รายงานวิจัยฉบับสมบูรณ์

โครงการ การแยกและการบ่งชี้สารที่มีฤทธิ์ต่อต้านเชื้อ *Clostridium difficile* จากเชื้อกลุ่ม *Bifidobacterium*

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พฤษภาคม 2559

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(Screening and identification of antimicrobial compound from *Bifidobacterium* with inhibitory activity against *Clostridium difficile*)

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

Abstract

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ชื่อโครงการ: การแยกและการบ่งชี้สารที่มีฤทธิ์ต่อต้านเชื้อ *Clostridium difficile* จากเชื้อกลุ่ม *Bifidobacterium*

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Clostridium difficile is spore forming and opportunistic anaerobic pathogen. It can cause *C. difficile* infection (CDI) by ingestion of spores. This bacterium has also been recognized as a major cause of antibiotic-associated diarrhea (AAD). There are several antibiotics used as treatment option for CDI, however, its use has evident limitations. It has been reported that antibiotic resistance of *C .difficile* continuously increase causing recurrent of CDI. Therefore, due to the adverse effects and limitations of the use of antibiotic for treatment CDI, using of microorganism called probiotics or its antimicrobial substances called bacteriocin as alternative treatment against *Clostridium difficile*-associated diarrhea (CDAD) is becoming interest.

From total 1,400 LAB isolates obtained from calf and piglet feces, there were 674 isolates exhibiting antimicrobial activity against *C. difficile*. Neutralized cell-

free supernatants (NCFS) of 10 isolates (5 from calves, and 5 from piglet) were selected to confirm antimicrobial activity, which showed antimicrobial activity not only against *C. difficile* but also against other Gram-positive and Gram-negative bacteria. Five from ten isolates were identified to be *Bifidobacterium* sp., which were *B. pseudolongum* (C-4-21, C-5-50, C-13-25) and *B. choerinum* (C-9-2, C-14-29). The strain C-14-29 was selected for further investigation based on its broad spectrum of antimicrobial activity. Production of antimicrobial substance from this strain was investigated. It was found that the antimicrobial activity was first detected at logarithm phase of growth (8-12 h) while the highest antimicrobial activity was observed at the stationary phase of growth, and it still remained stable throughout of cultivation period. The maximum relative percentage of inhibition was found to be at 59%. From physical and biochemical properties of antimicrobial substances, it revealed that antimicrobial compounds of the strain was protein, which was heat stable and exhibited wide range of pH stability (pH 3-9). The molecular weight was determined to be 10 kDa. Hence, antimicrobial substances obtained from this isolate may potential be a proteinaceous bacteriocin-like inhibitory substances, which can potentially be useful as an antimicrobial agent for treatment of *C. difficile* infection.

Keywords : *Bifidobacterium*, *Clostridium difficile*, Probiotics, Bacteriocin

INTRODUCTION

Clostridium difficile, an opportunistic anaerobic pathogen, is commonly found in the large intestine of healthy individuals. It has been isolated from different sources including environments, animal samples, human samples and foods [1]. When conditions become unsuitable such as deprivation of nutrients, exposure to acidic environment or high temperatures, *C. difficile* enters a dormant state and f5orms a resistant endospore. These spores contain characteristics with highly resistant to heat, radiation, chemicals, acidic or basic conditions, and oxygen. The remaining of *C. difficile* in spores form allows them to spread to other hosts via fecal-oral route. Therefore, the containing of spore form makes this bacterium overcomes other bacteria that it can survive within the acidic condition in stomach and then germinate and colonize to cause diseases[2]. Incidence and virulence of *C. difficile* infection (CDI) continuously increase over the past 20 years around the world. This bacterium has been recognized as a major cause of antibiotic-associated diarrhea (AAD) and pseudomembranous colitis (PMC) [3]. Although antibiotic therapy is used as the primary treatment option for CDI, its use has evident limitations. It has been reported that antibiotic resistance of *C .difficile* continuously increase causing recurrent of CDI. Moreover, the use of almost antibacterial drugs such as clindamycin, cephalosporins, and fluoroquinolones has been reported to cause AAD[4], which can suppress restoration and significantly decrease levels of normal gastrointestinal flora leading to the colonization and proliferation of *C. difficile* throughout in intestinal tract. Therefore, due to the adverse effects and limitations of the use of antibiotic for treatment CDI, using of microorganism called

probiotics or its antimicrobial substances called bacteriocin become to be interesting and have been investigated for primary and secondary prophylaxis against *Clostridium difficile*-associated diarrhoea (CDAD).

Nowadays, the studies of normal gastrointestinal flora and gut ecosystem expanded for their important role and mechanism to exert their effects on the gut of host. The gastrointestinal tract contains both friendly and pathogenic bacteria that exist in a complex symbiosis. For maintenance an optimal normal flora balance, friendly bacteria are required to oppose against pathogenic bacteria. In recent years, research and public interest in microorganism called probiotic have been risen[5]. The World Health Organization (WHO) defines “probiotics” as “living microorganisms that when administered in adequate amounts, confer a health benefit on the host”[6]. Most of lactic acid bacteria (LAB) such as *Lactococcus*, *Pediococcus*, *Lactobacillus*, *Enterococcus*, and *Bifidobacterium* were used as probiotics supplemented in several products[7]. The ideal of probiotics is they can remain viable in the intestine and adhere to the intestinal epithelium conferring a significant health benefit. The action mechanisms of probiotics are not clearly understood but the possible mechanisms include a competition for nutrients and competition for colonization sites of pathogens, immunomodulation, and production of antimicrobial substances called bacteriocin[8-11]. Among these, bacteriocin plays the most interesting role in pathogenic defense.

Bacteriocin is extracellular proteins secreted during growth involving in microbial defense system, which can inhibit the invasion of other microorganisms[12]. It has been reported that bacteriocin can also inhibit growth of similar or closely-related bacterial strains that they are typically considered to be narrow spectrum antibiotics[13,14]. Bacteriocin produced by LAB can be subdivided into 4 classes based on the

classification of Riley MA *et al*[15]: class I, lantibiotics, contain small heat-resistant peptides that consist of modified amino acids; class II, non-lantibiotics, contain small heat-stable peptides; class III is large heat-labile proteins, and class IV comprises of protein containing lipid and carbohydrate. The term of bacteriocin-like inhibitory substance (BLIS) has been referred to antimicrobial compound that do not fit to any class of bacteriocin and tend to have broad spectrum of activity[14]. To date, there were some of the bacteriocins such as nisin and pediocin, bacteriocins produced by *L. lactis* and *P. pentosaceus*, respectively, exhibited a potential properties to be used as naturally biopreservative agents in food industry which proven as generally recognized as safe (GRAS) status[16]. Recently, *Bifidobacterium*, a new probiotic candidate was expanded studied for its ability to possess probiotic properties including the production of antimicrobial substances that will be used in many applications. Nowadays, in-depth studies of bacteriocin produced by *Bifidobacterium* have been undertaken to get detailed information on their biochemical properties, chemical structures and mechanisms of action.

Bifidobacterium is an important Gram-positive normal gastrointestinal flora bacterium that mostly found in healthy breast-fed animals[17]. It is classified as one of the LAB members based on its ability to produce organic acid during growth. Moreover, it exhibits health beneficial effects that are potentially be used as probiotics in several applications. Among the many probiotic traits that have been attributed to bifidobacteria, the most interest is some of bifidobacteria are known to produce bacteriocin[18]. The first study on the production of bacteriocin by bifidobacteria has been reported by Anand *et al*[19] that it exhibited without any antimicrobial activity loss after refrigerating for 3 months. Meghrous *et al*[20] found that the inhibitory activity of bifilong, bacteriocin

produced from *B. longum*, inhibited the growth of both some Gram-positive and Gram-negative bacteria. Moreover, Saleh *et al*[21] revealed that bacteriocins produced by *B. lactis* Bb-12 and *B. longum* Bb-46 showed the strong inhibitory activity against *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella enterica* Thyphimurium. Currently, the study of bacteriocins from bifidobacteria including their production, spectrum of activity, physical and biochemical properties, and mechanisms of action are significantly interested. Although many bacteriocins produced by bifidobacteria showing antimicrobial activity were continuously reported, in-depth information data is still needed to be explored.

Therefore, antimicrobial compound produced by bifidobacteria exhibiting antimicrobial activity against *C. difficile* was studied in this research. Physical and biochemical properties of antimicrobial compound were also characterized. The knowledge from this research will fill more contents of information about bacteriocin from bifidobacteria. Moreover, we highlighted the potential of bacteriocin produced from the selected *Bifidobacterium* strain for use as antibacterial agent toward a range of bacterial pathogens. Hence, this bacteriocin could be potentially applied in many aspects such as food industrial, medical and pharmaceutical applications.

OBJECTIVES

1. To isolate *Bifidobacterium* with antimicrobial activity against *Clostridium difficile*

2. To characterize and partial purify antimicrobial substance produced by selected *Bifidobacterium* isolates

MATERIAL AND METHOD

Samples collection

Fresh fecal samples were collected from piglets and calves. Samples were kept in tightly closed tube containing 7 ml of transfer medium (1% peptone water supplemented with 0.25% L-cystein). Transfer medium was covered with mineral oil to prevent the dissolution of oxygen and cap was wrapped with parafilm. The sample tubes were then kept in anaerobic jar with an anaerobic pack during transport to the laboratory.

Isolation of lactic acid bacteria

Fecal samples were suspended and made 10-fold serial dilutions in 0.85% NaCl. Then, the appropriated serial dilutions (10^{-4}-10^{-5}) were spread on selective medium for lactic acid bacteria (de Man, Rogosa and Sharpe, MRS) and on selective medium for bifidobacteria (BSM) supplemented with 0.25% L-cystein and 0.75% (v/v) thioglycolate. After 2 days of incubation at 37ºC under anaerobic condition, fifty colonies of lactic acid bacteria grown on MRS agar and 12 bacterial colonies grown on BSM agar with different morphologies in same sample were selected and replicated onto new MRS agar as master plate and onto assay plate for further investigation. The assay plates were used for screening of bacterial isolates with antimicrobial activity.

Clostridium difficile culture

Clostridium difficile strain 630, 32g58 and R20291 were used as tested pathogenic bacteria (kindly provided by Prof. Nigel Minton, University of Nottingham).

The glycerol stock of *C. difficile* was picked and grown on Brain Heart Infusion (BHI) 1.5% agar supplemented with 0.1% (w/v) taurocholate (BHITA). After 2 days incubation under anaerobic condition, one or two colonies of *C. difficile* were then grown in BHI broth supplemented with 0.1% (w/v) taurocholate and 0.75% (v/v) thioglycolate. After overnight incubation, *C. difficile* culture was transferred to new BHI broth at 1-2% (v/v) of final concentration and continually cultivated to reach mid-logarithm phase of growth for 8-12 hours before antimicrobial assay.

Screening of *Bifidobacterium sp.* containing antimicrobial activity

The inhibitory effect of -*Bifidobacterium* isolates was investigated against *C. difficile* strain 630. Vegetative *C. difficile* culture with optical density of 1.0 at 600 nm was mixed with melt 0.85% agar of reinforced clostridial medium (RCM) supplemented with 0.1% (w/v) taurocholate and 0.75% (v/v) thioglycolate. Homogenized mixture was poured onto the assay plates as stated above. After incubation for 2 days at 37ºC under anaerobic condition, the inhibition zone was observed around the colonies indicating antimicrobial activity against *C. difficile*.

Bacterial identification and detection of bacteriocin-coding gene

Bacterial identification

To distinguish between Gram-positive and Gram-negative groups by coloring, all selected isolates were tested by gram staining.

To test the ability to motile of selected isolates, motility assay was performed using stabbing method in motility test medium. Bacterial motility is observed by a diffuse zone of growth spreading from the line of inoculation.

The catalase test is the method for detection of the enzyme catalase in bacteria. The catalase enzyme can neutralize the bactericidal effects of hydrogen peroxide. Hydrogen peroxide (H_2O_2) is breakdown into water and oxygen ($2H_2O_2$ + Catalase $\longrightarrow$ $2H_2O + O_2$) by catalase enzyme. The formation of bubbles was observed in positive strain. The catalase activity was tested using slide (drop) method. Colonies of bacteria was picked and placed onto glass slide. Hydrogen peroxide at 15% (for anaerobic bacteria) was then applied onto bacteria. The formation of bubbles was then observed.

Identification of *Bifidobacterium* isolates by polymerase chain reaction (PCR)

To confirm the selected isolates, genus-specific primers for *Bifidobacterium* were used. Amplification of partial 16s rDNA of *Bifidobacterium* was performed by PCR technique. PCR primers (Bif 164 and Bif 662) and conditions were modified from Kok RG [22]. The nucleotide sequences of primers were shown in Table…. The size of the PCR product from the primers is expected to be 523 bp. The 20 µl reaction contained 0.3µM of each primer, 150µM dNTP, 1X PCR buffer, 1.5mM $MgCl_2$ and 2.5 U of *Taq* polymerase. The thermocycle program used in genus-specific PCR consisted of the following time and temperature profile: (i) 95ºC for 5 min; (ii) 30 cycles of 1 min at 95ºC, 45s at 55ºC and 1 min at 72 ºC, and (iii) 10 min at 72ºC. DNA derived from

Bifidobacterium animalis was used as positive control and distilled water was used as negative control. After amplification, the PCR products were detected through electrophoresis in 1% agarose gel.

For the other isolates, DNA solutions were used as template for PCR with the universal primers, which are ULF500 and ULR500 (Table 1). These primers were designed by the presence of conserved sequence in 16s rDNA of general bacteria. These primers generate PCR fragment with the expected size of 450-500 bp[23]. The PCR mixture and conditions were used as described above. After amplification, the nucleotide sequenced of PCR products were investigated by DNA sequencing. The nucleotide sequences were compared with database using BLAST for bacterial identification.

Detection of known bacteriocin-coding gene

To explore that the selected isolates possess known bacteriocin-coding genes, which were pediocin- (PedF and PedR), nisin- (NisRF and NisRR) and enterocin- (EntAF and EntAR) gene (kindly provided by Prof. Watanalai Panbangred) [24] were used in this experiment. The sequences of those primers are shown in Table 1 and PCR conditions were used as described above. The PCR mixture contained final concentration of 0.4μM of primer each, 200 μM dNTP each, 3 mM $MgCl_2$, 2.5 U *Taq* polymerase, and 1X PCR buffer with final volume at 20 μl. Size of PCR products for detection of nisin-, enterocin-, and pediocin-coding genes are expected to be 608, 412, and 332 bp, respectively [24]. Bacterium containing of each bacteriocin-coding gene was used as positive control of PCR reaction. *Lactococcus lactis* was used as positive

control of nisin gene. *Enterococcus feacium* was used as positive control of enterocin gene. Also, *Pediococcus pentosaceus* was used as positive control of pediocin gene. After amplification, the PCR products were detected through electrophoresis in 1% agarose gel.

Table 1 PCR primers used in this study

Name	**Nucleotide sequence(5'→3')**	**Optimal annealing temp (ºC)**	**PCR amplicon size (bp)**	**Ref.**
***Bifidobacterium* genus specific primer**				
Bif164	GGGTGGTAATGCCGGATG	55	523	[22]
Bif662	CCACCGTTACACCGGGAA			
Bacterial universal primer				
ULF500	GCCTAACACTGCAAGTCGA	58	450-500	[23]
ULR500	CGTATTACCGCGGCTGCTGG			
Nisin primer				
NisRF	CTATGAAGTTGCGACGCATCA	55	608	[24]
NisRR	CATGCCACTGATACCCAAGT			
Enterocin primer				
EntAF	GGGTACCACTCATAGTGGAA	55	412	[24]
EntAR	CCAGCAGTTCTTCCAATTTCA			
Pediocin primer				
PedF	GGTAAGGCTACCACTTGCAT	55	332	[24]
PedR	CTACTAACGCTTGGCTGGCA			

Antimicrobial activity assay

Supernatant preparation of selected isolates

Selected isolates were cultivated on MRS agar supplemented with 0.75% (v/v) thioglycolate. After 2 days of incubation, bacterial colonies were then grown in MRS broth supplemented with 0.75% (v/v) thioglycolate. The bacteria were grown to reach early stationary phase of growth for 16 hours under anaerobic condition. Supernatants were collected after centrifugation. The supernatant was then filtrated through 0.45 µm membrane filter. In order to confirm antimicrobial activity of supernatant of selected *Bifidobacterium* which was not resulting from the organic acid production, the pH of supernatant was adjusted to be neutral (~pH7) using 5M NaOH. Neutralized cell-free supernatant (NCFS) then was kept at -20ºC until use.

Cultivation of bacterial indicator strains

The aerobic bacteria (*B. cereus* ATCC14579, *B. subtilis*, *E. coli* ETEC, *Pseudomonas aeruginosa*, *E. coli* ATCC1175*,* MRSA 1302, *Pectobacter carotovorum, Serratia marcescens, Salmonella* Typhi ATCC23566, *Shigella sonnei*, *Staphylococcus aureus* ATCC25923, *Citrobacter freundii*, *Enterobacter cloacea* were used as indicator bacteria. They were grown on Luria-Bertani (LB) agar at 37ºC under aerobic condition. After incubation for overnight, one or two colonies of tested bacteria were grown in LB broth at 37ºC with vigorously shake at 200 rpm for 4-6 hours before antimicrobial assay.

Four closely-related bacteria that are *E. faecium*, *L.lactis*, *L.plantarum*, and *P. pentosaceus* were routinely grown in MRS medium at 37ºC under anaerobic condition. After overnight incubation, one or two colonies of these bacteria were then

cultured in MRS broth at 37ºC in anaerobic condition for 16 hours before antimicrobial assay.

Antimicrobial activity assay of neutralized cell-free supernatant (NCFS) of selected *Bifidobacterium* isolates

Selected isolates showing inhibitory effect against *C. difficile* 630 were selected to confirm their inhibitory effects. NCFS were tested against three strains of *C. difficile* (630, QCD-32g58, and R20291) as tested pathogenic bacteria. These isolates were grown in MRS broth supplemented with 0.75% (v/v) thioglycolate for 16 hours. Supernatants were collected and adjusted the pH to be neutral (pH~7) using 5mM NaOH.

Agar well diffusion method was used to test inhibitory activity of supernatant from selected *Bifidobacterium* isolates. Six hours of *C. difficile* culture with optical density of 1.0 at 600 nm were mixed with melt 1% BHI agar supplemented with 0.1% (w/v) taurocholate and 0.75% (v/v) thioglycolate. Homogeneous mixture was poured onto Petri dish plate. After solidification, wells were made using cork borer with 6 mm of diameter. The assay was performed by adding 100 µl of NCFS of selected LAB isolates into well. Plates were incubated for overnight at 37ºC under anaerobic condition. Clear zone of inhibition around well (recorded in millimeters) was observed and measured.

Kinetics production of antimicrobial substance of *Bifidobacterium* strain C-14-29

Kinetics production of antimicrobial substance by selected *Bifidobacterium* isolates, C-14-29, was performed in MRS broth supplemented with 0.05% (v/v) L-cystein

(MRSC). The 1% of overnight culture was cultivated in MRS broth. The growth of bacteria, production of organic acid, and antimicrobial substance production were followed during 36 hours of incubation under anaerobic condition at 37ºC. Sample was taken every 2 hours in order to determine bacterial growth. Growth of *Bifidobacterium* was evaluated by measuring the turbidity of culture medium at OD 600 nm using spectrophotometer. Organic acid production was measured by detection of the reduction of pH of culture medium using pH meter. For determination of antimicrobial activity from antimicrobial substance, culture supernatants of each time intervals were collected and then neutralized to be neutral (pH7) prior assay. The inhibitory effect of neutralized cell-free supernatant (NCFS) was determined by measuring turbidity of *C. difficile* in culture broth after treatment as described below.

An antimicrobial activity of NCFS of each time intervals was determined against *C. difficile* strain 630. Eighty microlitters of NCFS were added into wells of a 96 well-flat bottomed polystyrene microplate. Each well was inoculated with equal volume (80μl) of 6-8 hours *C. difficile* strain 630 culture. Microplate was then incubated at 37°C under anaerobic condition for 20 hours and the optical density (OD) at 595±5 nm was then measured using a spectrophotometer. The MRS broth medium was used as control. The growth of *C. difficile* of control and treated *C. difficile* with NCFS were compared and expressed as percent relative of inhibitory effect as antimicrobial activity unit. The relative percentage of inhibition was calculated from the antimicrobial activity from NCFS compared with that of control (MRS broth).

$$\text{Relative percentage inhibition} = 100 - \left(\frac{100 \times OD_s}{OD_c}\right)$$

The OD_c referred to optical density of *C. diffcile* treated with MRS broth (control) and OD_s referred to optical density of *C. difficile* treated with NCFS from LAB (sample).

Physical and biochemical characterization of antimicrobial substance

Physical and biochemical properties of antimicrobial substance produced by *Bifidobacterium* strain C-14-29 were characterized into four aspects including temperature stability, pH stability, and effect of enzymatic treatment.

Temperature stability

To test whether antimicrobial compound presenting in NCFS of *Bifidobacterium* isolates that showed inhibitory effect against *C. difficile* is heat stable or heat labile, NCFS of *Bifidobacterium* strain C-14-29 was incubated at 65, 80 and 100ºC for 10, 20 and 30 min. After treatment, treated NCFS was tested for antimicrobial activity against *C. difficile* using turbidometric method as described above.

pH stability

The pH stability was determined by adjusting the pH of NCFS to be 3, 5, 7, and 9. The pH of NCFS was adjusted using 5M NaOH or 5M HCL to reach expected pH and then incubated at room temperature for 2 hours. After incubation, pH of NCFS was then adjusted to be neutral (pH~7) prior antimicrobial activity assay. The

antimicrobial activity against *C. difficile* was test using turbidometric method as described above.

Effect of enzymatic treatment

To confirm antimicrobial substance presenting in NCFS is the proteinaceous substance, protease K was used. The NCFS was incubated with protease K with final concentration at 1 mg/ml at 37ºC. After incubation for 2 hours, the proteolytic activity was stopped by heating at 65ºC for 15 min. After treatment, treated NCFS was tested for antimicrobial activity against *C. difficile* using turbidometric method.

Size determination of antimicrobial substance

The molecular weight of antimicrobial substance presenting in culture media of *Bifidobacterium* strain C-14-29 was measured using SDS-PAGE technique. The NCFS of C-14-29 was partial purified using molecular weight cut-off membrane (MWCO) concentrator. Concentrator membrane pore size cut-off of 3 kDa and 30 kDa was used. The protein fractions were concentrated to obtain 50 times concentration from the beginning volume. First, membrane was rinsed with sterilized distillated deionized water for 3 times. Then NCFS of *Bifidobacterium* C-14-29 was concentrated using MWCO at 30 kDa. Fraction containing proteins size less than 30 kDa that passed through the membrane was then subjected through concentrator with MWCO at 3 kDa. After processes, all fractions were collected and subjected for SDS-PAGE for determination of antimicrobial protein size.

Glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (glycine-SDS-PAGE) was performed using 4%, 3%C for stacking gel and 12%, 6%C for resolving gel with 1 mm thickness. Concentrated fractions were mixed with 5X sample buffer (5% SDS, 50% glycerol, 0.225M Tris-HCl pH 6.8, 0.25M dithiothreitol, 0.05% Bromophenol blue) and then boiled for 5 min. Running buffer (0.025M Tris, 0.192M glycine and 0.1% (w/v) SDS) was loaded into both upper and lower chamber. Samples were loaded and run at 60V constant. Prestained protein ladder (10-170 kDa) (Vivantis) was used as molecular weight mass standard marker. After electrophoresis, gel was separated into 2 parts and then performed gel staining and in-gel inhibitory assay as described above.

After electrophoresis, antimicrobial substance in acrylamide gel was localized using *P. pentosaceus* and *C. difficile* as tested bacterium in in-gel inhibitory assay. Gel was soaked in renaturation solution containing 200mM acetate buffer pH6, 1% triton X-100 and 5% NaCl at a final concentration for overnight at 37ºC with gently shaking at 60-80 rpm. Gel was then washed with sterilized distilled water and placed on thin layer of media containing 0.75% agar (MRS for *P. pentosaceus* and BHI supplemented with taurocholate for *C. difficile*). The strains were cultured for 6-8 hours and then mixed with media with 0.75% agar. Homogenized mixture was then overlaid onto the gel. After overnight incubation, clear zone of inhibition was observed on the protein band containing antimicrobial activity. Size of antimicrobial protein was then determined by comparing with that of molecular weight protein marker.

RESULTS

Sample collection and isolation of lactic acid bacteria (LAB)

Twenty eight fresh fecal samples were collected from 2 different sources that were piglets and calves. Twelve fresh feces from suckling piglet were collected from swine farm located in Chonburi province. Sixteen fresh calf feces were collected from natural farm at Nan province (kindly collected by Asst. Prof. Buntika Bucher). The total number of LAB isolates, which were randomly selected were 2,020 isolates (Table 2).

Table 2. Numbers of LAB isolates showing antimicrobial activity toward *C. difficile* strain 630

Source of sample	**Location**	**No. of sample**	**No. of isolated LAB**	**No. of LAB isolates containing antimicrobial activity**
Piglet	Chonburi	12	600	54
Calf	Nan	16	800	620
Total		28	1,400	674

Screening of *Bifidobacterium* containing antimicrobial activity

All isolated were then tested antimicrobial effect toward *C. difficile* strain 630. Among 1,400 isolates, there were 674 LAB isolates exhibiting antimicrobial activity against *C. difficile* strain 630, which formed zone of inhibition around their colonies. The width of inhibition zone was ranged from 0.5 cm to 2 cm (Fig. 1). From the result, all of

the isolates from calf and piglet feces exhibited antimicrobial activity (77.5%, and 9%, respectively), toward *C. difficile* (Table 2).

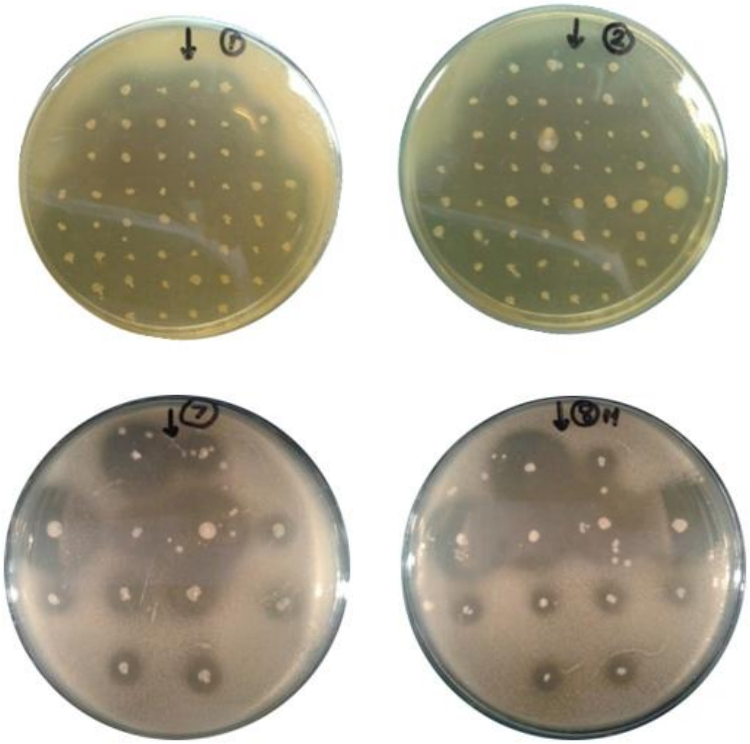


Figure 1 The representative of isolates containing antimicrobial activity against *C. difficile* strain 630

Ten isolates showing antimicrobial activity against *C. difficile* strain 630 were selected for further experiments shown in Table 2. Their names were designated as following by this schematic. First digit, **P**, represented to source of sample (P: Piglet feces and C: Calf feces). Second 2 digits, **10**, referred to sample number (sample number 10) and the last 2 digits, **40**, represented to isolate number (isolate number 40).

Table 2. List of selected isolates used in this study

Name	Source of sample	Collection place	Location
P-7-5	Piglet feces (P)	Swine farm	Chonburi province
P-7-24			
P-10-9			
P-10-22			
P-10-40			
C-4-21	Calf feces (C)	Natural farm	Nan province
C-5-50			
C-9-2			
C-13-25			
C-14-29			

Antimicrobial activity assay of neutralized cell-free supernatant (NCFS) of selected isolates

In order to confirm antimicrobial activity of selected LAB, which was not resulted from the reduction of pH in supernatant, the supernatant was then neutralized using 5M NaOH prior performing antimicrobial activity assay. All selected isolates were tested antimicrobial activity using their NCFS against 3 strains of *C. difficile* (630, QCD-32g58, and R20291). Clear zone of inhibition (mm) around the well was observed and measured. It was found that antimicrobial activity of NCFS from selected isolates was varied in tested *C. difficile* strains and bacterial isolates (Fig. 2). The average diameter of inhibition zone, including well diameter (6 mm), toward three strains of *C. difficile* was to be 9.4±0.4 mm, 9.3±0.3 mm and 10.4±0.6 mm for strain 630, QCD-32g58 and R20291, respectively. Therefore, when comparing antimicrobial activity of all isolates against each strain of *C. difficile*, there was no significantly different in the inhibitory effect among LAB isolates. Also, it was found that there was no significantly difference

in activity of each strain of *C. difficile* within each isolate. However, from the result, it could be summarized that all NCFS of selected isolates exhibited antimicrobial activity against all 3 strains of *C. difficile*, which do not resulting from the reduction of pH in culture supernatants. Therefore, it was indicated that the antimicrobial activity of selected isolates was may be due to the production of antimicrobial compound(s) represented in NCFS.

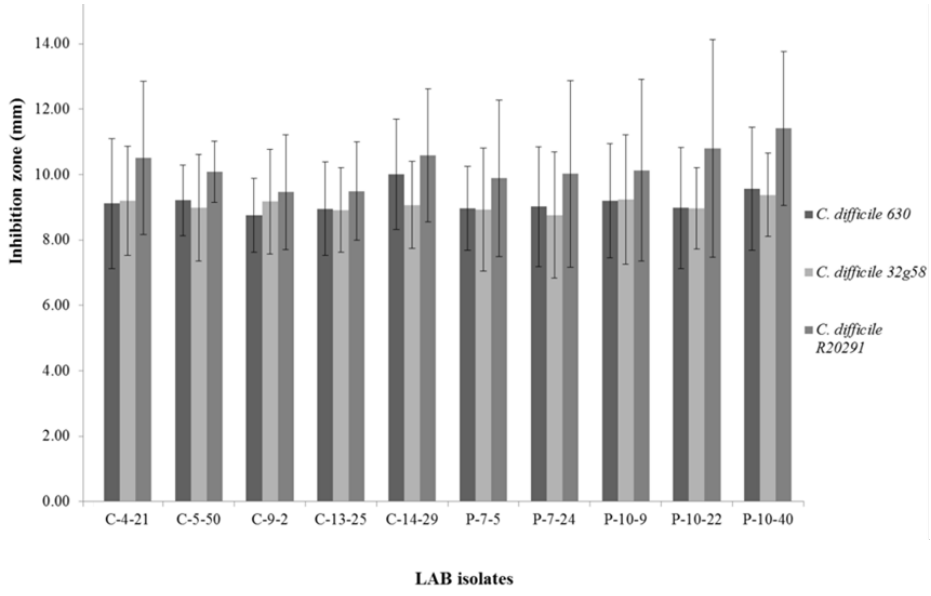


Figure 2. Inhibition zone (mm) of NCFS from 10 selected isolates showing antimicrobial activity against *C. difficile* strain 630 (■), *C. difficile* strain QCD-32g58 (■) and *C. difficile* strain R20291 (■) using agar well diffusion method.

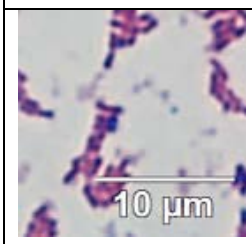
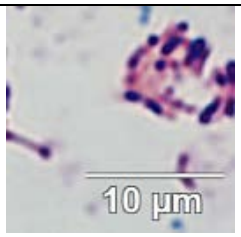
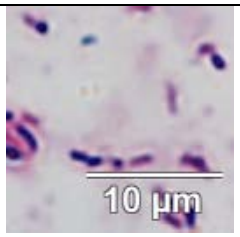
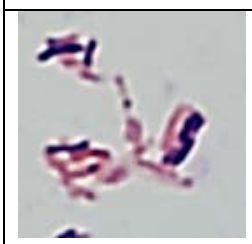
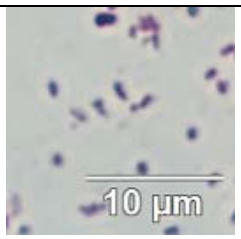
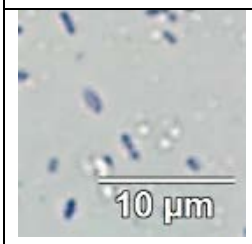
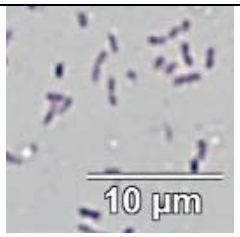
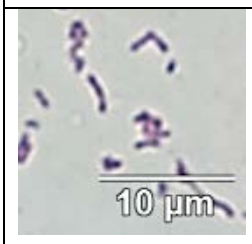
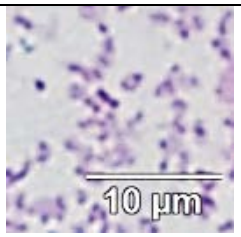
Bacterial identification

The bacterial identification by phenotypic characteristics was done by Gram staining, motility assay and catalase activity test. It was found that most of the selected bacteria were coccibacilli or bacilli. Cell shape of selected isolates under light microscope was shown in Table 3. Moreover, motility assay was performed in order to test the motile ability of selected bacteria. It was found that no diffuse zone of growth

spreading from the line of inoculation was observed in all 10 selected isolates. From the result, it indicated that 10 selected LAB isolates were non-motile bacteria. In addition, catalase activity assay was performed in order to detect the production of catalase, which acts as a cellular detoxify enzyme neutralizing bactericidal effect of H_2O_2. Result showed that no catalase activity (bubble formation) was observed in all 10 selected isolates indicating catalase negative in all selected isolate strains Table 4.

Moreover, all 10 selected isolates were identified using the genus-specific primer for bifidobacteria. The primers Bif164 and Bif662 amplified the fragment between region V2 and region V4 of 16s rDNA. The result showed that 5 out of 10 selected isolates generated the expected size of PCR amplicon at 523 bp (Fig. 3) indicating that the bacteria were identified as bacteria belonging to the genus *Bifidobacterium*.

Table 3. Cell shape of 10 isolates under light microscope

Image	Isolate	Cell shape	Image	Isolate	Cell shape
	C-4-21	Rod		C-5-50	Rod
	C-9-2	Rod		C-13-25	Rod
	C-14-29	Rod		P-7-5	Short-rod
	P-7-24	Rod		P-10-9	Short-rod
	P-10-22	Rod		P-10-40	Short-rod

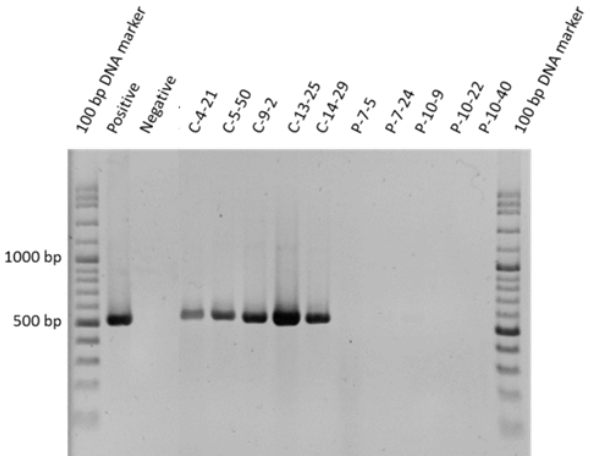


Figure 3. Visualized PCR product of isolates amplified using *Bifidobacterium* genus-specific primer.

In the other hand, the rest of isolates that were not belonging to genus *Bifidobacterium* were then amplified with universal primer (ULF500 and ULR500). After recovery of the PCR product from agarose gel, nucleotides of all selected isolates were then sequenced in order to species identification. After BLAST search, five isolates using genus-specific primer were identified as bacteria belonged to genus *Bifidobacterium*, which *Bifidobacterium pseudolongum* (3 isolates) and *Bifidobacterium choerinum* (2 isolates). The result indicated that this genus-specific primer was accurately specification primer for identification of bacteria in genus *Bifidobacterium*. In addition, it was found that all selected isolated from piglet feces were identified as *Lactobacillus reuteri* with 98-100% identity (Table 4).

Table 4. Bacterial identification of 18 selected LAB isolates

Primer used in sequencing	Bacterial identification	Isolates	Query cover	% identity	Charateristics	
					Motility	Catalase activity
Genus-specific primer for *Bifidobacterium* (Bif164, Bif662)	*Bifidobacterium pseudolongum*	C-4-21	96%	100	-	-
	Bifidobacterium pseudolongum	C-5-50	78%	99	-	-
	Bifidobacterium pseudolongum	C-13-25	100%	100	-	-
	Bifidobacterium choerinum	C-9-2	97%	99	-	-
	Bifidobacterium choerinum	C-14-29	97%	100	-	-
Universal primer (ULF500, ULR500)	*Lactobacillus reuteri*	P-7-5	100%	99		
	Lactobacillus reuteri	P-7-24	100%	99		
	Lactobacillus reuteri	P-10-9	99%	98		
	Lactobacillus reuteri	P-10-22	97%	100		
	Lactobacillus reuteri	P-10-40	97%	99		

Determination of antimicrobial activity spectrum

To study the spectrum of antimicrobial activity of NCFS from selected isolates, 10 NCFSs were tested for antimicrobial activity toward Gram-positive and Gram-negative bacteria. It was found that among 17 tested bacteria (13 aerobic tested bacteria and 4 closely-related LAB), 5 tested bacteria that were *B. cereus* ATCC14579, *B. subtilis*, *E. coli* ETEC, *P. pentosaceus*, and *P. aeruginosa* were inhibited their growth by NCFS of some isolates (Table 5). From the results, *P. pentosaceus* and *E. coli* ETEC

were the most sensitive tested bacteria, which were inhibited by 13 LAB isolate NCFS from 18 isolate NCFS (72.2%) following by *P. aeruginosa* (44.4%), *B. cereus* ATCC14579 (33.3%), and *B. subtilis* (33.3%). Moreover, the result revealed that the most effective NCFS, which showed the broadest spectrum of antimicrobial activity, was obtained by 4 isolates were I-8-2, P-7-5, P-7-24, and P-10-9. They showed antimicrobial effect toward 4 strains of tested bacteria. Eight isolates, which were I-8-6, I-8-9, I-8-12, I-2-47, I-5-4, C-14-29, P-10-22, and P-10-40 exhibited antimicrobial activity toward 3 tested bacteria. It was also found that NCFS of most of isolates contained antimicrobial activity toward at least a strain of tested bacteria, except isolate C-4-21 and C-13-25 that antimicrobial effect were not detected. Therefore, from the results, it could be summarized that the antimicrobial activity of NCFS from all 10 isolates were not resulted from the low pH caused by organic acid production but it might be due to the presence of some antimicrobial substance presenting in NCFS.

Table 5. Antimicrobial activity of NCFS from18 selected LAB against tested Gram positive and Gram negative bacteria

Tested bacteria	Selected isolates										
	C-4-21	C-5-50	C-9-2	C-13-25	C-14-29	P-7-5	P-7-24	P-10-9	P-10-22	P-10-40	**Total**
L. plantarum	-	-	-	-	-	-	-	-	-	-	**0**
L. lactis	-	-	-	-	-	-	-	-	-	-	**0**
E. faecium	-	-	-	-	-	-	-	-	-	-	**0**
P. pentosaceus	-	+	+	-	+	+	+	+	-	-	**6**
B. cereus ATCC14579	-	-	-	-	-	+	+	+	+	+	**5**
B. subtilis	-	-	-	-	+	-	-	-	-	-	**1**
S. aureus ATCC25923	-	-	-	-	-	-	-	-	-	-	**0**
MRSA 1302	-	-	-	-	-	-	-	-	-	-	**0**
C. freundii	-	-	-	-	-	-	-	-	-	-	**0**
E. cloacea	-	-	-	-	-	-	-	-	-	-	**0**
E. coli ATCC1175	-	-	-	-	-	-	-	-	-	-	**0**
E. coli ETEC	-	-	-	-	+	+	+	+	+	+	**6**
P. carotovorum	-	-	-	-	-	-	-	-	-	-	**0**
P. aeruginosa	-	-	-	-	-	+	+	+	+	+	**5**
S. marcescens	-	-	-	-	-	-	-	-	-	-	**0**
S. typhi ATCC23566	-	-	-	-	-	-	-	-	-	-	**0**
S. sonnei	-	-	-	-	-	-	-	-	-	-	**0**
Total	**0**	**1**	**1**	**0**	**3**	**4**	**4**	**4**	**3**	**3**	

+ referred to antimicrobial activity and - referred to antimicrobial activity was not detected.

Detection of known gene encoding bacteriocin

To detect the known bacteriocin genes, which were nisin, enterocin and pediocin, the specific primer of those genes were used to amplify with the selected isolates. The result showed that there was no any known bacteriocin genes were detected in selected isolates (data not shown). It could be indicated that the selected isolates might harbor a novel or other bacteriocin genes

Production kinetics of antimicrobial substance from selected *Bifidobacterium*

Among 5 *Bifidobacterium* isolates, C-14-29 were selected for further studies on the basis of their broad spectrum of antimicrobial activity. The production of antimicrobial substance of this strain was investigated. The result found that this strain grew satisfactorily in MRSC broth at 37ºC under anaerobic condition, the maximum growth was reached at 12 h with optical density at 600 nm approximately to be 5.8 (Fig. 4). It showed a short lag phase with 2-4 hours. Moreover, it was observed that it finished logarithm phase and then entered the stationary phase of growth at 12-14 hours of incubation period. After incubation for 36 hours, there was no reduction growth was observed in this isolates.

Acid production shown to be growth-associated where the pH of culture medium was dramatically decreased during logarithm phase from 6 to 4 and pH was remained stable at pH 4 during the stationary phase.

To study production kinetics of antimicrobial substance, supernatant of each time interval from 0 to 36 hours was collected and neutralized before performing antimicrobial activity assay against *C. difficile* strain 630. It was found that NCFS of C14-

29 contained antimicrobial substance, which can inhibit growth of tested bacterium. The antimicrobial activity was first detected at logarithm phase (8-12 h) while the highest antimicrobial activity was observed at early stationary phase and it remained stable until the end of experimental period. The relative percentage of inhibition was calculated from the antimicrobial activity from NCFS compared with that of control (MRS broth).

$$\text{Relative percentage inhibition} = 100 - \left(\frac{100 \times OD_s}{OD_c}\right)$$

The OD_c referred to optical density of *C. diffcile* treated with MRS broth (control) and OD_s referred to optical density of *C. difficile* treated with NCFS from LAB (sample).

Result revealed that the highest relative percentage of inhibition was observed to be 59% at 22 hours (Fig. 4).

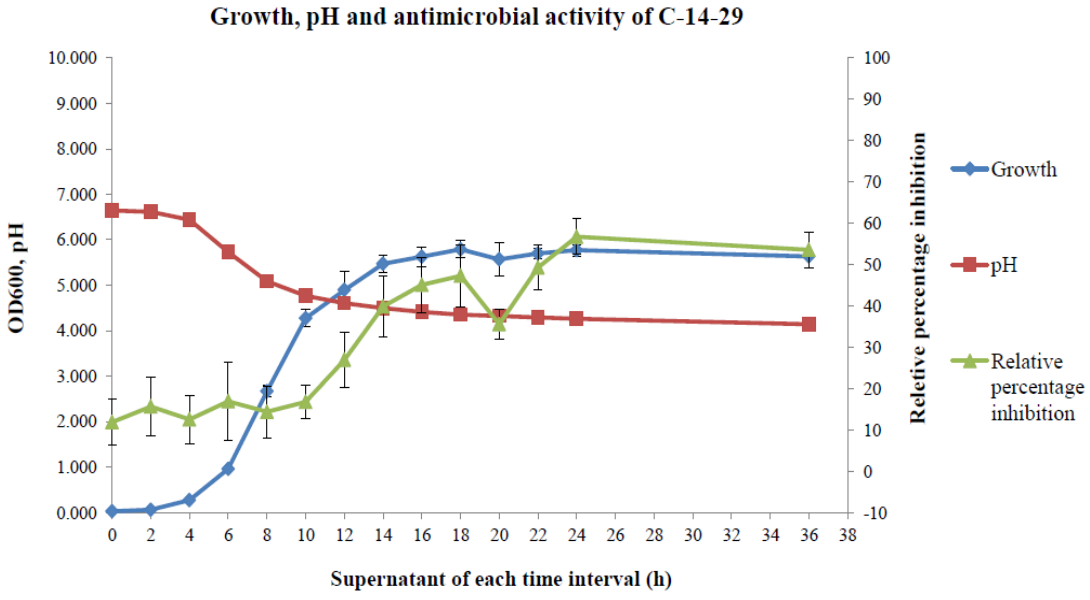


Figure 4. Growth of *Bifidobacterium* isolates C-14-29 (♦), acid production (■) and antimicrobial activity (▲).

Physical and biochemical characterization of antimicrobial substance

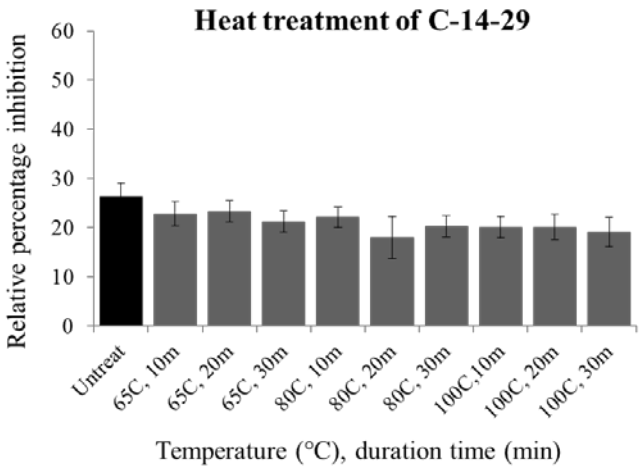
Temperature and pH stability

To test whether antimicrobial substance is heat stable or heat sensitive, NCFS of C14-29 was heated at various temperatures. The heat and unheated NCFS were further tested for antimicrobial activity against *C. difficile* by monitoring turbidity of culture medium. It was found that the antimicrobial activity of NCFS was also not significantly different after heating at 65, 80, and 100^{o}C for 30 min comparing with that of control. Interestingly, the result showed that the antimicrobial activity of heated NCFS still observed with the relative percentage of inhibition at 23% when NCFS was heated at 100^{o}C for 30 min (Fig. 5a). These results indicated that the antimicrobial substance in NCFS was heat stable substance.

Moreover, results also showed that NCFS from C-14-29 exhibited antimicrobial activity toward *C. difficile* strain 630 throughout the pH range of 3 to 9. Results revealed that antimicrobial activity of NCFS from isolate C-14-29 was slightly increased from untreated around 12% and 10% after treatment with pH 3 and 5, respectively (Fig. 5b). It could be summarized that the NCFS of C-14-29 was no any antimicrobial activity lost after treatment with the range of pH 3 to 9.

From the result, it indicated that antimicrobial substance presenting in NCFS of C14-29 was heat and pH stable that it still showed antimicrobial activity toward *C. difficile* strain 630 in all treated conditions.

a)



b)

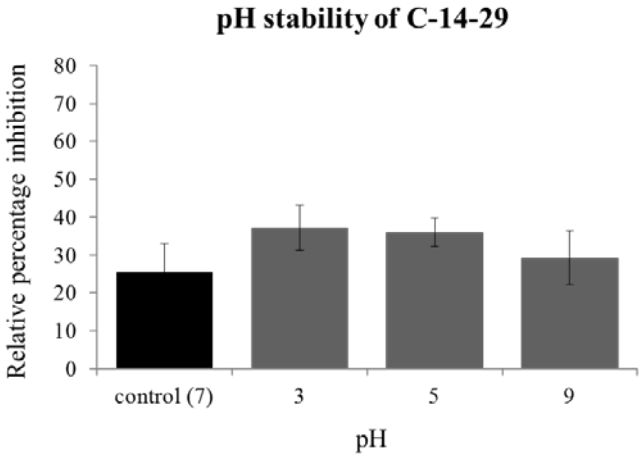


Figure 5 Antimicrobial activity against *C. difficile* strain 630 of NCFS after heat treatment (a) and pH treatment (b) from *Bifidobacterium* isolate C-14-29.

Effect of enzymatic treatment

To test the proteinaceous of antimicrobial substance presenting in NCFS of selected LAB, protease K was used. After treatment, treated NCFS with protease K was then tested for antimicrobial activity against *C. difficile* using turbidometric method. It was found that the antimicrobial activity of NCFS from *Bifidobacterium* isolate C-14-29 was significantly decreased its antimicrobial activity (P <0.05) after treatment with

protease K (Fig. 6). These results indicated that the antimicrobial substance presenting in NCFS this bifidobacteria isolates might be proteinaceous substance, which was heat and pH stable at tested conditions.

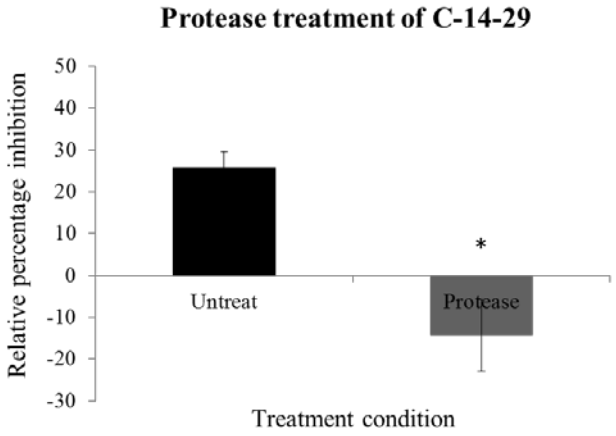


Figure 6. Antimicrobial activity against *C. difficile* strain 630 of NCFS after treatment with protease K from *Bifidobacterium* isolate C-14-29.

Size determination of antimicrobial substance by glycine SDS-PAGE

The partial purified concentrated NCFS was subjected to SDS-PAGE analysis for size determination. After electrophoresis, antimicrobial substance in polyacrylamide gel was localized using *P. pentosaceus* and *C. difficile* as tested bacterium. It was found that various bands of protein were clearly observed in different size shown in Fig. 7. Moreover, the result showed the inhibition zone at approximate 10 kDa of NCFS from C-14-29 isolates exhibiting growth inhibition against *P. pentosaceus*. It can be concluded that the size of antimicrobial substance in the NCFS of C-14-29 was approximated at 10 kDa.

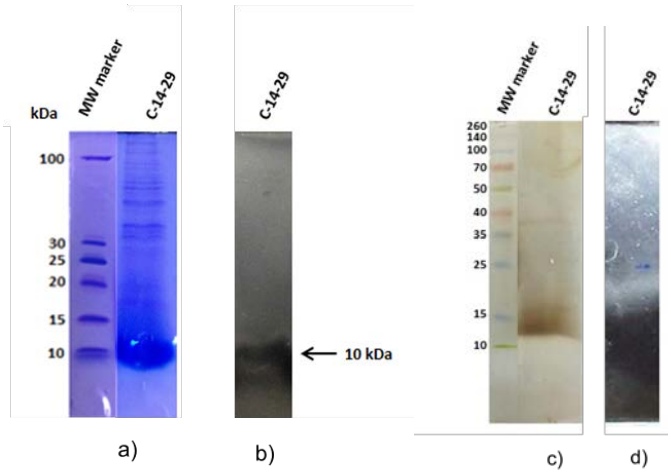


Figure 7. SDS-PAGE of concentrated NCFS of *Bifidobacterium* isolate C-14-29; (a) gel stained with Coomassie Blue G250, (b) gel overlaid with *P. pentosaceus***,** (c) gel stained with silver staining, (d) gel overlaid with *C. difficile*

DISCUSSION

In 1978, *C. difficile* was recognized as an opportunistic pathogenic bacterium for antibiotic-associated diarrhea. CDI has dramatically risen since 1990s with alarming increase since 2000. Moreover, the financial charge associated with *C. difficile* infection is substantial for hospital. The cost per case ranges from approximately 2,500-4,000 USD in the UK and almost 14,000 USD for a case of recurrent CDI with a total charge worldwide in the tens of billions. Therefore, the development of prevention and treatment methods has been expanded study. Although antibiotics therapy has been served as the first option for treatment patient with CDI, it has been reported that the use of antibiotics contains an increasing rate of treatment failure and recurrent of infection [25]. Since there were some adverse evidences of the use of antibiotics therapy for *Clostridium difficile*-associated diarrhea (CDAD) emerging, other alternative treatments were studied and investigated. One of the most interesting treatments is the use of living microorganism called probiotics, which contain health benefits to the host when ingested in sufficient numbers[6]. The administration of appropriate probiotic strains could be used as a strategy to protect and cure patients from CDAD by restoration of normal flora[26,27]. The possible mechanisms that they exert the positively effects to the host include the competition for nutrients, competition for epithelial or mucosal adherence of pathogens, stimulation of immunomodulation, decreasing pH of environment via the production of organic acids, and the production of antibacterial compounds. Ribosomally synthesized protein from LAB containing antimicrobial activity called bacteriocin has raised a growing interest for their applications in food industry and medical health as biopreservative or biotherapy agents.

In this research, we aim to isolate bacteriocin producing *Bifidobacterium*, which contains antimicrobial activity against *C. difficile*, fecal samples from beast-fed animals were collected. Many studies revealed that bifidobacteria belonging to LAB group are one of the most dominant normal microbiota of human gastrointestinal tract where they represent to 3-7% of the population of adults and reach up to 91% of population in newborns[28]. First, *Bifidobacterium sp*. isolates, which exhibited antimicrobial activity were screened from animal and human faces. There were 48% of total isolates showing anti-*C. difficile* activity. This antagonistic activity of selected isolates might be due to the production of organic acids, which can kill vegetative cell of tested bacterium. From the fact that all LAB including *Bifidobacterium* can produce organic acids such as lactic acid or acetic acid, and their antimicrobial activity has been usually reported to involve in the production of organic acids[29-31]. In the study of Fernando *et al* (2006) revealed that all 27 bifidobacteria produced supernatant containing pH at 4.1 and 5.0 which were able to produce strain-dependent growth inhibition of *C. difficile*[32]. After using HPLC for analysis the organic acid compositions presenting in supernatant, it was found that all strains produce acetic and lactic acid. This finding can be indicated that lactic or acetic acid was involved in the antibacterial activity. Also, the majority of antimicrobial activity toward food-borne pathogens of supernatant from all 11 LAB strains lost after neutralization under the study of Uraipan *et al* in 2015[33]. Moreover, this finding was in agreement with the study of Cheikhyoussef *et al* (2007) that the antagonistic effect against *E. coli* and *B. cereus* of the supernatants from *B. bifidum* and *B. adolescentis* were found to be significantly decreased after neutralizing. It may explain that their antibacterial effect is mainly related to the presence of organic acids[34].

Among 10 isolates, there was 5 isolates identified as *Bifidobacterium* spp. with characteristics of rod-shaped, non-motile and catalase negative. These all bacteria belonging to genus *Bifidobacterium* were selected to for the further experiments. To confirm their antibacterial activity was due to some antimicrobial compounds presenting in supernatant, the NCFS of from selected isolates was tested against *C. difficile* by agar well diffusion method. It was found that all 5 bifidobacteria showed antagonistic activity toward tested bacterium with different level of activity. This result was in agreement with the study of Yoon-Jong Lee *et al* (2003) that 5 NCFS obtained from bifidobacterial isolates exhibited antagonistic activity against *C. difficile* with more than 15% of inhibition rate[35]. Cheikhyoussef *et al* (2007) revealed that after neutralization of supernatant obtained from *B. infantis* and *B. longum*, it continued active against indicator strains[34]. Also, it was found under study of Gibson and Wang (1994) that the antagonistic effect of *B. infantis* toward *C. perfringens* and *E. coli* was not necessarily involved with the production of acids [36]. In addition, the observation of Zouhir *et al* (2011) indicated that the inhibitory effect of 2 *Bifidobacterium* spp. RBL 68 and 85 is due to the production of bacteriocin-like inhibitory substance presented in their spent culture[37]. On the other hand, Ibrahim and Bezorovainy (1993) reported that the antimicrobial effect of spent culture from bifidobacteria against tested bacteria was completely based on the production of acids[38]. However, from our findings could be summarized that the antibacterial effect of NCFS from 7 *Bifidobacterium* isolates was may be due to the effect of antimicrobial compound(s).

To study spectrum of antimicrobial activity of NCFS from *Bifidobacterium* isolates, only 4 bacteria (*B. cereus* ATCC14579, *B. subtilis*, *E. coli* ETEC, and *P. pentosaceus*) from 17 tested bacteria were inhibited their growth by NCFS from

bifidobacteria. - It was observed that isolate C-14-29 had the broadest spectrum of inhibitory activities towards 3 tested bacteria, while the other 2 isolates (C-5-50 and C-9-2) exhibited antimicrobial effect toward 1 strain of tested bacterium. From the results, it is an interesting phenomenon that most of antimicrobial substances produced by Gram-positive bacteria have no inhibitory activity toward Gram-negative bacteria[39] while the strains under our studied showed antibacterial against *E. coli* ETEC. However, their spectrum of antimicrobial activities of bifidobacteria isolates under our studied were found to be in agreement with the study of Cheikhyoussef *et al* (2007). Their study found that supernatant obtained from 4 strains of bifidobacteria significantly decreased growth of *E. coli* strains and *B. cereus* [34]. Also, the inhibitory test of 6 strains of *B. thermophilum* showed active not only against *E. coli* but also toward *L. nomocytogens*, *S. aureus* and *S.* Typhimurium[40]. Collado *et al* (2004) reported that 8 strains of bifidobacteria had the greatest inhibitory effects on *L. innocua* ATCC33090 and *L. lactis* subsp. *cremoris* MG1363, but they had the same activity as our finding that they were not active against *E. faecium* [41]. Moreover, many researches revealed the antimicrobial activity of *Bifidobacterium* strains toward many indicator strains such as *S. sonnei*, *S. flexneri*, *S. paratyphi*, *H. pylori*, and *C. sporogenes*[31,33,42,43]. The spectrum and level of antimicrobial activity of bifidobacteria found to be strain dependent, which can inhibit the growth of both Gram-positive and Gram-negative bacteria. However, no study observed the inhibitory effect of antimicrobial compound obtained from bifidobacterial strains towards yeast (*Sacchromyces cerevisiae*)[44,45]. From all findings, it can be confirmed that the production of bacteriocin-like inhibitory substances is a key factor in the inhibition of spoilage and pathogenic bacteria in *in vitro*. The ability of antimicrobial substance to not only prohibited the growth of Gram-positive but also active against Gram-negative

bacteria, which cause food spoilage and food-borne diseases, make it to be interesting in food applications.

From 5 *Bifidobacterium* isolates, isolates C-14-29 was then selected for further experiments on the basis of broad spectrum of antimicrobial activity. Its growth, production of organic acid and production of antimicrobial substance were kinetically measured. The acid production were shown to be growth-associated that the pH of culture medium was continuously decreased from 6 to 4. This result was in agreement with the study of Zouhir *et al* (2011) that *Bifidobacterium* spp. RBL 68 and 85 decreased the pH of culture medium from 6.5 to 4 related to their growth[37]. In case of the production of antimicrobial substance, it was found that the first antimicrobial activity was detected in the culture supernatant collected at logarithm phase of bifidobacterial growth (8-12 h). From the observation, the highest production of antimicrobial substance was observed at the late exponential phase of growth where the cell reached the maximum density. Same growth rate, acid production and production of antimicrobial substance were found in *B. animalis* subsp. *animalis* CICC 6165 under study of J. Pei *et al* (2013). Its antimicrobial activity was detected at the beginning of logarithm phase while the maximum activity was recorded after 20-30 h of growth. Moreover, the pH of culture medium continuously decreased from 6.5 to 3.5[46]. Also, the production of bacteriocin-like inhibitory substance from *B. infantis* BCRC 14602 reached the highest activity at 1600 AU/ml after 16-20 h of incubation like the highest cell density was detected after 18 h of incubation [45]. This finding indicated that the pH-independent inhibitory factor active toward indicator strain was produced, released or accumulated in sufficient amounts at exponential phase and reached the maximum antimicrobial activity then still stable at late exponential phase. There was some opposite evidence reported

by Cheikhyoussef *et al* (2009) and J. Pei *et al* (2013) that the reduction of antagonistic activity was observed in supernatant containing bificin C6165 after incubation for 33 h of *B. animalis* subsp. *animalis* CICC 6165, and 50% reduction of bacteriocin-like inhibitory substance titre was detected at 24 h of incubation of *B. infantis* BCRC 14602[45,46]. Moreover, the decreasing of antimicrobial activity of supernatant collected from *Bifidobacterium* strain after the late logarithm phase (10-12 h) was observed under studied of Collado *et al* (2005) [41]. On the other hand, there was no reducing of antagonistic activity phenomenon detected under our studied in supernatant obtained from bifidobacterial strains until the end of experimental period.

It was worth noting that antimicrobial substance produced from *Bifidobacterium* isolate C-14-29 was completely inactivated after treatment with proteinase K. Concentrated NCFS exhibited antimicrobial activity against indicator strains visualized at protein band size of 10 kDa. This result indicated that the antimicrobial substances presenting in NCFS of *Bifidobacterium* isolates C-14-29 was proteinaceous in nature, which can be called bacteriocin-like inhibitory substance (BLIS). Under study of Collado *et al* (2005), it was found that antimicrobial activity of NCFS of *Bifidobacterium* BIR-0304 was destroyed by all tested protease except proteinase K. The combination of NCFS and proteinase K (up to 500 μg/ml) resulted in wider inhibition zone (P <0.05). Although the addition only proteinase K at high concentration caused the antimicrobial activity toward indicator strain, its activity was less than mixed with the NCFS[43]. It indicated that the antimicrobial substance presenting in NCFS and proteinase K worked synergistic to produce inhibitory effect. Most of bacteriocins or BLIS derived from *Bifidobacterium* sp. contained molecular weight less than 10 kDa, which was degraded and loss its antimicrobial activity after treatment with proteinase K.

Bifidocin B, a bacteriocin derived from *B. bifidum* NCFB1454 contained molecular mass at 4.4 kDa, which was stable at high temperature and pH but it loss antagonistic activity when tested condition presented of proteinase K[47]. This phenomenon was also found in the study of bifidin I, a strong antilisterial bacteriocin with 2.8 kDa obtained from *B. infantis* BCRC14602 that its inhibitory effect loss after treatment with protease and proteinase K but still active in the presenting of α-amylase, lysozyme and lipase[44]. Moreover, a heat stable bificin C6165 contained 3.3 kDa loss its antimicrobial activity against indicator strain after adding proteinase K, pepsin, a chymotrypsin, papain, or trypsin. However, its activity was stable after treatment catalase, lipase and α-amylase[46].

The physical and biochemical characteristics of BLIS were determined by heat and pH treatments following by tested their antagonistic activity against *C. difficile* strain 630. It was found that treatment of bifidobacterial NCFS at either 65°C for 30 min or 80°C for 30 min did not elicit any loss of antagonistic activity. Interestingly, the antimicrobial activity of NCFS from C-14-29 was considerably stable after heating at 100°C for 30 min. In a similar fashion of heat stability of BLIS from bifidobacteria, antibacterial compounds produced by 6 strains of *B. thermophilum* showed resistant ability after heat treatment at 100°C for 10 min[40]. In addition, sample had antibacterial activity over a wide range of pH at 3, 5, 7, and 9 and was slightly risen at acid pH. This result had the same activity as the study of Collado *et al* (2004) and Collado *et al* (2005) that antibacterial effect of bifidobacterial strains was slightly increased under acidic conditions[41,43]. Our results were also in agreement of the finding of Cheikhyoussef *et al* (2007) who reported that the inhibitory substance from *B infantis* and *B. longum* was

stable in a wide pH range from 4 to 10 and 5 to 9, respectively[34]. Our BLIS showed the greater stability from bifidin, a bacteriocin obtained from *B. bifidum* NCDC 1452 that exhibited antimicrobial effect after heating at 100°C for 30 min but its activity was stable in only acid conditions at pH 4.8 to 5.5[19]. However, our observations had some conflict evidences from the study of J. Pei *et al* (2013) who has studied on heat and pH stability of bifidin C6165. They found that activity of bifidin C6165 was completely destroyed after heating at 100°C for 30 min and also inactive at pH 7.5[46]. Our finding data were also different from that of the pH stability studied on bifidobacterial BLIS that their BLIS were only active at pH range from 2 to 6. Nevertheless, their BLIS activities were maintained after autoclaving at 121°C for 15 min[37]. In the study of Cheikhyoussef *et al* revealed that the bifidin I obtained from *B. infantis* BCRC 14602 was still active against indicator strain after heating at 121°C for 15 min and also stable in a wide pH range from 4 to 10 [44]. The greater pH and heat stability of antimicrobial substance from our strains was also found in bifidocin B obtained from *B. bifidum* NCFB 1454 that its antagonistic activity was stable in both acid and base conditions from pH 2 to 12 and still worked after autoclaving[47,48]. Our findings showed that the growth-inhibiting factor produced by selected bifidobacteria under our studied could be identified as heat and pH stable bacteriocin-like inhibitory substance. Due to many industrial process procedures involve a heating step and high acidity condition can cause the reduction or loss of antimicrobial ability, the heat and pH stability of bacteriocin or BLIS produced from *Bifidobacterium* isolates can exhibit antagonistic activity toward spoilage and pathogenic bacteria and would be very beneficial for food or pharmaceutical application.

CONCLUSION

From total 1,400 LAB isolates obtained from calf and piglet feces, there were 674 isolates exhibiting antimicrobial activity against *C. difficile*. The isolates obtained from calf feces and piglet feces showed 77% and 9% of antagonistic activity toward tested bacterium, respectively. Among them, 10 isolates were selected based on size of inhibition zone for further experiments. To confirm antimicrobial activity of selected isolates, which was from antimicrobial substance(s) presenting in spent culture medium, their neutralized cell-free supernatants (NCFS) were used not only to test against *C. difficile* but also test toward both in other Gram-positive and Gram-negative bacteria. The spectrum of antimicrobial activity of selected isolates was found to be strain-dependent. However, all selected isolates showed antagonistic activity against all 3 tested strains of *C. difficile* (630, QCD-32g58 and R20291). *Bifidobacterium choerinum* (NN-14-29) was selected based on broad spectrum of antimicrobial activity for study of potential production of antimicrobial substance(s). It showed antimicrobial activity toward *B. subtilis*, *P. pentosaceus* and *E. coli* ETEC. Production of antimicrobial substance from this strain was investigated. It was found that the antimicrobial activity was first detected at logarithm phase of growth (8-12 h) while the highest antimicrobial activity was observed at the stationary phase of growth, and it still remained stable throughout of cultivation period. The maximum relative percentage of inhibition was found to be at 59%. Moreover, under the study of physical and biochemical properties of antimicrobial substances, it revealed that antimicrobial compounds obtained from selected bifidobacteria contained molecular weight at 10 kDa. They were extremely thermostable (100°C, 30 min) and exhibited wide range of pH stability (pH 3-9). It was suggested that antimicrobial substances obtained from this isolate may potential be a proteinaceous

bacteriocin-like inhibitory substances, which can potentially be useful as an antimicrobial agent for treatment of *C. difficile* infection.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

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