# Identification and characterisation of potential bacteriocins with biological activity against *Clostridium difficile* from environmental samples

**Sungchan Kim**

**BSc BDS**

**Thesis submitted to the University of Nottingham for the degree of Master of Research (MRes), June 2017**

***Clostridium* Research Group**
**Centre for Biomolecular Sciences**
**School of Life Sciences**
**University of Nottingham**

# Abbreviations

| | |
|---|---|
| AIDS | Advanced immune-deficiency syndrome |
| BHI | Brain Heart Infusion |
| BHIS | Brain Heart Infusion Supplemented |
| CCG | Clinical Commissioning Groups |
| CD | *Clostridium difficile* |
| CDAD | *Clostridium difficile* associated diarrhoea |
| CDI | *Clostridium difficile* infection |
| *C.difficile* | *Clostridium difficile* |
| *C. perfringens* | *Clostridium perfrigens* |
| CFS | Cell Free Saturation |
| CRG | *Clostridium* research group |
| *E. coli* | *Escherichia coli* |
| EDTA | Ethylenediaminetetraacetic acid |
| GRAS | Genetically recognised as safe bacteria |
| h | Hour |
| KDa | Kilo-Dalton |
| LAB | Lactic acid bateria |
| Mcc | Microcin |
| MES | 2-(N-morpholino) ethanesulfonic acid |
| MIC | Minimum Inhibition Concentration |
| Min | Minutes |
| mM | Millimolar |
| μM | Micromolar |
| nM | Nanomolar |
| pM | Picomolar |
| MRSA | Methicillin-resistant *Staphylococcus aureus* |
| OD | Optical density |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| RPM | Rotations per minute |
| rRNA | Ribosomal ribonucleic acid |
| SDS | Sodium dodecyl sulphate |
| *S.pneumoniae* | *Streptococcus pneumoniae* |
| Tris | 2-amino-2-(hydroxymethy)-1,3-propandiol |
| VRE | Vancomycin-resistant enterococci |

# Table of Contents

**Abstract** ..... **5**

**Acknowledgements** ..... **6**

**1. Introduction** ..... **7**

1.1. Overview ..... 8

1.2. Bacteriocins ..... 9

1.2.1. Classification of bacteriocins ..... 10

1.2.2. Mode of action of bacteriocins ..... 12

1.3. Applications of bacteriocins ..... 15

1.4. *Clostridium difficile* ..... 18

1.5. Implications of *C. difficile* infection and the need for novel treatments ..... 20

1.6. Bacteriocins as novel antimicrobials against *C. difficile* ..... 22

**2. Methods** ..... **23**

2.1. General chemicals, reagents and buffers ..... 24

2.2. Bacterial strains and media ..... 24

2.2.1. *Clostridium difficile* strains ..... 24

2.2.2. Active environmental isolates obtained and used ..... 24

2.2.3. Media used ..... 25

2.3. Screening environmental samples ..... 26

2.3.1. Isolating strains from environmental samples ..... 26

2.3.2. Screening ..... 26

2.4. Identification of environmental isolates ..... 27

2.4.1. Gram staining and microscopy ..... 27

2.4.2. Quantification of DNA, 16S rRNA PCR and Sequencing ..... 27

2.4.3. Tests for *Enterococcus* ..... 29

2.4.4. DNA extraction and Whole genome sequencing ..... 30

2.5. Growth curves ..... 31

2.5.1. Filtrate from environmental isolate S2 and its effect on CD630 ..... 31

2.5.2. Dilutions of filtrate of isolate S2 and its effect on CD630 ..... 31

2.6. Bacteriocin assay ..... 32

2.6.1. Spot assay using CFS of isolate S2 ..... 32

2.6.2. Concentrating the CFS of isolate S2 ..... 32

2.6.3. Induction with Mitomycin C/Hydrogen peroxide/co-culture with *E. hirae* ..... 32

2.7. Quantification of bacteriocin activity ..... 33
2.8. Analysis of nature of CFS of isolate S2 ..... 33
2.8.1. Effect of storage and temperature and storage time on CFS and its activity ..... 33
2.8.2. Effect of proteases on CFS and its activity ..... 33
2.8.3. Analysis of the protein profile of CFS by SDS-PAGE ..... 34
**3. Results and discussion ..... 35**
3.1. Isolation of a pool of environmental isolates active against *Clostridium difficile* ..... 36
3.1.1. Eight out of 12 active environmental isolates exhibited activity against more than three out of five clinically relevant *C. difficile* strains ..... 37
3.2. Isolate S2 exhibits slower rate of growth than other isolates and CD630 ..... 40
3.3. Identification of active environmental isolates indicated variety of different bacterial species showing inhibition of growth of *C.difficle* strains ..... 41
3.3.1. Results of Gram staining ..... 41
3.3.2. Results of 16s rRNA sequencing ..... 43
3.3.3. Series of tests confirmed that isolate S2 possibly belongs to *Enterococcus* ..... 45
3.3.4. Results of DNA extraction and whole genome sequencing ..... 47
3.4. Inhibition of growth of CD630 by CFS from isolate S2 ..... 49
3.4.1. Filtrate obtained from growing medium of isolate S2 in its stationary phase inhibited growth of both CD630 and isolate S2 ..... 49
3.4.2. Reduction in the activity of CFS from isolate S2 when incubated with proteases ..... 53
3.5. Bacteriocin assay using CFS of isolate S2 ..... 54
3.5.1. Concentrated CFS of isolate S2 exhibited inhibition of growth of *C. difficile* strains ..... 54
3.5.2. Concentrated CFS from isolate S2 induced with Mitomycin C, hydrogen peroxide, co-culture also exhibited zone of inhibition of growth on *C. difficle* ..... 55
3.5.3. CFS of isolate S2 is active against CD630 in lower concentrations ..... 57
3.5.4. Action of proteases inhibited on the activity of filtrate of isolate S2 on CD630 ..... 58
3.5.5. Activity of CFS of isolate S2 against CD630 is dependent on temperature and storage duration ..... 59
3.5.6. CFS of isolate S2 had many protein molecules of different sizes ..... 61
**4. Conclusion and further works ..... 63**
**5. References ..... 66**

# Abstract

Recently, the incidence of *Clostridium difficile* infection (CDI) has been decreasing but still presents a real threat as one of the common causes of nosocomial infection. It represents a real challenge in healthcare settings as one of the main causes of CDI is the use of commonly prescribed broad spectrum antibiotics. Effective infection control along with limiting the use of broad spectrum antibiotics can play their part in reducing CDI but fundamental need for an alternative to antibiotic treatment regime for CDI need to be addressed. Narrow spectrum, antimicrobial peptide bacteriocins can offer a viable alternative to antibiotics.

This research was designed to isolate and identify novel bacterocins from environmental samples that are active against *C. difficile* by a screening of heat treated, and non-heat treated sewage and faecal samples. 12 environmental isolates were found to have an inhibitory effect on indicator strain *C. difficile* strain CD630 out of which 8 environmental isolates (S2, S3, S6, S7, S8, S9, S10 and S11) exhibited inhibitory effect on growth of at least 3 of 5 clinically relevant *C. difficile* strains such as EK28, CD630, DH196, DH478 and R20291. Greatest activity was observed on isolate S2 on all five *C. difficile* strains. Growth curve analysis of all environmental isolates active against *C. difficile* revealed that isolate S2 showed significantly slower growth rate compare to other isolates that had growth rates similar to or faster than CD630. Gram staining and 16s rRNA sequencing revealed most of the environmental isolates obtained active against *C. difficile* belonged to the *Enterococcus* group (S2, S3, S4, S6, S7 and S8) whereas others (S9, S10 and S11) were different species of *Clostridium* group. Other investigations to determine the identity of isolate S2 confirmed isolate S2 as *Enterococcus*. Whole genome sequencing of S2 was done to identify the exact species of *Enterococcus* S2.

CSF or filtrate obtained from isolate S2 showed inhibition of the growth of CD630 in growing medium and increasing the percentage of the filtrate of isolate exhibited increased inhibition of the growth of CD630. Inhibitory effect of the filtrate of isolate S2 on indicator strain was proteases (proteinase K and trypsin) sensitive. The concentrated filtrate of isolate S2, when spotted on a lawn of CD630, exhibited the zone of inhibition of growth of CD630 as well as other strains whereas not concentrated CFS of isolate S2 didn't. CFS of isolate S2 was active on CD630 till critical dilution of 1 in 20ml. CFS of isolate S2 induced with hydrogen peroxide or co-culturing with different *Enterococcus* gave a more significant zone of clearance than that of Mitomycin C which is the well-known agent to induce a stress response that may increase the production of bacteriocin. The active ingredient in CFS of isolate S2 that gives a zone of clearance on CD630 is both effective in low and normal room temperature. However, the active component may be relatively unstable as its activity faded over a period of time. Concentrated CFS of isolate S2 did not have any zone of clearance when heat treated. SDS-PAGE analysis of CFS of isolate S2 has shown a presence of many protein molecules of size varying from 25-180 kDa from which active component of CFS of isolate S2 will need to be identified.

Most of the experimental data obtained from this investigation provide a good indication of the potential production of protein-based antimicrobial substance like bacteriocin active against *C. difficile* from environmental isolate S2, but further experiments in future are of paramount importance to confirm potential bacteriocin from isolate S2 active against *C. difficile* and its possible potential as antimicrobial substance.

# Acknowledgement

I am very grateful to my principal supervisor, Dr. Chris Penfold for providing me with the opportunity to carry out this research under his supervision and for giving me guidance, advice and continued support during my time at Nottingham Univerisity. I would also like to thank my second supervisor, Dr. Sarah Kuehne for supervisions and advice during the course this project. Thanks also to all members of CRG from for help and support.

# 1 Introduction

## 1.1 Overview

The identification and development of antibiotics therapy without argument represents one of the most significant scientific achievements of the 20th century that had the greatest impact on human life expectancy. However, our over-dependence and misuse of antibiotics brought several problems such as antibiotic resistance that cast doubt to future use and efficacy of antibiotics. Clinically important pathogens have emerged that are resistant to single antibiotics such as MRSA and VRE etc. (63) as well as resistant to multiple antibiotics like *Mycobacterium tuberculosis* which became the main cause of hospital-acquired infections in healthcare settings of developed countries. There is steadily increasing incidences of morbidity/mortality linked with them especially in long-term elderly patients and in patients that are immunocompromised due to treatment such as chemotherapy and bone marrow/organ transplantation etc. In addition to this challenge, high costs and risks associated with developing novel antibiotics have partly caused a shortage of new families of antibiotics that could potentially compensate for resistance to existing antibiotics (30, 150). Another adverse effect of using antibiotic has also been noted. The use of commonly prescribed broad-spectrum antibiotics has been confirmed to lead to significant disruption to the human commensal microbiota that plays several key roles in host health (13, 33). It has also been particular concern that the use of broad-spectrum antibiotics exhibited a potential association with the increasing incidence of atopic and autoimmune diseases (13). Consequently, there is an urgent requirement for the development of new antimicrobials that can be used in clinical settings to combat antibiotic resistance as well as to prevent further spread and development of new antibiotic-resistant bacterium. Potential alternatives have been explored such as antimicrobial compounds from the plant (135) or a variety of sources (81), probiotics- intake of beneficial commensals (139), bacteriophages and phage lysins (22) and RNA-based therapeutics (77). A subgroup of anti-microbial known as bacteriocins has been receiving much attention as a possible alternative to existing antibiotic therapy with significant potential advantages over antibiotics.

## 1.2 Bacteriocins

Due to limited resources and space, there is often fierce competition between various micro-organisms such as bacteria, fungi and Archaea within the ecological niche of highly diverse communities. Many species have thus evolved widely to produce a diversity of the antimicrobial molecules that can either inhibit (bacteriostatic) or kill (bactericidal) the competitors including broad-spectrum antibiotics, protein based exotoxins, lysozymes, and bacteriocins (128).

Ever since the discovery of the first bacteriocin named colicin nearly 90 years ago as a heat-labile product in *E. coli* V cultures active against *E.coli* S, bacteriocins are now considered as the most diverse and naturally abundant classes of antimicrobial molecules found in nearly all bacteria lineages and some Archaea (60, 125, 126). The first bacteriocin was named colicin after the species name of the producing organism, and a similar system of nomenclature was implemented for most bacteriocins discovered after that such as pyocin from *Pseudomonas aeruginosa*, Klebicin from *Klebsiella pneumoniae*, diffocins from *Clostridium difficile*, newly discovered thuricin 17 from *Bascillus thuringiensis* etc. It is thought that at least one bacteriocin is produced by 99% of all bacteria and the only reason they are not isolated is that few researchers have looked for them (75). Bacteriocins are a mixed family of antimicrobial molecules that encompass proteins differing in their molecular sizes and structures, mode of actions, mechanisms of release and immunity (59, 62). Up to date, bacteriocin family is characterised by ribosomally synthesised proteinaceous toxins (126) which are produced during the stressful conditions to target sensitive neighbouring microorganisms related to the producers (117,118). The production of bacteriocin may enable producing bacteria to invade an established bacterial community (57) as well as defend their territory against invaders by restricting advance of neighbouring cells (74). Unlike broad spectrum classical antibiotics, bacteriocins often have a narrow target range of action (126) and therefore are active against specific bacteria. Given their high specificity including the action against antibiotic resistant strains and their proteinaceous nature amendable to gene-based protein engineering, they have been suggested as a potential novel antibiotic. They are also thought to be involved in mediating the intra-specific or population-level interactions leading to and maintaining microbial diversity (125,126).

### 1.2.1 Classification of bacteriocins

Bacteriocins consist of a heterogenous group of pepetides and proteins which led to difficulties in classifying them. Klaenhammer et al., 1993 (75) first divided bacteriocins into four classes; Class I= lantibiotics; presence of lanthionine and its derivatives, thermostable. Example = nisin. Class II = up to 10KDa of size, thermo-stable without lanthionine derivatives, divided into three subclasses IIa-c as shown on the table. Class III= bigger size greater than 30KDa thermos-labile peptides. Class IV = large peptides with carbohydrate/ lipids (5) and it is suggested Class IV be excluded as they are artefacts of partial purification. Cotter et al., 2005 recommended two classes shown in figure 1: Class I (lantibiotics such as nisin and Class II (other peptides without lanthionine and largely unmodified peptides) (32) and exclusion of high molecular heat-labile peptides (group them as bacteriolysins) (112, 153). Drider et al, 2006 (40) using bacteriocins genetic and biochemical characteristics divided them into three classes as shown in figure 2. In this classification, class II include large thermos-labile bacteriocins with more than 30KDa, complex protein structure, induces lysis of cell wall of the target.

More recently, ribosomally-synthesized and post-translationally modified peptides (RiPPs) have been proposed in an attempt to classify bacteriocins into more distinct subgroups (92). However, the simplest approach is to divide bacteriocins regarding microbial origin: bacteriocins of Archaea, Gram-negative and Gram-positive (59, 62).
The Archaea produces archaeocins (bacteriocin-like antimicrobial peptides) when under a stressful condition such as limited resources, to kill target cells and reduce the competition within the habitat. First discovered archaeocin was the halocin S8 from halobacteria (a peptide consisting of 36 amino acids residues) (125, 126, and 128). As mentioned before, colicin produced by *E.coli* was the first bacteriocin to be identified by Gratia in 1925, following which many studies than have subsequently identified many other important bacteriocins produced by Gram-negative bacteria such as pyocins of *Pseudomonas* and cloacins of *Enterobacter cloacae* (125). *E. coli* produce two types of ribosomally synthesised bacteriocins; low molecular weight microcins (1-10KDa, MIC in nM) and larger proteins called colicin (over 40KDa, MIC in pM) (58). Most of the bacteriocins in Gram-negative bacteria share similarity to colicins in the way that they are relatively large, heat-labile whereas microcins share similar properties with the bacteriocin produced by Gram-positive bacteria; they are small, heat stable, resistant to some proteases and extreme pH (6, 52, 115).

Bacteriocins produced by Gram-positive bacteria, characterised by their diversity and natural abundance, are small (2-6KDa) and exist as cationic and permeabilizing membrane peptides resembling many of the eukaryotic antimicrobial peptides (62, 128). Although colicins produced by Gram negative *E.coli* remain one of the most well studied bacteriocins traditionally, many recent studies have emphasised a great food or therapeutic application potentials of Gram-positive LAB. The potential use of LAB is due to the GRAS status, valuable metabolic products such as bacteriocins, organic acids, antifungal peptides and a broader range of actions against both related and non-related species while safe to eukaryotic cells (5, 27). Some other key differences between bacteriocin from Gram-positive and Gram-negative bacteria (126) is that Gram-positive bacteriocins require many more genes for their production (101).They may not cause the suicide of producing cell as their synthesis is usually self-regulated and they have specific transport mechanisms that release bacteriocin toxin while some employ the sec-dependent pathway (40, 43, 88). However, for Gram-negative bacteria, bacteriocin relying solely on host's SOS regulatory system (103, 146).

| Group | Distinctive feature | Examples |
|---|---|---|
| *Class I (modified)* | | |
| MccC7-C51-type bacteriocins | Is covalently attached to a carboxy-terminal aspartic acid | MccC7-C51 |
| Lasso peptides | Have a lasso structure | MccJ25 |
| Linear azole- or azoline-containing peptides | Possess heterocycles but not other modifications | MccB17 |
| Lantibiotics | Possess lanthionine bridges | Nisin, planosporicin, mersacidin, actagardine, mutacin 1140 |
| Linaridins | Have a linear structure and contain dehydrated amino acids | Cypemycin |
| Proteusins | Contain multiple hydroxylations, epimerizations and methylations | Polytheonamide A |
| Sactibiotics | Contain sulphur–α-carbon linkages | Subtilosin A, thuricin CD |
| Patellamide-like cyanobactins | Possess heterocycles and undergo macrocyclization | Patellamide A |
| Anacyclamide-like cyanobactins | Cyclic peptides consisting of proteinogenic amino acids with prenyl attachments | Anacyclamide A10 |
| Thiopeptides | Contain a central pyridine, dihydropyridine or piperidine ring as well as heterocycles | Thiostrepton, nocathiacin I, GE2270 A, philipimycin |
| Bottromycins | Contain macrocyclic amidine, a decarboxylated carboxy-terminal thiazole and carbon-methylated amino acids | Bottromycin A2 |
| Glycocins | Contain S-linked glycopeptides | Sublancin 168 |
| *Class II (unmodified or cyclic)* | | |
| IIa peptides (pediocin PA-1-like bacteriocins) | Possess a conserved YGNGV motif (in which N represents any amino acid) | Pediocin PA-1, enterocin CRL35, carnobacteriocin BM1 |
| IIb peptides | Two unmodified peptides are required for activity | ABP118, lactacin F |
| IIc peptides | Cyclic peptides | Enterocin AS-48 |
| IId peptides | Unmodified, linear, non-pediocin-like, single-peptide bacteriocins | MccV, MccS, epidermicin NI01, lactococcin A |
| IIe peptides | Contain a serine-rich carboxy-terminal region with a non-ribosomal siderophore-type modification | MccE492, MccM |

Mcc, microcin.

**Figure 1: Showing the classification of bacteriocins into two classes: Class I and Class II according to Cotter et al., 2005 (31).**

| Classification | Features | Subcategories | Examples |
|---|---|---|---|
| Class I (Lantibiotics) | Lanthionine or peptides containing β-lanthionine | Type A (linear molecules)<br>Type B (globular molecules) | Nisin, subtilin, epidermine<br>Mersacidin |
| Class II | Heterogeneous class of small thermostable peptides | Subclass IIa (antilisterial-pediocine bacteriocins type)<br>Subclass IIb (composed of two peptides)<br>Subclass IIc (other bacteriocin) | Pediocin, enterocin, sakacin<br>Plantaricin, lactacin F<br>Lactococcin |
| Class III | Large thermolabile peptides | | Helveticin J, millericin B |

**Figure 2: Showing the classification of bacteriocins according to their genetic and biochemical characteristics (5, 40).**

### 1.2.2 Mode of action of bacteriocin

Different bacteriocins have been found to have many distinct modes of action on the target organism that differ from those of antibiotics (summarised in figure 3), and the mechanisms are broadly divided into:

(1) Disruption of cell wall synthesis (10) - Nisin and several other class I lantibiotics along with some class II bacteriocins target lipid II molecule which is a key intermediate in the peptidoglycan biosynthesis for bacterial cell wall (151). These bacteriocins bind specifically to lipid II at site other than the binding site for vancomycin and can inhibit peptidoglycan synthesis, even for vancomycin-resistant Gram-positive pathogens.

(2) Depolarisation of the lipid bilayer membrane (91, 106) - some class I bacteriocin like lantibiotics cause loss of the membrane potential (depolarization) and ultimately cell death by using lipid II as a docking molecule which facilitates pore formation on the cell membrane. Some other bacteriocins belonging to class IIa such as lactococcin A and microcin E492 can also damage or kill target cells through the formation of pore on the cell membrane (10, 104). These molecules bind to the cell membrane-associated mannose phosphor-transferase system (Man-PTS) that facilitates pore formation. A small subset of related lantibiotics like cinnamycin can also cause pore formation by binding to phosphatidyl-ethanolamine in the cell membrane which in turn inhibits the enzyme phospholipase A2.

(3) Inhibition of protein synthesis or degradation of host nucleic acids such as RNA and DNA (67) - bacteriocins can kill their target cells by interfering with the metabolism of protein and nucleic acid. For example, MccB17 move into Gram-negative cell via specific protein transporter molecules (OMpF and SbmA for outer and inner membrane respectively) and interfere with DNA replication by inhibiting DNA gyrase-mediated DNA supercoiling (109). Other bacteriocin like lasso bacteriocin MccJ25 inhibits transcription by blocking the secondary channel of RNA polymerase (148) whereas MccC7-C51 blocks transcription by blocking aspartyl-tRNA synthetase with generation of modified aspartyl-adenylate (95). Other bacteriocins such as nocathiacins, other thiopeptides target bacterial ribosome to prevent protein synthesis by binding to the 23s rRNA of the 50S ribosomal subunit (4, 152).

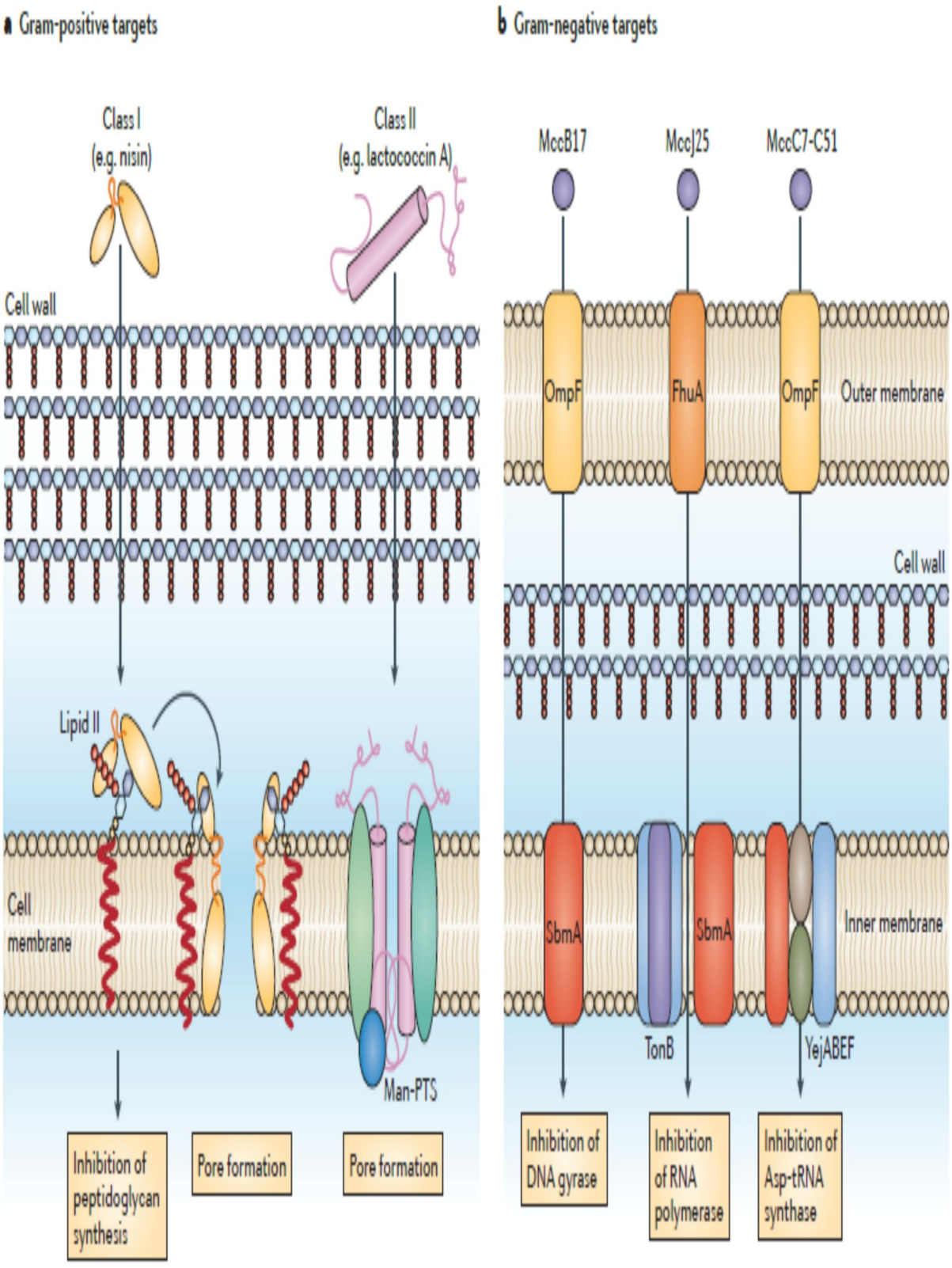



**Figure 3: Showing the summary of the mechanism of action of specific bacteriocins examples on (a) Gram-positive and (b) Gram-negative targets that is described above (34). The diagram also demonstrates the difference in the structure of Gram-positive and Gram-negative bacteria.**

The precise roles of bacteriocins are unclear; however, they are found in all surveyed lineages of bacteria and seem to exert strong selection in microbial communities (75). This foundings suggests that they may play a significant role in mediating microbial population or community interactions to maintain biodiversity possibly via the existence of intransitive fitness interactions as well as the spatial structure (147, 149).

Bacteriocins are also thought to act as offensive weapon or anti-competitors to enable the invasion of producing strains into an established microbial community (57, 83). They may also play a defensive role inhibiting the invasion of competing species into an occupied niche and therefore limit the advance of neighbours (25, 126).

Studies have shown that bacteriocin production improves the establishment success of the producing strains (93). However, it is also suggested that these toxins produced by cells can both be a weapon and a liability and hence a double – edged sword. Despite being a lethal offensive weapon to target cells, producers of bacteriocin face an energetic cost for carrying genes coding for bacteriocin production (27).

Gram-positive bacteriocins have recently found also to be involved in the mediation of quorum sensing and act as communication signals in bacterial consortia, e.g. biofilms (54, 76). Although the precise roles of bacteriocins, offence or defence or merely involved in diversity, are unclear, these seem to be dependent on surrounding conditions that bacteria are in. Various attempts were made to model the interplay between bacteriocin producing bacteria and continuous model for so called the rock-scissors-paper game was described as one of modes to explain competitive interaction between bacteriocin producing, resistant and sensitive bacteria (127).

## 1.3 Applications of bacteriocins

Many bacteriocins have inherent properties which suggest that they could be of a value and possible alternative to antibiotics in treatment of bacterial infection in clinical settings and their application has been gaining increased popularity (36).

These beneficial properties of bacteriocins include:

(1) Good potency in comparison to existing antibiotics (as determined both *in vitro* and *in vivo* conditions).

The activity of bacteriocins against clinically significant pathogens varies both across and within bacteriocin subclasses. In general, the class I bacteriocins such as lantibiotics and thiopeptides are most potent against Gram-positive pathogens. Lantibiotics including nisin, epidermin, mutacin and lacticin 3147, etc. exhibit potent *in vitro* activity against clinically significant pathogens like MRSA, VRE, *S. pneumoniae* and *C.difficile* etc. (9, 90, 112 and 113). Thiopeptide is also high potent against Gram-positive pathogens but not commercially developed due to their limited use as result of poor solubility (153). There are many examples of class II without modification that are potent against Gram-negative targets (42) such as class IIc peptides like enterocin As-48 and epidermicin NI01 that exhibit excellent potential for both non-clinical and clinical applications (134). Although there are many examples of bacteriocins with significant *in vitro* activity against Gram-negative bacteria, bacteriocins are known to show less potential against Gram-negative bacteria in comparison to Gram-positive. Microcins, bacteriocin produced by Gram-negative bacteria typically exhibit the greatest potential against Gram negative pathogens with a spectrum of activity. Microcins such as MccB17 exhibit a wide range of Gram-negative pathogens including *Escherichia, Klebsiella, Salmonella, Shigella* and *Pseudomonas spp.* etc. (6, 96). Purified lantibiotics such as nisin and epidermin have been discovered to kill some of Gram-negative bacteria even though they are known to show reduced activity against them (80). The lantibiotics and thiopeptides are the most extensively investigated for their *in vivo* activity and nisin has been demonstrated to target *S. pneumoniae* with 8-16 times more potency than vancomycin in intravenous regimen (55). Moreover, the lactibiotic B-Ny266 (25) and Nisin F (87) were found to be active *in vivo* against *S. aureus* comparable to that of vancomycin which further support potential of bacteriocin as a viable substitute for antibiotics. However, *in vivo* activity of bacteriocins is not as well investigated as that of *in vitro* activity.

(2) Low toxicity (especially low oral toxicity for the treated host).

Bacteriocin produced by LAB, in particular, has been consumed by humans in the form of fermented food without problems for an extended period (89). Nisin which is most extensively studied in this regard along with other lantibiotics exhibits a lack of toxicity and has been used widely as a food preservative (87) with the only exception of *Enterococcus spp.*-associated cytolysin (a lantibiotic) which does exhibit cytotoxic activity against both bacterial and mammalian cells and is considered as a virulence factor.

(3) Availability as both broad and narrow-spectrum peptides.

Bacteriocins commonly show a narrow range of activity, but many bacteriocins also exhibit a broad-spectrum antimicrobial activity which can be an attractive trait as it allows targeting infections of unknown aetiology. However, broad spectrum antimicrobials are known to damage beneficial commensal human microbiota, and as a result, narrow range of activity shown by bacteriocin could be of great use.

(4) Possible use as a probiotic due to in situ production of bacteriocin.

Bacteriocins have potential to be produced at the site of infection by probiotic bacteria (53). Many healthy gut microbiotas have previously been known to need bacteriocin production for gut colonisation as mentioned earlier. In term of probiotic gut microbiota, an ability to produce bacteriocin has long been considered as a beneficial character, but most of the evidence for this has often been indirect and depended heavily on circumstances (37). The application of bacteriocin produced by probiotic bacteria to target the specific bacterial populations will need to be further investigated for their clinical treatment with advanced genetic technologies as disruption to 'balanced' gut microbiota was revealed to contribute to many diseases such as coeliac disease, obesity and diabetes, etc. (136).

(5) Can be genetically modified and bioengineered to reinforce and enhance its property as an antimicrobial.

Due to the peptide nature of bacteriocin, as genes directly encode them, bacteriocins are often more amenable to improve its property by genetic or bioengineering than traditional antibiotics. Engineering of bacteriocin can be done by manipulating the bacteriocin gene involving *in vitro* harnessing of biosynthetic enzymes used in protein synthesis as well as partial or complete chemical synthesis of the antimicrobial (34). Bioengineering of bacteriocin was mostly carried out as a way to further our understanding of the fundamentals of bacteriocin activity and structure-function relationships but also there is an increased attempt to produce engineered bacteriocins that exhibit enhanced activity or stability which make them more useful from a clinical perspective. For example, a bioengineered derivative of the lantibiotics generated *in vitro* such as nisin, actagardine, and derivative of lacticin. 481 etc. exhibits enhanced specific activity against both Gram-positive and Gram-negative targets (45). Synthetic forms of lactocin S are more stable than their natural counterpart (14, 15,130) and hybrids of nisin-vancomycin are potent against VRE (1). Semi-derivatives of thiopeptides such as nocathiacin derivatives have also been generated that have increased water solubility (28). Engineering could also be done to produce derivatives with enhanced properties such as broadened spectrum, increased cell binding or increased trypsin resistance to allow oral intake in addition to increased activity and stability which are all important to make bacteriocin more clinical applicable instead of antibiotics (85).

Many bacteriocins and their derivatives (both natural and engineered) are currently used in many different non-clinical and clinical setting. Even though bacteriocin offers great promise as a clinical treatment option from bacterial infections, the primary use of bacteriocins has been on animals rather than human health. Most notably, Nisin and Lacticin3147 has been introduced as a preservative in the food industry (132, 133) and as a prophylactic in veterinary healthcare along with other bacteriocins such as enterocin from *Enterococci* (35, 98).

Clinical application of bacteriocins so far has been progressing slowly due to a lack of sufficient investments. However, recently the issue with investment seem to be addressed with several bacteriocins are now being developed to (or "intending to") human application and they are currently undergoing clinical trials at different phases as potential chemotherapeutic agent. These include many thiopeptides such as LFF571 (derived from GE2270 A) as well as lantibiotics such as Mu1140-S (a synthetic form of the mutacin 1140) and NVB302 (a semi-synthetic derivative of deoxyactagardine B) (44). Bacteriocin called microbisporicin is being developed as an injectable therapy to control multi-antibiotic resistant Gram-positive pathogens (38). Colicins have recently been shown to have good activity against pathogenic *E.coli* existing within biofilm communities (20), and colicin M (derivative of colicin) has recently been suggested as a candidate for a novel antimicrobial agent as result of its mode of action being common to all bacteria. Probiotics synthesising bacteriocin has also been developed commercially such as the bacteriocin produced by the BLIS K12 probiotics used in oral hygiene products.

The clinical application of bacteriocins will ultimately depend on our understanding of their mechanism of action along with strategies to prevent the development of resistance against bacteriocin in future as possible resistance mechanism through mutation and immune mimicry against bacteriocin such as lipid II-targeting lantibiotics that are primarily targeting the cell membrane has been identified (40, 112). There are vast arrays of different bacteriocin available that could also be engineered to enhance functionality along with various structures and mechanisms of action. Widespread use of bacteriocin in a clinical setting would not be a complete surprise shortly as a narrow spectrum, strong alternative or upgrade on antibiotics with less adverse effects to humans in treating various bacterial infections.

## 1.4 *Clostridium difficile*

*Clostridium difficile* are a Gram-positive, rod-shaped motile bacterium, ubiquitous in nature especially commonly found in soil and is also a bacterium that's found established in human colon. It can be found in healthy people, where it causes no symptoms (up to 3% of adults and 66% of babies) (97). *C. difficile* are obligate anaerobic bacteria, and they exhibit maximum growth in human body temperatures in the absence of oxygen. They, therefore, survive well in the colon (bowel) under anaerobic condition.

*C. difficile* characteristically forms vegetative endospores when stressed that can tolerate extreme conditions that the active bacteria cannot tolerate (71) and this allows the bacteria to endure in unfavourable conditions for extended periods of time up to five months (70).

*C. difficile* can be a significant opportunistic human pathogen, especially in nosocomial environments in elderly patients. *C. difficile* was first discovered and described by Hall and O'Toole (61) as a bacillus (rod-shaped) type bacterium isolated from neonatal faeces as a harmless inhabitant of the gut and later identified as the causative agent for pseudomembranous colitis (50). CDI is the primary cause of severe complications such as iatrogenic colitis/pseudomembranous colitis in 90-100% of cases which has signs and symptoms ranging from mild diarrhoea to severe, life-threatening inflammation of the colon (70). It is also the biggest cause of infectious diarrhoea known as CDAD, responsible for 20-30% of hospital acquired and antibiotic-associated diarrhoea in hospital patients (131). It is about 10-fold more prevalent compared to other common hospital-acquired infection such as MRSA.

*C. difficile* is transmitted by faecal-oral route when the bacterium or its spores are ingested through contact with a contaminated environment or person. Once *C. difficile* starts producing spores infection spreads quickly as spores leave the body in an infected person's diarrhoea. Spores produced by *C. difficile* are heat resistant and are also not killed by alcohol-based hand cleansers or routine surface cleaning which make cross infection control of clinical surfaces challenging (99). Thus, these spores endure in clinical environments for long periods and get transmitted to other individuals, especially when people are in close contacts with each other, such as hospitals and care homes. Spores' acid-resistance protects them from damage by stomach acid when ingested and when exposed to bile acids, they germinate and multiply into vegetative cells in the colon, where they cause infection.

*C. difficile* causes disease when the healthy gut microflora or commensals are compromised by *C. difficile* bacteria (dysbiosis), usually by taking broad spectrum antibiotics as a treatment for an unrelated infection (70). The commensals are usually closely related microbes sharing same ecological niche and competing for similar resources such as space and nutrients. Use of antibiotics allows opportunistic pathogen like *C. difficile* to flourish to overrun or replace the healthy gut microflora (46). As a result, healthy gut microflora that controls the growth of other pathogenic bacteria such as *C. difficle* is disturbed significantly or may be killed which prevents it from providing enough protective function against opportunistic pathogens of the gut (63).

People are more at risk of suffering *C. difficile* infection when:

- They have been treated with broad-spectrum antibiotics such as amoxicillin, fluoroquinolones, cephalosporins, carbapenems, and clindamycin, etc.
- They have had to stay in healthcare settings for a long time. The rate of *C. difficile* acquisition is estimated to be 13% in patients with hospital stays of up to two weeks and increases up to 50% with stays longer than four weeks (29).
- They are over 65 years old
- They have a serious underlying illness or condition or have a compromised immune system as a result of a condition such as diabetes/AIDS or as a side effect of a treatment such as chemotherapy/organ transplant
- They have had surgery on the digestive system or conditions like ulcerative colitis etc.

Human pathogenesis by *C. difficile* depends greatly on the toxins produced by *C. difficile* which are the main virulence factors along with the production of endospores. Pathogenic *C. difficile* strains produce various toxins: the most well-characterized and researched being enterotoxin (*C. difficile* toxin A) and cytotoxin (*C. difficile* toxin B) (11). However, relative contributions of toxin A and B in causing pseudomembranous colitis is still being debated, but without either toxin A or toxin B, *C. difficile* may colonise the gut, but is unlikely to cause pseudomembranous colitis (78). Toxins A and B are enzyme molecules belonging to glucosyltransferases that target and inactivate the Rho family of GTPases. The Rho family of GTPases consists of a family of small (~21 kDa) signalling G proteins that belong to the Ras superfamily as a subfamily (23). The members of the Rho GTPase family are found in all eukaryotic kingdoms and have been known to regulate many aspects of intracellular actin dynamics (23). All G proteins are "molecular switches", and Rho proteins play a role in organelle development, mitosis, wound healing, cytoskeletal dynamics, cell movement, and other standard cellular functions (7). Toxin B (cytotoxin) induces actin-depolymerisation by a mechanism correlated with a decrease in the ADP-ribosylation of the low molecular mass GTP-binding Rho proteins (71). Another toxin, binary toxin, also has been discovered, but its role in CDI or pseudomembranous colitis is not yet fully understood (7).

## 1.5 Implications of *C. difficile* infection and the need for novel treatments

The appearance worldwide of a more virulent strain of *C. difficile* (PCR ribotype 027/NAP-1) has caused increased morbidity/mortality (69, 79). Many notable outbreaks of CDI throughout the developed world such as two outbreaks in Montreal, Quebec, and Calgary, Alberta in 2003 (111) and outbreak at Stoke Mandeville Hospital in the United Kingdom between 2003 and 2005 (79). Outbreaks of CDI since then were also documented in several developed countries such as UK, US, Australia, and Scandinavian countries such as Denmark, Sweden, and Finland, etc. (86). This strain is characterised by several differences to other *C. difficile* strains that make it more virulent such as binary toxin production, mutation of the tcdC gene that results in increased toxin A/B output and resistance to antibiotic fluoroquinolone (66, 141).

Recently, CDI has been on the decrease in the developed countries but still presents a real threat as one of the common causes of nosocomial infection (51). Data from Public Health England exhibited that within the last year (July 2016-July 2017) there were 14294 cases of CDI according to CCG which represent an decrease in comparison to the previous years, 14698 cases (July 2015-July 2016) and 15742 cases (July 2014-July 2015). 5113 cases of CDI in Acute Trust also represent a decrease in comparison to the previous years, 5339 cases (July 2015-July2016) and 5806 cases (July 2014-July2016) (116). Efforts have been made to limit transmission of *C. difficile* and to prevent CDI by more appropriate prescription of antibiotics, implementation of a range of more efficient infection control measures such as intensive cleaning methods, use of bleach (sodium hypochlorite), peroxide vapours in closed wards and increased surveillance of epidemiological data (68). However, the ability of *C. difficile* to produce its protective spores as well as resistance to conventional desiccants used for infection control have made CDI challenging to prevent despite control measures and precautions (3, 72).

Complications of a CDI are uncommon, occurring in around 1 in 35 cases especially in elderly patients in healthcare and care home settings with chronic diseases such as cardiovascular or chronic obstructive pulmonary disease, etc. or patients with compromised immune system. But when they do occur, they can be severe in addition to other signs and symptoms of CDI such as dehydration, acute kidney failure, peritonitis (perforation of the colon) and toxic megacolon, which can be fatal. In 2011, CDI resulted in about half a million infections and 29,000 deaths in the United States (84). Also *C. difficile* was mentioned as cause of death in 1,646 cases in England and Wales in 2012 which fell for the fifth consecutive year and deaths involving CDI accounted for 0.5% of all hospital deaths during 2010-12 in comparison to 2.0% during 2007-09 (68).

Carrying *C. difficile* without symptoms is common as mentioned previously and in general, mild cases do not require specific treatment other than oral rehydration therapy to prevent dehydration through diarrhoea. Therapeutic choices of CDI are the limited and current treatment of CDI includes antibiotic therapy, use of probiotics, stool transplant (faecal bacteriotherapy) (39, 142). Antibiotics have long been the choice of treatment for CDAD (107), and broad spectrum vancomycin and metronidazole has been the first line antibiotics to treat CDAD (2, 19). However, problems associated with the use of broad-spectrum have been highlighted recently despite their effectiveness. As mentioned previously, CDI often caused

by the use of broad-spectrum antibiotics that moderate healthy gut microbiota as *C. difficile* competitively benefits and flourishes under antibiotic-induced disruption of the microbiota. Use of antibiotic can address the acute infection, but can also lead to further damage to normal microbiota. As a result, treatment failures and recurrence of the disease often take place at 2-38% and 8-50%, respectively (2, 102). In addition to this drawback of using antibiotic therapy, *C. difficile* strains with increased resistance to these antibiotics have been emerging in the healthcare setting (17, 18 and 110) which enhanced the need for new antimicrobial therapy against *C. difficile*. Efforts have been made to develop novel antibiotics against multiple antibiotic-resistant *C. difficile* such as rifaximin and ramoplanin etc. which are in their clinical trial stage (26, 138). The vast amount of investments required to generate new, effective antibiotic/antimicrobial with a novel mechanism of activity due to antibiotic resistance as well as annual bill of hospital treatment of patients with CDI placed a significant financial burden on the healthcare systems of the developed world. It is estimated that within the European Union alone the annual expenditure amounts to three billion euros for the hospital treatment of patients with CDAD (79, 110).

Other treatment option such as stool transplant is documented to be approximately 85% to 90% effective in those for whom antibiotics have not worked with minimal side effect (21) which involves introduction of microbiota acquired from the faeces of a healthy donor to reverse the disruption of microbiota responsible for the recurring nature of CDI and to re-establish healthy microbiota that resist colonisation by *C. difficile* (16). Use of probiotics as a treatment option also appears to have promise as probiotics produce specific bacteriocins that target specific pathogen other than beneficial gut microflora (24, 56, 89 and 100). However, evidence to support the use of probiotics and stool transplant alone as an alternative for antimicrobials in the treatment of CDI is still insufficient and not practical enough to completely replace the use of antibiotics altogether (39, 64, 94 and 114). Bovine antibody enriched whey has been made and trialled as a treatment method to prevent a relapse of CDAD with varying degree of success (145).

In recent years, an increasing number of CDI have occurred outside a healthcare setting, known as community-acquired CDI (144). Research has found that these infections are most common in middle-aged women and those having medications to suppress gastric acid production which is one type of commonly prescribed drug (65) and the symptoms tend to be milder than a "standard" *C. difficile* infection. As a consequence, there is a resurging interest in the discovery of novel antimicrobial agent active against *C. difficile* to overcome the problems of broad spectrum antibiotics and bacteriocins are widely accepted as the best potential candidate due to its advantages over the existing antibiotic treatment as discussed before.

## 1.6 Bacteriocins as novel antimicrobials against *C. difficile*

Currently, many studies have highlighted the potential of bacteriocins as a novel treatment for CDI (34). Gebhart et al., 2012 (49) have identified an R-type diffocin (Diffocin 43593) produced by non-toxicogenic *C. difficile* ATCC 43593 and this exhibited a potent and specifically targeted inhibitory effect on 13 strains of clinically relevant, hyper-virulent *C. difficile* ribotype 027. Lantibiotics such as nisin (8) and lacticin 3147 (48, 113) were also found to exhibit important activity against *C. difficile* comparable to antibiotics such as metronidazole and vancomycin (120). However, lacticin 3147 has been shown not to survive transit through the gut as well as having a broad range of activity against many other bacteria, affecting beneficial gut microbiota such as *Lactobacillus* and *Bifidobacterium* which limits its possible clinical use against *C. difficile*. LFF571, a derivative of the thiopeptide bacteriocin GE2270A, was found to be more effective than vancomycin in an experimental model of both primary and recurring CDI (143). The advantage of LFF571 over lacticin 3147 is that it exhibits potent activity against many Gram-positive organisms including *C. difficile* except normal gut microbiota such as *Lactobacillus* and *Bifidobacterium* (82). More in vivo investigations using LFF571 would be required. A study by Rea et al. found a novel narrow-spectrum; two peptides based bacteriocin termed Thuricin CD from *Bacillus thuringiensis* by screening faecal samples (121). Further studies by Rea et. al. (122, 123, and 124) confirmed that thuricin CD was at least as effective as antibiotics vancomycin and metronidazole at killing *C. difficile* with negligible changes to gut flora which makes it a plausible alternative to conventional antibiotic therapy. However, one peptide component of Thuricin CD, Trn-β found to be degraded by the gastric enzymes both *in vitro* and *in vivo* which make oral delivery of thuricin CD difficult. Rectal administration of thuricin was found to be a promising mode of delivery of thuricin CD to the colon. Identification of efficient delivery method along with more clinical tests will determine clinical use of thuricin CD which has the greatest promise among bacteriocins so far.

Discovery of promising thuricin CD from a screening of environmental and faecal sample in the study by Rea et al, 2010 (121) formed the foundation of research carried out in this paper. The main aim of this study was to screen environmental samples to isolate and characterise novel bacteriocins with biological activity against *C. difficile*. This research was intended to isolate bacteria producing novel bacteriocins that have a narrow spectrum of activity to have minimal effect on beneficial microbiota with a wide range of therapeutic activity against many clinically relevant *C. difficile* strains which possibly in future can provide a targeted alternative to existing antimicrobial treatment against *C. difficile* infection.

# 2. Methods

## 2.1 General chemicals, reagents and buffers

The general laboratory chemicals were purchased from Sigma unless otherwise stated. Buffers were prepared in distilled water as aqueous solutions according to CRG protocol. Solutions were sterilised by autoclaving.

## 2.2 Bacterial strains and media

### 2.2.1 *Clostridium difficile* strains used

| Strain Name | Description | Reference |
|---|---|---|
| **R20291** | *C. difficile* ribotype 027 | CRG culture collection |
| **EK28** | *C. difficile ribotype 078* | CRG culture collection |
| **DH196** | *C. difficile* ribotype 027 | CRG culture collection |
| **DH478** | *C. difficile* ribotype 027 | CRG culture collection |
| **CD630** | *C. difficile* ribotype 012 | CRG culture collection |

### 2.2.2 Active environmental isolates obtained and used

| Environmental isolates | Description | Reference |
|---|---|---|
| **S2** | Colony of bacteria identified from environmental sample | Environmental sample from sewage sample |
| **S3** | Colony of bacteria identified from environmental sample | Environmental sample from sewage sample |
| **S6** | Colony of bacteria identified from environmental sample | Environmental sample from sewage sample |
| **S7** | Colony of bacteria identified from environmental sample | Environmental sample from sewage sample |
| **S8** | Colony of bacteria identified from environmental sample | Environmental sample from sewage sample |
| **S9** | Colony of bacteria identified from environmental sample | Environmental sample from sewage sample |
| **S10** | Colony of bacteria identified from environmental sample | Environmental sample from sewage sample |
| **S11** | Colony of bacteria identified from environmental sample | Environmental sample from sewage sample |

### 2.2.3 Media used

| Media name | Composition | Source |
|---|---|---|
| **BHIS broth** | 14.8g BHI (brain heart infusion)<br>2.0g yeast extract<br>0.4g L-cysteine<br>400ml distilled water | Oxoid<br>Oxoid<br>Sigma-Aldrich<br>CRG lab |
| **BHIS agar** | 18.8g BHI agar<br>2.0g yeast extract<br>0.4g L-cysteine<br>400ml distilled water | Oxoid<br>Oxoid<br>Sigma-Aldrich<br>CRG lab |
| **Fructose agar** | 27.6g fructose agar<br>2.0g yeast extract<br>400ml distilled water | Oxoid<br>Oxoid<br>CRG lab |
| **Top agar** | 7.4g of BHIS<br>1.0g yeast extract<br>0.2g L-cysteine<br>1.0g Bacterial agar<br>200ml distilled water | Oxoid<br>Oxoid<br>Sigma-Aldrich<br>Oxoid<br>CRG lab |
| **Mannitol Salt agar** | 44.0g MS agar<br>400ml distilled water | Oxoid<br>CRG lab |
| **Slanetz and Bartley agar** | 8.0g Tryptose<br>2.0g Yeast extract<br>0.8g Glucose<br>1.6g Dipotassium hydrogen phosphate<br>0.16g Sodium azide<br>0.04g Tetrazolium chloride<br>4.0g agar<br>400ml distilled water | Oxoid<br><br><br><br><br><br><br>CRG lab |
| **MacConkey broth** | 16.0g MacConkey broth<br>400ml distilled water | Oxoid<br>CRG lab |
| ***Brucella* Blood agar** | Ordered | Sigma-Aldrich |

*Clostridium* Research Group protocols were followed for handling and carrying out all experiments using all bacterial strains, environmental samples and media stated above unless otherwise indicated and aseptic technique was conducted throughout the investigations. Graph-pad Prism6 was used to perform all statistical analysis of data obtained from the experiments.

## 2.3 Screening environmental samples

### 2.3.1 Isolating strains from samples

CRG provided the protocol for growing colonies from environmental samples under aerobic conditions, which is similar to that described by (120). Environmental samples used in this study included that of sewage, enriched sewage, etc. For each cycle of Isolation: 1 ml of environmental samples were placed into Percelly Lysing tubes and homogenised at 1:5000 for 30 s. Cells were then centrifuged at 6,000x g (Beckman Coulter Allegra) for two min at room temperature. The supernatant was transferred into new tubes. One was kept as non-heat treated sample, and the other was placed onto the heat block for 30 minutes at 65°C as heat treated sample to select for spore-forming bacteria. 100µl of the sample was then spread onto fructose agar plates and then allowed to incubate for 24-72 hr inside the anaerobic chamber (Don Whitley Scientific MG1000) in an atmosphere of 5% hydrogen, 5% carbon dioxide and 90% nitrogen.

### 2.3.2 Screening

Fifty isolates were selected from grown colonies (both non-heat treated and heat treated) and were patch plated identically on to two fructose plates each and left to incubate overnight anaerobically at 37°C. One of the two plates was overlaid with 3 ml of molten top agar (0.5% weight per volume) which was seeded with 200µl of an overnight culture of *C. difficile* strain CD630 left to set and incubated overnight anaerobically at 37°C. The plate that was not overlaid with CD630 was kept as a temporary bank of isolates during screening. Colonies that showed an inhibitory effect on CD630 by forming a zone of clearance were cultivated from the matched position on the bank plates and harvested. Stocks of active environmental isolates were made for storage at -80°C in BHIS 10% glycerol. These steps of obtaining positive environmental isolates were carried out numerous times to get a large number of active environmental isolates that could be used for further tests. The environmental isolates that showed an inhibitory effect on initial screening were re-screened on five clinically relevant *C. difficile* strains: CD630, R20291, EK28, DH196, DH478 and those environmental isolates that show activity against more than 3 of 5 these strains of *C. difficile* have been identified and selected for future experiments.

## 2.4 Identification of environmental isolates

### 2.4.1 Gram staining and microscopy

Environmental isolates were grown overnight anaerobically at 37°C in BHIS broth, and then 10µl of each overnight was pipetted onto a separate slide. Gram staining was then performed using a standard CRG Gram staining protocol. Smear containing environmental sample was heat fixed onto the slide and was bathed in crystal violet for 1 minute. Crystal violet was rinsed with distilled water and slide was flooded with iodine solution for 1 minute. Iodine solution was rinsed with distilled water and alcohol was used to decolourize stain for few seconds. Slide was then bathed in safranin and was allowed to stand for 30 seconds. Safranin was rinsed with distilled water and the slide was left to dry in upright position. Gram stained samples were visualised at 100x magnification to study the morphology of environmental isolates as well as for their identification.

### 2.4.2 Quantification of DNA, 16S rRNA PCR and Sequencing

DNA of active environmental isolates was extracted using the Genomic DNA kit according to manufacturer's instructions, and NanoDrop ND-1000 spectrophotometer was then used to check for sufficient DNA quantity for PCR by measuring absorbance at 260 nm. The purity was estimated by the ratio of absorbance of nucleic acids (260 nm) to amino acids (280 nm). PCR reaction for 16s rRNA was set up and amplified using 16S rRNA primers (primers used are 27F and 1525R from Sigma-Aldrich given in Table 1-1). The summary of reagents used in a standard 50 µl reaction and the typical reaction conditions are shown in Table 1-2 and Table 1-3 respectively.

| Target genes | Oligo name | Primer sequence | Ampliconsize |
|---|---|---|---|
| **16S rRNA** | 27F | 5'-AGAGTTT GATCMTGGCTC AG-3' | 1.2-1.5Kbp |
| | 1525R | 5'-AAGGAGGTGAT CCAGCC-3' | 1.2-1.5Kbp |

**Table 1-1: List of Primers (Sigma-Aldrich) used in this study. Same primers 27F and 1525R was used for 16s rRNA sequencing of all environmental isolates active against *C. difficile.***

| Volume | Components |
|---|---|
| 5 µl | Template DNA (50ng/µl) |
| 5 µl | Forward primer (10 mM) |
| 5 µl | Reverse primer (10 mM) |
| 5 µl | dNTPs (2mM) |
| 5 µl | x10 PCR reaction buffer |
| 25 µl | Pure water (Sigma) |
| 0.5-1 µl | DNA polymerase |
| 50 µl | Total reaction volume |

**Table 1-2: A standard PCR reaction mixtures.**

| Steps | Cycles | Temperature | Time |
|---|---|---|---|
| Step 1 | 1 | 95°C | 2 min |
| Step 2 | 34 | 95°C<br>50°C<br>72°C | 30 s<br>30 s<br>40 s |
| Step 3 | 1 | 72°C | 10 min |
| Step 4 | | 4°C | HOLD |

**Table 1-3: A standard PCR reaction conditions**

PCR products were isolated by gel electrophoresis using 1 % (w/v) agarose gel in 50 ml TAE buffer (242 g Tris base, 57.1ml acetic acid and 100 ml 5 M EDTA). 5µl of SYBR® Safe DNA gel stain was then added for visualisation. DNA samples were loaded into the gel with 5x loading dye along with DNA ladder (1 kb; NEB). The DNA bands were then visualised via a Safe Imager™ Blue-Light Trans illuminator, and digital images were saved on the computer. DNA bands of interest of length 1.5kb were excised and purified using the QIAquick ® gel extraction kit (QIAGEN) according to the manufacturer's recommendations. CD 630 was a used as a positive confirmatory control with same PCR conditions and a PCR with distilled water instead of template DNA was used as negative control. For 16s rRNA sequencing, extracted DNA samples were sent to Source BioScience. Sequences were then put through a sequence match of the Ribosomal Database Project (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) and NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Only results that had highest percentage similarity (over 97% only) were taken into account for the active environmental isolates.

### 2.4.3 Tests for *Enterococcus*

#### (a) Growth at both aerobic and anaerobic condition

Overnight cultures were grown anaerobically at 37°C in BHIS broth with isolate S2 in triplicate. They are incubated at a temperature of 37°C both in aerobic and anaerobic conditions respectively. OD600 was taken every two h over a period of -12 h and then at 24, 48, 72 h to monitor the growth of the isolate using spectrophotometer (Thermo Scientific Biomate 3).

#### (b) Growth at temperature of 45°C

An overnight culture of isolate S2 was set up in BHIS broth in triplicate. Sample was incubated in a heat block with temperature controlled at 45°C for three days and OD600 was taken on 0h, 24h, 48h and 72h to monitor the growth of isolate S2 at a temperature of 45°C. Isolate S2 was also grown in 37°C as an experimental control for comparison.

#### (c) Growth in medium containing 6.5% NaCl

The overnight cultures were grown anaerobically at 37°C in 10ml BHIS broth with isolate S2 in triplicate. They are incubated at a temperature of 37°C, and OD600 was for intervals of 0h, 24h, 48h, and 72h to monitor the growth of isolate S2 in medium containing 6.5% weight per volume NaCl (by adding 0.65g of NaCl salt from Sigma-Aldrich to 10ml BHIS broth and mixing it thoroughly with homogeniser). Isolate S2 was also grown at the same temperature in clear BHIS broth as an experimental control for comparison.

#### (d) Growth in medium containing 40% bile salt

The overnight culture was done in 10ml BHIS broth with isolate S2 in triplicate and bile salt was added to make the culture medium with bile salt concentration of 40% weight per volume (by adding 4g of bile salt from Sigma-Aldrich to10ml BHIS broth and mixing it thoroughly with homogeniser). They are incubated at a temperature of 37°C, and OD600 was taken for intervals of 0h, 24h, 48h, and 72h to monitor the growth of isolate S2 in medium containing 40% bile salt. Isolate S2 was also grown at the same temperature in clear BHIS broth as an experimental control for comparison.

#### (e) Growth on blood agar

Isolate S2 was streaked on Brucella blood agar plate in triplicates and were incubated in aerobic condition at 37°C. Plates were then observed for signs and pattern of haemolysis on the blood agar plate.

**(f) Growth on selective medium for Enterococcus**

- Slanetz and Bartley agar

Isolate S2 was streaked on Slanetz and Bartley agar plate in triplicates and was incubated in aerobic conditions at 37°C. Plates were then observed for growth and change in colour of isolate S2 colonies.

- Mannitol salt agar

Isolate S2 was streaked on mannitol salt agar plate in triplicates and was incubated in aerobic condition at 37°C. Plates were then observed for growth and change in colour of isolate S2 colonies.

- Growth in MacConkey broth

Overnight cultures were done in 10ml MacConkey broth with isolate S2 in triplicates. They are incubated at temperature of 37°C and OD600 was taken for intervals of 0h, 24h, 48h and 72h to monitor growth of isolate S2 in MacConkey broth medium. Growth and colour of bacterial colonies observed.

### 2.4.4 DNA extraction and whole genome sequencing

DNA sample of isolate two was extracted from an overnight culture of isolate S2 using spooning method involving the use of chloroform:phenol:isoamyl alcohol given using obtained DNA sample of isolate S2 for whole genome sequencing to identify isolate 2. RNase was used to eliminate RNA from the DNA sample of isolate S2. Using the nano-drop quantity of DNA present in the sample was measured and was found to contain 1845.4ng/µl and A260/A280 value of 1.92 which falls within the range of 1.8-2.0 that indicates that the DNA sample is not contaminated. Then the DNA sample was run on the gel to check one large DNA sample hasn't been broken down into fragments. DNA samples were loaded into the gel with 5x loading dye along with DNA ladder (1000bp or 1 kb; NEB). The DNA bands were then visualised via a Safe Imager™ Blue-Light Trans illuminator, and digital images were saved on the computer. One clear DNA band of interest of length greater than 1kb were excised and purified using the QIAquick ® gel extraction kit (QIAGEN) according to the manufacturer's recommendations. For the whole genome sequencing, extracted DNA sample was sent to Source BioScience for analysis.

## 2.5 Growth curves

Cell growth was measured using a spectrophotometer (Thermo Scientific Biomate 3), measuring optical density at wavelength 600nm (OD600). Overnight cultures of each environmental isolates were diluted individually to 1:1000 in 20ml BHIS broth. 1ml of samples were taken, and the OD600 measured every 2h over a period of -12 h and then at 24, 48, 72 h. CD630 was used as positive control, and BHIS broth was used as a blank. Each environmental isolates were tested 3 times for the growth curve.

### 2.5.1 Filtrate from environmental isolate S2 and its effect on CD630

Several 10ml BHIS overnight cultures of isolate S2 were allowed to grow until the stationary phase of growth. They were firstly centrifuged for three mins at 8,200x g and then using a syringe the supernatant was passed through a filter (Minisart syringe filter, 0.22 μm) into other 10ml centrifuge tubes.

The filtrate obtained from isolate S2 was used to test for the production of bacteriocin by isolate S2 by growing CD630 inside filtrate of isolate S2. Growth was monitored by taking OD600 every 2 hours till 12 hours, 24h, 48h and 72h. Isolate S2 was also grown in the filtrate as a positive control. This investigation was repeated three times.

### 2.5.2 Dilutions of filtrate of isolate S2 and its effect on inhibition of growth of CD630

The difference in inhibition of growth of CD630 by different percentage of the filtrate of isolate S2 in the growth medium was monitored by taking OD600 reading similar to that suggested previously. Different dilutions of the filtrate of isolate S2 used were 0%, 25%, 50%, 75% and 100% which were produced by adding a different volume of filtrate from isolate S2 to BHIS growth medium. Again this investigation was triplicated and isolate S2 was used as a positive control as well.

## 2.6 Bacteriocin Assay

For all spot assays test, BHIS plates were overlaid by spreading 100 µl of CD630 overnight culture, which was allowed to set for 2 h before spotting. For all the listed methods, the extract was serially diluted to $10^{-7}$ using distilled water as a buffer. 5µl of each dilution were spotted on the overlaid BHIS plates and incubated anaerobically at 37°C. The putative bacteriocin inhibitory effects were visualised by a clearing or decrease in opacity compared to spots of BHIS blank controls.

### 2.6.1 Spot assay using filtrate (CFS) of environmental isolate S2

A spot assay was carried out on BHIS agar plate. An overnight culture of CD630 strain was set up in 1 ml BHIS broth. 200ml of overnight culture was mixed with 3ml of molten top agar and was used to overlay BHIS agar plate. Overlaid plates incubated overnight under anaerobic conditions at 37°C to obtain consistent overlay of CD630. 24-h culture tubes of isolate S2 were concentrated and purified by centrifuging culture at 15,000x g for 5 min at room temperature to precipitate cells. . To obtain lysed culture, the pellet from centrifuge was resuspended in 500 µL PBS and sonicated alternatively on and off every 30s for 10 min using a Sonicator Diagenode. The lysate was centrifuged at 15,000x g for 5 min to separate the cell debris, and the lysate supernatant (LS) was then filter-sterilised using a 0.45µm cellulose acetate membrane (49, 137). 5µl of filtrate from isolate S2 was spotted onto prepared BHIS plated and incubated for 24 h. After incubation, any zone of clearance was inspected for activity.

### 2.6.2 Concentrating the CFS of isolate S2

Concentrated solutions of CFS of isolate S2 were produced by ultrafiltration. 500µL samples were loaded in spin-concentrating columns (Corning Spin-X UF 500µL concentrators) with a membrane of 5 kDa molecular weight cut-off and centrifuged at 15,000x g for 15 min. 100µL of concentrated sample was retained for further experiments involving CFS of isolate 2 by storing it in fridge at temperature 2°C. CFS of isolate S2 was not kept longer than a week and fresh CFS was obtained almost every week to maintain its potency/activity.

### 2.6.3 CFS of isolate S2 obtained from induction with Mitomycin C, hydrogen peroxide and co-culturing using *Enterococcus hirae*

Isolate S2 was cultured overnight without the addition of Mitomycin C in 1:100 dilutions in 10ml BHIS broth. Samples are grown till OD600 of 0.1-0.3/cm. After five hours of growth, Mitomycin C was added to give a final concentration of 3µg/ml to induce a stress response that may increase the production of bacteriocin by isolate 2 (49, 137). Isolate S2 with Mitomycin C added was incubated overnight anaerobically at 37°C till isolate S2 reached the stationary phase of growth when filtrate of isolate two was obtained. 5µl of the filtrate obtained from isolate 2 with induction using Mitomycin C was spotted onto prepared BHIS plated overlaid with CD630 and incubated for 24 h. After incubation, any zone of clearance

was inspected for activity. The same method was used with other inducing reagent such as hydrogen peroxide to investigate its inducing effect in comparison to that of Mitomycin C. Production of potential bacteriocin by isolate two was also induced by co-culturing isolate S2 with related bacterial species such as *Enterococcus hirae*. Isolate S2 was initially incubated to grow for 5hrs and *Enterococcus hirae* was introduced after to induce stress response of competition for resources to aid potential increased production of bacteriocin by isolate S2.

## 2.7 Quantification of bacteriocin activity

CFS from isolate S2 were assayed for bacteriocin activity by the critical dilution method using ten-fold serial dilution with PBS -1ml of CFS of isolate 2 in 10ml, 1 in 20ml, 1 in 30ml, etc. and 5µL spotted on solidified soft agar overlaid with CD630. One arbitrary unit per millilitre (AU/mL) of bacteriocin was defined as the reciprocal of the highest dilution showing growth inhibition of the indicator organism. The critical dilution method was also carried out with the following modification; samples were one-fold serially diluted with PBS as well to check for activity in lower concentration to improve the reliability of the result.

## 2.8 Analysis of nature of CFS of isolate S2

### 2.8.1 Effect of storage temperature and storage time on CFS and its activity

CFS obtained from isolate S2 was concentrated using the spin columns mentioned previously for 15 mins. The growth of isolate S2 was not induced. CFS was stored in both 2°C, and 25°C (room temperature) and their activity were monitored over three weeks through observing zone of clearance on an overlay of *C. difficile*. CFS was also placed into tubes to be incubated at 60°C for an hour. Heat treated CFS was cooled to normal temperature and was checked for its activity on CD630. This experiment was repeated 3 times to obtain reliable result.

### 2.8.2 Effect of protease on CFS and its activity

Proteinase K was added to filtrate of isolate S2, CD630 and isolate S2 (positive control) were grown in the filtrate of isolate S2 with or without proteinase K. Amount of enzyme added was calculated according to the volume of CFS to obtain a suitable final concentration of protease enzyme to be about 3µg/ml. OD reading was taken to monitor inhibition of growth at a time interval of every 2 hours until -12 h and at 24 h.

CFS of isolate S2 was treated with different protease enzymes such as proteinase k, trypsin. The amount of enzyme added was calculated according to the volume of CFS to obtain a suitable final concentration of protease enzyme to be about 3µg/ml. After incubation of several hours with different protease treated CFS was spotted on the overlay of CD630 to check for activity and compare activity and protease treated and non-treated CFS. The control used for the experiment was just different protease of final concentration about 3µg/ml in BHIS instead of CFS of isolate S2.

### 2.8.3 Analysis of the protein profile of CFS by SDS-PAGE

To determine the protein profiles of CFS of isolate S2, sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) was used. The concentrated filtrate of isolate S2 (both induced and non-induced) was used with the non-concentrated filtrate of isolate S2 for comparison.4µL of 4xLDS sample buffer, and dithiothreitol to a final concentration of 0.2 M was added to 12 µL of samples to be analysed. The treated samples and the protein marker (ColorPlus Prestained Protein Ladder 11-245 kDa) were heated at 90°C for 1 min, followed by centrifugation at 15,000x g for 1 min. 15µL of each sample were loaded into wells of a NuPAGE Novex 4-12% Bis-Tris polyacrylamide gel, and the gel electrophoresed in MES running buffer at 150 Volts for one h. The gel was stained with SimplyBlue Safestain (Invitrogen) for one h at room temperature with gentle shaking and washed with distilled water overnight. The SDS-PAGE analysis was carried out with safestain staining method as well as silver staining method that have a better sensitivity (using and following instructions according to Pierce Silver stain kit from Thermo Scientific).

# 3. Results and discussion

## 3.1 Isolation of a pool of environmental isolates active against *Clostridium difficile*

Sewage, enriched sewage and faecal samples were used as environmental samples to isolate a pool of environmental isolates active against *C. difficile*. They were used as *C. difficile* is commonly found in gut and human excretions and other bacterial species that shares similar ecological niche are most likely to produce bacteriocin against *C. difficile* to gain competitive advantage. In total 12 active environmental isolates against *C. difficile* strain; CD630 were found from a screening of environmental samples, patch plating individual colonies and observing for a zone of inhibition of growth by overlaying them with CD630. Most of them came from a non-heat treated sewage sample, and only one environmental isolate (S9) showed activity from the heat-treated sample. There were no active environmental isolates from the enriched sewage and faecal samples.

**Table 2: List of active environmental isolates obtained from the environmental samples.**

| Date of isolation | Source | Annotation of active environmental isolates |
|---|---|---|
| 10/12/2014 | Non-heated treated sewage sample | S1 |
| 10/12/2014 | Non-heated treated sewage sample | S2 |
| 12/11/2014 | Non-heated treated sewage sample | S3 |
| 28/11/2014 | Non-heated treated sewage sample | S4 |
| 16/01/2015 | Non-heated treated sewage sample | S5 |
| 16/01/2015 | Non-heated treated sewage sample | S6 |
| 16/01/2015 | Non-heated treated sewage sample | S7 |
| 16/01/2015 | Non-heated treated sewage sample | S8 |
| 22/01/2015 | Heat-treated sewage sample | S9 |
| 22/01/2015 | Non-heated treated sewage sample | S10 |
| 22/01/2015 | Non-heated treated sewage sample | S11 |
| 28/01/2015 | Non-heated treated sewage sample | S12 |

### 3.1.1 Eight out of 12 active environmental isolates exhibited activity against more than three out of five clinically relevant *Clostridium difficile* strains

When active environmental isolates (S1-S12) were tested for their activity against five different clinically relevant *C. difficile* strains; EK28, CD630, R20291, DH478, DH196 by overlaying active environmental isolates with those five different *C. difficile* strains, 8 environmental isolates out of 12 exhibited activities against more than three out of five clinically relevant *C. difficile* strains. These environmental isolates were S2, S3, S6, S7, S8, S9, S10 and S11. This process was carried out more than five times to confirm that their activities against *C. difficile* were replicable and they formed a pool of active isolates against a wide range of *C. difficile* strains for future investigations.

Environmental isolate S2 especially showed very good activity against all five *C. difficile* strains and showed better activity than 27 which was used commonly as experimental control as it was environmental isolate identified as *Clostridium beijerinckii* that showed good activity against *C. difficile* strains in previous CRG investigations. Isolate 3,6 and 7 exhibited a varying zone of inhibition of growth on five different strains of *C. difficile* and did not given significant results like that of isolate S2 or experimental control 27. Isolate 8, 9, 10 and 11 exhibited a varying zone of inhibition of growth on five different strains of *C. difficile* and did not give significant result like that of experimental control 27. Isolate 8 and 9 appears to exhibit a zone of inhibition of growth of all five different strains of *C. difficile* but not as clear and broad like that of isolate 2 or 27.

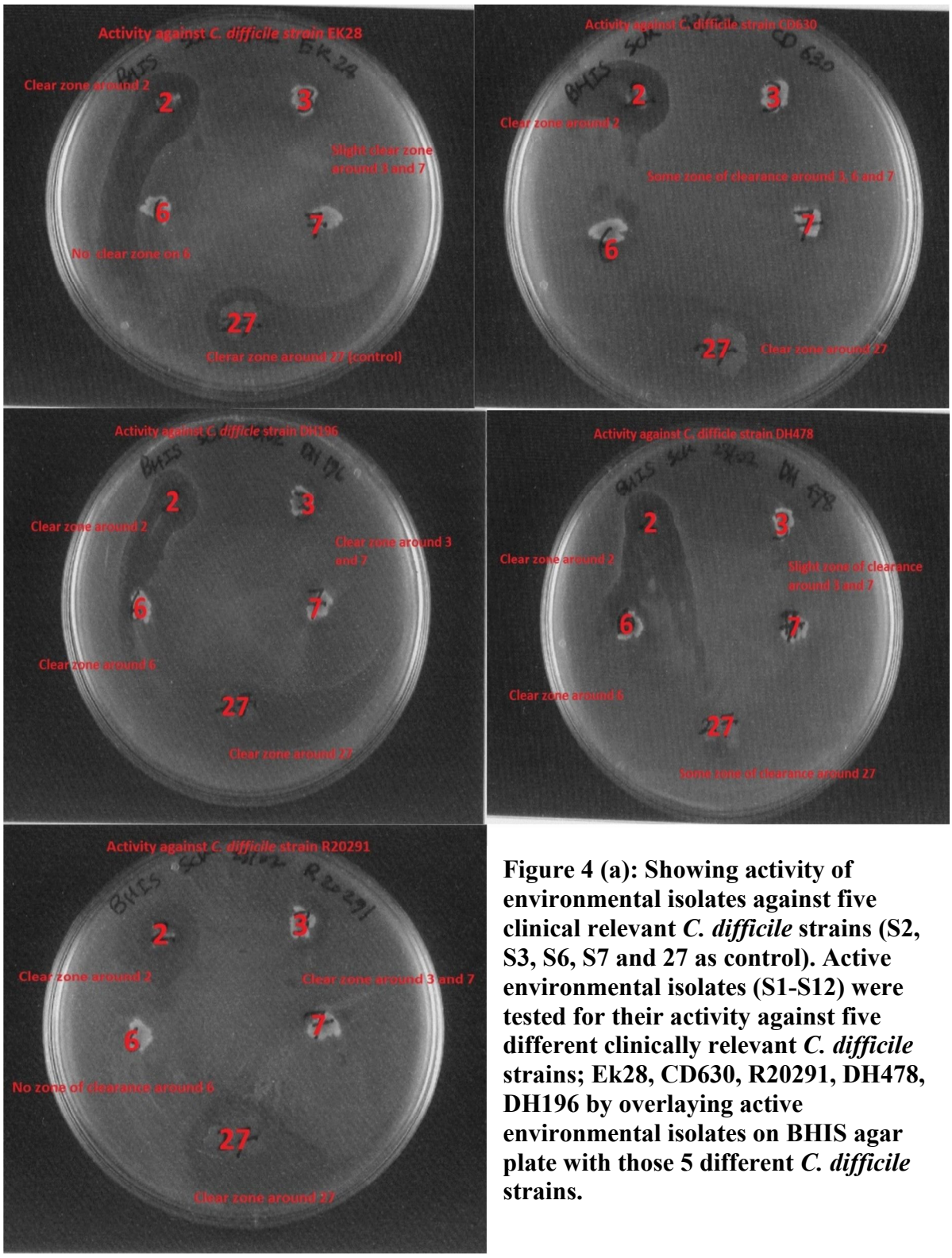



**Figure 4 (a): Showing activity of environmental isolates against five clinical relevant *C. difficile* strains (S2, S3, S6, S7 and 27 as control). Active environmental isolates (S1-S12) were tested for their activity against five different clinically relevant *C. difficile* strains; Ek28, CD630, R20291, DH478, DH196 by overlaying active environmental isolates on BHIS agar plate with those 5 different *C. difficile* strains.**

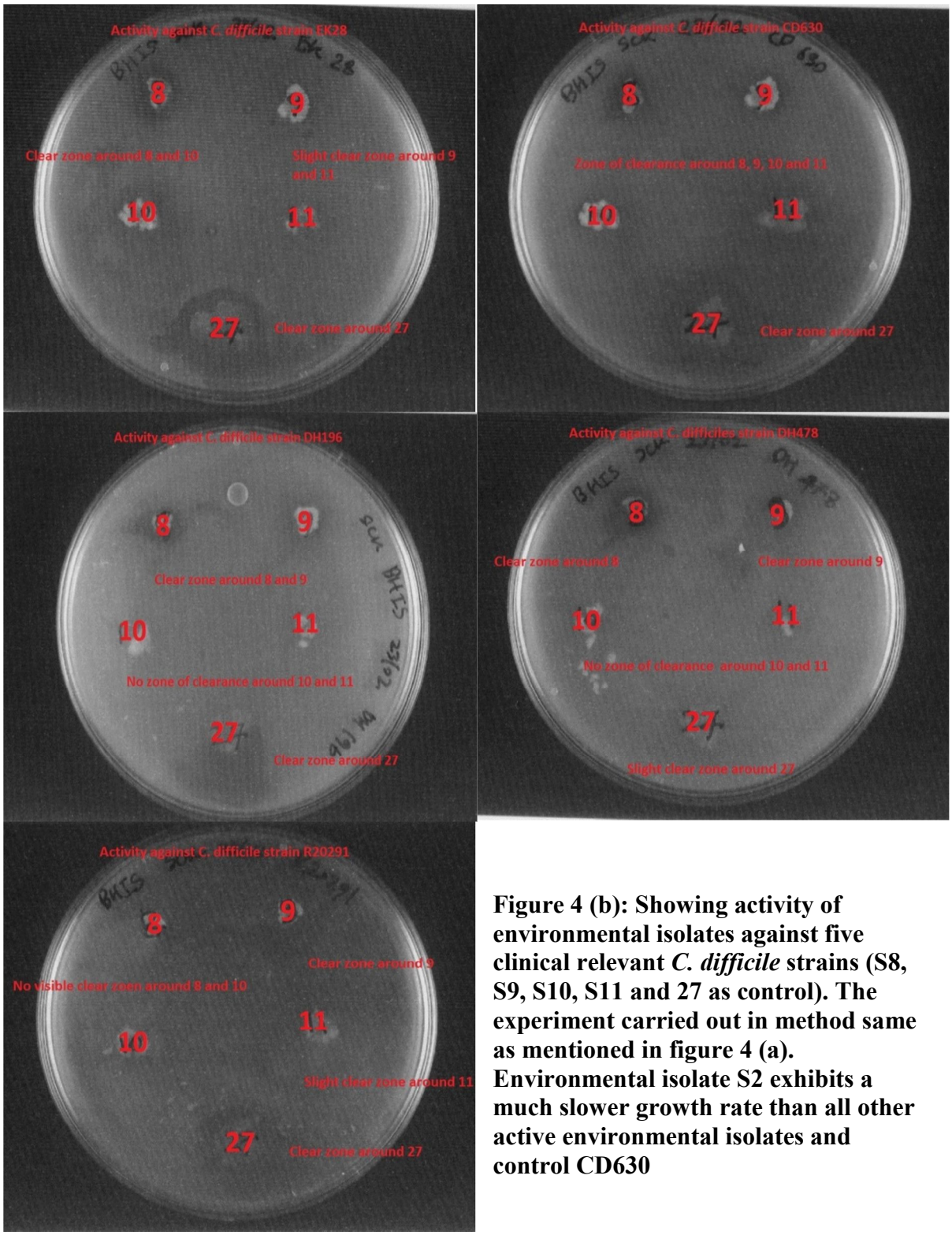



**Figure 4 (b): Showing activity of environmental isolates against five clinical relevant *C. difficile* strains (S8, S9, S10, S11 and 27 as control). The experiment carried out in method same as mentioned in figure 4 (a). Environmental isolate S2 exhibits a much slower growth rate than all other active environmental isolates and control CD630**

## 3.2 Isolate S2 exhibits slower rate of growth than other isolates and CD630 as control

Growth rates were measured for all active environmental isolates to compare their growth rate. This was done to check whether the activity against *C. difficile* strains shown by the active environmental isolates was the result of the possible production of substance like a soluble bacteriocin by the isolates or simply due to a faster-growing rate which could cause inhibition of growth of *C. difficile* due to competition for nutrients etc. It is important to select good potential candidates among many active environmental isolates that appear to have the most potential as possible producers of a novel bacteriocin and assessing the growth rate was a major step in deciding on those candidates that are suitable for further experiments.

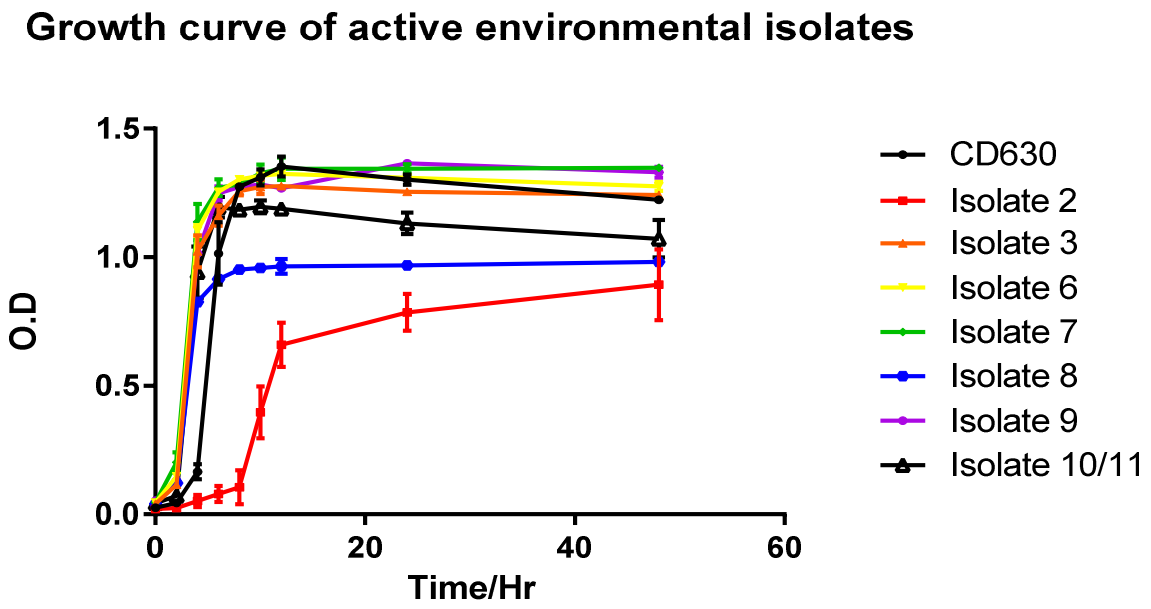



**Figure 5: Graph showing growth curves for all active environmental isolates that showed as a wide range of activities against *C. difficile*. This analysis was carried out by measuring the OD reading from environmental isolates all grown in BHIS growth medium under anaerobic condition with the temperature of 37°C at intervals every 2 hours from 0 hrs to 12 hrs and then 24hrs and 48hrs.**

Isolates S3, S6, S7, S8, S9, S10/11 all show initial growth rates slightly faster than the control *C. difficile* strain CD630. This may hint that the inhibition of growth of *C. difficile* strain CD630 could be a result of out-competing CD630 due to a faster growth rate. Isolates S8, S10, S11 reached stationary phase quicker than CD630 whereas S3, S6, S7, S9 reached stationary phase in a similar time to that of CD630. Isolate S2 has the greatest variation in growth rate but generally exhibited a significantly slower growth rate compared to other environmental isolates and the control CD630. Isolate 2 entered the exponential and stationary growth phases (figure 5) much slower than other isolates as well as stationary phase of growth. Since isolate 2 exhibited the most significant inhibition of the growth of *C. difficile* with the slowest growth rate, it is likely that it may not inhibit the growth of *C. difficile* by out-competing it with faster growth rate but may be due to production of

bacteriocin like substance that has anti-microbial activity against *C. difficile* which will need to be investigated by further experiments. As result, isolate S2 was selected for further investigations to determine whether isolate S2 indeed produces an anti-microbial substance that inhibits the growth of *C. difficile*.

## 3.3 Identification of active environmental isolates indicated variety of different bacterial species showing inhibition of growth of *C. difficile* strains

### 3.3.1 Result of Gram staining

| Active environmental isolates | Result of Gram staining |
|---|---|
| S2 | Gram positive cocci (diplococci, short chain shape) |
| S3 | Gram positive cocci (diplococci, cluster shape) |
| S6 | Gram positive cocci (diplococci. short chain shape) |
| S7 | Gram positive cocci (monococci, cluster shape) |
| S8 | Gram positive cocci (smaller in size, diplococci like) |
| S9 | Gram positive rod (shorter rod shape) |
| S10 | Gram positive rod (longer rod, chain like shape) |
| S11 | Gram positive rod (longer rod, chain like shape) |

**Table 3: Showing summary of Gram staining of active environmental isolates observed under microscope with magnification of x100. S2, S3, S6, S7 and S8 are Gram positive cocci whereas S9, S10, S11 are Gram negative rod like control CD630.**

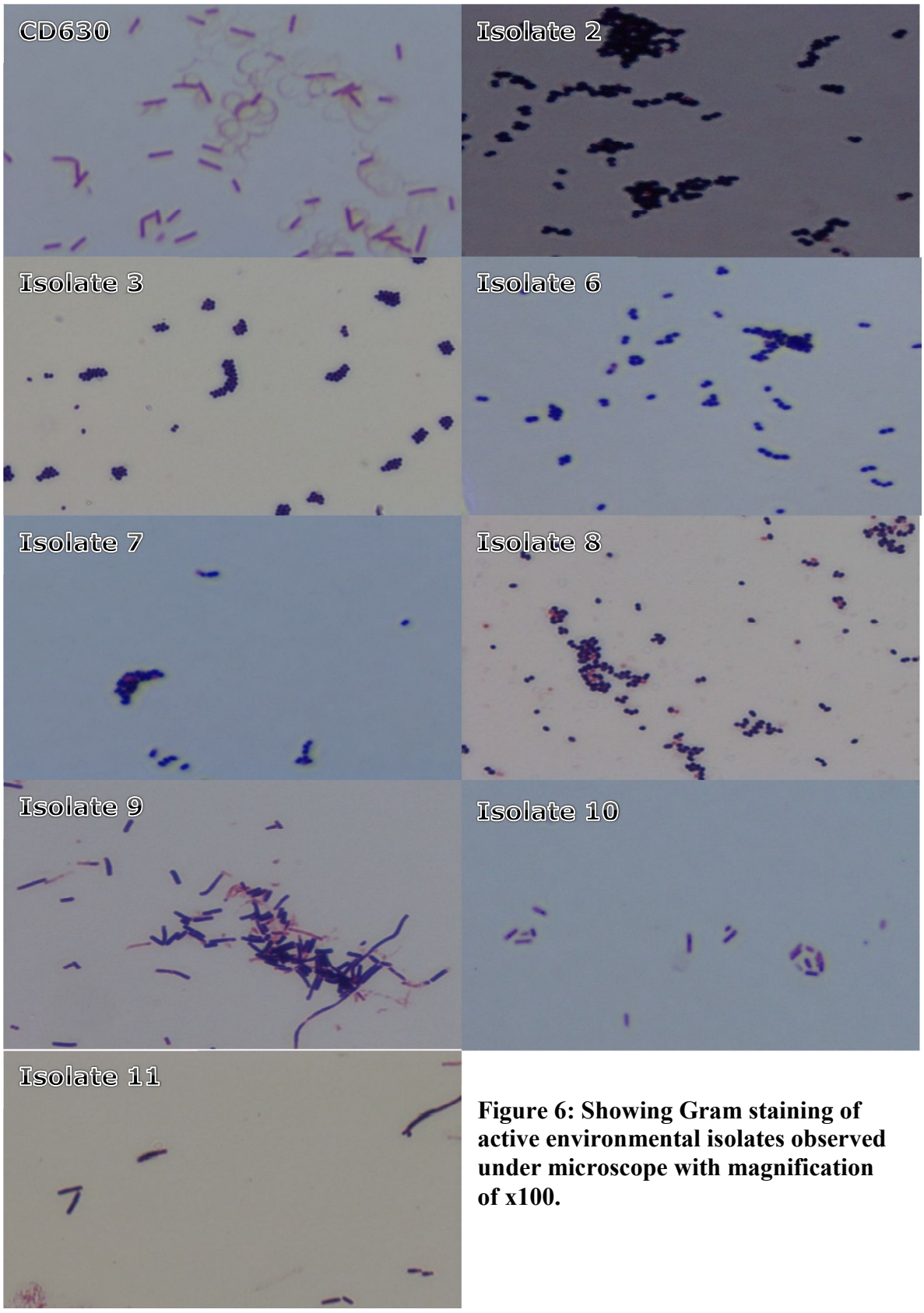



**Figure 6: Showing Gram staining of active environmental isolates observed under microscope with magnification of x100.**

### 3.3.2 Result of 16s rRNA sequencing

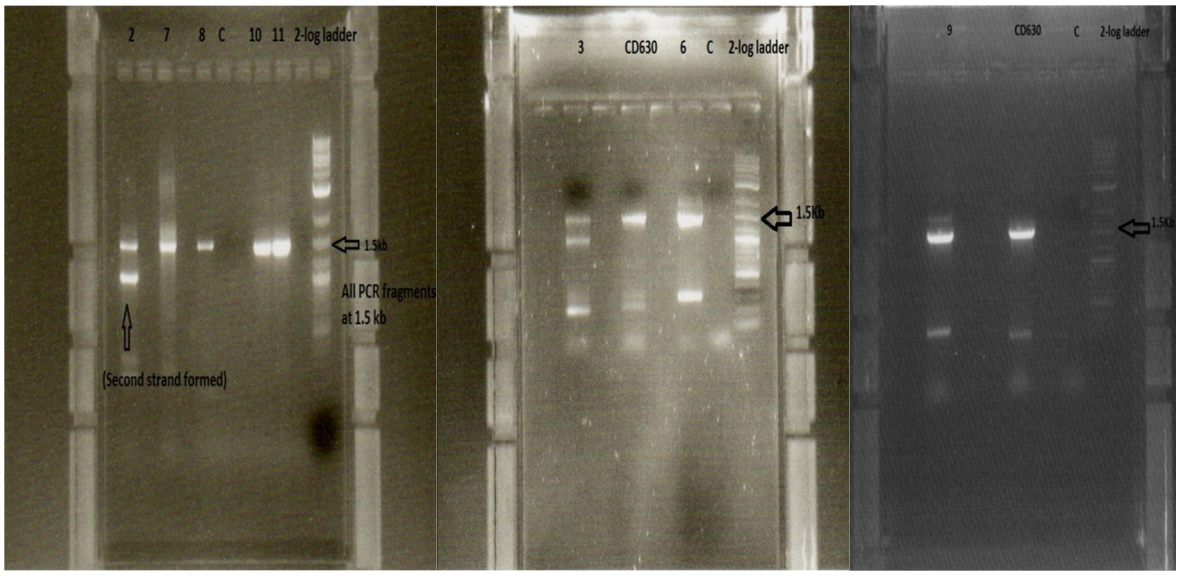



**Figure 7: Showing the separation of DNA fragments on 1% agarose gel after PCR amplification of DNA samples extracted from active environmental isolates. DNA samples were extracted from overnight cultures of environmental isolates active against *C. difficile* and were amplified using PCR for 16s rRNA sequencing. Amplified DNA samples of environmental isolates were separated by gel electrophoresis. DNA fragment of size 1.5kb was extracted from 1 % agarose gel to be use in 16s rRNA sequencing. CD630 was used as positive control and PCR mixture with distilled water instead of DNA sample © was used as negative control for this experiment.**

DNA samples of isolates S2, S3, S6 exhibited another clear band of smaller size apart from target 1.5 kb fragment which are all of different sizes. This could be because PCR procedure used for 16s rRNA sequencing was universal one for all bacteria which works well for *C. difficile* strains rather than specific for unknown environmental isolates under investigation. More specific PCR procedure may be required for individual environmental samples to obtain more representative and accurate data from 16s rRNA sequencing as all bacteria have different bases sequence for 16s rRNA that may result in varying physical properties such as length, possible structure and melting point etc.

| Active environmental isolates | Result of 16s rRNA sequencing |
|---|---|
| S2 | 99% *Enterococcus faecium/lactis/durans/faecalis* |
| S3 | 99% *Enterococcus faecium/lactis/durans* |
| S6 | 99% *Enterococcus faecium/lactis/durans* |
| S7 | 99% *Enterococcus faecium/lactis/durans* |
| S8 | 99% *Enterococcus hirae* |
| S9 | 98% *Clostridium difficile* strain CD630 |
| S10 | 97% *Clostridium perfringens* |
| S11 | 97% *Clostridium perfringens* |

**Table 4: Showing results of 16s rRNA sequencing of all active environmental isolates exhibiting inhibition of growth of different C. difficile strains. 16s rRNA sequences of all environmental isolates were compared with online database for identification.**

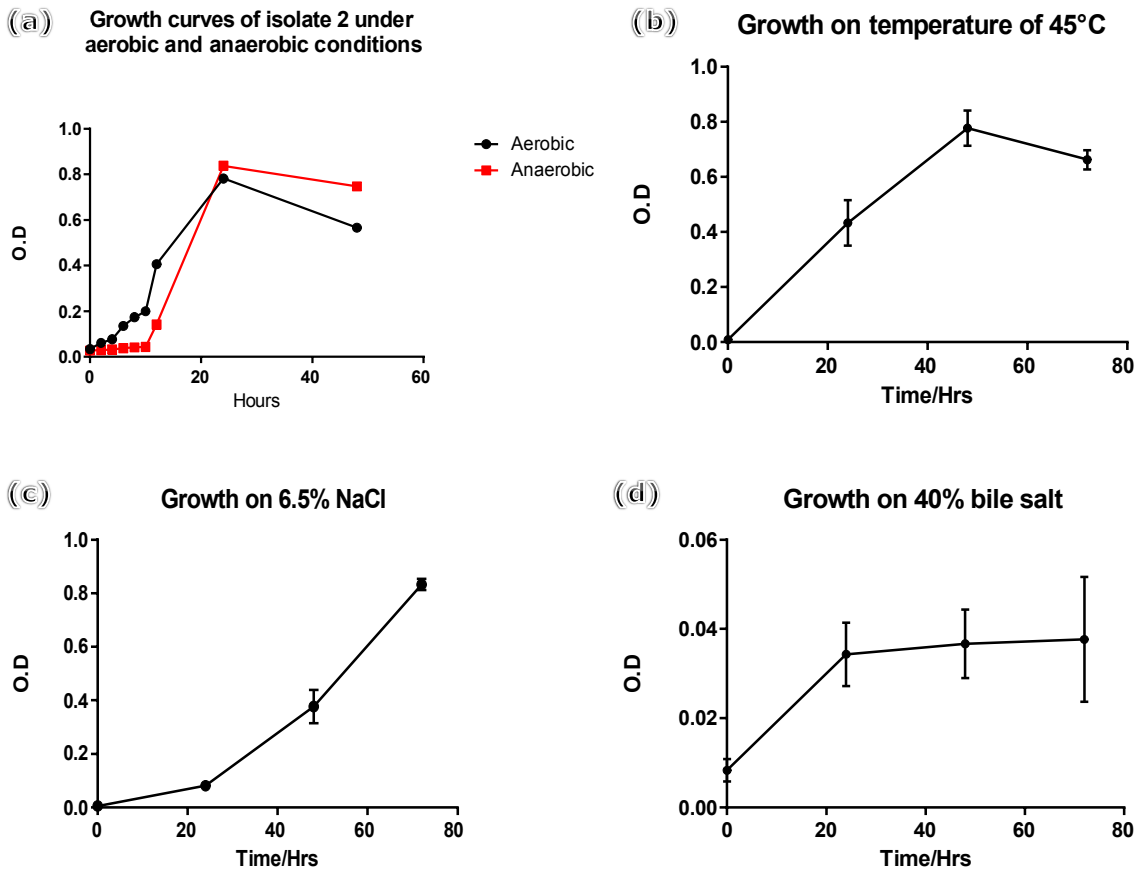
Most of the isolates identified by commonly used 16s rRNA sequencing belonged to the *Enterococcus* group; S2, S3, S6 and S7 were designated as either *Enterococcus faecium* or *Enterococcus lactis* or *Enterococcus durans* or *Enterococcus faecalis* with same percentage similarity with all these three species. Isolate S8 was identified as *Enterococcus hirae* with no other Enterococcus species with higher percentage similarity. It was interesting to find that S9 belonged to *C. difficile* strain CD630 and that it was able to inhibit the growth of other *C. difficile* strains. Inhibition could be due to competition for nutrient between similar species supported by the growth curve result as they had similar growth rate to control but also could be due to the production of bacteriocin like chemical that is usually produced as a narrow spectrum antimicrobial substance to kill closely related organisms to gain competitive advantage. Both *Enterococcus sp.* and *Clostridium sp.* have long been known producers of bacteriocin which may explain their activity against different strains of *C. difficile* (73, 105). S10 and S11 both belonged to *Clostridium perfringens*. It was noted in previous studies done within CRG that *C. perfringens* exhibited the inhibition of growth of *C. difficile*. Since previous studies involving *C. perfringens* is still ongoing, the decision was made to further explore *C. perfringens* along with S9 in future and focus work on isolate S2 for further experiments as it exhibited the greatest inhibition of growth on all five *C. difficile* strains tested (EK28, CD630, DH196, DH478 and R20291). Isolate S2 also had the slowest growth rate compared to other active environmental isolates and the control CD630 which could allow the exclusion of the possibility of inhibition of growth of *C. difficile* as result of competition due to faster growth rate. Therefore, after identification of all active environmental isolates, isolate S2 has shown the greatest possibility as potential bacteriocin producer with good effectiveness in inhibiting the growth of *C. difficile* and was determined as the most suitable candidate for the further experiment to investigate for the production of anti-microbial substance against *C. difficile*.

### 3.3.3 Series of tests confirmed that isolate S2 possibly belonged to *Enterococcus*

*Enterococci* are characteristically facultative anaerobe that can both survive in aerobic and in anaerobic conditions. Isolate S2 exhibit growth in both conditions and shows faster growth rates under aerobic condition confirming that can grow in both conditions.

*Enterococci* are known to be able to grow in wide range of temperatures, including high temperature like 45°C as well as other unfavourable conditions such as high salt concentration etc. Isolate S2 was able to grow in temperature of 45°C even though slower than that compare to growth of isolate 2 at temperature of 37°C where all other growth curve of isolate 2 was carried out. Isolate S2 was able to grow in the high salt concentration of 6.5% NaCl, confirming that it's an *Enterococcus*. *Enterococci* are known to be able to grow in the high bile salt concentration up to 40% as they are often found in gut. Isolate S2 did not exhibit sufficient growth in 40% bile salt concentration. This may be due to a greater concentration of bile salt in a small volume of 10ml or incubation time may have not been sufficient.

**Figure 8: Showing the results of different investigations to establish and identify environmental isolate S2 belonging to the Enterococcus group.**
**(a): Showing isolate S2 growing under both aerobic and anaerobic conditions.**
**(b): Showing isolate S2 growing in high temperature of 45°C.**
**(c): Showing isolate S2 growing in high salt concentration of 6.5% NaCl.**
**(d): Showing isolate S2 not growing well in bile salt concentration of 40%.**



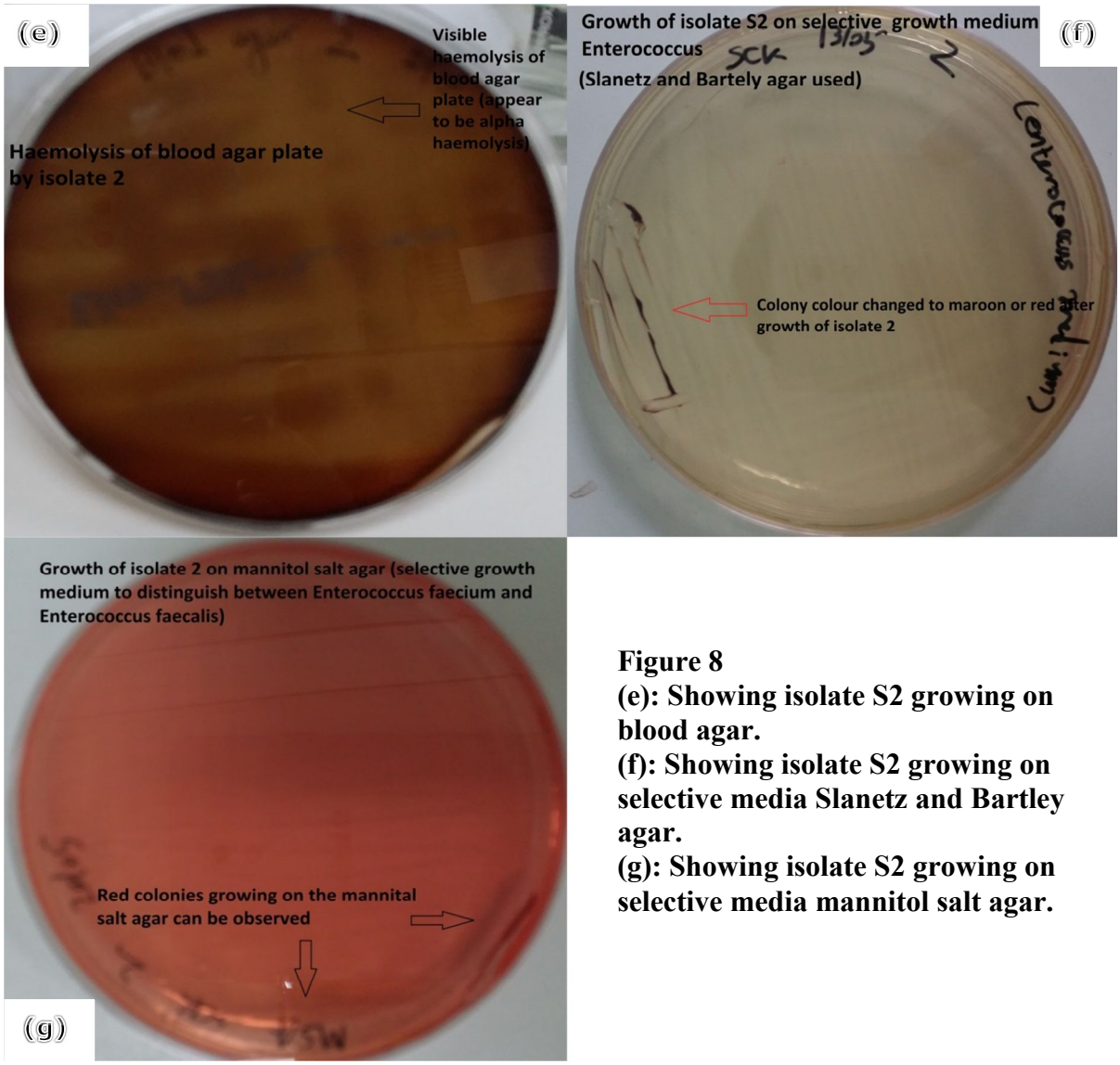



**Figure 8**
**(e): Showing isolate S2 growing on blood agar.**
**(f): Showing isolate S2 growing on selective media Slanetz and Bartley agar.**
**(g): Showing isolate S2 growing on selective media mannitol salt agar.**

Isolate S2 was grown in selective mediums for Enterococcus for confirmation and identification. Isolate S2 grew sufficiently well on the blood agar, and it caused haemolysis of blood agar around the area where isolate S2 colony was growing (Alpha-haemolysis). *Enterococcus faecium* and *durans* exhibit Alpha haemolysis compared to beta haemolysis by *Enterococcus faecalis*. *Enterococcus lactis* is known to be either alpha or non-haemolytic. It's hard to identify exact species of isolate S2 by observing haemolysis of blood agar plate. Slanetz and Bartley agar were used as a selective medium to grow *Enterococcus* (140). Isolate S2 grew relatively well, and the region around bacterial colonies of isolate S2 changed colour to maroon or red confirming isolate S2 being an *Enterococcus*. **.** Mannitol salt agar acts as selective media to distinguish between *Enterococcus faecium* and *Enterococcus faecalis* as observing haemolysis was not conclusive. *Enterococcus faecalis* produces organic acids from mannitol that turns phenol red in mannitol salt agar into yellow colour, and *Enterococcus faecium* does not (119). Mannitol salt agar did not turning yellow confirms that isolate S2 is more likely to be *Enterococcus faecium/durans /lactis* rather than *faecalis* along with haemolysis result.

### 3.3.4 DNA extraction and whole genome sequencing was carried out

When DNA sample extracted from isolate S2 for whole genome sequencing was run on the gel, one distinct DNA band of large size was obtained which indicates DNA hasn't been broken down into fragments and is suitable for whole genome sequencing. The clear band obtained had a size greater than the highest molecular band size of the ladder that is 1kb. There is some sign of radiolucency stretching on both DNA samples which could be due to some degradation of DNA, possibly due to prolonged incubation time than necessary. The DNA sample extracted from the gel was sent to Source BioScience for whole genome sequencing. The whole genome sequencing result for isolate S2 could not be presented on results of this investigation due to the time constraint of the project as it required a long time for the sequencing result to come out for the sequence lab. As the result of experiments done so far, the identity of the current environmental isolate S2 could be narrowed down to it being *Enterococcus faecium* or *lactis* or *durans*.

*Enterococcus* belonging to LAB along with *Streptococcus* have been long scrutinised for bacteriocin production in food, and veterinary industry and many types of research have shown that they are producers of a wide range of antimicrobial peptides and bacteriocins given in figure 9 (105). Most peptide bacteriocins have been obtained from *E. faecium* and *E. faecalis* and exhibit good activity against a wide range of bacteria. There has not been any important research done on bacteriocin production by *Enterococcus* that is active against *C. difficile*. However, *E. faecium* produces known enterotoxins; most researched bacteriocin from *Enterococcus* which is usually active against other enterococci, LAB, *Listeria monocytogenes*, *S. aureus*, and *Clostridium spp.* Including *C. botulinum*, *C. perfringens*, and *C. tyrobutyricum* (47). Therefore, isolate S2 belonging to *Enterococcus* supports its potential as bacteriocin producing bacteria which could produce bacteriocin against *C. difficile* which has not been well documented before. Since *Enterococci* are one of most common human intestinal lactic acid bacteria usually present as commensals, it is possible that *Enterococci* may encounter other commensal or opportunistic pathogen like *C. difficile* also present in the human intestine. Therefore, it is not strange idea for *Enterococci* to produce bacteriocin active against *C. difficile* to gain a selective advantage as the part of gut microflora. Also, isolate S2 may belong to *E. lactis* or *durans* rather than well researched *E. faecium* and may be producing novel bacteriocin against *C. difficile*. The fact that other active environmental isolates obtained in this experiment such as S3, S6, S7 and S8 apart from S2 belonged to the *Enterococcus* group would explain their activity against different strains of *C. difficile* even though their antimicrobial effect was not as profound as that of isolate S2. Also environmental isolates were obtained from faecal, sewage samples which are likely to contain bacterial species of common gut microflora.

| Organism | Bacteriocin | Type | Mass (Da) (amino acids) |
|---|---|---|---|
| *E. faecalis* | Cytolysin $Cyl^{r}_{L}$, $Cyl^{r}_{S}$ | Class I two-peptide lantibiotics | 3,458 (38), 2,032 (21) |
| *E. faecium* | Enterocin A | Class IIa pediocin-like | 4,829 (47) |
| *E. faecium* | Enterocin P | Class IIa pediocin-like | 4,493 (44) |
| *E. faecium* | Bac 32 | Class IIa pediocin-like | 7,998 (70) |
| *E. faecium* | Bacteriocin GM-1 | Class IIa pediocin-like | 4,630 (44) |
| *E. faecalis* | Bac 31 | Class IIa pediocin-like | (43) |
| *E. mundtii* | Mundticin ATO6, mundticin KS, enterocin CRL35, mundticin QU2 | Class IIa pediocin-like | 4,287 (43) |
| *E. faecalis* | Enterocin SE-K4 | Class IIa pediocin-like | 5,356.2 (43) |
| *E. faecium* | Bacteriocin T8 | Class IIa pediocin-like | 5,090 (44) |
| *E. faecium* | Enterocin B | Class II (no subclass) | 5,479 (53) |
| *E. faecalis* | Enterocin 1071A, enterocin 1071B | Class IIb two-peptide bacteriocin | 4,285 (39), 3,897 (35) |
| *E. faecalis* | MR10A MR10B | Class IIc, leaderless | 5,202 (44), 5,208 (43) |
| *E. faecium* | Enterocin L50; L50A, L50B | Class IIc, leaderless | 5,190 (44), 5,178 (43) |
| *E. faecium* | Enterocin Q | Class IIc, leaderless | 3,980 (34) |
| *E. faecalis* | Enterocin EJ97 | Class IIc, leaderless | 5,328 (44) |
| *E. faecium* | Enterocin RJ-11 | Class IIc, leaderless | 5,049 (44) |
| *E. faecalis* | AS-48 | Class IId circular bacteriocin | 7,166 (70) |

**Figure 9: Summary of a list of examples of different bacteriocins produced by *Enterococcus* (105). *E. faecalis* and *E. faecium* are two most well documented producer of various types of bacteriocin even though others also do exhibit bacteriocin production in some studies such as pediocin like Avicin A from *Enterococcus avium* (12).**

## 3.4 Inhibition of growth of CD630 by CFS from isolate S2

### 3.4.1 Filtrate (CFS) obtained from growing medium of isolate S2 in its stationary phase inhibited growth of both *C.difficile* strain CD630 and isolate S2

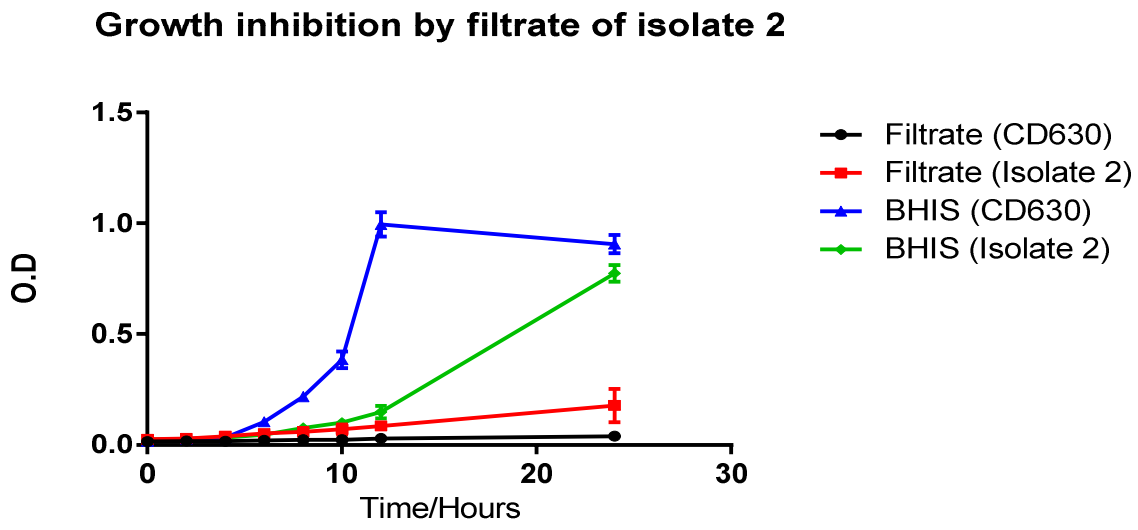



**Figure 10: Showing inhibition of growth of *C. difficile* strain CD630 by filtrate obtained from isolate S2 that may contain the antimicrobial substance. The filtrate (cell free supernatant) was obtained from isolate S2 grown till stationary phase in BHIS growth medium by centrifuging and filtering to remove lysed cell debris, and CD630 was grown in filtrate as culture medium to see if there is any inhibition in growth of CD630. OD reading was taken to monitor inhibition of a growth over time interval of every 2 h until -12 h and at 24 h. Isolate S2 was used as positive control to see the effect of filtrate on growth in comparison to that of CD630.**

A marked reduction in the growth rate for both CD630 and isolate S2 was observed if they were both cultured on the filtrate of isolate S2. When both CD630 and isolate S2 were grown in BHIS, they showed similar growth rate and pattern to that mentioned previously for growth curves of all active environmental isolates. However, for first 24 hours, both CD630 and isolate S2 did not grow much, and CD630 appeared to be inhibited more than isolate S2 as isolate S2 showed some growth even though it was much reduced. Reduction in growth of CD630 and isolate S2 in filtrate could be the result of using spent medium (filtrate) to grow to isolate S2 to stationary phase or it could also be due to secretion of an antimicrobial substance like bacteriocin from isolate S2 which may have inhibited or slowed the growth of CD630 as well as isolate S2. It is unlikely for the isolate S2 to be sensitive to the potential active ingredient of filtrate which may be causing the inhibition of growth for CD630. It is more likely for the reduced growth could be as a result of decrease in nutrients on growth medium or changes in medium during growth of isolate S2 such as changes in pH, presence of waste products etc. which could have caused reduction/inhibition of growth for both CD630 and isolate S2.

To investigate whether the inhibition of growth of CD630 and isolate S2 is due to lack of nutrients rather than the production of antimicrobial substance that inhibits growth, incubation time for growth curve was increased to 72 hours to see if there is further growth of

CD630 and isolate S2 in filtrate obtained from isolate S2 from the very slow growth exhibited in after 24 hours of incubation.

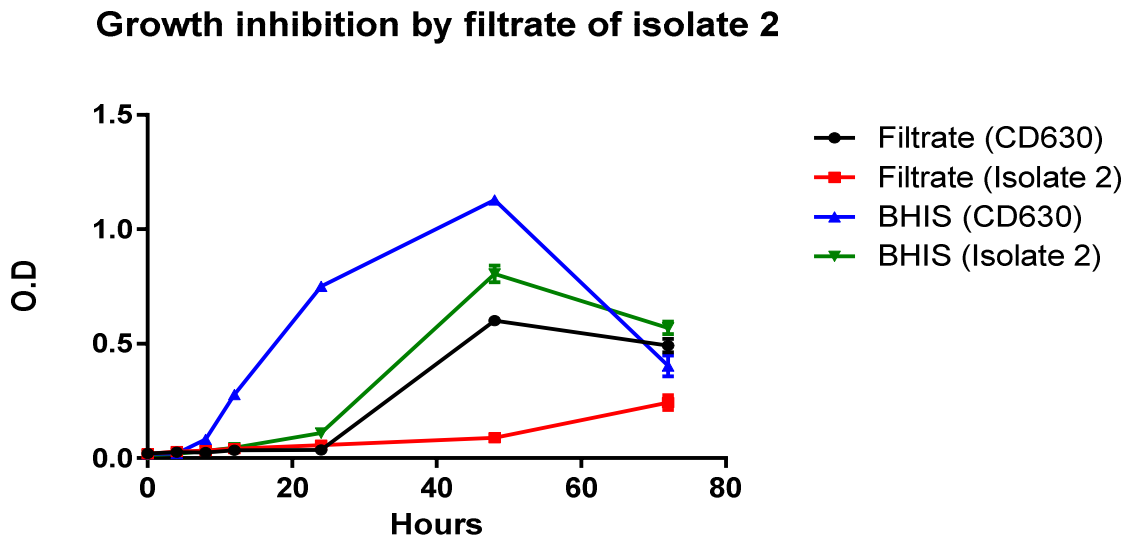



**Figure 11: Showing inhibition of growth of C. difficile strain CD630 by filtrate obtained from isolate S2 for an incubation period of 72 h. This experiment was carried out with the same method used in figure 7 but for an incubation period of 72 h rather than for first 24 h. This was done to see if the inhibition of growth of CD630 and isolate S2 was due to lack of nutrient in the filtrate or due to a substance present in the filtrate of isolate S2.**

When CD630 was incubated for 72 hours in the filtrate of isolate S2, the growth of CD630 was inhibited compared to CD630 grown in BHIS growth medium for first 24 hours similar that obtained previously (figure 10). However, after 24 hours, CD630 was able to grow in the filtrate of isolate S2 similar to that grown on BHIS growth medium. This result could be used to rule out the fact that inhibition of growth of CD630 by filtrate of isolate S2 is not due to using spent medium as a growth medium, as otherwise, CD630 wouldn't have resumed near normal growth rate if nutrients were lacking in the growth medium in the first place. Isolate S2 did not grow as much compared to CD630, but it also started growing steadily after the 48 hours mark which may mean that the amount of nutrient couldn't have been the limiting factor in inhibiting the growth of both CD630 and isolate S2 when they were grown in the filtrate obtained from isolate S2 growing in it. This result does support the possibility that isolates S2 may be potentially secreting or producing a substance which may be inhibiting the growth of CD630 and may be the reason why isolate S2 is exhibiting an obvious and diffuse zone of inhibition on the lawn of five different *C. difficile* strains. It is not certain why the growth of isolate S2 may be inhibited by growing it in the filtrate of isolate S2. However, it could be due to the fact that the substance present in filtrate of isolate S2 could have inhibitory effect or toxic to closely related bacterial species including itself or it could be due to the fact that particular nutrient in BHIS growth media may have been in little amount owing to growing isolate S2 against in filtrate where isolate S2 was grown previously. Isolate S2 if it's is a bacteriocin producer should be immune to the bacteriocin it produces. Thus the inhibition of the growth of isolate S2 will need to be explained with further experiments by doing the same experiment but in concentrated BHIS growth medium which will confirm whether the result was due to lack of nutrient or not for inhibition of growth of isolate S2 in its filtrate.

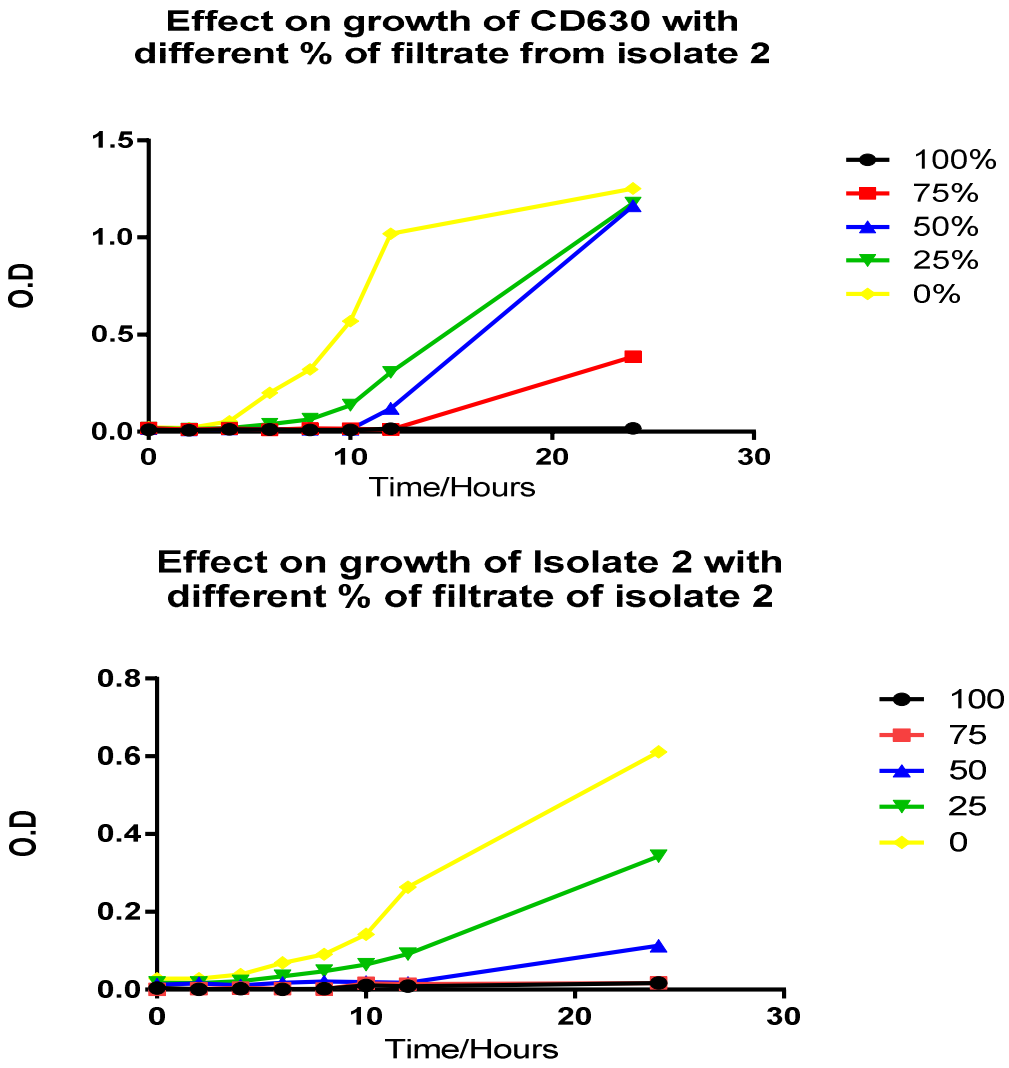



**Figure 12: Showing inhibition of growth of *C. difficile* strain CD630 and isolate S2 respectively by different percentage concentrations of the filtrate obtained from growing isolate S2. An experimental method carried out was same as that used in figure 10 and 11, but the different concentration of filtrate was used by adding a different amount of filtrate of isolate 2 in BHIS growth medium mentioned in table 2.**

Different percentage of filtrate from isolate S2 as the growth media had differing effects on the growth of CD630. The smaller the amount of filtrate used then the less inhibition of growth of CD630 was. As a result, it took less time for the CD630 to start growing with a decrease in the percentage of the filtrate of isolate S2 in the growth medium. This result further supports the previous experiments that indicated that isolate S2 might produce anti-microbial substance like bacteriocin which inhibited the growth of CD630 as the inhibitory effect of the filtrate of isolate S2 on the growth of CD630 appears to be concentration dependent. Concentration dependent inhibition of growth of CD630 would potentially mean that isolate S2 may be producing a soluble substance that may be inhibiting the growth of CD630. This result also supports the fact that more new media supports increased growth of CD630 which will need to be further confirmed.

Different percentages of filtrate from isolate S2 had a similar effect on the growth of isolate S2 which was used as a positive control. Isolate S2 has growth rate curve that is much slower in comparison to CD630. As a result, over 24hrs, isolate S2 did not reach the stationary phase of growth even in 0 % filtrate (only BHIS). Also an increase in the percentage of filtrate within growth medium inhibited the growth of isolate S2 more, and it took longer for isolate S2 to exhibit growth in the higher percentage of filtrate in the growth medium. Both 75% and 100% filtrates almost inhibited the growth of isolate S2 completely for 24 hours incubation period. It is important to note that the filtrate obtained from the growing medium of isolate S2 could potentially contain a substance that inhibited the growth of both CD630 and isolates S2 itself. Since the growth of CD630 and isolate S2 appears to increase to normal levels (like in 100% BHIS or 0% filtrate from isolate S2) if percentage of filtrate in growth medium decreases, we could conclude that it may not be filtrate obtained from isolate S2 making the growth medium unfavourable for growth like lowering pH of growth medium by producing acids, etc. that would not support healthy growth if amount of filtrate decreased. These findings are suggestive that isolate S2 could be a potential producer of antimicrobial substance like bacteriocin that is active against *C. difficile* which will require further investigations.

### 3.4.2 Reduction in the activity of CFS from isolate S2 when incubated with protease

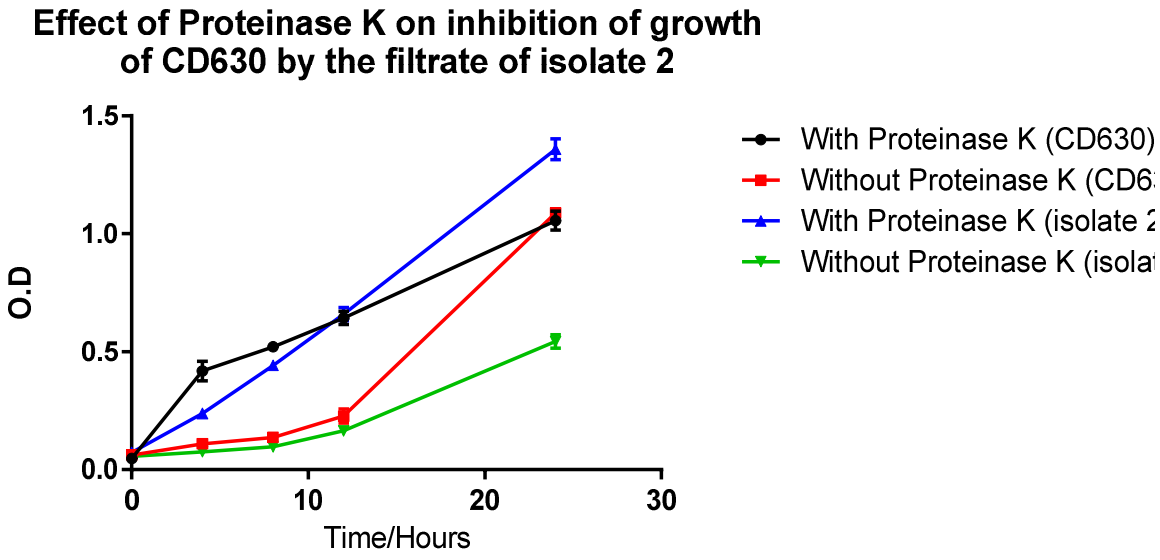



**Figure 13: Showing the effect of Proteinase K on the inhibition of growth of CD630 and isolate S2 by the filtrate obtained from isolate S2. Proteinase K was added to the filtrate of isolate S2 and CD630 and isolate S2 (positive control) was grown in filtrate of isolate S2 with or without proteinase K. OD reading was taken to monitor inhibition of growth at the time interval of every 2 hours until -12 h and at 24 h. This was done to see if the inhibition of growth of CD630 was a result of protein based substance present in the filtrate of isolate S2.**

In an effort to determine whether the isolate S2 was possibly producing a proteinaceous substance similar to ribosomally synthesized bacteriocin, sensitivity to a general protease was tested by adding proteinase K to the filtrate obtained from isolate S2 to determine the effectiveness of filtrate obtained from isolate S2 in inhibiting the growth of CD630 was due to secreted protein. Growth of CD630 in filtrate of isolate S2 as growth medium with proteinase K exhibited increased growth of CD630 in comparison to growth of CD630 in filtrate of isolate S2 without proteinase K. However, after 24 hours of incubation, growth of CD630 became similar for both with or without Proteinase K, even though initially growth was higher with the proteinase K treated sample. Isolate S2 also showed a similar result to that of CD630 as isolate S2 grown in its filtrate with added proteinase K exhibited greater growth in comparison to that grown in filtrate without proteinase K (figure 13).

## 3.5 Bacteriocin assay using filtrate of isolate S2 (CFS)

### 3.5.1 Concentrated CFS of isolate S2 exhibited inhibition of growth of *C. difficle* strains

Protocols for bacteriocin assays were developed to cover most referenced tests for bacteriocins analysis (49, 137). The protocol developed for the spot assay was a modified method employed by Sell et al., 1983 (137). After confirming the inhibition of growth of CD630 by the filtrate obtained from isolate S2, spot assays were done on BHIS agar plates overlaid with CD630. CFS obtained from isolate S2 was used as well as concentrated CFS. Concentrated CFS from isolate 2 exhibited zone of inhibition of growth on BHIS plate overlaid with CD630 whereas CFS without concentrating didn't. This may be because 5 µl of filtrate or cell free supernatant was not concentrated enough and may not have contained enough dissolved CFS that inhibited the growth of CD630 in previous experiments. Methods to induce more production of a potential antimicrobial substance by isolate S2 such as the use of DNA damaging agents such as Mitomycin C, hydrogen peroxide will need to be explored. Bacteriocin assay using other *C.difficle* strains also exhibited inhibition of growth with concentrated CFS.

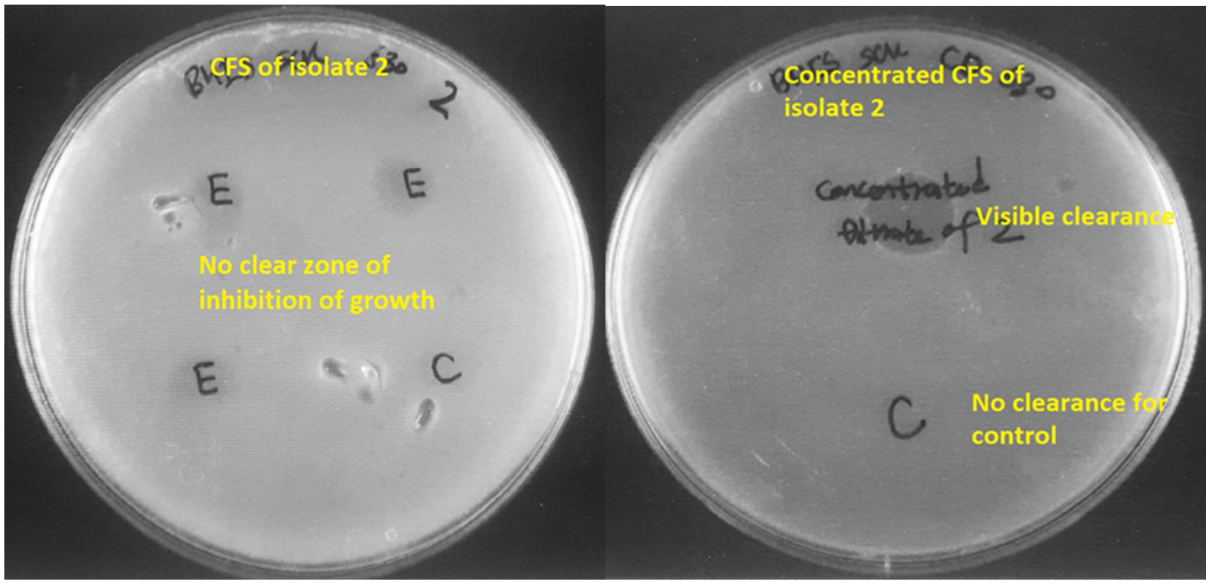



**Figure 14: Showing inhibition of growth of CD630 by concentrated CFS of isolate S2. Control used was plain BHIS. Non-concentrated CFS of isolate S2 did not give clear zone of inhibition of growth of CD630. However, there are some signs of inhibition of growth of CD630.**

### 3.5.2 Spot assay using concentrated CFS from isolate S2 induced with Mitomycin C, hydrogen peroxide, co-culture also exhibited zone of inhibition of growth on BHIS agar plates overlaid with CD630

As a common method to cause increased production of a potential antibacterial substance by inducing a stress response, Mitomycin C was used to a final concentration of 3µg/ml to induce increased production of antibacterial substance against CD630 by isolate S2 (49, 137). The filtrate of isolate S2 induced using Mitomycin C was used to carry out spot assay to check its activity against CD630. The concentrated filtrate of isolate S2 (CFS) induced with Mitomycin C gave a clear zone of inhibition of growth on BHIS agar plates overlaid with CD630. Using Mitomycin C to induce production of R-type bacteriocin by *C. difficile* is well documented in Gebhart et al., 2012 (49) and is commonly used method to induce increased bacteriocin production by increasing stress stimuli to the growing organism. However, in this experiment, Mitomycin C may not have been most suitable inducer to induce potential production of an antimicrobial substance by isolate S2. When induced by hydrogen peroxide and co-culturing with *E. hirae* as a competitor, zone of clearance on CD630 was more significant by concentrated CFS of 2 in comparison to that induced by Mitomycin C. This could be that isolate S2 could have reduced sensitivity to Mitomycin C or may have been killed by it.

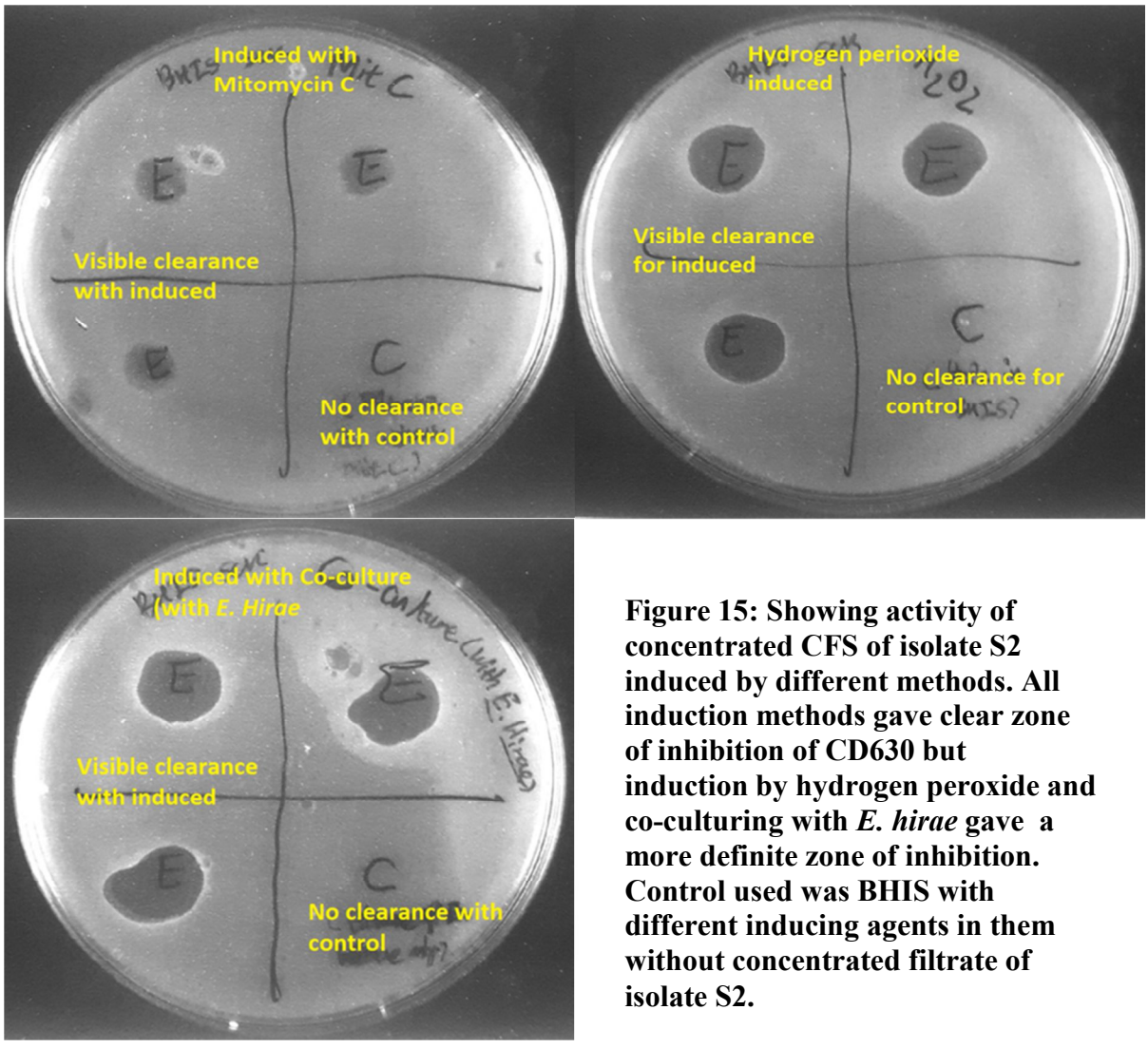



**Figure 15: Showing activity of concentrated CFS of isolate S2 induced by different methods. All induction methods gave clear zone of inhibition of CD630 but induction by hydrogen peroxide and co-culturing with *E. hirae* gave a more definite zone of inhibition. Control used was BHIS with different inducing agents in them without concentrated filtrate of isolate S2.**

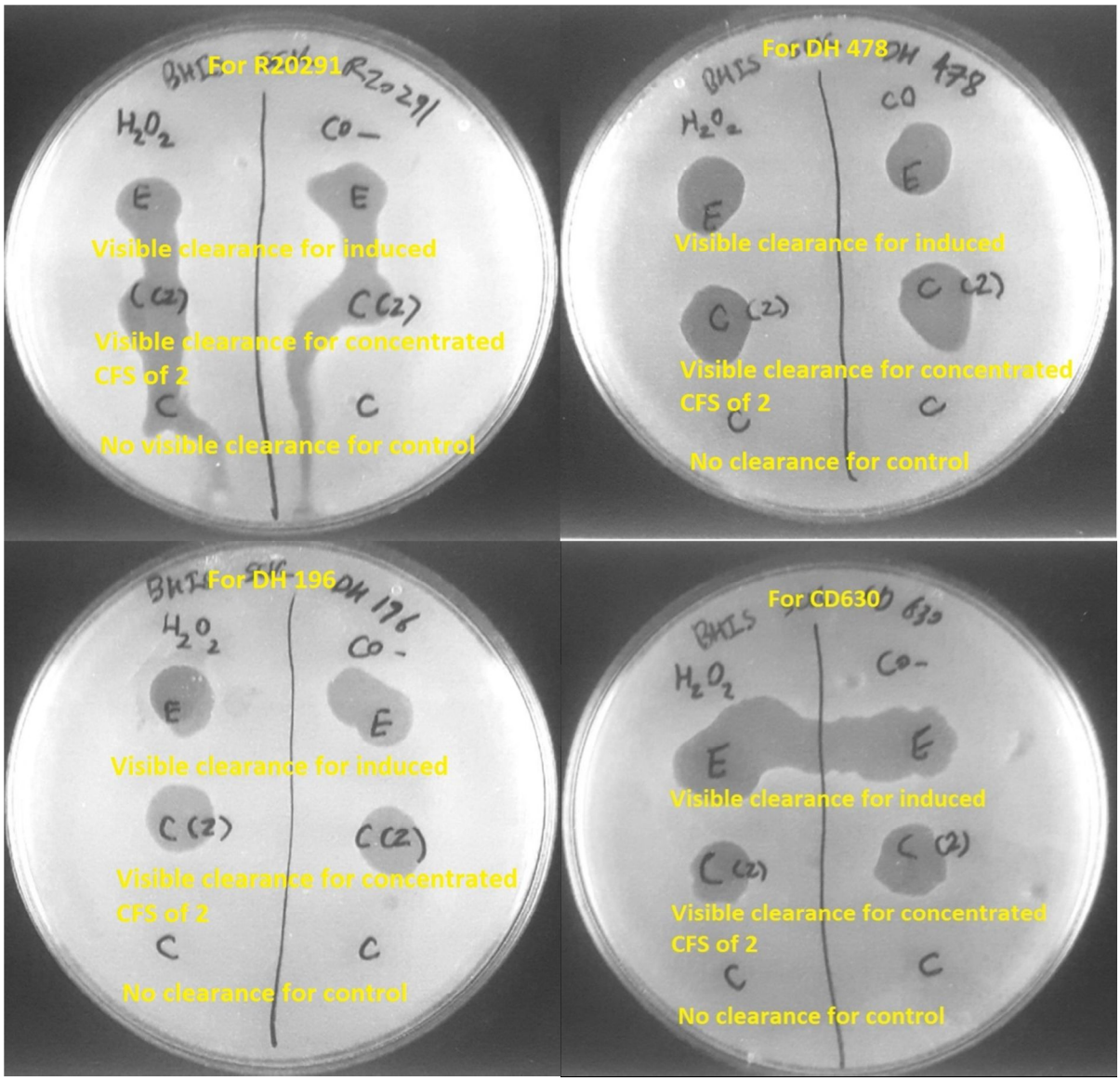



**Figure 16: Showing clear inhibition of the growth of different strains of *C. difficle* (R20291, DH478, DH196 and CD630) by concentrated CFS of isolate S2 induced by hydrogen peroxide and co-culturing with *E. hirae.* Induction was done with hydrogen peroxide and co-culturing with *E. hirae* as it was discovered to be more effective in inducing production of active component of CFS of isolate S2 against *C. difficle* than Mitomycin C. The control used in this investigation was BHIS with different inducing agents in them without concentrated filtrate of isolate S2.**

### 3.5.3 CFS of isolate S2 is active on CD630 in lower concentrations

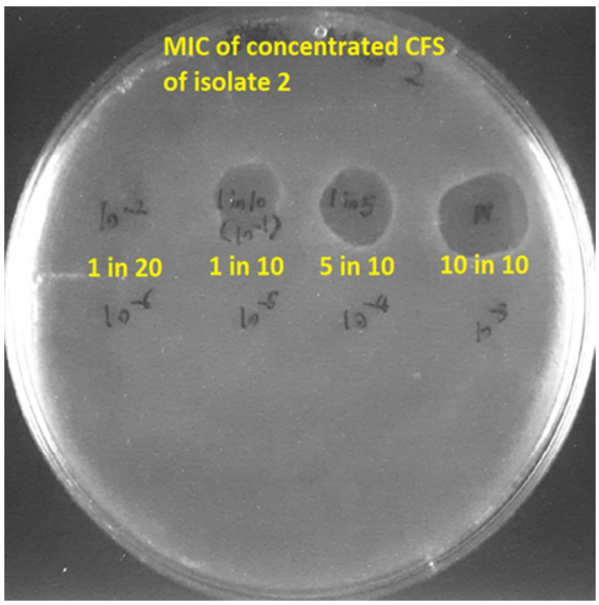



**Figure 17: Showing the MIC of concentrated CFS of isolate 2 on CD630. CFS of isolate 2 shows consistently significant activity against CD630 till 1 in 10 (1ml of CFS of isolate 2 in 10ml). MIC of concentrated CFS isolate 2 is estimated to be 1 in 20 (20 AU/ml) as CFS of isolate 2 exhibited inhibition of growth of CD630 even though up to a small extent. As it can be seen on the figure, area of clearance of growth of CD630 gradually decreased from 10 in 10ml to 1 in 20ml**.

Zone of clearance was visible in concentrations: from no dilution 10 in 10ml (as control) to 1 in 10ml dilution. A small zone of clearance was visible for 1 in 20ml dilution and after1 in 20ml; no zone of clearance could be visible. This was repeated few times and was concluded that MIC of the concentrated filtrate of isolate S2 might lie between dilutions of 1 in 10ml and 1 in 20ml. The titre was expressed as the highest dilution showing inhibition and was estimated to be 20 AU/ml. According to Corbett et al., MIC of vancomycin against CD630 has a range of 0.5-10µg/ml and according to Pelaez et al. MIC of metronidazole against 415 strains of *C. difficile* has a range of 0.5-4.0µg/ml. Direct comparison with CFS of isolate S2 with other commonly used antibiotics against CDI such as metronidazole and vancomycin is difficult by results obtained from this investigation due to not being able to correctively quantify amount of active ingredient of filtrate of isolate S2 against CD630 for accurate comparison. However, this result allows us to know that the CFS of isolate S2 has an active component that is relatively potent against the growth of CD630 as it is active in small quantity. The activity of filtrate of isolate S2 could be compared with commonly used antibiotics against CDI such as metronidazole and vancomycin by susceptibility testing and possibly time kill curves.

### 3.5.4 Action of protease inhibited on the activity of filtrate of isolate S2 on CD630

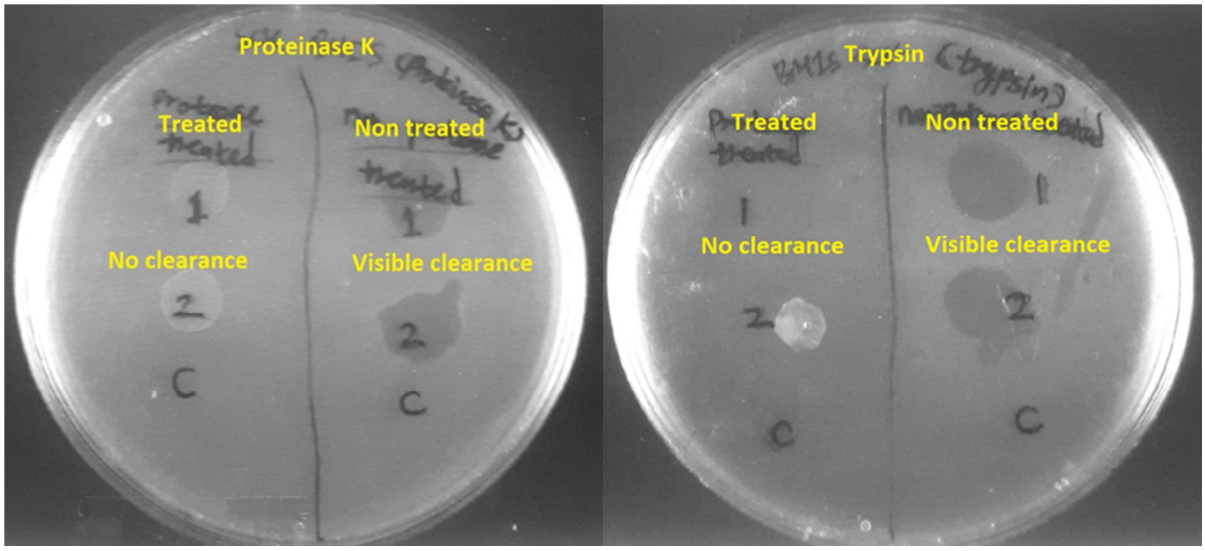



**Figure 18: Showing activity on CD630 of concentrated CFS of isolate 2 which is treated and non-treated with proteinase K and trypsin. Both non-treated CFS inhibited the growth of CD630 as there is a clear zone of growth inhibition whereas treated CFS did not give the area of clearance. Control used was a drop of proteinase K and trypsin mixed with water respectively.**

The concentrated filtrate obtained from isolate S2 was treated with different protease enzymes such as proteinase k and trypsin. After incubation with various proteases, the filtrate was spotted on the overlay of *C.difficile* to check for activity. Filtrate treated with proteinase K and trypsin did not show any zone of clearance whereas filtrate untreated with proteinase K and trypsin exhibited activity on the overlay. This experiment indicates that the active component of filtrate in isolate S2 that is active against *C. difficile* could be of protein nature like ribosomally synthesised small antimicrobial such as bacteriocin.

### 3.5.5 Activity of CFS of isolate S2 against CD630 is dependent on temperature and storage duration

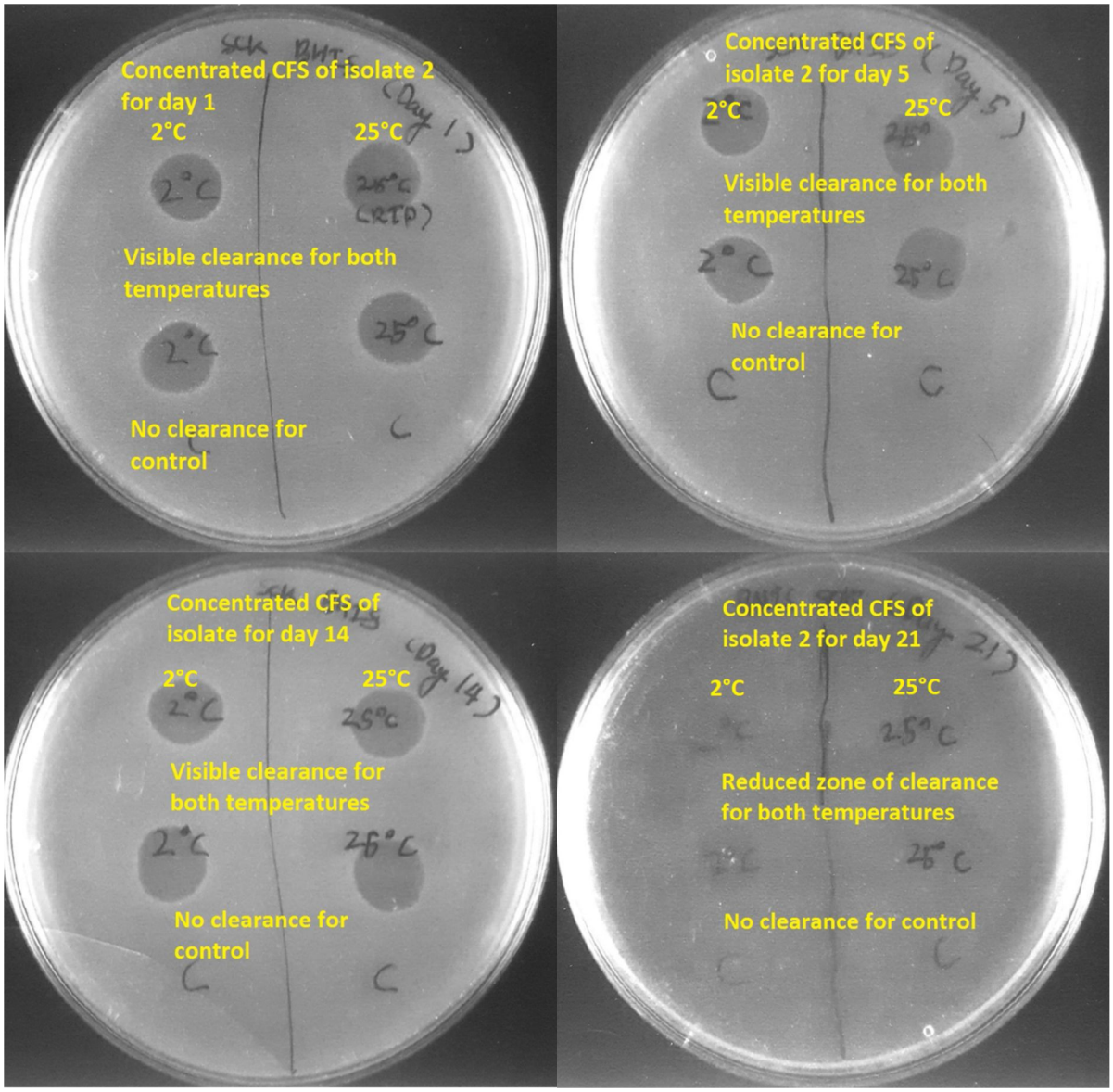



**Figure 19: Showing the activity of filtrate of isolate S2 under the storage condition of 2°C and 25°C (room temperature). For both temperatures, the activity of filtrate of isolate S2 remained roughly same on day one as well as day 14 (after two weeks). Very little or no activity was visible for both 2°C, and 25°C after the CFS of isolate S2 was stored for three weeks.**

Zone of clearance was observed clearly from concentrated filtrate of isolate S2 over 2 weeks without much change in the activity against CD630 in both 2°C and 25°C (room temperature). When the concentrated CFS of isolate S2 was incubated at the high temperature of 60°C, no zone of clearance was observed on CD630. As a result, I could conclude that the CFS of isolate S2 could be stored in 25°C or lower temperature for storage but loses its activity in high temperature. This result reinforces the possibility that the active component of CFS of isolate S2 against *C. difficle* could be protein based like bacteriocin as the activity could have been lost in higher temperature due to denaturation of protein structure. To further reinforce this idea, investigation on the effect of pH on the activity of CFS of isolate S2 could be carried out for the further physical characterisation of potential bacteriocin.

The activity of CFS of isolate S2 also did not last for more than 3 weeks as little or no zone of clearance visible on CD630 after an incubation period of 3 weeks. This could be because the active component of CFS of isolate S2 could be quite unstable and may not have long storage live. This could be because potential bacteriocin present in CFS of isolate S2 could be of a low molecular size which could make it thermally and physically unstable. Also interestingly, during many repeated experiments I have noticed that when CFS of isolate S2 was spotted on an already grown overlay of CD630, no zone of clearance of growth was visible which potentially indicates that the effect of an active component of isolate S2 on *C. difficle* could be of bacteriostatic in nature-preventing bacterial growth rather than bactericidal. More carefully designed experiment may be required to verify this observation made during the investigation as even though the bacteriocin could have killed the bacteria after it had grown, there could still be dead cells suspended in the agar which may have prevented formation of zone of clearance of growth.

### 3.5.6 CFS of isolate S2 had many protein molecules of different sizes

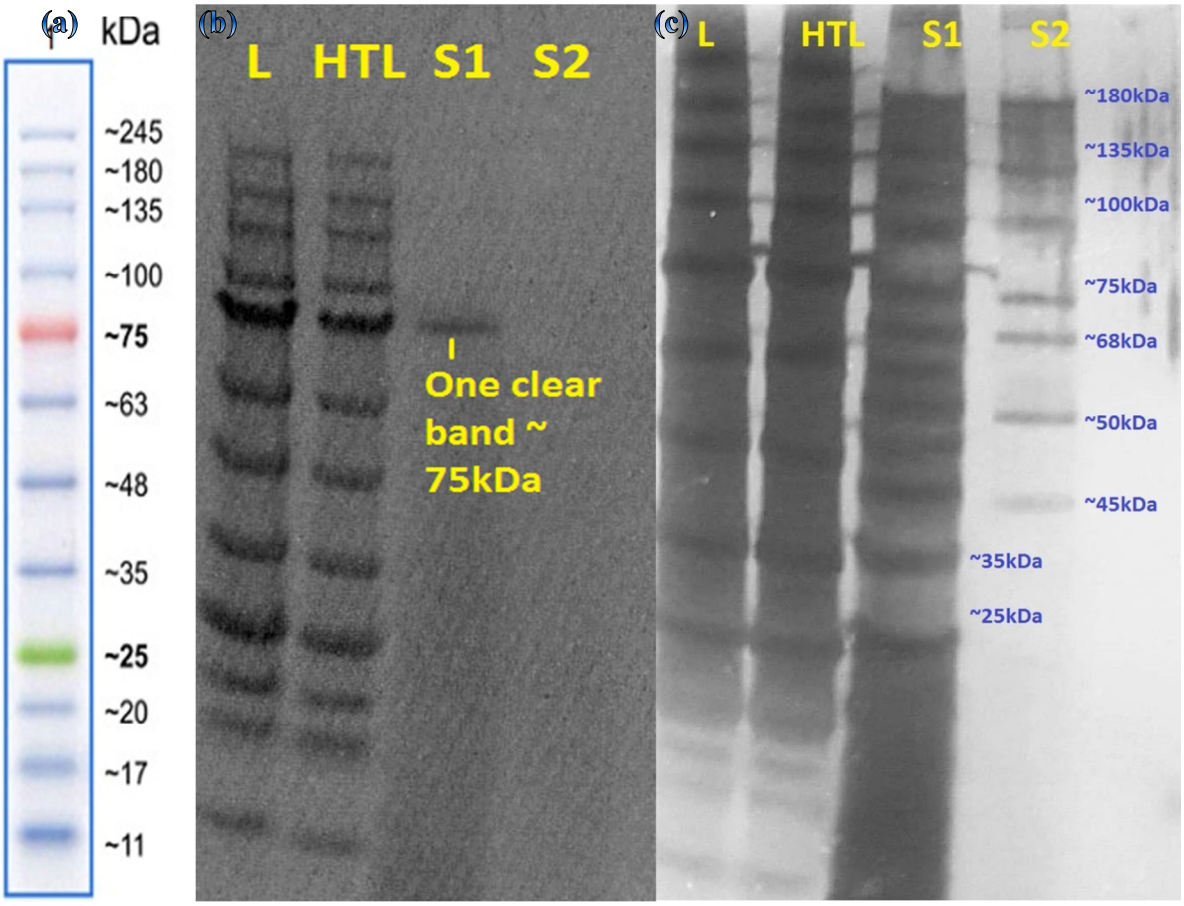



**Figure 20: Showing result obtained from SDS-PAGE analysis of CFS of isolate S2.**

**(a) The protein ladder used in this investigation (ColorPlus Prestained Proteinladder 11-245 kDa)**

**(b) Showing the SDS-PAGE analysis carried out using NuPAGE Novex 4-12% Bis-Tris polyacrylamide gel stained with SimplyBlue Safestain (Invitrogen). Gel was loaded in the order of protein marker (L), heat treated protein marker (HTL), concentrated CFS of isolate 2 (S1) and not concentrated CFS of isolate 2 (S2). Only one protein band of size approximately 75 kDa was found on S1 and none on S2.**

**(c) Showing the SDS-PAGE analysis carried out using NuPAGE Novex 4-12% Bis-Tris polyacrylamide gel gel stained with Pierce Silver stain kit from Thermo Scientific for better sensitivity. Gel was loaded in the order of protein marker (L), heat treated protein marker (HTL), concentrated CFS of isolate 2 (S1) and not concentrated CFS of isolate 2 (S2). Many bands of different sizes were detected for both S1 and S2. Both S1 and S2 have bands at same protein sizes of 180kDa, 135kDa, 100kDA, 75kDa, 68kDA, 50kDa and 45kDa. Many more bands of different protein size visible on concentrated CFS of isolate 2 (S1) especially of protein sizes of approximately 63kDa, 48kDa, 35kDa and 25kDa.**

From the SDS-PAGE analysis, it could be concluded that CFS of isolate S2 consists of a mixture of many different proteins of various sizes. Only one protein band of size approximately 75 kDa was found in gel stained with SimplyBlue Safestain (Invitrogen) due to lower sensitivity in comparison to the Pierce Silver Staining. However, this also tells us that in 12 µL of CFS sample of isolate S2 did not contain much amount of protein in general. A protein band of size ~75kDa was also visible in the gel stained with silver staining, and this allows us to conclude that ~75kDa protein molecule could be present in the most amount in CFS of isolate S2 in comparison to others as it was stained in low sensitivity as well. Given that 75kDa protein molecule may be most abundant in CFS of isolate S2, it may be the active component of CFS that has an antimicrobial property like bacteriocin against *C. difficle* or it could be standard protein material present in the cytoplasm of the cell. More in depth investigation will be required to identify and analyse this protein molecule further.

For gel stained with silver stain kit, many bands of different sizes were detected for both concentrated and not concentrated CFS of isolate S2. Both have bands at same protein sizes of 180kDa, 135kDa, 100kDA, 75kDa, 68kDA, 50kDa and 45kDa. Many more bands of different protein size visible on concentrated CFS of isolate S2 especially of protein sizes of approximately 63kDa, 48kDa, 35kDa and 25kDa. Small peptides could only been seen with silver stain here as the stain has greater sensitivity and was over-exposed which allowed small peptides of small amount to be detected. The silver stained gel had a high background as stained gel could have been overdeveloped, and it could be resolved by reducing development time. Also, stop solution may not have been as effective in stopping development gel as it should have been and this could be avoided by preparing new 5% acetic acid and replace it twice in the first mins of incubation with the gel in the future use of silver stain for SDS-PAGE. To improve visibility, SDS-PAGE could be carried out again with silver staining to obtain more distinct bands for investigation. Since there are many bands of protein molecules of varying size from ~180kDa to ~25kDa, it's hard to pin point what protein molecule could be the possible active component of CFS of isolate S2 against *C. difficle*. As result, further investigations will be required to isolate and identify very likely protein based antimicrobial substance or substances produced by isolate S2 and good starting point would be to study proteins of molecular size ~180kDa, 135kDa, 100kDA, 75kDa, 68kDA, 50kDa and 45kDa that is observed in both concentrated and not concentrated CFS. As mentioned previously, bacteriocins from Gram-positive bacteria are typically classified into Class I, Class IIa/b/c, and Class III, and they produce bacteriocins with different molecular sizes (31). Class, I are small peptide inhibitors like nisin and the class II bacteriocins are small (<10 kDa), heat-stable proteins (32). Class III bacteriocins are large (>10 kDa), heat-labile protein bacteriocins (32) which best described the protein molecules observed on SDS-PAGE analysis of CFS of isolate S2 which was confirmed heat-labile before. However, as figure 10 shows, *Enterococcus* are known to produce class I or II type bacteriocin rather than type III. It may be possible that active component of CFS of isolate S2 could be unknown class III type bacteriocin from Enterococcus which need further investigation. The SDS-PAGE analysis could be carried out to specifically look for protein molecules of a size smaller than 11kDa and thermostability of CFS of isolate S2 could be further tested by a range of temperatures not as high as 60°C.

# 4. Conclusion and further works

Among 12 environmental isolates by the screening of heat treated and non-heat treated sewage and faecal samples were found to have an inhibitory effect on *C. difficile* strain CD630 commonly used as the experimental control/indicator strain. 8 out of 12 of these environmental isolates (S2, S3, S6, S7, S8, S9, S10 and S11) exhibited the inhibitory effect on the growth of at least 3 of 5 clinically relevant *C. difficile* strains such as EK28, CD630, DH196, DH478 and R20291. Growth curve analysis of all environmental isolates active against *C. difficile* revealed that isolate S2 showed significantly slower growth rate compare to other isolates that had growth rates similar to or faster than CD630. All active environmental isolates were identified using Gram staining and 16s rRNA sequencing and most of the active isolates (S2, S3, S4, S6, S7 and S8) belonged to the Enterococcus group whereas others (S9, S10 and S11) were different species of *Clostridium group* such as *C. perfringens* and *C. difficile*. Both *Enterococcus sp.* and *Clostridium sp*. have been known producers of bacteriocin which may explain their activity against different strains of *C. difficile* (73, 105). Isolate S2 was given particular focus in this investigation as it exhibited the greatest activity on all five *C. difficle* strains and had slowest growth rate which suggests that it could potentially inhibits the growth of *C. difficle* by antimicrobial substance production like bacteriocin rather than out-competing them for resources through faster growth. Tests to determine the exact identity of isolate S2 along with 16s rRNA sequencing result confirmed isolate S2 as either *Enterococcus faecium/lactis/durans* which are intimately related species with similar properties. The total DNA was extracted from isolate S2 and whole genome sequencing was carried out for its identification. The result of whole genome sequencing was not obtained from the lab in time for this thesis but can confirm isolate S2 belongs to Enterococcus. Knowing the exact identity of isolate S2 could be useful for designing further experimental methods specific to isolate S2 as well as researching for more past literature on the organism about bacteriocin research. Since Enterococci are well documented to be the producer of bacteriocin by many studies, it would be important to investigate if environmental isolate S2 is producing novel bacteriocin active against *C. difficile* or bacteriocin which has been discovered previously but activity against *C. difficile* that has not been documented well before.

CFS obtained from the growing medium of isolate S2 exhibited inhibition of the growth of CD630 when CD630 was grown within it in comparison to growth in BHIS broth as the growth medium. Increasing the percentage of CFS of isolate S2 in growth medium also showed increased inhibition of the growth of CD630 which is suggestive of potential production of antimicrobial substance against C. difficile by isolate S2. Decreased growth inhibitory effect on CD630 by the addition of proteinase K to the filtrate of isolate S2 may also indicate that isolate S2 may be producing protein based antimicrobial substance active against *C. difficile*. The concentrated filtrate of isolate S2 when spotted on a lawn of CD630, exhibited a distinct zone of inhibition of growth of CD630 whereas not concentrated didn't. Zone of inhibition of growth of CD630 also was visible when isolate S2 was induced using Mitomycin C as well as hydrogen peroxide and co-culturing *Enterococcus* such as *E. hirae*. Hydrogen peroxide and co-culturing with other *Enterococcus* such as *E. hirae* induced the activity of CFS of isolate on CD630 and other *C. difficile* more than Mitomycin C by giving a more distinct zone of clearance. According to Birri et al., 2009 (12), for isolation and induction of bacteriocin from *Enterococcus* species such as *E. avium*, they have used MRS (De man, Rogosa, Sharpe) growth medium to grow *Enterococcus* as it acted as good non-selective medium for profuse growth of LAB at specific pH of 6.2 and temperature of 25°C.

Culture medium specific for *Enterococcus* could be used in conjunction to inducing agents to aid growth and production of potential bacteriocin for isolate S2.

Concentrated CFS of isolate S2 was treated with protease enzymes such as proteinase K and trypsin, and the activity of CFS of isolate S2 on the CD630 was inhibited when treated in comparison to CFS untreated with protease. The concentrated filtrate of isolate S2 did not show any change in its activity against CD630 over two weeks in both 2°C and 25°C (room temperature). When the concentrated CFS of isolate S2 was incubated at the high temperature of 60°C, no zone of clearance was observed on CD630. As a result, it can be concluded that the activity of CFS of isolate S2 was affected by storage temperature and could be stored in 25°C or lower temperature for storage but loses its activity in high temperature. The potential loss of activity due to denaturation under heat reinforces the possibility that the active component of CFS of isolate S2 against *C. difficle* could be protein based like bacteriocin along with other results. To further investigate this idea, investigation on the effect of pH and other enzymes such as lipase, beta glucosidase, papain, lysozyme, etc. could be carried out on the activity of CFS of isolate S2 for the further physical characterisation of potential bacteriocin as well as to provide more reliability of data (108). The activity of CFS of isolate S2 also did not last for more than three weeks as little or no zone of clearance visible on CD630 after an incubation period of 3 weeks. This could be because the active component of CFS of isolate S2 could be quite unstable and may not have long storage life, possibly due to a low molecular size which could make it thermally and physically unstable.

From the SDS-PAGE analysis, it could be concluded that CFS of isolate S2 consists of a mixture of many proteins of different sizes ranging from ~25kDa to ~180kDa. Both concentrated and not concentrated CFS of isolate S2 have bands at same protein sizes of 180kDa, 135kDa, 100kDA, 75kDa, 68kDA, 50kDa and 45kDa. Many more bands of different protein size were visible on concentrated CFS of isolate S2 especially of protein sizes of approximately 63kDa, 48kDa, 35kDa and 25kDa. Since there are many bands of protein molecules of varying sizes, it's hard to pin point what protein molecule could be the possible active component of CFS of isolate S2 against *C. difficle*. As result, further investigations will be required to isolate and identify very likely protein based antimicrobial substance or substances produced by isolate S2. *Enterococcus* is known to produce class I or mainly II type bacteriocins that are small (<10 kDa), heat-stable proteins. The SDS-PAGE analysis could be carried out to specifically look for protein molecules of a size smaller than 11kDa and thermostability of CFS of isolate S2 could be further tested by a range of temperatures not as high as 60°C.

Most of the experimental data obtained from this investigation provide solid indications rather than conclusive results for the potential production of protein based antimicrobial substance like bacteriocin active against *C. difficile* from environmental isolate S2. Therefore, experiments carried out so far provides a good foundation for further experiments in future which is of paramount importance to confirm and isolate potential bacteriocin from isolate S2 by more in depth molecular techniques such as mass spectrometry investigation of protein molecules as well as confocal assay of activity. Also, genetic analysis technique such as site specific mutagenesis of bacteriocin producing gene from isolate S2 could be done to determine whether bacteriocin produced by environmental isolate S2 is modifiable or can be used as a soluble substance or as probiotic as a potential novel alternative treatment for *C. difficile* infection

# 5. Reference

1. Arnusch, C. J. et al. (2008). The vancomycin-nisin (1-12) hybrid restores activity against vancomycin resistant *enterococci*. Biochemistry 47, 12661–12663.
2. Aslam, S., Hamill, R. J. & Musher, D. M. (2005). Treatment of *Clostridium difficile*-associated disease: old therapies and new strategies. Lancet 5, 549–557.
3. Badger, V. O. et al. (2012). *Clostridium difficile*: epidermiology, pathogenesis, management, and prevention of a recalcitrant healthcare-associated pathogen. J Parent Ent Nutri 36, 645-662.
4. Bagley, M. C., Dale, J. W., Merritt, E. A. & Xiong, X. (2005). Thiopeptide antibiotics. *Chem. Rev.* 105, 685–714.
5. Balciunas, E. M. et al. (2013). Novel biotechnological applications of bacteriocins: A review. Food Control 32(1), 134-142.
6. Baquero, F. & Moreno, F. (1984). The microcins. FEMS Microbiol. Lett. 23, 117–124.
7. Barth, H. et al. (2004). Binary Bacterial Toxins: Biochemistry, Biology, and Applications of Common *Clostridium* and *Bacillus* Proteins. Microbiology and Molecular Biology Reviews 68(3), 373–402.
8. Bartoloni, A. et al. (2004). In-vitro activity of nisin against clinical isolates of *Clostridium difficile*. J Chemother 16, 119–121.
9. Begley, M., Cotter, P. D., Hill, C. & Ross, R. P. (2009). Identification of a novel two-peptide lantibiotic, lichenicidin, following rational genome mining for Lan M proteins. Appl. Environ. Microbiol. 75, 5451–5460.
10. Bierbaum, G. & Sahl, H. G. (2009). Lantibiotics: mode of action, biosynthesis and bioengineering. Curr. Pharm. Biotechnol. 10, 2–18.
11. Binion, D. G. (2010). *Clostridium difficile* and IBD. Inflammatory Bowel Disease Monitor 11(1), 7–14.
12. Birri, D. J. et al. (2010). Molecular and genetic characterisation of a novel bacteriocin locus in *Enterococcus avium* isolates from infants. AEM 76(2), 483-492.
13. Blaser, M. (2011). Antibiotic overuse: Stop the killing of beneficial bacteria. Nature 476, 393–394.
14. Boakes, S. et al. (2012). Generation of an actagardine A variant library through saturation mutagenesis. Appl. Microbiol. Biotechnol. 95, 1509–1517.
15. Brand, A. M., de Kwaadsteniet, M. & Dicks, L. M. (2010). The ability of nisin F to control *Staphylococcus aureus* infection in the peritoneal cavity, as studied in mice. Lett. Appl. Microbiol. 51, 645–649.
16. Brandt, L. J. et al. (2012). Long-term follow-up of colonoscopic faecal microbiota transplant for recurrent *Clostridium difficile* infection. The Am J Gastro 107, 1079-1087.
17. Brazier, J. S., Fawley, W., Freeman, J. & Wilcox, M. H. (2001). Reduced susceptibility of *Clostridium difficile* to metronidazole. J Antimicrob Chemother 48, 741–742.
18. Brazier, J. S. et al. (2008). Distribution and antimicrobial susceptibility patterns of *Clostridium difficile* PCR ribotypes in English hospitals, 2007-08. Euro Surveill 13.
19. Bricker, E. et al. (2005). Antibiotic treatment for *Clostridium difficile*-associated diarrhoea in adults. Cochrane Database Syst Rev 1, CD004610.
20. Brown, C. L. et al. (2012). Colicin-like bacteriocins as novel therapeutic agents for the treatment of chronic biofilm-mediated infection. Biochem Soc Trans 40, 1549-1552.

21. Burke, K. E. & Lamont, J. T. (2013). Faecal transplantation for recurrent *Clostridium difficile* infection in older adults: a review. Journal of the American Geriatrics Society 61(8), 1394–1398.
22. Burrowes, B., Harper, D. R., Anderson, J., McConville, M. & Enright, M. C. (2011). Bacteriophage therapy: potential uses in the control of antibiotic resistant pathogens. Expert Rev. Anti Infect. Ther. 9, 775–785.
23. Bustelo, X. R., Sauzeau, V. & Berenjeno, I. M. (2007). GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions in vivo. BioEssays 29(4), 356–370.
24. Can, M. et al. (2006). Prophylactic *Saccharomyces boulardii* in the prevention of antibiotic-associated diarrhoea: a prospective study. Med Sci Monit, 12, 119–122.
25. Cascales, E. et al. (2007). Colicin biology. Microbiol Mol Biol Rev 71, 158-229.
26. Chatterjee, S. et al. (1992). Mersacidin, a new antibiotic from *Bacillus*. In vitro and in vivo antibacterial activity. J. Antibiot. (Tokyo) 45, 839–845.
27. Cintas, L. M. (2001). Review: bacteriocins of lactic acid bacteria. Food Sci Technol Int 7, 281-305.
28. Citron, D. M., Tyrrell, K. L., Merriam, C. V. & Goldstein, E. J. (2012). Comparative in vitro activities of LFF571 against *Clostridium difficile* and 630 other intestinal strains of aerobic and anaerobic bacteria. Antimicrob. Agents Chemother. 56, 2493–2503.
29. Clabots, C. R. (1992). Acquisition of *Clostridium difficile* by hospitalized patients: evidence for colonized new admissions as a source of infection. The Journal of Infectious Diseases 166 (3), 561–567.
30. Cooper, M. A. & Shlaes, D. (2011). Fix the antibiotics pipeline. Nature 472, 32.
31. Cotter, P. D. et al. (2005a). Posttranslational conversion of L-serines to D-alanines is vital for optimal production and activity of the lantibiotic lacticin 3147. Proc Natl Acad Sci U S A 102, 18584–18589.
32. Cotter, P. D., Hill, C. & Ross, R. P. (2005b). Bacteriocins: developing innate immunity for food. *Nature Rev. Microbiol.* 3, 777–788.
33. Cotter, P. D., Stanton, C., Ross, R. P. & Hill, C. (2012a). The impact of antibiotics on the gut microbiota as revealed by high throughput DNA sequencing. Discov. Med. 13, 193–199.
34. Cotter, P. D., Ross, R. P. & Hill, C. (2012b). Bacteriocin- a viable alternative to antibiotics? Nature Reviews 11, 95-105.
35. Depardieu, F. et al. (2004). Van D-type vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis*. Antimicrob Agents Chemother 48, 3892–3904.
36. Dischinger, J. et al. (2014). Lantibiotics: Promising candidate for future application in healthcare. Int J Med Microbiol. 302 (1), 51-62.
37. Dobson, A., Cotter, P. D., Ross, R. P. & Hill, C. (2012). Bacteriocin production: a probiotic trait? Appl. Environ. Microbiol. 78, 1–6.
38. Donadio, S. et al. (2010). Sources of novel antibiotics—aside the common roads. Appl. Microbiol. Biotechnol. 88, 1261–1267.
39. Drekonja, D. M. et al. (2011). Comparative effectiveness of *Clostridium difficile* treatments: a systematic review. Annals of Internal Medicine 155 (12), 839–47.
40. Drider, D. et al. (2006). The continuing story of class IIa bacteriocins. *Microbiol. Mol. Biol. Revs* 70, 564–582.
41. Drummond, L. J. et al. (2003). Changes in sensitivity patterns to selected antibiotics in *Clostridium difficile* in geriatric in-patients over an 18-month period, J Med Microbiol, 52, 259–263.
42. Eijsink, V. G. et al. (1998). Comparative studies of class IIa bacteriocins of lactic acid bacteria. Appl. Environ. Microbiol. 64, 3275–3281.

43. Eijsink, V. G. et al. (2002). Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication, *Antoine Van Leeuwenhoek* 81, 639-654.
44. Febbraro, S., Hancock, A., Boyd, A. & Dawson, M. J. (2012). A phase I, double-blind, randomised, placebo-controlled, dose escalating study to assess the safety, tolerability, and pharmacokinetics of single and multiple doses of NVB302 administered orally to healthy volunteers. 52nd Interscience Conference on Antimicrobial Agents and Chemotherapy.
45. Field, D., Hill, C., Cotter, P. D. & Ross, R. P. (2010). The dawning of a 'Golden era' in lantibiotic bioengineering. Mol. Microbiol. 78, 1077–1087.
46. Fooks, L. J. & Gibson, G. R. (2003). Mixed culture fermentation studies on the effects of synbiotics on the human intestinal pathogens *Campylobacter jejuni* and *Escherichia coli*. Anaerobe 9, 231–242.
47. Franz, C. M., Holzapfel, W. H. & Stiles, M. E. (1999). *Enterococci* at the crossroads of food safety? Int J Food Microbiol 47, 1–24.
48. Gardiner, G. E. et al. (2007). Fate of the two-component lantibiotic lacticin 3147 in the gastrointestinal tract. Appl Environ Microbiol 73, 7103-7109.
*49.* Gebhart, D. et al. (2012). Novel High-Molecular-Weight, R-Type Bacteriocins of *Clostridium difficile* Journal of Bacteriology 194, 6240–6247.
50. George, R. H. et al. (1978). Identification of *Clostridium difficile* as a cause of pseudo-membranous colitis. Br Med J 1, 695.
51. Gerding, D.N. (2010). Global epidemiology of *Clostridium difficile* infection in 2010. Infect Control Hosp Epidemiol 31 (1), 32-34.
52. Gillor, O., Kirkup, B. C. & Riley, M. A. (2004). Colicins and microcins: The next generation antimicrobials. Adv Appli Microbiol 54, 129-146.
53. Gillor, O., Etzion, A. & Riley, M. A. (2008). The dual role of bacteriocins as anti- and probiotics. Appli Microbiol Biotechnol 81, 591-606.
54. Gobbetti, M. et al. (2007). Cell–cell communication in food related bacteria. Int J Food Microbiol, 120 (1–2), 34–45
55. Goldstein, B. P., Wei, J., Greenberg, K. & Novick, R. (1998). Activity of nisin against *Streptococcus pneumoniae*, in vitro, and in a mouse infection model. J. Antimicrob. Chemother. 42, 277–278.
56. Goossens, D. et al. (2003). Probiotics in gastroenterology: indications and future perspectives. Scand J Gastroenterol 38 (239), 15–23.
57. Gordon, D. M. & Riley, M. A. (1999) A theoretical and empirical investigation of the invasion dynamics of coliciongeny. Microbiol 145, 655-661.
58. Gordon, D. M. & O'Brien, C. L. (2006) Bacteriocin diversity and the frequency of multiple bacteriocin production in *Escherichia coli*. Microbiol 152, 3239-3244.
59. Gordon, D. M. et al. (2007). The diversity of bacteriocins in Gram-negative bacteria, Chavan M (eds) Bacteriocins: ecology and evolution. Springer, Berlin, 5-18.
60. Gratia, A. (1925) Sur un remarquable exemple d'antagonisme entre deux souches de coilbacille. Comp Rend Soc Biol 93, 1040-1041.
61. Hall, I. C. & O'Toole, E. (1935). Intestinal flora in new-born infants with a description of a new pathogenic anaerobe, *Bacillus difficilis*. Am J Dis Child 49, 390–402.
62. Heng, N. C. K. et al. (2007). The diversity of bacteriocins in Gram-positive bacteria. Chavan M (eds) Bacteriocins: ecology and evolution. Springer, Berlin, 45-92.
63. Hiramatsu, K. (2001). Vancomycin-resistant *Staphylococcus aureus*: a new model of antibiotic resistance. Lancet Infect Dis 1, 147–155.

64. Hopkins, M. J. & Macfarlane, G. T. (2003). Non-digestible oligosaccharides enhance bacterial colonization resistance against *Clostridium difficile* in vitro. Appl Environ Microbiol 69, 1920–1927.
65. Howell, M. D. et al. (2010). Iatrogenic gastric acid suppression and the risk of nosocomial *Clostridium difficile* infection. Archives of Internal Medicine 170(9), 784–790.
66. Huang, H., Weintraub, A., Fang, H. & Nord, C.E. (2009). Antimicrobial resistance in *Clostridium difficile*. Int J Antimicrob Agents 34, 516-522.
67. James, R., Penfold, C. N., Moore, G. R. & Kleanthous, C. (2002). Killing of *E. coli* cells by E group nuclease colicins. Biochimie 84, 381–389.
68. Jones, A. M., Kuijper, E. J. & Wilcox, M. H. (2013). *Clostridium difficile*: A European perspective. J Infect. 66, 115-128.
69. Joseph, R. et al. (2005). First isolation of *Clostridium difficile* PCR ribotype 027, toxin type 111 in Belgium. Eurosurveillance 10, 274–275.
70. Joshi, N. M., Macken, L. & Rampton, D. (2012). Inpatient diarrhoea and *Clostridium difficile* infection. Clinical Medicine 12(6), 583-588.
71. Just, I. et al (1995). The low molecular mass GTP-binding protein Rh is affected by toxin a from *Clostridium difficile*. The Journal of Clinical Investigation 95(3), 1026–1031.
72. Kelly, C. P. & LaMont, J. T. (2008). *Clostridium difficile*-more difficult than ever. New Eng J Med. 359, 1932-1940.
73. Kemperman, R. et al. (2003). Identification and characterisation of two novel clostridial bacteriocins, circularin A and closticin 574. Appl Environ Microbiol 69, 1589-1597.
74. Kerr, B. et al. (2002). Local dispersal promotes biodiversity in a real-life game of rock-paper-scissors. Nature 418, 171-174.
75. Klaenhammer, T. R. (1993). Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Reviews 12, 39-85.
76. Kleerebezem, M., Quadri, L. E., Kuipers, O.P. & de Vos, W.M. (1997). Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. Mol. Microbiology 24, 895–904.
77. Kole, R., Krainer, A. R. & Altman, S. (2012). RNA therapeutics: beyond RNA interference and antisense oligonucleotides. Nature Rev. Drug Discov. 11, 125–140.
78. Kuehne, S. A. et al. (2010). The role of toxin A and toxin B in *Clostridium difficile* infection. Nature 467(7316), 711–713.
79. Kuijper, E. J. et al. (2008). Update of *Clostridium difficile* infection due to PCR ribotype 027 in Europe, Euro Surveil 13, 1-3.
80. Kuwano, K. et al. (2005). Dual antibacterial mechanisms of nisin Z against Gram-positive and Gram-negative bacteria. Int. J. Antimicrob. Agents 26, 396–402.
81. Li, Y., Xiang, Q., Zhang, Q., Huang, Y. & Su, Z. (2012). Overview on the recent study of antimicrobial peptides: origins, functions, relative mechanisms and application. Peptides 37, 207–215.
82. Leeds, J. A. et al. (2012). Mechanism of action of and mechanism of reduced susceptibility to the novel anti- *Clostridium difficile* compound LFF571. *Antimicrob. Agents Chemother.* 56, 4463–4465.
83. Lenski, R. E. & Riley, M. A. (2002). Chemical warfare from an ecological perspective. Proc Natl Acad Sci U S A 99, 556-558.
84. Lessa, F. C. et al. (2015). Burden of infection in the United States. New England Journal of Medicine 372 (9), 825–834.

85. Lohans, C. T. & Vederas, J. C. (2012). Development of Class IIa bacteriocins as therapeutic 2012, 386410.
86. Loo, V. G. et al. (2005). A predominantly clonal multi-institutional outbreak of *Clostridium difficile*-associated diarrhoea with high morbidity and mortality. The New England Journal of Medicine 353(23), 2442–9.
87. Maher, S. & McClean, S. (2006). Investigation of the cytotoxicity of eukaryotic and prokaryotic antimicrobial peptides in intestinal epithelial cells in vitro. Biochem. Pharmacol. 71, 1289–1298.
88. Maqueda, M. et al. (2008). Genetic features of circular bacteriocins produced by Gram-positive bacteria. FEMS Microbiol Rev 32, 2-22.
89. Marteau, P. R. (2002). Probiotics in clinical conditions. Clin Rev Allergy Immunol 22, 255–273.
90. Martin, N. I. et al. (2004). Structural characterization of lacticin 3147, a two-peptide lantibiotic with synergistic activity. Biochemistry 43, 3049–3056.
91. McAuliffe, O. et al. (1998). Lacticin 3147, a broad-spectrum bacteriocin which selectively dissipates the membrane potential. Appl Environ Microbiol 64, 439–445.
92. McAuliffe, O., Hill, C. & Ross, R. P. (2000). Each peptide of the two component lantibiotic lacticin 3147 requires a separate modification enzyme for activity. Microbiology 146, 2147–2154.
93. McCormick, B. A., Franklin, D. P., Laux, D. C. & Cohen, P. S. (1989). Type 1 pili are not necessary for colonization of the streptomycin-treated mouse large intestine by type 1-piliated *Escherichia coli* F-18 and *E. coli* K-12. *Infect. Immun.* 57, 3022–3029.
94. McFarland, L. V. (2005). Alternative treatments for *Clostridium difficile* disease: what really works? J Med Microbiol 54, 101–111.
95. Meijerink, M. et al. (2010). Identification of genetic loci in *Lactobacillus plantarum* that modulate the immune response of dendritic cells using comparative genome hybridization. PLoS One 5, 10632.
96. Metlitskaya, A. et al. (2006). Aspartyl-tRNA synthetase is the target of peptide nucleotide antibiotic microcin C. J. Biol. Chem. 281, 18033–18042.
97. Moreno, M. A. et al. (2013). *Clostridium difficile*: a cause of diarrhoea in children". JAMA Pediatrics 167(6), 592.
98. Morgan, S. M. et al. (1999). Development of a lacticin 3147-enriched whey powder with inhibitory activity against foodborne pathogens. J Food Prot 62, 1011–1016.
99. Mulligan, M. E., Rolfe, R. D., Finegold, S. M. & George, W. L. (1979). Contamination of a hospital environment by *Clostridium difficile*. Curr Microbiol 3, 173–175.
100. Na, X. & Kelly, C. (2011). Probiotics in *Clostridium difficile* infection. J Clinic Gastro 45, 154–158.
101. Nagao, J. L. et al. (2006). Lantibiotics: Insight and foresight for new paradigm. J Biosci Bioeng 102, 139-149.
102. Nelson, R. L. et al. (2011). Antibiotic treatment for *Clostridium difficile*-associated diarrhea in adults. The Cochrane Database of Systematic Reviews (9): CD004610.
103. Nes, I. F. et al. (1996). Biosynthesis of bacteriocins in lactic acid bacteria. Antonie Van Leeuwenhoek 70, 113-128.
104. Nes, I. F. & Holo, H. (2000). Class II antimicrobial peptides from lactic acid bacteria. Biopolymers 55, 50-61.
105. Nes, I. F., Yoon, S. S. & and Dzung, B. D. (2007). Ribosomally synthesiszed antimicrobial peptides (bacteriocins) in lactic acid bacteria: A Review. Food Sci. Biotechnol. 16 (5), 675-690.

106. Nissen-Meyer, J. et al. (2009). Structure-function relationships of the non-lanthionine-containing peptide (class II) bacteriocins produced by Gram-positive bacteria. Curr Pharm Biotechnol 10, 19-37.
107. Ohl, M. E. et al. (2005). What are effective therapies for *Clostridium difficile*-associated diarrhoea?. J Fam Pract 54, 176–178.
108. Park, S. H. et al. (2003). Characteristics and identification of enterocins produced by *Enterococcus faecium* JCM 5804. J A Microbiol 95, 294-300.
109. Parks, W. M. et al. (2007). The action of the bacterial toxin, microcin B17, on DNA gyrase. *Biochimie* 89, 500–507.
110. Pelaez, T. et al. (2002). Reassessment of *Clostridium difficile* susceptibility to metronidazole and vancomycin. Antimicrob Agents Chemother 46, 1647–1650.
111. Pepin, J. et al. (2004). *Clostridium difficile* associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. CMAJ 171, 466–472.
112. Piper, C., Cotter, P. D., Ross, R. P. & Hill, C. (2009a). Discovery of medically significant lantibiotics. Curr. Drug Discov. Technol. 6, 1–18.
113. Piper, C. et al. (2009b). A comparison of the activities of lacticin 3147 and nisin against drug-resistant *Staphylococcus aureus* and *Enterococcus* species. J. Antimicrob. Chemother. 64, 546–551.
114. Plummer, S., Weaver, M. A., Harris, J. C., Dee, P. & Hunter, J. (2004). *Clostridium difficile* pilot study: effects of probiotic supplementation on the incidence of *C. difficile* diarrhoea. Int Microbiol 7, 59–62.
115. Pons, A. M. et al. (2004). Genetic analysis and complete primary structure of microcin L. Antimicrob. Agents Chemother. 48, 505–513.
116. Public Health England. 2016-2017. Summary Point on *Clostridium difficile* Infection (CDI). https://www.gov.uk/government/collections/clostridium-difficile-guidance-data-and-analysis#epidemiology
117. Pugsley, A. P. (1985). *Escherichia coli* K12 strains for use in the identification and characterization of colicins. J Gen Microbiol 131, 369-376.
118. Pugsley, A. P. & Oudega, B. (1987). Methods of studying colicins and their plasmids. IRL, Oxford, 105-161.
119. Quiloan, M. L. G. et al. (2012). *Enterococcus faecalis* can be distinguished from *Entercoccus faecium* via differential susceptibility to antibiotics and growth and fermentation characteristics on mannitol salt agar, Front. Biol. 7(2), 167-177.
120. Rea, M. C. et al. (2007). Antimicrobial activity of lacticin 3147 against clinical *Clostridium difficile* strains. J Med Microbiol 56, 940-946.
121. Rea, M.C. et al. (2010). Thuricin CD, a post-translationally modified bacteriocin with a narrow spectrum of activity against *Clostridium difficile*. Proc Natl Acad Sci U S A. 107, 9352-9357.
122. Rea, M C., Ross, R. P., Cotter, P. D. & Hill C. (2011a). Classification of Bacteriocins from Gram-Positive Bacteria p 29-52. *In* Drider D, Rebuffat S (eds) Prokaryotic antimicrobial peptides. From genes to applications. Springer, New York, Dordrecht, Heidelberg, London.
123. Rea, M. C.et al. (2011b). Effect of broad- and narrow-spectrum antimicrobials on *Clostridium difficile* and microbial diversity in a model of the distal colon. Proc Natl Acad Sci U S A. 108 Suppl 1, 4639-4644.
124. Rea, M. C. et al. (2012). *Clostridium difficile* carriage in elderly subjects and associated changes in the intestinal microbiota. J Clin Microbiol 50, 867-875.
125. Riley, M. A. & Wertz, J. E. (2002a). Bacteriocin diversity: ecological and evolutionary perspectives. Biochimie 84, 357-364.

126. Riley, M. A. & Wertx, J. E. (2002b). Bacteriocins: Evolution, ecology, and application. Annual Rev Microbiol 56, 117-137.
127. Riley, M. A. et al. (2003). A phylogenetic approach to assessing the targets of microbial warfare. J Evol Biol 16, 690-697.
128. Riley, M. A. (2009). Bacteriocins, Biology, Ecology, and Evolution. Encyclopedia of Microbiology. (Moselio Schaechter, Editor), Oxford, Elsevier, 32-44.
129. Ritchie, J. M. et al. (2011). An *E. coli* O157-specific engineered pyocin prevents and ameliorates infection by *E. coli* O157:H7 in an animal model of diarrheal disease. Antimicrob Agents Chemother 55, 5469–5474.
130. Ross, A. C., McKinnie, S. M. & Vederas, J. C. (2012). The synthesis of active and stable diaminopimelate analogues of the lantibiotic peptide lactocin S. J. Am. Chem. Soc. 134, 2008–2011.
131. Rupnik M., Wilcox, M. H. & Gerding D. N. (2009). *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. Nat Rev Microbiol. 7, 526-536.
132. Ryan, M. P., Rea, M. C., Hill, C. & Ross, R. P. (1996). An application in Cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. Appl Environ Microbiol 62, 612–619.
133. Ryan, M. P., Ross, R. P. & Hill, C. (2001). Strategy for manipulation of cheese flora using combinations of lacticin 3147-producing and -resistant cultures. Appl Environ Microbiol 67, 2699–2704.
134. Sanchez-Hidalgo, M. et al. (2011). AS-48 bacteriocin: close to perfection. Cell. Mol. Life Sci. 68, 2845–2857.
135. Savoia, D. (2012). Plant-derived antimicrobial compounds: alternatives to antibiotics. Future Microbiol. 7, 979–990.
136. Sekirov, I., Russell, S. L., Antunes, L. C. & Finlay, B. B. (2010). Gut microbiota in health and disease. Physiol. Rev. 90, 859–904.
137. Sell, T. L., Schaberg, D. R. & Fekety, F. R. (1983). Bacteriophage and bacteriocin typing scheme for *Clostridium difficile.* J Clin Microbiol 17,1148–1152.
138. Shah, D. et al. (2010). *Clostridium difficile* infection: update on emerging antibiotic treatment options and antibiotic resistance. Expert Review of Anti-Infective Therapy 8 (5), 555–564.
139. Shanahan, F. (2010). Probiotics in perspective. Gastroenterology 139, 1808–1812.
140. Slanetz L. W. and Bartley C. H. (1957) J Bact 74, 591-595.
141. Stabler, R. A. et al. (2009). Comparative genome and phenotypic analysis of *Clostridium difficile* 027 strains provides insight into the evolution of a hyper-virulent bacterium. Genome Biol 10, 102.
142. Surawicz, C. M. et al. (2013). Guidelines for diagnosis, treatment, and prevention of *Clostridium difficile* infections. The American Journal of Gastroenterology 108 (4), 478–498.
143. Trzasko, A. et al. (2012). The efficacy of LFF571 in the hamster model of *Clostridium difficile* infection. Antimicrob. Agents Chemother. 56, 4459–4462.
144. Valiquette, L., Low, D. E., Pepin, J. & McGeer, A. (2004). *Clostridium difficile* infection in hospitals: a brewing storm. CMAJ 171, 27–29.
145. van Dissel, J. T. et al. (2005). Bovine antibody-enriched whey to aid in the prevention of a relapse of *Clostridium difficile* associated diarrhoea: preclinical and preliminary clinical data. J Med Microbiol 54, 197–205.
146. van Hemert, S. et al. (2010). Identification of *Lactobacillus plantarum* genes modulating the cytokine response of human peripheral blood mononuclear cells. BMC Microbiology 10, 293.

147. Vellend, M. & Geber, M. A. (2005). Connections between species coexistence: a critique of the coexistence program. Ecol Lett 8, 767-781.
148. Vincent, P. A. & Morero, R. D. (2009). The structure and biological aspects of peptide antibiotic microcin J25. Curr. Med. Chem. 16, 538–549.
149. West, S. A. et al. (2007). The social lives of microbes. Annu Rev Ecol, Evol Syst 38, 53-77.
150. White, A. R. (2011). Effective antibacterials: at what cost? The economics of antibacterial resistance and its control. J. Antimicrob. Chemother. 66, 1948–1953.
151. Wiedemann, I. et al. (2006). The mode of action of the lantibiotic lacticin 3147 – a complex mechanism involving specific interaction of two peptides and the cell wall precursor lipid II. Mol Microbiol 61, 285–296.
152. Zhang, C. et al. (2008). Isolation, structure, and antibacterial activity of philipimycin, a thiazolyl peptide discovered from *Actinoplanes philippinensis* MA7347. J. Am. Chem. Soc. 130, 12102–12110.
153. Zhang, C. et al. (2009). Thiazomycins, thiazolyl peptide antibiotics from *Amycolatopsis fastidiosa*. J. Nat. Prod. 72, 841–847.