. larvae* across antibiotic-reliant apiculture systems such as those in North

America (39,75,76,100,121). The purpose of this thesis was to bridge these critical information gaps through the establishment of widespread surveillance, while delivering a convenient and accessible risk assessment tool to allow all beekeepers, including large-scale, commercial operations, to improve their evidence-based decision-making regarding the prevention and control of AFB.

### 5.2 Paradigm Shift from Individual to Herd Health Management

A significant amount of research has investigated the detection of *P. larvae* spores in various matrices as a means of establishing risk for AFB disease (15–17,66,69,90,92,100,114–116). As a result of these studies, most currently available prognostic services offered by diagnostic laboratories require the submission of samples of either brood chamber honey or adult bees collected from individual hives (12–21,90). Although practical for those beekeepers managing a very small number of hives, the strategy of hive-by-hive sampling is laborious and logistically impossible for large-scale, commercial beekeeping operations in North America that manage thousands of hives at a time. In Chapter 3, we demonstrated that spores are not homogenously distributed amongst hives within a yard, even in those yards with a recently reported case of AFB. This heterogeneous spore distribution is consistent with findings of previous studies (14,120). Instead, only a small proportion of hives carried the majority of spores. Therefore, even if commercial operations attempted to adopt an individual hive approach to AFB risk assessment by sampling a selected subset of their colonies, they would still be required to sample a very large number of hives to ensure they included those relative few with high numbers of spores presumably at the greatest risk of disease (14,120). In light of these insurmountable procedural obstacles in AFB risk assessment, most North American beekeeping operations instead rely on the relatively more convenient approach of indiscriminate antibiotic metaphylaxis.

If North American apiculture is to have any hope of making progress towards the implementation of evidence-based decision-making in the management of AFB, we must strive to identify those risk assessment strategies that are operationally feasible for large-scale commercial operations. In other words, risk assessment strategies must undergo a paradigm shift from an individual health approach to one of herd health management. Within the context of apiculture, the information gathered through risk assessment strategies must be representative of entire yards of hives rather than individual hives themselves, thereby allowing beekeepers to make evidence-

based decisions at the level of entire yards (i.e., "herds") within their operations. In addition, these strategies must maintain comparability to the current standard of the sampling and testing of individual hives.

In this thesis, we have developed a risk assessment tool that meets the above criteria in the form of pooled, extracted honey. In Chapter 3, we demonstrated that pooled honey, conveniently collected during routine, end-of-season extraction and representing multiple hives within a single sample, was predictive of the degree of spore contamination we identified through individual hive sampling. Collection of these samples was rapid and presented minimal disturbance to normal workflow of the beekeepers, a significant improvement over the relatively time-consuming practice of sampling many individual hives. Furthermore, we found that pooled honey samples may be predictive of the risk of AFB in the subsequent year at the yard or operation level (15,115,116). With this foundation, we expanded our testing in Chapter 4, surveying pooled honey from beekeepers representing the majority of Saskatchewan's registered honey bee colonies over the study period. Through follow-up with participating beekeepers, we were able to correlate spore concentrations with the future occurrence of AFB and establish reliable, prognostic reference ranges denoting the future risk of disease. By incorporating the collection of pooled honey samples into their routine honey extraction workflow, beekeepers are now able to determine the imminent risk of AFB at the yard or operation level, providing invaluable evidence to direct their managerial decision-making.

### 5.3 The Role of a Collaborative Health Management Team

In addition to obtaining prognostic data regarding their yards and operations, beekeepers in regions of antibiotic-reliant apiculture also require knowledge of relevant management practices correlated with an increased risk of AFB (26,92). Together, an understanding of the current risk status of representative yards within an operation, along with management practices to avoid in order to mitigate the risk of disease, can provide beekeepers with sufficient information to confidently and safely make decisions regarding the use of antibiotics in their IPM strategies for the prevention and control of AFB. In Chapter 4, participating beekeepers were asked to complete questionnaires regarding their current management practices and historical experience with AFB. Through the analysis of this questionnaire data using logistic regression, we identified variables that were important predictors of high categories of AFB risk. These variables centered around

different aspects of biosecurity related to the indoor overwintering of hives, the movement of honey-producing colonies between different yards, and the purchase and integration of used equipment from other beekeepers. These practices represent key targets for intervention and subsequent mitigation of risk, regardless of the test results of a beekeeper's pooled honey samples.

With the enactment of regulatory changes on December 1, 2018, requiring increased veterinary oversight of all medically important antimicrobials in animals, Canadian veterinarians have only recently become recognized as a necessary component of health management within the apiculture industry (104). Accordingly, in Chapter 2, we characterized four outbreaks of AFB as not only a foundational groundwork for our subsequent research, but as a means of continuing education for veterinarians who may have been previously unfamiliar with apiculture management and AFB. Rather than be relegated to simple administrators of prescriptions for antibiotics, veterinarians instead have a significant opportunity to establish themselves as integral members of a beekeeper's health management team. Veterinarians, given their training and strong fundamental knowledge regarding herd health, biosecurity, and epidemiology, can provide essential expertise in the mitigation of AFB risk that a beekeeper may identify through the testing of their pooled, extracted honey. Through mutual collaboration, veterinarians and apiculture specialists can form effective health management teams for beekeepers, working together to install effective biosecurity solutions with consideration to practicality. If a beekeeper identifies yards at high risk of AFB, consultation with their health management team can ensure the judicious prescription of antibiotics, thorough evaluation of management practices, and implementation of both short- and long-term remedial action to improve IPM strategies against AFB.

### 5.4 Limitations

Several limitations of this research require discussion. First, there are inherent constraints in our overall, collective ability to cultivate *P. larvae* from spores on artificial media (66,71). It is widely recognized that a relatively small proportion of spores within any given sample will readily germinate and produce visible colony growth (42,71,128). As a result, we may inadvertently underestimate the true numbers of spores present within a sample or, at worst, fail to detect clinically viable spores and report a false negative result. That said, all samples across all studies in this thesis were processed in an identical manner and would therefore be equally affected by suboptimal spore germination. As such, comparisons made for the purposes of risk assessment

would be valid within the context of our enhanced cultivation protocols. Additionally, our ability to detect spores of *P. larvae* from pooled honey was relatively comparable to Pernal and Melathopoulos (2006) who, using a modified PLA medium, detected a similar magnitude range of spore concentrations in Manitoba as we did in Saskatchewan (100). We also detected spores in 51% of our pooled, extracted honey samples, whereas as Von der Ohe and Dustmann (1997) identified spores in approximately 10.5% of samples using CSA, Hornitzky and Clark (1991) in 12.5% of samples using blood agar supplemented with ovine blood, and Hansen and Rasmussen (1986) in 9.5% of samples using J-agar (15,115,116). Although Hansen and Rasmussen (1986) detected a much higher upper range of spore concentrations (i.e., over 600,000 spores per 5 grams of honey) this is likely at least partially attributable to differences in methodology, particularly the amount of honey used for cultivation (approximately 20 grams in the work of this thesis compared to 0.08 grams in Hansen and Rasmussen's) (115). Finally, we found that 36% of all beekeepers (7% of large-scale beekeepers and 59% of small-scale beekeepers) had no detectable spores of *P. larvae* in any of their honey samples. Only 10.5% of beekeepers in Pernal and Melathopoulos' study had no detectable spores, although these were commercial operations comparable to 7% of large-scale operations without detectable spores in our work (100). In contrast, approximately 83.5% of beekeepers enrolled in Hornitzky and Clark's study had no detectable spores (116).

In an effort to improve our overall culture sensitivity, we compared and contrasted the performance of multiple media formulations prior to commencement of outbreak (Chapter 3) and surveillance (Chapter 4) sample analysis in order to identify the formulation capable of germinating the greatest proportion of spores within a given sample (see APPENDIX). Different formulations of PLA media, blood agar, and MYPGP were tested with and without the inclusion of antibiotic additives (i.e., Nalidixic acid and/or Pipemidic acid) and with or without reported activators of spore germination (i.e., uric acid and L-tyrosine) (6,66,71,75,79). Through comparative testing, we discovered that MYPGP media with added Nalidixic acid, L-tyrosine, and uric acid (i.e., enhanced MYPGP) was superior to other formulations with regard to its germination ability and was consequently used throughout the entirety of our investigations and surveillance in Chapter 3 and Chapter 4.

Importantly, the work in this thesis does not extensively characterize the genotypes of the *P. larvae* isolates we obtained from different samples across the province of Saskatchewan. Different strains of *P. larvae*, particularly when categorized by ERIC type, have variable responses

to cultivation protocols and exhibit different levels of virulence in the field (43,58,70). Specifically, the germination of ERIC I *P. larvae* strains tend to be stimulated by heat treatment during sample processing, whereas the germination of ERIC II *P. larvae* strains are inhibited by heat treatment protocols using higher temperatures (70). The protocols that we developed and used throughout this research were specifically designed to permit the germination and subsequent growth of all ERIC strains of *P. larvae* expected to be present in field conditions (6). Despite this, all cultivated bacterial colonies across our studies that were confirmed as *P. larvae* by MALDI-TOF MS had similar morphological features characteristic of ERIC I (6,127). Furthermore, representative isolates of *P. larvae* derived from samples in Chapter 2 and Chapter 3 were confirmed to be ERIC I through repetitive element PCR fingerprinting (31). Our exclusive cultivation of *P. larvae* isolates compatible with ERIC I morphology is fairly consistent with our understanding of the distribution of ERIC genotypes across the Americas (22,132). Regardless, a lack of *P. larvae* isolates consistent with ERIC II does not rule-out their presence in Saskatchewan, although their potential significance in Saskatchewan beekeeping is unclear.

In retrospect, our questionnaire, which was used for predictive modelling in Chapter 4 to identify management variables correlated with a higher risk of AFB, could have been better designed to capture and identify more relevant risk factors. This conclusion is supported by the relatively low pseudo R-squared value of our final model, as well as the model's overall poor ability to differentiate moderate from high risk beekeepers. Our original questionnaire attempted to capture data demonstrably important for AFB risk in other studies, but poor response rates of the relevant questions precluded meaningful evaluation (23,26,51,92). Regardless, we successfully recognized several key management practices related to biosecurity that could represent targets for risk mitigation, especially if addressed through the concerted effort of a dedicated health management team.

Remaining limitations of this research center around the voluntary recruitment and retention of participating beekeepers. We question if the disparity of AFB risk between large-scale and small-scale beekeepers may be partially explained by selection bias. Large-scale beekeepers were primarily identified and recruited through publically available and mandatory registration information. Consequently, we were able to contact a broad demographic spectrum of commercial operations across the province. In contrast, recruitment of small-scale beekeepers was heavily reliant on solicitation at municipal beekeeping clubs, and recruited small-scale beekeepers was

skewed towards those with a high level of interest in apiculture and honey bee health. Due to the voluntary nature of this study, continuation and follow-up with some participating beekeepers was unreliable.

### 5.5 Future Directions

Although our presented method of risk assessment – whereby we use quantitative microbiological cultivation from pooled honey – provides a reliable prognostic decision-making tool for beekeepers, the process of *P. larvae* cultivation using artificial media is not without its drawbacks (69–71,82,83,127,128). Other than strain-dependent responses to cultivation protocols (see section 5.4), quantitative microbiological cultivation is a relatively time-intensive and laborious technique, requiring at least a week of processing and incubation to appropriately quantify spores within a sample (70,82,83,127). Additionally, the recognition and quantification of *P. larvae* colonies grown on media may be obscured by contaminant bacterial and/or fungal overgrowth (66,93,127). In order to streamline laboratory workflow and improve turnaround times for health management teams, our cultivation and risk assessment protocols could be correlated to alternative, more rapid quantification techniques, such as quantitative PCR (qPCR) (69,82,83,127). The use of qPCR is not without its own set of challenges (69,83,133). The extraction of DNA from *P. larvae* spores (as opposed to vegetative bacteria) is difficult due to the impervious nature of the spore wall, and results may be highly dependent on the DNA extraction protocol and kits used (69). The intrinsic properties of honey itself may also significantly inhibit the effectiveness of PCR (127,133). Fortunately, recent work by Rossi *et al.* (2018) appears to have overcome many of these obstacles by developing a reliable qPCR protocol for the detection of *P. larvae* spores that can be applied to both honey and hive debris (83). If our established prognostic thresholds from the cultivation of pooled, extracted honey could be correlated to qPCR results, this more rapid molecular test could improve the overall capacity and throughput of *P. larvae* surveillance and risk assessment in the province of Saskatchewan.

To improve our growing understanding regarding the distribution and concentration of *P. larvae* across the province of Saskatchewan, future work should concentrate on the genotypic characterization of our collected *P. larvae* isolates. Studies evaluating the genotypic distribution of *P. larvae* throughout other geographic regions, especially those within Europe, have been instrumental in improving understanding of the genomic epidemiology and transmission of this

pathogen both within and between beekeeping operations in these areas (67,127,132,134–139). Given the relatively limited categorization of ERIC genotyping, studies have worked toward the development of techniques that increase the resolution of genotypic analysis and allow for a greater degree of strain differentiation for spatiotemporal mapping (67,136,137). Most recently, some researchers are exploring the use of whole genome sequencing as a means to improve our understanding of *P. larvae* genetic diversity and tracing outbreaks of AFB and the transmission of spores (132,134). In addition to these applications, the use of whole genome sequencing for *P. larvae* isolates in Saskatchewan may also have utility in identifying and mapping the origin and transmission of AMR genes, which could further improve the industry's ability to make evidence-based decisions regarding antibiotic use, when deemed necessary (7,99).

### 5.6 Conclusion

The surveillance for spores of *P. larvae* in pooled, extracted honey represents a convenient, reliable, and effective tool for risk assessment of AFB in Saskatchewan. By shifting sampling and managerial decision-making from an individual hive to a herd-level perspective, this approach to AFB risk assessment is accessible for beekeepers of all demographic groups, especially those managing hundreds to thousands of hives in large-scale, commercial operations. Through the establishment of prognostic thresholds of 2 spores per gram of honey and 100 spores per gram of honey, we have provided categorical degrees of low, moderate, and high AFB risk that correspond to the degree of interventive, mitigative action required by a beekeeper's health management team to prevent and control outbreaks of disease. Our identification of specific management practices related to higher risk of AFB in antibiotic-reliant management systems provides beekeepers and their health management teams with tangible targets for intervention, and veterinarians can play an integral role in the implementation of improved biosecurity strategies.

Through improved evidence-based decision-making, those beekeepers with a low risk of future AFB that are simultaneously implementing appropriate biosecurity strategies may temporarily cease antimicrobial metaphylaxis while maintaining other components of their IPM strategies. By reducing their personal reliance on antibiotic use, beekeepers will promote a shift toward more judicious antimicrobial use and stewardship throughout North American apiculture, thereby ensuring the continued health and sustainability of the industry while reducing the threat of AMR in human and animal medicine.

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## APPENDIX

Supplemental material for this thesis is provided in this appendix (one figure total). Data includes a comparison of the ability of various formulations of culture media to cultivate spores of *Paenibacillus larvae* (unpublished).

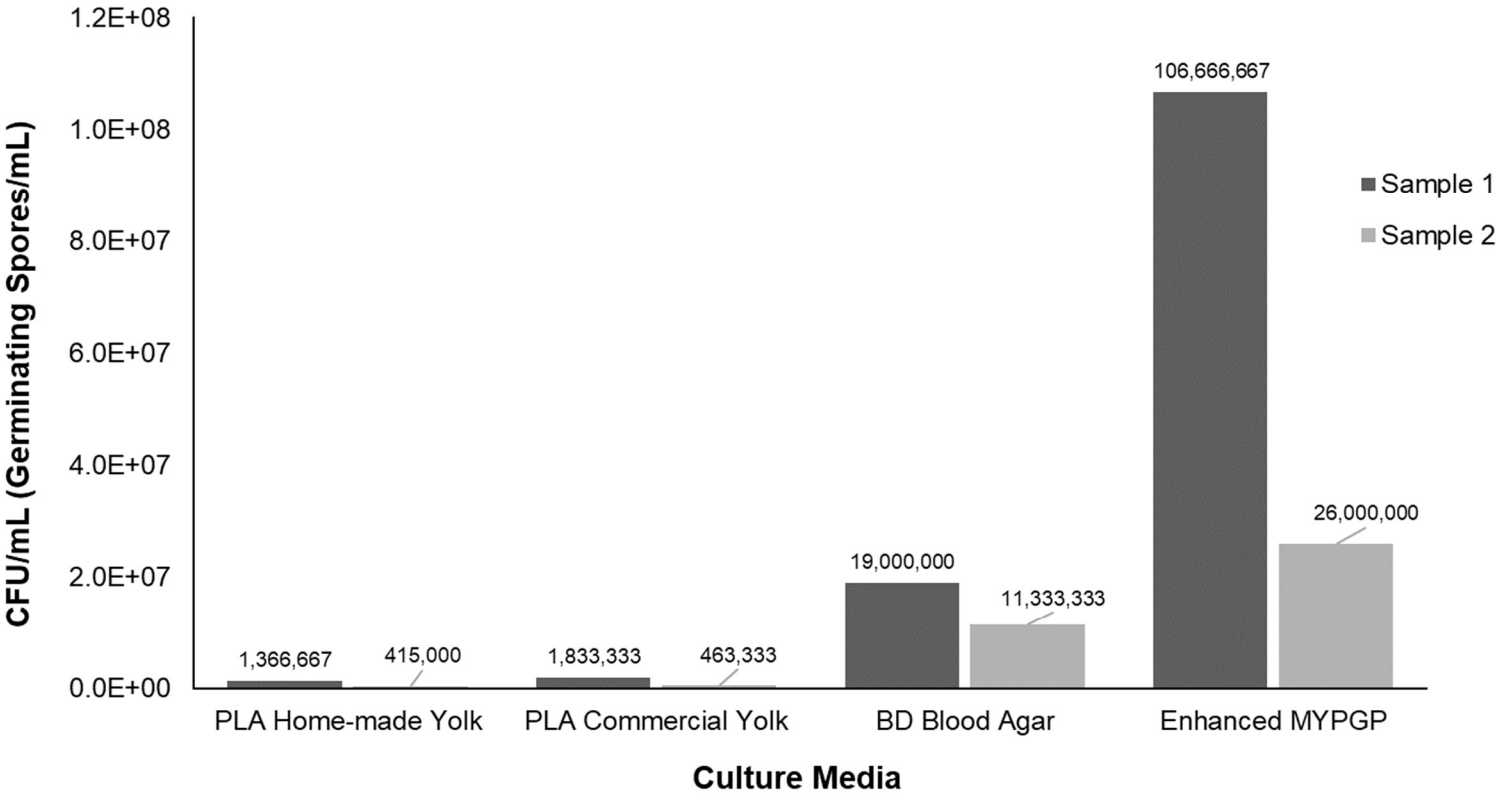


**Figure A1. A comparison of *Paenibacillus larvae* spore cultivation on various formulations of culture media.** Samples represent pooled, extracted honey contaminated with spore suspension derived from larval scale. PLA = *Paenibacillus larvae* agar (Schuch *et al.*, 2001) (75); BD = Becton Dickinson (BD Biosciences, San Jose, California, USA); Enhanced MYPGP = agar comprised of Mueller Hinton broth, yeast extract, potassium phosphate, glucose, and sodium pyruvate combined with L-tyrosine and uric acid (Zabrodski *et al.* 2019, unpublished).