Original Article

JHSMR Journal of Health Science and Medical Research

Effects of Postbiotic from Bacteriocin-Like Inhibitory Substance Producing *Enterococcus faecalis* on Toxigenic *Clostridioides difficile*

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Received 11 August 2022 ● Revised 23 October 2022 ● Accepted 2 November 2022 ● Published online 6 January 2023

Abstract:

Objective: To determine the activities of postbiotics, prepared from bacteriocin-like producing *Enterococcus faecalis* (*E. faecalis*) against *Clostridioides difficile* (*C. difficile*) and its spores, and to assess the safety of postbiotics in vivo.

Material and Methods: Bacteriocin production of *E. faecalis* PK1201 was screened by proteolytic enzyme treatment, and bacteriocin-encoding genes were characterized by whole genome sequencing. A postbiotic of *E. faecalis* PK1201 was prepared using neutralized cell-free supernatant or bacterial cell lysate, which was then used to screen for antimicrobial activity via agar well diffusion. The lyophilized cell-free supernatant (LCFS) was further determined for its minimum inhibitory concentration (MIC) against *C. difficile* 630. The morphological changes of *C. difficile* were observed under scanning electron microscopes (SEM). Subsequently, the LCFS at sub-MIC and MIC were used to evaluate anti-spore germination activity. Finally, the safety of postbiotics was accessed using the Galleria mellonella model.

Results: *E. faecalis* PK1201 carried enterolysin A encoding gene. For postbiotic preparation, only LCFS exhibited antimicrobial activity, and the activity was completely lost after proteinase K treatment; indicating the existence of bacteriocin. LCFS showed anti-*C. difficile* with MIC of 18.2±6.9 mg/mL. The SEM images demonstrated shorter destruction of *C. difficile* cells after being treated with LCFS. Interestingly, LCFS at the MIC and sub-MIC revealed anti-spore germination

This paper was from the 1st Annual Health Research International Conference (AHR-iCON, August 1-2, 2022).

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J Health Sci Med Res 2023;41(4):e2023918
doi: 10.31584/jhsmr.2023918
www.jhsmr.org

activity against toxigenic *C. difficile* compared to the control. LCFS showed no acute toxicity in *G. mellonella* at the tested concentration.

Conclusion: The powerful activity and safety of LCFS shed light on the role of postbiotics in pharmaceutical products to control CDI.

Keywords: bacteriocin, *Clostridioides difficile*, *Enterococcus faecalis*, postbiotics, spore

Introduction

Clostridioides difficile infection (CDI) is considered the main cause of nosocomial diarrhea. CDI affects almost half a million illnesses in the United States every year[1]. *C. difficile* is a Gram-positive, spore-forming, toxin-producing anaerobic bacteria, which can produce toxin A and/or toxin B that initiates diarrhea and colitis[2]. The current standard of care for CDI involves antibiotic treatments with vancomycin or fidaxomicin, which not only kills *C. difficile*, but also affects other gut benefit bacteria; causing up to 12–64% recurrence risk[3]. Thus, novel adjunctive therapies that can improve outcomes are required.

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host", which can defeat diseases and combat gut pathogens[4]. They are considered to defend gut health against infections through various mechanisms; including the generation of antimicrobial compounds; such as hydrogen peroxide, organic acids, and bacteriocins or bacteriocin-like substances[5]. Among them bacteriocins, proteinaceous or peptidic toxins produced by bacteria have great attractiveness as they are safe, effective and natural inhibitors against various pathogens[6]. Moreover, systematic reviews have elucidated that some probiotic bacteria; especially lactic acid bacteria (LAB) and *Bifidobacterium* spp., are able to prevent 17% of antibiotic-associated diarrhea as well as treat 3% of CDI in a clinical trial[7,8]. Some *Enterococcus* strains, which are LAB, have been proven to inhibit *C. difficile*. *E. faecium* NM1015, *E. faecalis* NM815, and *E. faecalis* NM915 have been shown to inhibit *C. difficile* in vivo[9]. Additionally, the study of Romyasamit et al., 2020 has found anti-*C. difficile* activity in *E. faecalis* isolates. These could be promising probiotics with potential applications in preventing CDI[10]. Although, probiotics might be effectively used to treat CDI, their clinical application in immunocompromised patients is limited due to the possibility of bacteremia and sepsis[11].

Hence, postbiotics were invented to reduce the risk of using probiotics. They refer to inanimate microorganisms and/or their components that confer a physiological benefit on the host[12]. Otherwise, postbiotics are products or metabolic byproducts that are secreted into cell-free supernatant (CFS) of bacterial suspension during the growth of probiotics[13,14]. Therefore, several postbiotic compounds, such as bacterial cell lysate, enzymes, organic acids and bacteriocins, have demonstrated antimicrobial activity against Gram-positive and Gram-negative microorganisms[15]. For instance, *Enterococcus* avium's cells lysate retained antibacterial effects against *Pseudomonas aeruginosa*[16]. In addition, LAB strains commonly produce bacteriocins; including, nisin A, secreted from *Lactococcus* and *Streptococcus* species have a broad spectrum of activity against other LAB and other Gram-positive organisms, including *C. difficile*, by causing changes in the bacterial membrane[17].

The studies regarding postbiotics are now of interest; however, few reports concerning postbiotics being used in treating or preventing CDI, and the activity of postbiotics

from *E. faecalis* against *C. difficile* have not been elucidated. Thus, this study aimed to characterize and verify the activity of bacteriocin-like inhibitory substances producing *E. faecalis's* postbiotic against toxigenic *C. difficile*, and assess the safety of postbiotic to provide a novel, alternative therapeutic option for CDI patients.

Material and Methods

Materials

Brain heart infusion agar (HiMedia, India), Brain heart infusion broth (HiMedia, India), deoxyribonucleic acid (DNA) extraction kits (Tiangen Biotech, China), EDTA (Amresco, USA), Ethanol (JT Baker, USA), HEPES buffer (Sigma-Aldrich, USA), L-cysteine (Merck Millipore, Germany), Tris (Thermo Fisher Scientific, UK), Phosphate Buffered Saline (PBS), pH 7.4 (HiMedia, India), Proteinase K (Amresco, USA), Taurocholic acid sodium salt (Sigma-Aldrich, USA), Tryptose Broth, (HiMedia, India), Sarcosyl (Amresco, USA), Yeast extract (Sigma-Aldrich, USA)

Methods

1. Characterization of bacteriocin-producing *E. faecalis* by proteolytic treatment

1.1 Proteinase K treatment

A culture medium or 500 mg/mL of postbiotic containing protein, having respective anti-*C. difficile* activity was tested by treating with proteinase K at a final concentration of 10 mg/mL and compared with those untreated. The mixture was then incubated at 30 °C for 2 hours. The reaction was stopped by heating the culture at 80 °C for 10 minutes: the antimicrobial activity was evaluated by agar spot on lawn assay.

1.2 Agar spot on lawn assay

The agar spot-on lawn assay was performed to screen for anti-*C. difficile* activity. Briefly, 10 µL of culture medium, treated with or without proteinase K, was dropped on the agar lawn of *C. difficile* 630, purchased from the American Type Culture Collection (ATCC). This was followed by incubation at 37 °C for 48 hours in an anaerobic environment. After the incubation time, the inhibitory zone was observed[18]. The inhibitory zone disappeared, indicating that the active compound is a protein or the postbiotics might be composed of bacteriocin or bacteriocin-like inhibitory substances[19].

2. Characterization of bacteriocin-producing *E. faecalis* by genome analysis

2.1 DNA extraction and whole genome sequencing

Genomic DNA of *E. faecalis* PK 1201 was extracted with commercial DNA extraction kits: TIANamp Genomic DNA kit (Tiangen Biotech, Beijing, China). Then, the genomic DNA was sequenced using the BGISEQ-500 (BGI, Beijing, China) to generate 150-bp paired-end reads.

2.2 Genome assembly, annotation, and bioinformatics analysis

The raw sequence reads of the sample were first cleaned and trimmed by Trimmomatic v0.40[20] using default parameters. The trimmed reads were then assembled using spades v3.13.1[21] to generate draft assemblies of all three samples. The assembly quality evaluations were performed by Quast v5.0.2[22]. The genome was then annotated using Prokka v1.12[23] to identify genomic features for use in further downstream analysis. Bacteriocins were predicted using the Bacteriocin Genome mining tool (BAGEL) 4 server[24].

3. Postbiotic preparation

Firstly, a postbiotic was prepared using both bacterial cell lysate and CFS from *E. faecalis* PK 1201.

3.1 Bacterial cell lysate preparation

The sonication-kill cell method was used for the preparation of bacterial cell lysate. Briefly, the overnight culture of *E. faecalis* was centrifuged for 10 minutes at 5,714 g at 4 °C to remove the supernatant and resuspend it in HEPES buffer. Then, the cell suspension was killed by sonication (60% amplitude, pulse 45s, pause 1 min)

for 5 min to receive bacterial cell lysate and utilized as a postbiotic[25].

3.2 LCFS

The culture supernatant of *E. faecalis* from 3.1 was neutralized to pH 6.5 with 1 N NaOH before it was filtered through a 0.22 µm microfilter to eliminate bacterial cells. The CFS was then lyophilized (Vacuum pressure at 0.128 millibars, 21 °C for 24 hours) to obtain LCFS. This was then diluted in 20 mM HEPES buffer to the starter concentration of 500 mg/mL and utilized as a postbiotic.

3.3 Agar spot on lawn assay

Both postbiotics were screened for their antimicrobial activity by agar spot-on lawn assay; as mentioned above, in 1.2. using postbiotics instate of cell suspension. An effective postbiotic was considered when the inhibition zone was presented.

4. Minimum inhibitory concentration (MIC) testing

Only the LCFS of *E. faecalis* PK1201 showing an inhibition zone in agar spot on lawn assay was further used to determine the quantitative antimicrobial activity of postbiotics on *C. difficile*. The MIC of the postbiotics was evaluated using the broth microdilution method: described by CLSI. The postbiotics were prepared in concentrations ranging from 500 to 0 mg/mL, in 96-well plate. Each well was inoculated with 100 µL (1×10^6 CFU/mL) of an overnight culture of *C. difficile* in an anaerobic condition at 37 °C for 48 hours. After 48 hours of incubation, each well was inspected for the presence and absence of turbidity, so as to determine the microorganism's growth. The MIC is the lowest concentration in which no turbidity was detected, implying that postbiotic inhibits *C. difficile* growth. Vancomycin was used as the positive control. Three independent experiments, with duplication samples, were performed.

5. Morphological changes of *C. difficile*

C. difficile's morphological changes were observed by scanning electron microscopy (SEM). Firstly, *C. difficile* $1x10^6$ CFU/mL was cultured in a BHI broth with LCFS at the MICs, then the glass coverslips were added to the mixture and incubated at 37 °C for 24 hours under anaerobic conditions. After this, the glasses were prepared for SEM by washing with PBS then fixed in 2% glutaraldehyde for 2 hours. These glasses were then serial dehydrated in 25%, 50%, 75%, and 100% ethanol for 30 minutes for each concentration, respectively. Specimens were photographed at a magnification ranging from x1,000 to 10,000 under a Scanning Electron Microscope (JEOL CarryScope, JEOL Ltd., Tokyo, Japan) with an accelerating voltage of 20 k[26].

6. Effect of postbiotics on anti-spore germination activity of *C. difficile*

6.1 Spore purification

C. difficile spores were generated by cultivating a single colony in 0.5% yeast extract and 0.1% L-cysteine, which were then incubated anaerobically at 37 °C overnight. The culture was then subculture into BHI agar with 0.1% L-cysteine, and incubation was continued at 37 °C for seven days. After the incubation period, spores were harvested and washed in PBS twice, followed by being suspended in PBS; containing 125 mM Tris, 200 mM EDTA, 0.3 mg/ml proteinase K, and 1% sarcosyl. The suspension was then incubated at 37 °C for two hours. After this, the spore suspension was centrifuged ten times in PBS at 6,500 g for 10 minutes each time. After the last wash, the spores were heated at 60 °C for 20 minutes to kill residual cells. Then, the amount of spores was calculated by plating an aliquot of spore dilution on BHI agar supplemented with 0.1% sodium taurocholate and incubating anaerobically for 48 hours before counting the colony on the plate[27].

6.2 Anti-spore germination test

The purified spores were used to evaluate the anti-spore germination activity of LCFS. The spore suspension, at a final concentration of 5×10^6 spores/mL, was added to the BHI broth supplement with 0.1% taurocholate together with postbiotics at 0.5 or 1xMICs.

These were then incubated anaerobically at 37 °C for 30 minutes. Following incubation, the taurocholate level was stopped by diluting the mixture. The germinated spores were counted by plate for the dilution on BHI agar and compared to total spores, which were determined by plating on BHI agar supplement with 0.1% taurocholate[28]. Assays were performed in triplicate, and the percentage of germination was calculated as:

Percentage of germination = (CFU/mL on BHI plate)/(CFU/mL on BHI agar supplement with 0.1% taurocholate plate) x 100)

7. Safety of LCFS on *Gelleria mellonella*

G. mellonella larvae at the final larval stage (body weight of 200–300 mg) were divided into two groups (10 larvae/group); 1) control (HEPES buffer); 2) LCFS group. The larvae were injected with 10 µL of LCFS to the last left proleg of each larva. Following injection, the larvae were stored in the plate at 37 °C, and the number of killed *G. mellonella* was recorded daily for seven days to analyze the survival rate[29]. This experiment had been approved by the animal ethical committee: animal EC no. Ref.AI004/2022.

8. Statistical analysis

Statistical analyses were performed using GraphPad PRISM software. One-way ANOVA was performed for group difference comparison, and a p-value<0.05 indicated a statistically significant difference. The Kaplan–Meier survival function of Stata software and log-rank test was applied to the *G. mellonella* survival rate analysis.

Results

1. Identification and characterization of bacteriocin-producing *E. faecalis* strains

The probiotic *E. faecalis* PK1201, characterized by a previous study, exhibited both phenotypic and genotypic characteristics as a bacteriocin-producing strain. The anti-*C. difficile* activity attributed to the bacteriocin-like substance was abolished by proteinase K treatment. The whole genomic analysis showed that after genome assembly, the total length of *E. faecalis* PK1201 was 2,678,694 bp. Moreover, the presence of a bacteriocin-encoding gene was explored. There was *enl*A coding for enterolysin A in *E. faecalis* PK1201genome (Table 1).

2. Postbiotic preparation

From two postbiotic preparation methods, after screened activity by agar spot on lawn assay, the presence of an inhibition zone against *C. difficile* is shown in Table 2. From this result, we found that only LCFS presented with an inhibition zone; hence, LCFS was decided to be used in further steps.

3. Minimum inhibitory concentration (MIC) testing

Quantitative investigation of anti-*C. difficile* activity was carried out by evaluating MIC of postbiotics on *C. difficile* 630, using broth microdilution methods. Vancomycin was used as a positive control. The results are presented in Table 3: MIC of postbiotic was 18.2±6.9 mg/mL, while the MIC of vancomycin against *C. difficile* 630 was 0.7± 0.3 µg/mL.

Table 1 Bacteriocins identified in bacterial strains by Bacteriocin Genome mining tool (BAGEL) 4

Strain	Bacteriocin	Scaffold number	Location	Identity (%)
PK1201	Enterolysin A	1	746,986-767,658	100.0

Table 2 Presentation of inhibition zones of postbiotics

Postbiotic	Inhibition zone
Bacterial cell lysate	-
LCFS	Present

LCFS=lyophilized cell free supernatant

Table 3 minimum inhibitory concentration (MIC) of postbiotic on toxigenic *C. difficile* 630

Postbiotic	MIC (mean±S.E.)
LCFS 1201	18.2±6.9 mg/mL
Vancomycin	0.7±0.3 μg/mL

MIC=minimum inhibitory concentration, LCFS=lyophilized cell free supernatant, S.E.=standard error, mg=milligram, μg=microgram, mL=milliliter

4. Morphology changes of *C. difficile*

The morphology changes of *C. difficile* cells were determined using SEM. The SEM results are illustrated in Figure 1. LCFS at the MIC affects *C. difficile* after being treated for 24 hours by shortening the size of the cells compared to the control group, HEPES. Most cells were severely destroyed, and the formation of pores in the cell walls was observed.

5. Anti-spore germination activity

The activity of postbiotics on anti-spore germination is represented in Figure 2. The percentage of spore germination of *C. difficile* 630 was significantly reduced after being treated with LCFS of *E. faecalis* PK1201, at both 0.5x and 1x the MICs, compared to the untreated one indicating the great anti-spore germination activity of this postbiotic.

6. Safety of LCFS on *G. mellonella*

The safety of postbiotics prepared from potential probiotic *E. faecalis* PK1201 was tested using the *G. mellonella* model. All larvae injected with LCFS at the MIC survived within the 7-day period of incubation compared to that of negative control: this indicates the safety of certain LCFS (Figure 3).

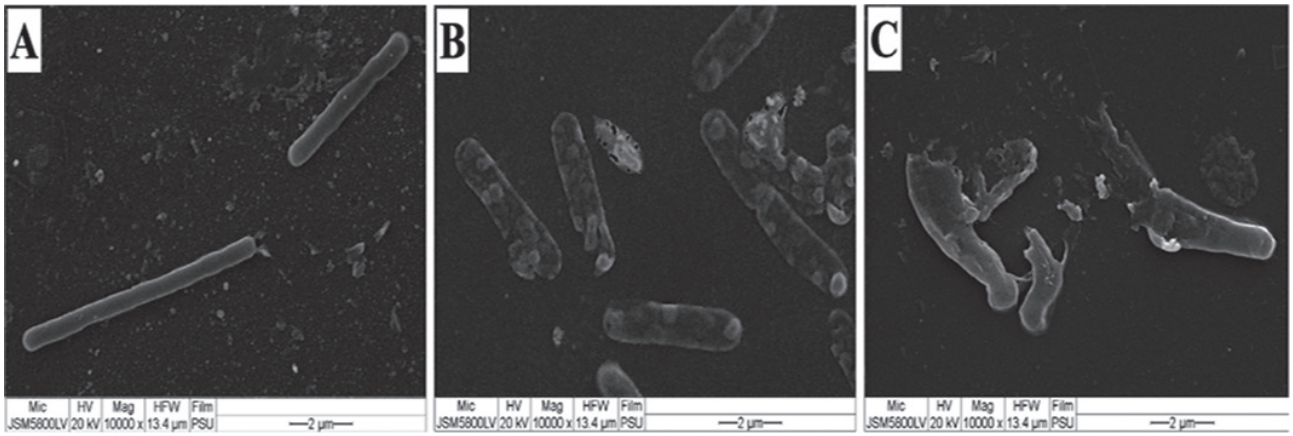

Figure 1 Representative images of *C. difficile* 630 after treatment with lyophilized cell free supernatant (LCFS) of *E. faecalis* PK1201, or vancomycin under SEM. A; HEPES, B; LCFS PK1201, C; vancomycin. The bar represents 2 μm scale.

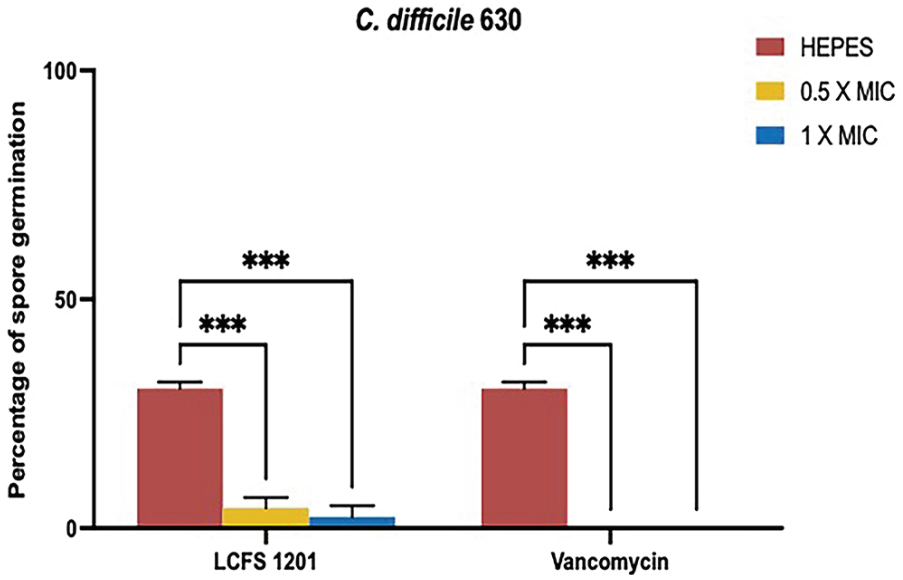

Figure 2 Effect of lyophilized cell free supernatant (LCFS) PK1201 postbiotic and vancomycin on the anti-spore germination activity of *C. difficile* 630. The results are the means of three independent experiments (mean±SEM) ***p-value< 0.001 vs the control group, HEPES

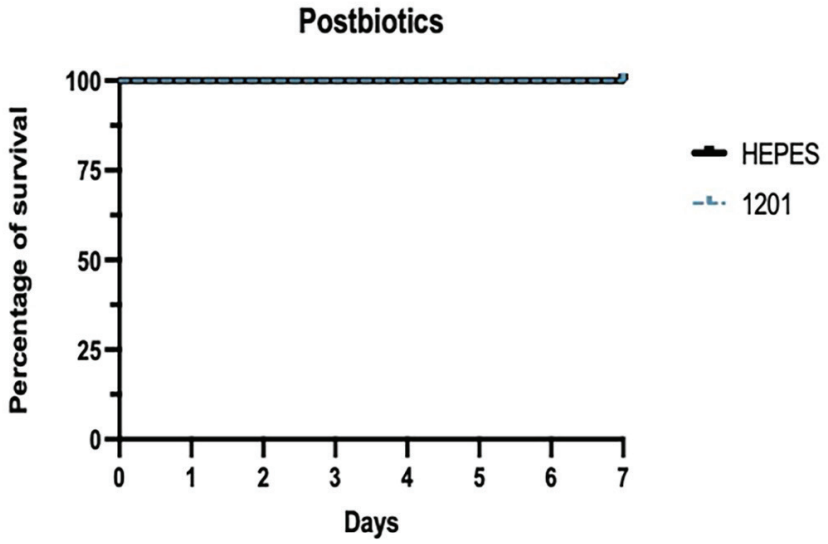

Figure 3 Toxicity of postbiotic lyophilized cell free supernatant (LCFS) PK1201 in *G. mellonella* model at the 1xMIC

Discussion

Antibiotic treatment for CDI is limited, due to the disruption of gut flora that might lead to a high rate of CDI recurrence[30]. Postbiotics have recently gotten a lot of attention for treating bacterial infection diseases, because of their positive effects on the host without the risk of bacteremia, especially in immunocompromised individuals.[11] One exciting finding is the LCFS, postbiotic, established from potential probiotics *E. faecalis* strain PK 1201 that elucidated suppressive effects on *C. difficile* growth and caused cell damage. Another important finding is that postbiotics inhibit spore germination of *C. difficile* spores. Moreover, LCFS showed no acute toxicity in via *G. mellonella* model. These findings suggest a potential alternative strategy for preventing or treating CDI.

Postbiotics can be prepared from both nonviable bacteria components and bacterial metabolism substances that confer a health benefit on the host[31]. Thus, CFS which is prepared from probiotics during the growth of bacteria contains the secreted metabolites; including, antimicrobial substances, and should be considered as postbiotics, and able to be used as an alternative; thereby, replacing live bacteria[32]. Caly et al., 2017, found that *E. faecalis* strain 14, isolated from meconium, was able to inhibit *C. perfringens* by synthesis of enterocin DD14: the bacteriocin which was then purified from the culture supernatant[33]. This work used LCFS produced from the neutralized CFS of bacteriocin-producing *E. faecalis* probiotic strains as postbiotics, and demonstrated the feasibility of using this postbiotic to prevent CDI.

To control CDI, one option is destroying *C. difficile* cells; therefore, postbiotic inhibitory activity was first investigated. The results showed that LCFS inhibited *C. difficile* 630 growth with MIC of 18.2±6.9 mg/mL. Even though the MIC of the postbiotic was higher than that of the positive control, vancomycin, it is possible that the postbiotic contains many molecules that have not been purified. The possible mode of action in cell destruction may possess by bacteriocin, the ribosomally synthesized antimicrobial substances. Previous studies demonstrated that *Enterococcus* spp. produce bacteriocin, including, enterocin A, enterocin AS-48, and enterocins L50A and L50B, that can form pores in the cell membrane of targeted bacteria. This makes them release important organelles that are followed by killing the desired bacterium[34].

In this present study, the activity in anti-*C. difficile* of bacteriocin-like inhibitory substances was destroyed after exposure to the proteolytic enzyme, indicating bacteriocin is the major active component. Furthermore, the genome of the *E. faecalis* strain was analyzed and it was found that the genes encode bacteriocin in the bacteria, including enterolysin A. As a result, these strains may produce bacteriocin. The enterolysin A showed antimicrobial activity against targeted bacteria, by pore formation in the membranes and the cell wall breakdown process, respectively[35]. It is cell wall-degrading proteins that can degrade the targeted cell by hydrolyzing specific peptides of the bacterial cell wall[35]. In this study, postbiotics might kill *C. difficile* cells through pore formation and cell wall disintegration. This was supported by the SEM results that show the morphology changes of *C. difficile* after being treated with postbiotics.

Another strategy that can directly minimize CDI is suppressing germination processes; when, *C. difficile* cells are exposed to specific environments, such as food restriction, quorum sensing and other stressful environments. This commences sporulation to create enough dormant spores to survive under those conditions. Spores are thought to cause recurrence, if they remain in the patient's stomach and germinate into the vegetative after the antibiotic treatment is finished or stopped. This is followed by producing the toxins that cause the primary symptoms of this disease[36]. The spore germination process is induced by primary bile acids, such as cholic acid whice is synthesized in human livers[37]. On the other hand, the bile salt hydrolase (BSH) enzyme, which is found on the cell

surfaces, of many bacteria especially probiotics, catalyzes the deconjugation of primary bile acids to unconjugated bile salts resulting in reduced spore germination[38].

Interestingly, the results of this study found that *E. faecalis* PK1201 postbiotic dramatically inhibited the toxigenic *C. difficile* spore germination process; induced by taurocholic bile salt, compared to that of control. Thus, the anti-spore germination activity might be facilitated by the action of BSH enzymes. Romyasamit et al., 2020, also found that *E. faecalis* PK 1201 probiotics can produce BSH enzymes, which break down bile salt and are further implicated in the decline of *C. difficile* spore development[10]. In this study, the BSH enzymes might secrete out into the CFS used to prepare LCFS that shows anti-spore germination activity.

The effectiveness of postbiotics in *C. difficile* inhibition in vitro led to its possible application use as pharmaceutical product to prevent and treat CDI. However, the safety of postbiotic use is still required. In this study, acute toxicity was observed using the *G. mellonella* model. *G. mellonella* is a popular, primitive in vivo model for toxicity, antimicrobial activity and antivirulence tests[39]. This model was used as an experimental model for the toxicity test of postbiotics against candidiasis[40]. This present study demonstrated that LCFS PK1201 at the concentration of 1xMIC did not experience toxicity effects on G. mellonella comparable to that of the negative control: this indicates the safety of certain LCFS.

The overall results were able to conclude that LCFS from *E. faecalis* PK1201 that produced bacteriocin-like inhibitory substances inhibited *C. difficile* growth and the spore germination processes. LCFS showed no acute toxicity in *G. mellonella* at a concentration tested in this study. Although, the specific mechanism relating to the postbiotic effect in anti-spore germination is still not elucidated, LCFS might be employed as a possible, alternative approach to prevent and treat CDI.

Conclusion

LCFS from bacteriocin-like producing *E. faecalis* had antibacterial action against *C. difficile*, as well as the capacity to inhibit spore germination. In addition, LCFS at the MIC showed no acute toxicity at the tested concentration. It is possible that bacteriocin in LCFS is responsible for its antibacterial efficacy against *C. difficile*. As a result, LCFS might be used as a possible postbiotic against CDI.

Funding sources

The study was funded by the Development and Promotion of Science and Technology Talents Project (DPST), first placement 2588 [grant number 023/2558], through K.S. and P.L.

Conflict of interest

There are no potential conflicts of interest to declare.

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