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RESEARCH

Detection of microbial organisms on *Apis mellifera* L. beehives in palm garden, Eastern Thailand

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Background: Honey bees play a crucial role in pollination and ecological balance. *Apis mellifera* L. colonies, especially those located in specific geographic regions, such as the palm garden in Eastern Thailand, are susceptible to potential threats from microbial contaminants. Understanding and detecting microbial organisms in these beehives is essential for the preservation of bee health, honey production, and the broader ecosystem. However, the problem of microbial infection and antibiotic-resistant bacteria is more severe and continuously increasing, resulting in a health, economic, and social crisis. The purpose of this study is to determine the prevalence of microorganisms in *A. mellifera* beehives in palm gardens in Rayong province, Eastern Thailand.
Results: Ten swabs in transport media were swabbed and obtained from different parts of each beehive (1 swab per beehive), for a total of 10 hives. Traditional microbial culture-based methods, biochemical tests, and antimicrobial susceptibility (disc-diffusion) tests were used to detect microbial organisms and antibiotic resistance in bacteria. The swab tests from nine beehives resulted in the detection of Gram-positive bacteria (63.64%), Gram-negative bacteria (27.27%), and fungi/yeast (9.09%). These microorganisms are classified as a group of coagulase-negative *Staphylococcus* spp. and made up 40.91% of the bacteria discovered. Other bacteria found were Coryneform bacteria (13.64%), *Pantoea* spp. (13.64%), *Bacillus* spp. (9.09%), yeast (9.09%), glucose non-fermentative Gram-negative bacilli (9.09%), and *Pseudomonas* spp. (4.55%). However, due to the traditional culture-based and 0biochemical tests usually used to identify the microbial organisms in clinical specimens and the limitation of identifying some environmental microbial species, the results of the antimicrobial susceptibility test cannot reveal if the organism is resistant or susceptible to the drug. Nevertheless, drug-sensitive inhibition zones were formed with each antibiotic agent.
Conclusions: Overall, the study supports prevention, healthcare, and public health systems. The contamination of microorganisms in the beehives may affect the quality of honey and other bee products or even the health of the beekeeper. To avoid this kind of contamination, it is therefore necessary to wear personal protective equipment while harvesting honey and other bee products.

Keywords: antibiotic resistance, bacteria, beehive, honey, microbial organism, microorganism

Introduction

People worldwide extensively engage with honey bees and their products. Beekeeping has gained popularity in the agricultural sector in Thailand due to its high return on investment, low costs (as beekeepers do not need to purchase agricultural land), and its minimal environmental impact. Beekeeping serves as an additional income

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source, contributing to poverty reduction. Furthermore, the pollination services provided by beekeepers are crucial for sustaining crop cultivation and pollination.

However, a bee colony has unique characteristics, involving warmth, humidity, and a diverse range of ecological niches, which make it attractive to infectious pathogens. Examples of potential pathogen targets encompass both the members of the colony and the various developmental stages of the bees (McAfee 2020). Furthermore, when collecting pollen and nectar, honey bee foraging poses a risk of transmitting pathogens to and from other pollinators. Notably, many pathogen infections frequently occur at the same time in the beehive, significantly impairing colony health and rendering it susceptible to other dangers (Lannutti et al. 2022).

Nevertheless, the problem of bacterial infection and the antimicrobial resistance of bacteria is more severe and tends to increase continuously, and this is a serious threat to health security. Nowadays, the problem of drug resistance is not only affecting public health but also causing economic and social losses (Dadgostar 2019; Serwecińska 2020). Both national and international efforts have long been made to address this problem. Most investigations have concentrated on the functions of gut-associated microorganisms and how microbes transform pollen into bee bread (Foote 1957; Haydak 1958).

From previous studies, the microbial community connected to pollen and bee bread kept in hives was viewed from a fresh angle (Anderson et al. 2014) but the role of accompanying bacterial and fungal communities were not examined, which instead solely examined the bacterial populations in fresh pollen and bee bread that had been preserved in hives.

In the present, to ensure food safety in the food industry, foodborne pathogen detection is required. Before a serious outbreak, research is required to identify and control the spread of pathogens. In Thailand, the study of the microbial contamination of beehives in agriculture fields is limited. For the agricultural sector in Thailand, apiculture, or beekeeping, is a popular practice, especially when growing plants like rambutan, longan, lychee, palm, rubber, and other flowering plants. Therefore, this study examined the prevalence of microorganisms with antibiotic resistance in beehives located in a palm garden, which is one of the favorite plantations in several regions of Thailand.

To prevent or reduce the spread of resistant microorganisms from nature to humans, in this study, we investigated the microbial community found in beehives; both the bacterial and fungal communities were determined by conventional culture methods. In addition, this study was aimed at determining the prevalence of resistant microorganisms in the *Apis mellifera* beehives that may affect the quality of honey and bee products available on the market and also continue to affect the quality of human life and economic conditions in the future.

Materials and Methods

Study area

This study collected *A. mellifera* beehive swab specimens from a palm garden in Rayong province, Eastern Thailand. The swab specimens were brought to perform the laboratory testing at the Research Institute for Health Sciences, Chiang Mai University, and the Microbiology Unit, Diagnostic Laboratory, Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

Sample collection

In March 2023, Amies swabs were swabbed on the outer surface area of the *A. mellifera* beehives located in palm gardens, Rayong province, Eastern Thailand (1 swab per 1 beehive). A total of ten Amies agar gel transport swabs were obtained and transported to the laboratory at room temperature.

Microbial culture and identification

All ten Amies agar gel transport swabs that were swabbed on beehives were used to perform bacterial culture and identification by a standard culture-based method in accordance with the laboratory standard operating procedures adopted by the Clinical and Laboratory Standards Institute (CLSI 2022). These swab specimens were cultured on sheep blood and MacConkey agar and incubated at 37°C for 18–24 hours. Then, all suspected colonies were isolated and further identified by using Gram's staining technique and biochemical analyses, including catalase, coagulase, oxidase, indole, motility tests, sugar fermentation tests, triple sugar iron (TSI) agar, citrate utilization, and urease production. For quality control of microbial culture and identification, *Escherichia coli* ATCC® 25922, *Staphylococcus aureus* ATCC® 25923, and *Pseudomonas aeruginosa* ATCC® 27853 were used.

Antimicrobial susceptibility test

The antimicrobial susceptibility was performed on the suspected isolated colonies using ten antibiotic discs from the glycopeptide, β-lactams, fluoroquinolone, 3rd generation cephalosporins, and carbapenem antibiotic group. They included vancomycin (VA30), cefoxitin (oxacillin) (CX30), ciprofloxacin (CIP5), levofloxacin (LEV5), ceftazidime (CAZ30), cefotaxime (CTX30), imipenem (IMI10), and meropenem (MEM10).

The antimicrobial susceptibility tests were performed on Mueller–Hinton agar (MHA) plates using the disc diffusion Kirby–Bauer technique with 0.5 McFarland turbidity standard methods. After being incubated at 37°C for 18–24

hours, the results were interpreted according to the standards for antimicrobial susceptibility of the CLSI protocol (CLSI 2015). For susceptibility results, the zones of inhibition were measured to the nearest millimeter at the back of the inverted culture plate. The measurements were then compared with a standard chart as adopted by the CLSI to determine susceptibility or resistance.

Data analysis

Data were described as frequencies (counts and percentages). The percentage of prevalence of microbial organisms found on *A. mellifera* beehives was calculated by measuring the number of microorganisms found/total organism found (n = 22) × 100.

Results

Microbial culture and identification

Ten Amies agar gel transport swabs were swabbed on *A. mellifera* beehives and used to perform bacterial culture and identification by standard methods (Table 1) Results provided by swab no. 5 exhibited no growth (10%), whereas the other 9 swabs found organism growth (90%). The results from 9 swabs found 22 microorganism colonies, including Gram-positive bacteria (63.64%, 14/22), Gram-negative bacteria (27.27%, 6/22) and fungi (yeast) (9.09%, 2/22). The results of the identification of a microbial organism are shown in Table 1. Several types of organisms were found, including yeast, coagulase-negative *Staphylococcus* spp., coryneform bacteria, *Pseudomonas* spp., non-fermentative Gram-negative bacilli, *Pantoea* spp., and *Bacillus* spp.

From Figure 1, the prevalence of microbial organisms is shown. The coagulase-negative *Staphylococcus* spp. was found the most (40.91%, 9/22); followed by coryneform bacteria and *Pantoea* spp., found at 13.64% (3/22); *Bacillus* spp., non-fermentative Gram-negative bacilli, and yeast, which were found at 9.09% (2/22); and *Pseudomonas* spp., which was found at 4.55% (1/22), respectively.

Antimicrobial susceptibility test

From the overall 10 *A. mellifera* swabs, we isolated 22 microorganism colonies and used 11 suspected isolated colonies to perform an antimicrobial drug susceptibility test on MHA plates using the disc diffusion Kirby–Bauer technique. Gram-positive bacteria such as coagulase-negative *Staphylococcus* spp. were tested using antibiotic agents including vancomycin (VA30) and cefoxitin (oxacillin) (CX30). Whereas, for Gram-negative bacteria such as *Pantoea* spp., glucose non-fermentative Gram-negative bacilli, and *Pseudomonas* spp., antibiotic agents such as ciprofloxacin (CIP5), levofloxacin (LEV5), ceftazidime (CAZ30), cefotaxime (CTX30), imipenem (IMI10), and meropenem (MEM10) were used. The results of the inhibition zone sizes for each antibiotic agent are shown in Table 2.

Table 1 Identification of microbial organism from *Apis mellifera* beehives

Swab No.	Morphology	Microbial organism identification (n = 22)
1	Not done	Yeast
2	Gram-positive cocci	Coagulase-negative *Staphylococcus* spp.
	Gram-positive cocci	Coagulase-negative *Staphylococcus* spp.
	Gram-positive cocci	Coagulase-negative *Staphylococcus* spp.
3	Gram-positive bacilli	Coryneform bacteria
4	Gram-positive cocci	Coagulase-negative *Staphylococcus* spp.
	Gram-positive cocci	Coagulase-negative *Staphylococcus* spp.
5	No growth	No growth
6	Gram-positive bacilli	Coryneform bacteria
	Gram-negative bacilli	*Pseudomonas* spp.
	Gram-negative bacilli	Non-fermentative gram-negative bacilli
7	Gram-positive cocci	Coagulase-negative *Staphylococcus* spp.
	Gram-positive cocci	Coagulase-negative *Staphylococcus* spp.
	Gram-negative bacilli	*Pantoea* spp.
8	Gram-positive bacilli with spore	*Bacillus* spp.
	Gram-positive bacilli	*Bacillus* spp.
	Not done	Yeast
9	Gram-positive cocci	Coagulase-negative *Staphylococcus* spp.
	Gram-positive cocci	Coagulase-negative *Staphylococcus* spp.
	Gram-negative bacilli	*Pantoea* spp.
10	Gram-positive bacilli	Coryneform bacteria
	Gram-negative bacilli	*Pantoea* spp.
	Gram-negative bacilli	Non-fermentative Gram-negative bacilli

Discussion

The intricate relationship between agriculture and the cycle of life is epitomized through the essential process of pollination. This process, vital for sustaining the ecological balance of ecosystems, serves as the foundation for global food production. Recognizing pollinators as indispensable contributors to the agricultural yield process, especially in the context of cross-pollination, underscores their pivotal role in crop production. As crucial contributors to crop pollination, bees play a significant role in enhancing both the quality and quantity of a diverse range of crops, fruits, vegetables, and oilseeds. Therefore, bee pollination not only enhances the quality and quantity of these crops but

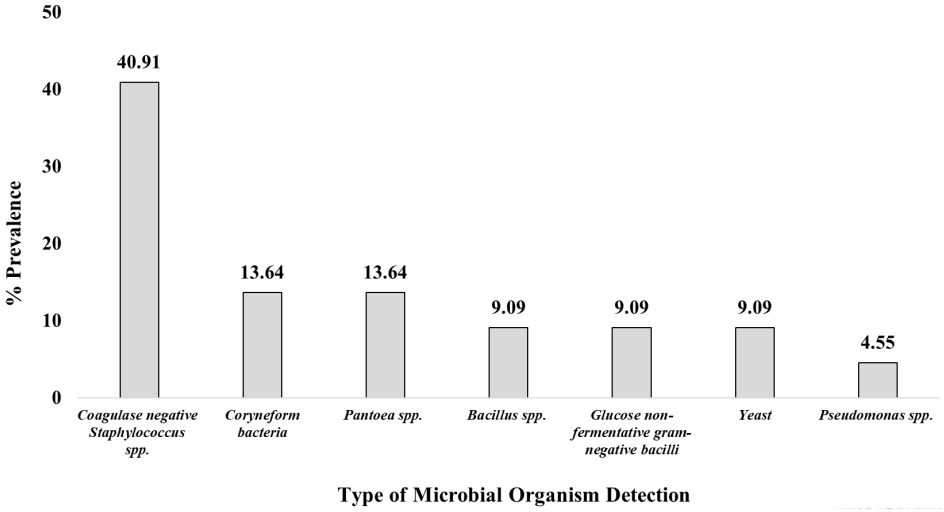

Fig. 1 Prevalence of microbial organism on swab specimens from *Apis mellifera* beehives.

Table 2 Antimicrobial susceptibility testing of microorganisms from *Apis mellifera* beehive swabs

Swab No.	Isolated colony No.	Microorganism	Antibiotic inhibition zone size (mm)[a]							
			VA30[b]	CX30[c]	CIP5[d]	LEV5[d]	CAZ30[e]	CTX30[e]	IMI10[f]	MEM10[f]
2	1	Coagulase-negative *Staphylococcus* spp.	21	23	ND	ND	ND	ND	ND	ND
	2	Coagulase-negative *Staphylococcus* spp.	19	22	ND	ND	ND	ND	ND	ND
4	1	Coagulase-negative *Staphylococcus* spp.	20	24	ND	ND	ND	ND	ND	ND
6	1	*Pseudomonas* spp.	ND	ND	38	32	18	18	19	19
	2	Glucose non-fermentative Gram-negative bacilli	ND	ND	34	34	32	32	40	34
7	1	Coagulase-negative *Staphylococcus* spp.	19	21	ND	ND	ND	ND	ND	ND
	2	Coagulase-negative *Staphylococcus* spp.	19	20	ND	ND	ND	ND	ND	ND
		Pantoea spp.	ND	ND	40	36	34	30	30	34
9	1	Coagulase-negative *Staphylococcus* spp.	18	22	ND	ND	ND	ND	ND	ND
	2	Coagulase-negative *Staphylococcus* spp.	19	23	ND	ND	ND	ND	ND	ND
		Pantoea spp.	ND	ND	36	36	28	34	30	32
10	1	Glucose non-fermentative Gram-negative bacilli	ND	ND	34	32	28	30	26	30
	2	*Pantoea* spp.	ND	ND	22	20	21	21	34	22

ND: not done; VA30: vancomycin; CX30: cefoxitin; CIP5: ciprofloxacin; LEV5: levofloxacin; CAZ30: ceftazidime; CTX30: cefotaxime; IMI10: imipenem; MEM10: meropenem.
[a]Clinical and Laboratory Standards Institute® M100, performance standards for antimicrobial susceptibility testing, 32nd edition guideline (CLSI 2022), [b]glycopeptide antibiotic, [c]β-lactams antibiotic, [d]fluoroquinolone antibiotic, [e]3rd generation cephalosporins antibiotic, [f]carbapenems antibiotic.

also contributes to the world's food security (Khalifa et al. 2021).

Nevertheless, there are numerous obstacles that affect the development, reproduction, and sustainability of bee colonies, in particular pesticides, land usage, management effectiveness, and climate change. Consequently, it is crucial to emphasize these elements in order to promote profitable pollination.

At the present, antibiotic resistance among bacteria has gotten worse and is continuing to rise, posing a serious threat to public health. In addition, drug resistance is not only harming public health but also resulting in social and economic costs (Foote 1957; Haydak 1958).

The alternative, cost-effective method for identifying foodborne pathogens in food samples must start with the culture-based method (Bell et al. 2016). Culture-based methods for the identification of foodborne pathogens are selective and unique, suppress the growth of unneeded bacteria, and use a differential medium to identify specific pathogenic microbes.

A biochemical test is a growth-promoting method where compounds are used as signals that indicate the presence of pathogens and inhibit the growth of competing microbes. There are many biochemical assays that can be used to confirm the presence of specific pathogens in food samples, including the urase test, citrate utilization test, oxidase test, catalase test, indole production test, triple sugar iron agar, blood agar plates, motility agar, mannitol salt agar, etc. (Saravanan et al. 2021). These conventional bacterial cultures, identification by culture-based standard methods, and biochemical tests can be performed in the routine microbiology laboratory.

For microbial culture and identification tests

From the overall 10 *A. mellifera* beehive swabs, no microbial organism growth was found in 1 of the 10 swab specimens (10%) (Table 1). Since microbial contamination or very limited microbial contamination, and with the limitation of the sensitivity of this culture-based method, it may not be able to detect contamination of the bacteria in this swab sample.

For the other 9 beehive swab specimens (90%), this study could not identify the isolated microbial organisms to the species level of the bacteria. Due to the limitations of traditional culture-based and biochemical tests that are suitable for detecting pathogens in humans, animals, or clinical specimens such as blood and urine, as a result, it is not possible to distinguish microbes isolated from environmental or natural specimens up to the species level.

Nevertheless, according to the results of microbial culture and identification from 9 *A. mellifera* beehive swab specimens, various types of microbial organisms were found, including Gram-positive bacteria and coagulase-negative bacteria. *Staphylococcus* spp., coryneform bacteria, and *Bacillus* spp. were found to be the most common (63.64%), followed by Gram-negative bacteria including *Pantoea* spp., non-fermentative Gram-negative bacilli and *Pseudomonas* spp., which were found at 27.27% and fungi (yeast) at 9.09%, respectively (Fig. 1).

Consequently, people who work in these businesses are significantly affected by certain newly discovered bacteria in terms of their health. *Staphylococcus* spp., one of the main nosocomial pathogens, is a typical opportunist. The two most important species are *S. epidermidis* and *S. haemolyticus*. They contribute significantly to infections caused by foreign bodies and infections in premature babies. While *S. saprophyticus* has been linked to acute urethritis, *S. lugdunensis* is a special case that shares some characteristics with *S. aureus* in its ability to infect the heart, causing infectious endocarditis (Becker et al. 2014).

For coryneform bacteria, or corynebacterium, a thermoduric bacteria, which is an important bacterium in food and is the cause of spoilage of many types of food (microbial spoilage), such as meat and poultry products (Sandot et al. 2023).

Pantoea spp. are bacteria isolated from soil, water, plants (e.g., epiphytes or endophytes), seeds, fruits (e.g., pineapples, mandarin oranges), and gastrointestinal tracts of humans and animals, as well as in dairy products, blood, and urine. *Pantoea* spp. causes infections in humans and plants. Some are plant pathogens, and some are opportunistic in immunocompromised humans, causing wounds, bleeding, and inflammation of the urinary tract (Layla and Darweesh 2016).

Bacillus spp. is a thermoduric bacterium that can produce endospores (spore-forming bacteria) resistant to numerous conditions, pollutants, and drought. *Bacillus* is a major cause of food spoilage (microbial spoilage) and causes spoiled food to smell bad. Some strains of *Bacillus*, including *Bacillus cereus*, cause food poisoning intoxication through the consumption of food that produces toxins (Hölzel et al. 2018; Nguyen and Tallent 2019).

Bacteria in the group glucose non-fermentative Gram-negative bacilli are bacteria that do not ferment glucose to acidify bacteria and cause disease in humans. Bacteria in this group include *Pseudomonas* spp., *Burkholderia* spp., *Alcaligenes* spp., *Moraxella* spp., etc. This infection causes disease in humans only when their immunity is low, such as in those who are taking immunosuppressive drugs, patients with cancer, diabetes, scalds, or through contaminated medical equipment such as surgical instruments or respirators, etc. They are considered opportunistic infections or opportunistic pathogens, so this group is often associated with infectious diseases in health care facilities (Yadav et al. 2020). Currently, researchers have discovered that this group of bacteria frequently exhibits resistance to antimicrobial agents, posing a significant public health concern.

Overall, in this study, we discovered a number of organisms that could harm the health of beekeepers and other related workers. Consequently, these microbial organisms have the potential to degrade honey quality and infect honey bees and beekeepers, who mostly use their hands and lack proper hygiene. Therefore, the potential and danger of these pathogen infections when handling or dealing with them may worry the beekeepers who set their beehives among palm gardens.

Antimicrobial susceptibility test

Based on the susceptibility tests of bacteria to antimicrobial agents, the advantage of these laboratory tests is that they are used to guide physicians in selecting effective antibiotic agents to treat patients.

This study provided the results of a microbial susceptibility test of microbial organisms on *A. mellifera* beehives, which aims to identify antibiotic-resistant bacteria by using the disc diffusion Kirby–Bauer technique. Ten antibiotic discs from the glycopeptide, β-lactams, fluoroquinolone,

3rd generation cephalosporins, and carbapenem antibiotic groups; these groups of antibiotics are the most commonly prescribed drugs in hospitalized patients. They include vancomycin 30 mg (VA30), cefoxitin (oxacillin) 30 mg (CX30), ciprofloxacin 5 mg (CIP5), levofloxacin 5 mg (LEV5), ceftazidime 30 mg (CAZ30), cefotaxime 30 mg (CTX30), imipenem 10 mg (IMI10), and meropenem 10 mg (MEM10).

Based on the susceptibility test of bacteria to antimicrobial agents, this study could not identify the microorganism species, so the antimicrobial susceptibility test results cannot be interpreted as resistant or susceptible to the drug. The results provided only the inhibition zones of drug-sensitivity for each antibiotic agent, which represent that the antibiotic agents can inhibit bacterial growth. Measuring the size of its inhibition zone determines an antibiotic's efficacy. The destruction of more bacteria within the zone of inhibition suggests that the drug was more effective (LibreText 2023).

In this study, of the overall 9 beehive swabs and 22 microorganisms found, 11 suspected isolated colonies were used to perform an antimicrobial drug susceptibility test.

The susceptibility result showed that for Gram-positive bacteria groups such as coagulase-negative *Staphylococcus* spp., the inhibition zone sizes against VA30 and CX30 vary by 18–21 mm and 20–24 mm, respectively.

For Gram-negative bacteria groups such as *Pantoea* spp., glucose non-fermentative Gram-negative bacilli and *Pseudomonas* spp., antibiotic agents such as CIP5, LEV5, CAZ30, CTX30, IMI10, and MEM10 were used.

For the *Pantoea* spp. group, the inhibition zone sizes of fluoroquinolone antibiotic agents for CIP5 and LEV5 vary by 22–40 mm and 20–36 mm, respectively. Whereas for the 3rd generation cephalosporin drugs such as CAZ30 and CTX30, the inhibition zone size varies from 21–34 mm. And for carbapenem antibiotic agents, the inhibition zone sizes of IMI10 and MEM10 vary by 30–34 mm and 22–34 mm, respectively.

As for the non-fermentative Gram-negative bacilli bacteria group, the inhibition zone sizes of fluoroquinolone antibiotic agents for CIP5 and LEV5 vary by 34 mm and 32–34 mm, respectively. Whereas for the 3rd generation cephalosporin drugs such as CAZ30 and CTX30, the inhibition zone size varies between 28–32 mm and 30–32 mm. And for carbapenem antibiotic agents, the inhibition zone sizes of IMI10 and MEM10 vary by 26–40 mm and 30–34 mm, respectively.

As for the *Pseudomonas* spp. group, the inhibition zone sizes of fluoroquinolone antibiotic agents for CIP5 and LEV5 vary by 38 mm and 32 mm, respectively. Whereas for the 3rd generation cephalosporin drugs such as CAZ30 and CTX30, the inhibition zone size was 18 mm. And for carbapenem antibiotic agents, it provided the inhibition zone size of IMI10 and MEM10 as 19 mm.

Regarding the bacteria in the group *Bacillus* spp. and coryneform bacteria, which are Gram-positive. The disc diffusion method is not suitable for testing the susceptibility of an antibiotic. The minimal inhibitory concentration method, also known as the MIC method, is used to determine the minimal concentration of antimicrobial medication needed to inhibit or kill bacteria in order to assess the bacterium's susceptibility to antimicrobials for the optimum treatment outcome.

The overall result of this study provided the results of microbial detection on *A. mellifera* beehives, which have the potential to infect the human body, including beekeepers or related people in this business. However, this study provides the results from beehives limited to one of several types of plantations; therefore, further study in other areas will provide more advantage knowledge of health, safety, and security.

Conclusions

In this study, bacterial contamination was detected in beehives located in a palm garden in Rayong province, Eastern Thailand. This study's findings can be used to inform beekeepers, healthcare organizations, and other parties involved in the beekeeping industry to be concerned while handling or working with these materials. Beekeepers must have a thorough awareness of microbial diseases. A key component of maintaining the safety of beekeepers and their colonies is the use of personal protective equipment and good hygiene in beekeeping boxes throughout the bee management and honey harvesting processes.

Abbreviations

CLSI: Clinical and Laboratory Standards Institute
TSI: Triple sugar iron
MHA: Mueller–Hinton agar

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Authors' contributions

Conceptualization: Surat Hongsibsong, SD, and BC. Methodology: SD, SY, Sayamon Hongjaisee, and Surat Hongsibsong. Validation: SD, BC, and Surat Hongsibsong. Formal analysis: SD, PJ, PK, and SY. Investigation: KD, BC, and JM. Writing – original draft preparation: SD, BC, and Surat Hongsibsong. Writing – review and editing: SD, BC, and Surat Hongsibsong. Visualization: SD and PK. Supervision: Surat Hongsibsong. Project administration: SY. Funding acquisition: BC. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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