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Research article

Efficacy of cyclic lipopeptides obtained from *Bacillus subtilis* to inhibit the growth of *Microsporum canis* isolated from cats

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ABSTRACT

Background and aim: *Microsporum canis* (*M. canis*) is a dermatophyte fungal pathogen that causes ringworms. Cats are considered to be a dominant reservoir host enabling *M. canis* transmission to humans. The concerns of dermatophyte resistance were raised among the usage of antifungal drugs to treat the ringworm. This study aimed to evaluate the fungal activity of cyclic lipopeptides (CLPs) obtained from *Bacillus subtilis* (*B. subtilis*) as an alternative method for the inhibition of *M. canis* growth.

Materials and methods: The culture plate of *M. canis* from confirmed cats with ringworm infection was provided. The purification of CLP extract, fengycin, iturin A, and surfactin was carried out from *B. subtilis* by preparative thin-layer chromatography (PTLC) coupled with solid-phase extraction (SPE) methods. Half-maximal effective concentration (EC_{50}) and agar well diffusion assays were performed to determine the efficacy of *Bacillus* CLP extract, fengycin, iturin A, and surfactin to inhibit the growth of *M. canis* isolated from cats.

Results: All purified *Bacillus* substances displayed antifungal activity to control the growth of *M. canis* when compared with 80% ethanol (control). EC_{50} values for CLP extract, fengycin, iturin A, and surfactin were 0.23, 0.05, 0.17, and 0.08 mg/mL, respectively. In agar well diffusion assay, the ability of CLP extract, fengycin, iturin A, and surfactin on fungal inhibition had no statistically significant difference at 24 and 48 h after treatment ($p < 0.05$). However, CLP extract showed a statistically significant difference on *M. canis* inhibition at 62.21% followed by surfactin with 59.04% at 72 h after treatment.

Conclusion: *In vitro*, *Bacillus* CLPs revealed an inhibitory effect on *M. canis* growth which is a zoonotic pathogen that causes ringworms. This study suggests an alternative approach to control the growth of *M. canis* using substances obtained from *B. subtilis* as a biomedicine agent with antifungal activity.

1. Introduction

M. canis is one of the most common fungal pathogens causing dermatophytosis (ringworm) by invading the host animals at stratum corneum of the epidermis and keratinized tissues [1]. Contagious zoonosis disease caused by *M. canis* is commonly found in dogs and cats which are considered carriers of diseases in epidemiology [2]. Humans could become the *M. canis* infected patients through contact with their pets in the condition of both asymptomatic and symptomatic characterization [3]. The regular appearance of ringworm includes inflamed lesions in single or multiple areas, which are mainly localized on the head and certain parts of the body [4]. To heal *M. canis* infection on the skin, various clinical treatments comprised of oral and topical antifungal drugs such as fluconazole, griseofulvin, itraconazole, ketoconazole, and terbinafine are applied to infected patients [5]. Among the drug strategies for antifungal therapy, the apprehension of drug tolerance is exhibited [6].

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An approach to biological treatment is a modern trend to reduce the usage of antifungal drugs in the manipulation of the fungal infection [7]. The application of biological chemicals synthesized by antagonistic bacteria, which can produce metabolites in an antifungal manner, was utilized to control the fungal growth [8, 9]. *B. subtilis* is one kind of bacteria used as an antagonist to suppress the growth of fungi. *B. subtilis* illustrated the potential of antifungal activity against dermatophytes such as *Trichophyton* spp. and *M. fulvum* by agar well diffusion assay [10]. Moreover, a previous study demonstrated that the application of *B. subtilis* had the ability to hinder the growth of *M. canis in vitro* [11].

B. subtilis can produce secondary metabolites, called cyclic lipopeptides (CLPs) [12]. CLPs can help control the germination of fungal pathogens with their antifungal property by defacing the cellular membranes of fungal pathogens affecting the fungal hyphae and spore development [13]. Fengycins, iturins, and surfactins are three main families of CLPs which reveal antifungal action [14, 15, 16]. Fengycin was not only reported as a possible biomedicine agent [17] but also exhibited the ability to control the fungal pathogens causing plant diseases such as *Fusarium* and *Colletotrichum* genus [18] by causing leakage and lysis of the fungal cell membrane [19]. Iturin A, one of the iturins derivatives, has shown the ability to obstruct the growth of fungal pathogens such as *Aspergillus carbonarius* by generating the incidence of osmotic imbalance in fungal cells affecting the disruption of energy, transport and osmotic pressure metabolisms [20]. Surfactin functions as a biocontrol agent to retard the growth of *Fusarium* species such as *F. moniliforme*, which is related to mycotoxicosis in animals and humans [21]. Furthermore, surfactin has been suggested for use as a potential agent in the biotechnological and pharmaceutical fields [22]. While CLPs display the antifungal property, the concerns of their toxicity were reported [23, 24, 25]. The previous reports revealed that fengycins, iturins and surfactin are safe and show no toxicity on mammal cells following the experimental designs [23, 24, 25]. However, understanding concerning the application of *Bacillus* CLPs to inhibit *M. canis* causing ringworm remains. This study aimed to evaluate the fungal activity of CLPs obtained from *B. subtilis* on the control of *M. canis* growth as an alternative treatment to reduce the factors of antifungal drug resistance.

2. Materials and methods

2.1. Ethical approval

This research was approved by the Research Committee of Kasetsart University and the Kasetsart University Institutional Animal Care and Use Committee. All the other experiments were conducted *in vitro* using the collection of samples from *M. canis* culture plate.

2.2. Study period and location

This study was performed from November 2020 to May 2021. Mycological approaches were conducted at the Department of Companion Animal Clinical Sciences, Faculty of Veterinary Medicine, Kasetsart University, Thailand. The bacteriological procedures were carried out at the Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Thailand. The mass spectrometry method was executed at the National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathum Thani.

2.3. Culture of microorganisms

The culture plate of *M. canis* from confirmed cats with ringworm infection that visited the Kasetsart University Veterinary Teaching Hospital (KUVTH), Bangkok, Thailand was provided. In KUVTH, the dermatophyte test kit (Cat No. 290610, KRUUSE, Denmark) was used as a selective media to isolate *M. canis* and identification of *M. canis* was performed under KUVTH's Fungal Laboratory before providing. Prior to experimenting, the fungal pathogen was brought to the laboratory to verify the mycology by microscopic examination and fungal culture following the previous methods [26] in Sabouraud dextrose agar (SDA). The fungal isolates with the *M. canis* microscopic appearance (spindle shaped with 5–15 cells with rough surface, a thick outer wall, and a terminal knob) were selected as a fungal pathogen. *B. subtilis* ABS-S14, which was used as the antagonistic bacteria to perform the extraction of CLPs, was cultured as described in previous work [27]. The identification of *B. subtilis* ABS-S14 was performed using the biochemical tests [27, 28, 29] coupled with peptide mass fingerprinting (PMF) analysis using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, USA) compared to the PMF of *B. subtilis* ssp *subtilis* DSM 5660 DSM (Supplementary Figure-1).

2.4. Extraction, purification, and identification of CLPs

The preparation of *Bacillus* CLP extract, fengycin, iturin A, and surfactin was conducted from the stock solution of crude extract (50 mg/mL) obtained from *B. subtilis* ABS-S14 in 80% ethanol according to a previous report [27]. Briefly, PTLC was used to purify fengycin, iturin A, and surfactin. Increasing the purity of fengycin, inturin A, and surfactin was executed by SPE using the step gradients of acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) as a mobile phase. Concentrations of 40%–55%, 25%–35%, and 60%–80% ACN in 0.1% TFA was used to purify fengycin, iturin A, and surfactin, respectively. MALDI-TOF MS was carried out to determine the molecular mass of fengycin, iturin A, and surfactin compared to their commercial standards (Sigma-Aldrich, USA) as detailed in published work [27].

2.5. Determination of EC_{50} values

Bacillus CLP extract, fengycin, iturin A, and surfactin were each tested for the ability to control the growth of fungal pathogen at the half-maximal effective concentration on two well concave slides by EC_{50} following a previous report [30]. The 900 μL of the pre-warmed SDA were mixed with 100 μL of each purified *Bacillus* chemicals at different concentrations. A two-fold serial dilution in the range of 0.625–10 mg/mL for CLP extract and 0.156–2.5 mg/mL for fengycin, iturin A, and surfactin concentration was performed. 80% ethanol was used as a control. The different concentrations between CLP extract and individual CLP treatments were used to interact with *M. canis* based on the preliminary experiments. The mixed SDA media and each substance solution were each dropped into each well of concavity slides. The 0.1 cm diameter of actively growing mycelial plug earned from 9 day-old *M. canis* [11] was deposited on the center of each well. The radial growth of the mycelium was observed using an ocular micrometer under a stereo-microscope after 48 h of incubation at room temperature. The inhibition percentage was calculated according to the previous equation [31]. The linear regression equation was created by Microsoft Excel 2019 software for the determination of EC_{50} values.

2.6. Agar well diffusion assay

To indicate the antifungal properties of CLP extract and individual CLP, the agar well diffusion assay was carried out following the method described in previous work [32]. An actively growing mycelial plug (0.1 cm diameter) obtained from 9 day-old *M. canis* [11] was placed on the center of a SDA plate and incubated at room temperature for 48 h before starting the test. Two cavities with 0.5 cm diameter were generated away from the edge of growing mycelium on the SDA plate (approximately 1 cm). Concentrations of CLP extract, fengycin, iturin A, and surfactin in 80% ethanol at 10, 0.5, 2, and 1 mg/mL, respectively, were added to the cavities. The radial extension of *M. canis* mycelium was measured at 24, 48, and 72 h. The percentage of inhibition was measured following a previous study [31]. 80% ethanol was used as a control.

2.7. Statistical analysis

The data sets were examined for significant differences by ANOVA. Differences at $p < 0.05$ were considered to be significant. Tukey's range test was used to indicate the significant differences in mean values which were reported as the mean ± standard error (n = 3).

3. Results

CLPs which were synthesized by *B. subtilis* ABS-S14 contain three main substances, fengycin, iturin A and surfactin [27]. Prior to evaluating the antifungal ability of individual CLP, purification by PTLC, separation by C18-SPE, and verification by MALDI-TOF MS were performed. It was found that fengycin, iturin A, and surfactin had m/z values of MALDI-TOF MS spectra in the ranges of 1436.796–1546.855, 1067.605–1081.997, and 1016.641–1084.668, respectively which were found to match with their commercial standards (Sigma-Aldrich, USA) (Supplementary Figure-2).

3.1. EC_{50} values

To indicate the ability of CLP extract on the inhibition of *M. canis* growth, the range of CLP extract concentration at 0.625–10 mg/mL and 80% ethanol (control) were applied to interact with *M. canis*, while the range was 0.156–2.5 mg/mL for fengycin, iturin A, and surfactin concentration. The radial extension of *M. canis* mycelium was measured by an ocular micrometer under a stereo-microscope after 48 h of incubation. Inhibition percentage and EC_{50} values were calculated according to a linear regression equation. The EC_{50} values of CLP extract, fengycin, iturin A, and surfactin obtained from *B. subtilis* were 0.23, 0.05, 0.17, and 0.08 mg/mL, respectively.

3.2. Inhibitory effect of Bacillus CLPs on growth of M. canis

To determine the antifungal efficacy of CLPs, concentrations of CLP extract, fengycin, iturin A, and surfactin at 10, 0.5, 2, and 1 mg/mL, respectively, were used to interact with *M. canis* by agar well diffusion assay. The measurement of radial mycelium growth was performed at 24, 48, and 72 h followed by the calculation of inhibition percentage. In Figure 1, the antifungal activity of CLP extract and surfactin was increased from 24 to 72 h when compared with the control. Among the treatments for CLPs, fengycin demonstrated the greatest antifungal percentage at 48 h, while iturin A showed the increasing ability of inhibition percentage from 24 to 48 h, but remained at 72 h. According to statistical analysis, the antifungal ability of CLP extract, fengycin, iturin A, and surfactin showed no statistically significant difference at 24 and 48 h after treatment. However, CLP extract showed a statistically significant difference in *M. canis* inhibition at 62.21% followed by surfactin with 59.04% at 72 h after treatment. In Table 1, CLP extract, fengycin, iturin A, and surfactin presented the inhibition percentage at 37.02 ± 2.57, 41.26 ± 6.03, 38.58 ± 4.47, and 44.75 ± 5.81, respectively, at 24 h after treatment. The ability of CLP extract, fengycin, iturin A, and surfactin could control the *M. canis* mycelial growth with inhibition percentages of 52.29 ± 8.65, 53.53 ± 3.66, 47.51 ± 3.68, and 49.97 ± 3.11 at 48 h after treatment, respectively (Table 1). Inhibition percentages of 62.21 ± 6.48, 41.95 ± 8.07, 47.21 ± 3.94, and 59.04 ± 2.69 were observed in the treatments of CLP extract, fengycin, iturin A, and surfactin, respectively, at 72 h after treatment (Table 1 and Figure 2).

4. Discussion

The use of antagonistic bacteria such as *Bacillus* species, which synthesize antifungal metabolites, is an influential method to suppress the growth of fungal pathogens [8, 9]. The application of antagonistic *B. subtilis* as a biological treatment against dermatophyte pathogens was elucidated by *B. subtilis* cell approaches [10, 11]. However, the utilization of antifungal metabolites produced by *B. subtilis* on the inhibition of *M. canis* is still limited. In this study, *Bacillus* CLP extract, fengycin, iturin A, and surfactin were applied to interact with *M. canis* to observe the growth of fungal pathogen and evaluate the antifungal activity. However, the antifungal experiments throughout this study used only one *M. canis* isolate which was the limitation of the current work because the antimicrobial activity of different isolates may be not similar.

In microbiological methods, there are several ways to examine the effectiveness of substances, chemicals, and drugs such as the analysis of concentration–response which relates to the measurement of the concentration of the agent which gives 50% of the maximum response such as EC_{50} [33]. EC_{50} was described as the chemical agent concentration

Table 1. Inhibition percentage of each treatment on *Microsporum canis* growth. The standard errors of the mean value for three trials are shown. The same letter above shows no significant difference between them at $p < 0.05$ according to Tukey's range test.

Treatments	Time after treatment (h)		
	24	48	72
	Inhibition (%) ± S.D.	Inhibition (%) ± S.D.	Inhibition (%) ± S.D.
Cyclic lipopeptide extract (10 mg/mL)	37.02 ± 2.57^{a}	52.29 ± 8.65^{a}	62.21 ± 6.48^{c}
Fengycin (0.5 mg/mL)	41.26 ± 6.03^{a}	53.53 ± 3.66^{a}	41.95 ± 8.07^{a}
Iturin A (2 mg/mL)	38.58 ± 4.47^{a}	47.51 ± 3.68^{a}	47.21 ± 3.94^{ab}
Surfactin (1 mg/mL)	44.75 ± 5.81^{a}	49.97 ± 3.11^{a}	59.04 ± 2.69^{bc}

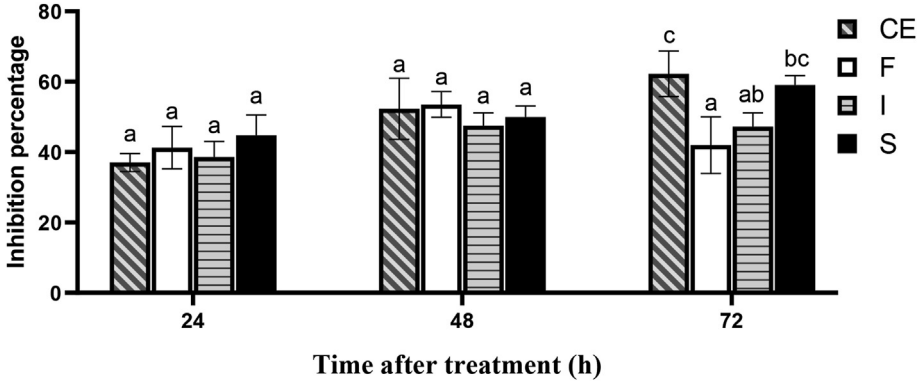

Figure 1. Inhibitory effect of each treatment on *Microsporum canis* growth. Vertical bars represent standard errors of the mean value of three trials; columns with the same letter above show no significant difference between them at $p < 0.05$ according to Tukey's range test. Abbreviations: CE; cyclic lipopeptide extract (10 mg/mL), F; fengycin (0.5 mg/mL), I; iturin A (2 mg/mL), S; surfactin (1 mg/mL).

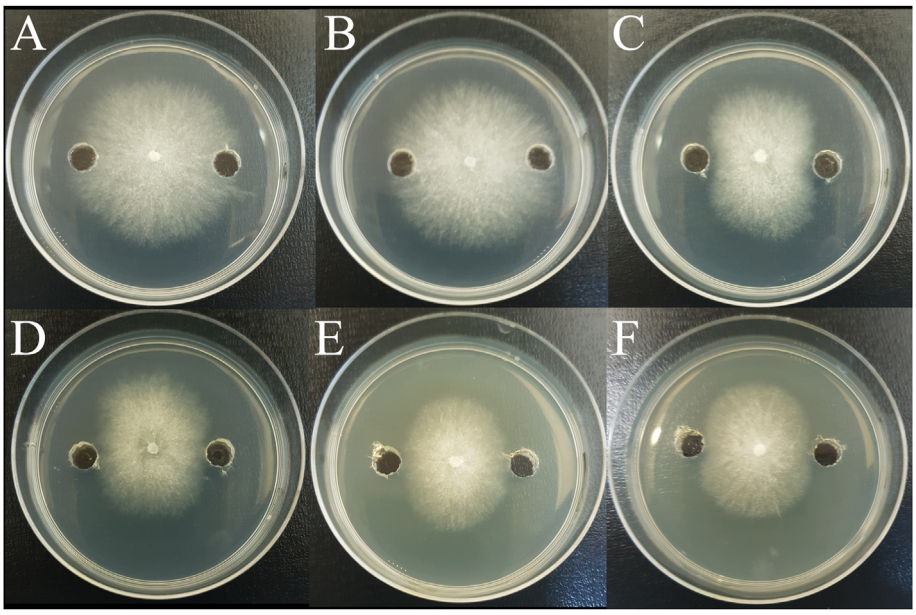

Figure 2. Antifungal efficacy of each treatment on *Microsporum canis* growth at 72 h by agar well diffusion assay. *Microsporum canis* (A), 80% ethanol (control) (B), 10 mg/mL cyclic lipopeptide extract (C), 0.5 mg/mL fengycin (D), 2 mg/mL iturin A (E), and 1 mg/mL surfactin (F).

which was added to an *in vitro* culture of pathogens that decreases their density to 50% of the control produced without adding the chemical agent [34]. The EC_{50} method was conducted to investigate the effect of substances on the suppression of fungal pathogens in previous studies [35, 36]. To determine the effective dose of CLPs that caused a 50% growth rate reduction of *M. canis*, EC_{50} assay was carried out *in vitro*. In this study, the EC_{50} values indicated that *Bacillus* CLP extract, fengycin, iturin A, and surfactin had antifungal activity to suppress *M. canis* growth when compared with the control. Moreover, each purified CLP demonstrated a greater effect on the inhibition of *M. canis* than CLP extract. Activity of CLPs on the control of fungal pathogen by EC_{50} assay was in the same manner as previous works [27, 28, 29]. This could be suggested that *Bacillus* CLP extract, fengycin, iturin A, and surfactin had antifungal properties to inhibit the growth of *M. canis*. However, the synergistic effect of each CLP on antifungal activity should be focused.

To clarify the antifungal properties of *Bacillus* CLPs, an agar well diffusion assay was performed. When CLPs obtained from *B. subtilis* were applied *in vitro* to the culture plate of *M. canis*, the size of the *M. canis* colony was limited and unable to spread through the plate. The agar well diffusion assay illustrated the slow rate of fungal growth when compared with the control plate. This indicated that *M. canis* could not grow normally when exposed to CLP extract, fengycin, iturin A, and surfactin. It is the same result as a previous study that reported on the properties of *B. subtilis* extract for the inhibition of *M. canis* [11].

Although the inhibition percentage of CLPs on *M. canis* growth was greater than the control and the antifungal ability of all CLPs had no statistically significant difference at 24 and 48 h after treatment, the inhibitory effect of individual treatment was different at 72 h after treatment. This study demonstrated that the treatment of CLP extract showed the greatest action of *M. canis* inhibition with a statistically significant difference at 72 h. The activity of fengycin on inhibition of *M. canis* growth decreased from 48 h to 72 h after treatment. This phenomenon might occur because of the instability of fengycin. Fengycin was reported as a chemically unstable substance. The action of fengycin to control the growth of *F. graminearum* which is a fungal pathogen at 48 h–72 h was reduced because of the unstable effect of fengycin [37] following the same manner as this study. Surfactin demonstrated a higher inhibitory effect than iturin A and fengycin, but a lower effect than CLP extract treatment with a statistically significant difference at 72 h. CLP extract displayed the strongest ability because it is a mixture of fengycin, iturin A, and surfactin [29, 38]. Each CLP has its own antifungal property. When each CLP was prepared as a mixture, it might have the synergistic action affecting the antifungal activity. CLP extract might use this advantage to overcome the individual CLP action on fungal inhibition because the individual CLPs each reported antifungal activity [18, 19, 20, 21, 22] by causing the leakage of fungal cellular contents and inhibition of fungal branch formation and growth [13, 39]. Furthermore, surfactin displayed greater ability against fungal pathogen by the induction of defensive gene (*lipoxygenase* gene) accumulation which relates to the defensive mechanism than iturin A and fengycin [27] following the same manner as this study. However, to study the real synergistic action of individual CLP on fungal inhibition, combination of CLP such as fengycin and iturin A, fengycin and surfactin, as well as, iturin A and surfactin might be investigated in further study. Even fengycin and iturin A could suppress the mycelium of fungal extension, though their inhibitory action might not be maintained for 72 h, causing a lower inhibition percentage than CLP extract and surfactin. However, the treatments of CLP extract, fengycin, iturin A, and surfactin obtained from *B. subtilis* were established in this study to reduce the mycelium growth of *M. canis*. It could be concluded that the application of *Bacillus* CLPs *in vitro* expressed their antifungal action to decrease the growth of *M. canis* which is the main pathogen causing skin disease in cats.

5. Conclusion

The current *in vitro* study revealed the antifungal properties of CLP extract, fengycin, iturin A, and surfactin obtained from *B. subtilis* on the inhibition of *M. canis* growth which is considered a zoonotic fungus causing ringworm disease. In the further study, the mechanism of each purified *Bacillus* CLPs on *M. canis* inhibition should be investigated. This study suggests an alternative approach to control the growth of *M. canis* using the metabolites obtained from *B. subtilis* with antifungal activity as a biomedicine agent for the development of ringworm treatment.

Declarations

Author contribution statement

Paiboon Tunsagool: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Sekkarin Ploypetch: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Janthima Jaresitthikunchai: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Sittiruk Roytrakul: Analyzed and interpreted the data; Wrote the paper.

Kiattawee Choowongkomon: Conceived and designed the experiments; Wrote the paper.

Jatuporn Rattanasrisomporn: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Competing interest statement

The authors declare no conflict of interest.

Additional information

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