UiT The Arctic University of Norway

Faculty of Health Science

Characterization of commensal enterococcal membrane vesicles and their cytotoxic effect on various host cells

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Master's thesis in Biomedicine MBI-3911-1 May 2020

Acknowledgements

The present work was done to qualify for a Master of Science in Biomedicine at the Research Group for Host-Microbe Interactions, Faculty of Health and Science, UiT, the Arctic University of Norway, from August 2019 to May 2020.

First of all, I would like to express my gratitude to my supervisors Theresa Wagner, Professor Mona Johannessen, Professor Kristin Hegstad and Kjersti Julin for excellent guidance and support throughout my thesis project. Thank you very much for sharing your knowledge and experience and encouraging me in every step.

I would like to give special thanks to my supervisor Theresa Wagner without whom my thesis work would be incomplete. Your guidance, knowledge and encouragement help me a lot from the first day of my laboratory work and also this would guide me in the future to explore my knowledge.

I am also grateful to Prof. Natasa Skalko-Basnet from the Drug Transport and Delivery Research Group (Institute for Pharmacy, UiT), Augusta Hlin Aspar Sundbø from the Advanced Microscopy Core Facility (AMCF, IMB, Helsefak, UiT) and Dr. Jack-Ansgar Bruun and Toril Anne Grønset at the Tromsø University Proteomics Platform (TUPP, IMB, Helsefak, UiT) for their role in different part of my thesis work.

I would also like to thank all the members from the Research Group for Host-Microbe Interactions for their kind support and providing me the pleasant atmosphere to work and sharing your ideas.

And finally, I would like to thank my family for the enormous support and their guidance throughout my study. And special thanks for my wife Anjana Neupane, for your encouragement and holding my hands throughout this period.

Ishan Bhandari, May 2020.

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Abstract

Enterococcus are a group of bacteria growing on different environmental condition behaving as a commensal as well as opportunistic pathogen. Among the enterococci, *Enterococcus faecium* is recently emerged as a nosocomial multi-resistance pathogen especially in the severely ill and immunocompromised patients causing a wide range of diseases like endocarditis, bacteraemia and urinary tract infections. Membrane vesicles are released by these bacteria and are the membranous structure carrying different proteins and some virulence factors. This present study focuses on the isolation of membrane vesicles released by the commensal *E. faecium* E1007, to study the morphological characteristics and measure the host responses in different cells. First in preliminary observations, *E. faecium* E1007 was grown on different cultural plates and their colony characteristics were observed. Gram staining from blood agar and growth curve of *E. faecium* in BHI medium was monitored. *E. faecium* grown on BHI from both exponential and stationary growth phase releases membrane vesicles which were further purified using the density gradient centrifuge. The purified vesicles were analysed by Transmission electron microscope that showed their broad size range from 30-182 nm and 20-174 nm in stationary and exponential phases respectively and they look small circular structures. Dynamic light scattering shows an average size of 55.1 nm in crude and 100.1 nm in OptiPrep samples exponential growth phase whereas 73.7 nm in crude and 114 nm in OptiPrep samples from Stationary growth phase. The proteomic analysis shows a total of 1160, 1012, 497 proteins from Stationary growth phase OptiPrep fractions 1, 2 and 3 respectively, 425 proteins from Stationary growth phase crude samples, 362 proteins from exponential phase OptiPrep and 1044 proteins from exponential phase crude samples. Membrane vesicles were inoculated in HaCaT cells, CaCo-2 cells and neutrophil cells at different concentration and cytotoxicity in these cells were observed. Membrane vesicles from *E. faecium E1007* showed high toxic to HaCaT cells and Neutrophil cells but not cytotoxic to CaCo-2 cells. This study provides a better understanding of Gram-positive *E. faecium* membrane vesicles and their cytotoxic nature to different host cells.

Key words: *E. faecium*, Membrane vesicles, Transmission electron microscope, Dynamic light scattering, Cytotoxicity

Abbreviations

BA	Blood Agar
BHI	Brain Heart Infusion
CBB	Coomassie Brilliant Blue
CC	Clonal Complex
BCA	Bicinchoninic Acid
CV-I	Crystal Violet-Iodine
DCG	Density Gradient Centrifugation
DLS	Dynamic Light Scattering
DTT	Dithiothreitol
EVs	Extracellular Vesicles
HGT	Horizontal Gene Transfer
LDH	Lactate Dehydrogenase
LB	Luria-Bertani
LPS	Lipopolysaccharides
MLST	Multilocus Sequence Typing
MsrR	Membrane Associated Global Regulators
MV	Membrane Vesicle
OD	Optical Density
OMVs	Outer Membrane Vesicles
PG	Peptidoglycan
TCA	Trichloroacetic Acid
TEM	Transmission Electron Microscopy
SDS	Sodium Dodecyl Sulfate

1. INTRODUCTION

1.1 The genus *Enterococcus*

The *Enterococcus* are the diverse group of bacteria that can grow in widely different conditions and adopt themselves to these conditions. They can behave as commensals and as opportunistic pathogens (1). The genus *Enterococcus* contains more than 50 species that reside in different environments from soil, fresh and marine water sediments, gastrointestinal tract of humans and animals to the hospital environment (2, 3). The genus *Enterococcus* is Gram-positive, catalase negative, non-spore forming and facultative anaerobic bacteria which can be found as both single cocci or in chains (4). They are identified by their low G+C content, ability to grow in broth containing 6.5% NaCl, and are capable of hydrolysing the esculin in presence of bile salt (Bile-Esculin test) (1, 5).

The term "*Enterococcus*" was first used by Thiercelin when he described the diplococcus that had intestinal origin and were capable of causing infection (6). In 1899 the first member of this genus was isolated from a lethal case of endocarditis and is now known as *Enterococcus faecalis (E. faecalis)* (7). In 1906 the name *Streptococcus faecalis* was given by Andrews and Horder when they isolated the bacteria from a patient with endocarditis (8). In 1919 Orla-Jensen found a bacteria from an animal and human faeces and named the bacterium as *Streptococcus faecium* (9). Streptococci were classified by Sherman in 1937 into four groups: pyogenes, viridians, lactic and *Enterococcus* (10).

Until 1984, the enterococci were classified as group D Streptococci (10) and after the reclassification *Streptococcus faecalis* and *Streptococcus faecium* were assigned as *E. faecalis* and *Enterococcus faecium* (*E. faecium*) (11). *E. faecalis* and *E. faecium* are the two species of *Enterococcus* that are mostly found in the gastrointestinal tract of humans and comprise up to 1% of adult intestinal microbiota (12, 13). These two enterococci are the most reported commensals of human. In early 1990's, it was reported that *E. faecalis* account for 80-90% whereas *E. faecium* accounts for 5-10% of the clinical isolates (14). However recently, the rate of infection by *E. faecium* has risen and are responsible for more than 30% of enterococcal infections (15). Other enterococcal species responsible for causing diseases are *Enterococcus*

avium, Enterococcus gallinarum, Enterococcus casseliflavus, Enterococcus durans, Enterococcus raffinosus and *Enterococcus mundtii* (16).

1.2 General features of *Enterococcus faecium*

E. faecium is a Gram-positive bacterium from the family *Enterococcaceae* and phylum *Firmicutes* (17). Their colonies on blood agar and nutrient agar are circular, smooth and entire and are non-pigmented (18). They can grow in wide ranges of temperature from 10°C to 45ºC, can survive heat at 60°C for 30 minutes and also have the ability to grow in 6.5% NaCl at pH 9.6 (11, 19). These bacteria can be present in human faces in numbers between 10^4 to 10^6 per gram weight, hence they are also considered as indicators of environmental faecal contamination (20).

1.2.1 Clade structure of *E. faecium*

Different types of molecular methods were used previously for the study of epidemiology of *E. faecium* but the development of Multilocus Sequence Typing (MLST) helped finding the population structure of E. faecium (21). MLST was first introduced for *E. faecium* in 2002. MLST allelic profiles are based on the sequence of seven housekeeping genes (22). It showed that the hospital lineages were from genetic lineage known as Clonal Complex 17 (CC17) that are different from that of the community isolates based on eBRUST (23). Recently, Bayesian Analysis of Population Structure (BAPS) has been introduced as a different way to identify related groups of bacteria where the nosocomial *E. faecium* isolates mainly clustered in group 3-3 whereas the animal isolates clustered in 2-1 (24).

Based on the whole genome sequence of *E. faecium* three different clade structure has been described; Clade A1, Clade A2, and Clade B (25). Clade A1 includes the clinical isolates, Clade A2 includes majority of isolates that are derived from animals and Clade B includes the human commensals isolates (25). It has been calculated that it is approximately 3000 years since the first split of the *E. faecium* took place into Clade B and Clade A (25). The main reason behind this split may be difficult to know but it coincides with the rise of urbanization, increase in the domestication of livestock and also elevated hygiene measures (25). Another split in clade occurred approximately 75 years ago forming Clade A1 and Clade A2 where the time coincides with the use of antibiotics in clinical medicine and agriculture (25, 26).

1.2.2 Infections caused by *E. faecium*

For many years it was thought that the *Enterococcus* species were harmless to human and much of the importance was not given. They were widely used in the food industry as a probiotic or as starter culture due to their ability to produce bacteriocin (19). However, it was also known for the cause of endocarditis in the past (27). The earliest known case of endocarditis was in 1899 (7) and causative organism was *Micrococcus zymogenes* but later it was thought to be *S. faecalis var zymogenes* (10). Now in recent time *E. faecium* has emerged as a multi-drug resistant nosocomial pathogen causing infections in blood stream, urinary tract, and surgical wounds (28, 29). Most of the enterococcal infections were caused by *E. faecalis* however in recent time infections by *E. faecium* is increasing and they account for almost 40% of enterococcal infections (30). The current rise in *E. faecium* infections is mainly due to the rise in the antibiotic resistance and majority of clinical *E. faecium* isolates are Ampicillin and/or Vancomycin resistant (31-33).

1.2.2.1 Urinary tract infection

The most common nosocomial infection caused by enterococci in adults is urinary tract infection (34). In older men, lower tract infections like cystitis, prostatitis, and epididymis are commonly due to enterococci (35). Apart from this some cases of bacteraemia that leads from the upper urinary tract infections have also been reported in some older men (36). A study from National Health Safety Network in U.S shows enterococci are the third most common bacteria that causes infections in patients with urinary catheters of which 81% of these are *E. faecium* with vancomycin resistance (34).

1.2.2.2 Bacteraemia

Bacteremia is one of the common infections caused by *E. faecium*. The routes for the entry of *E. faecium* are the gastrointestinal tract, urinary tract, intravascular catheters and wounds (37). Bacteraemia caused by *E. faecium* has higher mortality rate than that by *E. faecalis* (38). Older adults with multiple underlying diseases and patients with immunocompromised conditions are at high risk of bacteraemia with *E. faecium*, most probably because of the underlying complicating factors. (35, 37). Also, a new research from Australia show that one-third of cases

of bacteremic enterococcal infections are caused by *E. faecium* and the 90% of these are the ampicillin resistant CC-17 strains of which 50% are the vancomycin resistant (39).

1.2.2.3 Endocarditis

Enterococcus are responsible for the serious endocarditis infections and they are responsible for the 5-15% of cases of infective endocarditis (40). *E. faecalis* remains the most common organism and *E. faecium* is less frequent, for the endocarditis infection and *E. faecium* is also responsible for all community-acquired, nosocomial-acquired and healthcare-associated endocarditis (41-43). The inner lining of the heart and the heart valves are mainly associated with the biofilm associated infections that can be caused by multi-resistant *E. faecium* (44, 45).

1.3 Extracellular vesicles

Bacteria, Eukarya, and Archaea, all produce membranous spherical structures known as the extracellular vesicle (46). These extracellular vesicles (EVs) are formed from the membrane either as consequence of a physiological process or due to some mechanical disruption of the membranes (47). The EVs contains different types of lipids from the cellular membranes along with the membrane, periplasmic and cytoplasmic proteins (48). EVs plays role in different microbes differently from nutrition, physio-pathogenesis to cell to cell communications (46). The size of EVs may vary from species to species which depends upon the mechanism of biogenesis, but they range between 30-1000 nm (46, 49-51). EVs can be referred as differently for different microbes, for bacteria and archaea it is called as membrane vesicles (MVs), for fungal and parasitic vesicles it is referred as exosomes or shedding vesicles (46, 52).

1.3.1 Archaeal vesicles

Archaea releases the MVs which are normally coated with the S-layer proteins (49). Archaea like *Sulfolobus spp., Ignicoccus spp., Thermococcus kodakarensis* are able to produce the membrane vesicles (49, 53, 54). These MVs are derived from the cell surface and are released through the budding process playing their role in genetic exchange and inter-microbial communication (55). The hyper-thermophilic archaea *Sulfobus* species releases the MVs that are 90-230 nm in size, containing sulfolobicins toxin that suppresses the growth of other species (49, 55). *Ignicoccus* species releases the vesicles through the budding-off process and mainly found between the space of inner and outer membrane (54). Another archaea, from strain

Thermococcales produces MVs of various shape including the unusual filamentous structure (56) that contains the DNA helping in the gene transfer (57).

1.3.2 Eukaryotic vesicles

Eukaryotic cells also release the membrane vesicles, but their mode of biogenesis may vary according to the type of the cell. These eukaryotes release MVs as apoptotic bodies, shedding microvesicles or exosomes (58). The release of these vesicles in eukaryotes usually follow two different paths: one describes that the shedding vesicles pinch off directly from plasma membrane, and the other explains that the intraluminal vesicles of multivesicular bodies are exported out as the exosomes (59).

The apoptotic bodies are generally 1-5 µm in diameter and are released as the blebs of cells from the process of apoptosis, containing the fragmented DNA (58, 60). Exosomes are the vesicles that are surrounded by the phospholipid bilayer having the diameter of 40-100 nm (61). These are mainly released by the exocytosis and play a role in horizontal mRNA and miRNA transfer (62), intercellular communication and regulation of immune response (61). The shedding microvesicles are 100-1000 nm in diameter and are made of the phospholipid bilayer that are formed by budding or blebing of plasma membrane (63). These microvesicles have pro-coagulant activity (64), and can contribute to the pathogenesis of rheumatoid arthritis (65) as well as feto-maternal communications (66).

Some parasites like *Leishmania* spp. and *Trypanosoma cruzi* releases vesicles that functions in protein export pathway and inflammatory responses (67, 68). In *Plasmodium falciparum* the vesicles play in role transferring the genetic information (69). Eukaryotic fungi release the unique vesicles as they need to be released from the cell wall. Fungi like *Histoplasma capsulatum, Saccharomyces cerevisiae, Candida albicans, Cryptococcus neoformans* have shown to release the extracellular vesicles (70-73).

1.3.3 Bacterial Membrane Vesicle

MVs are released by the bacteria and their vesicles size varies from 20-400 nm in diameter (74). They are the lipid bilayer structures that may contain the various macromolecules like protein, lipopolysaccharides, phospholipids, nucleic acids and other different metabolites (47). These membrane vesicles have different role including horizontal gene transfer, cell to cell

communication, virulence, and carrying the cellular metabolites (75, 76). The first MVs were described in the Gram-negative bacteria *Escherichia coli* in 1960 (77). After that researches were focused on bacterial MVs and later different research explains the presence of MVs on both Gram-negative as well as Gram-positive bacteria (78).

1.3.3.1 Membrane vesicle of Gram-negative bacteria

The MVs released by Gram-negative bacteria are generally called as the Outer-Membrane vesicles (OMVs) because these vesicles are released from their outer membrane (OM) (74). These OMVs usually ranges between 20-250 nm in diameter (79). The first observation of MVs was from the cell free supernatant culture of *E. coli*. It was grown under the lysine-limiting growth conditions which shows the secretion of LPS in forms of bags that looks spherical (77). Later in 1967 it was observed that from *Vibrio cholerae* some small spherical vesicles were released by pinching off the outer membrane (80).

The OMVs not only contain the OM of the bacteria, it also contains the inner membrane constituents and the cytoplasmic elements. The structure of Gram-negative bacterial envelope contains the two membranes that is the cytoplasmic membrane and the OM and in between these membranes consists the periplasmic space containing the peptidoglycan layer (81). These two membranes differ in protein and lipid contents however the OM constitutes the lipopolysaccharides along with the phospholipid. The inner or the cytoplasmic membrane has the phospholipid bilayer. (82). The OM is porous to the small molecules however larger molecules like proteins, sugar and vitamins cannot pass through it (79, 82). The periplasmic space is approximately 13 nm and makes the 7-40% of total cell volume that contains the peptidoglycan layer and anchors both membranes (83).

The OMVs are formed from the OM of the bacterial wall by bulging out or by pinching off process (76). The process involves entrapping of the portions of the periplasm along with cytosolic proteins, DNA and RNA (84, 85). In some of the pathogenic bacteria these vesicles carry virulence factors, adhesins, and toxins (86, 87) whereas in non-pathogenic bacteria they functions as cell to cell communication, surface modification and removal of undesirable molecules (46).

In some of the Gram-negative bacteria an unusual type of vesicles were noticed which was later known as the outer-inner membrane vesicles (88). The formation of outer-inner membrane vesicles is when the peptidoglycan layer of the bacteria becomes weak. During autolysin the inner membrane protrudes out in the periplasm allowing the cytoplasmic contents enter into the vesicles then it is pinch off from the cell surface along with the surrounding outer membrane (88). Such type of double bilayer vesicles were first discovered in Gram-negative bacterium *Shewanella vesiculosa M7T* (89).

The formation of OMV is a fundamental and conserved process (90) and does not require the ATP, NADPH or any energy sources (91). Some models eliciting the formation of OMV includes the disruption of the outer membrane and PG linkage, altered turgor pressure of the periplasmic space, and anionic charge repulsion between LPS molecules (74). When the cross linking between the outer membrane and peptidoglycan layer is disrupted some region of the outer membrane protrude out leading to the formation of the membrane vesicles (92). Antibiotic and autolysin also plays role in budding of vesicles as they help in disruption of the peptidoglycan (93). Apart from this, during the cell division also the outer membrane-peptidoglycan complexes are temporarily disrupted leading to the formation of the vesicles at the site of division (92). When the turgor pressure is induced in the periplasmic space, it induces the membrane curvature which result in the budding process leading to the formation of the vesicles. This pressure is generated in the periplasmic space due to the accumulation of peptidoglycan fragments and misfolded outer membrane proteins (94).

1.3.3.2 Membrane vesicle of Gram-positive bacteria

After the discovery of OMV in *E.coli*, research were mainly focused on Gram-negative bacteria and it was assumed that due to their thick cell wall of Gram-positive bacteria they does not produce MVs as it is difficult to cross such a barrier (47). Until 1990 there were no reports suggesting the presence of MVs in Gram-positive bacteria and first reports were on *Bacillus cereus* and *Bacillus subtilis* (95). The first detailed study of MV in Gram-positive bacteria was on *Staphylococcus aureus* (*S. aureus*) in 2009 where protein composition of the MV through the mass spectrometry was performed (96). Studies have shown that Gram-positive bacterium including *Bacillus anthracis, Listeria monocytogenes, Clostridium perfringenes, E. faecium* and *Streptococcus pneumonia* produce MVs (97-101).

MVs produced by Gram-positive bacteria are 20-400 nm in diameter with spherical bilayer structure (47). The MVs released by Gram-positive bacteria differs from those of Gram-negative bacteria because of the structure of the cell wall envelope (102). The outer membrane is absent in Gram-positive bacteria. They contain the thick peptidoglycan layer and the inner plasma membrane. The peptidoglycan layer is around 30-100 nm thick and their chemical structure is similar to that of Gram-negative bacteria (103). Apart from this it contains teichoic acids and lipoteichoic acids in the cell wall. The teichoic acids are the anionic polymers emerging through the peptidoglycan layer and are attached covalently whereas the lipoteichoic acids arises from the plasma membrane and protrudes through peptidoglycan layer (104).

The membrane vesicles from Gram-positive bacteria contain proteins and DNA. *Clostridium perfringens* contains the DNA that encodes the 16 ribosomal RNA. Apart from this, it also contains other DNA components like alpha-toxins and perfringolysin-O (105). *Staphylococcus aureus* produces the MVs that contains the cytoplasmic proteins including the DNA polymerase, tRNA synthetases and also some metabolic enzymes. Also, they contain the peptidoglycan degrading enzymes such as Sle1, and proteins like IgG binding proteins (96). *Bacillus anthracis* produces membrane vesicles containing the toxins containing lethal factor, edema toxin, anthrolysin (100).

The biogenesis of MV in Gram positive bacteria is little known however there are several factors explaining the formation of MVs and how they can cross the thick peptidoglycan layer. Some Gram-positive bacteria releases the cell wall modifying enzymes that help in loosening cell wall resulting in releasing of the MVs. In *S. aureus*, Sle1 which is the peptidoglycan degrading enzyme, is released degrading the thick peptidoglycan layer of the cell wall facilitating the release of MVs (96). Turgor pressure on the cell wall also facilate in the release of MVs from the plasma membrane. Apart from this, the MVs are actively transited through the protein channels (47).

1.3.3.3 Functions of membrane vesicles in bacteria

MVs produced by both Gram-positive and Gram-negative bacteria has several functions. The major functions of MVs are shown in figure 1 and some important roles are described below.

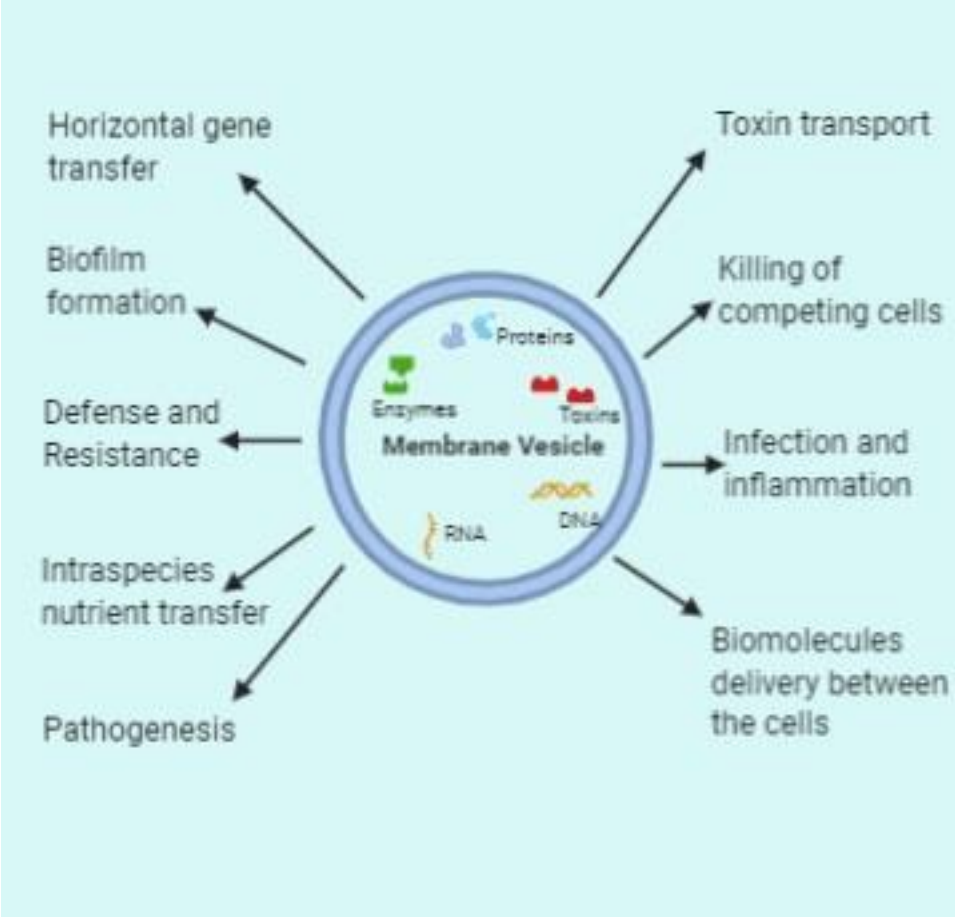


Figure 1: Overall function of membrane vesicles in bacteria. The figure was created using BioRender tool.

1. Membrane vesicles associated with biofilm formation.

Biofilms are the surface adhering substances produced by bacteria which consists of the polysaccharides, proteins , DNA, and many other molecules, that helps the bacteria for their survival and protection including the antibiotic resistance (106). Biofilms are produced by bacteria during the stressed condition (79). Also during the stressed condition, bacteria produces higher number of MVs providing their evidence of relationship (107). The role of membrane vesicles in biofilm production is important and has been well studied in Gram-positive bacteria like *Bacillus subtilis, Streptococcus mutans*, and *Mycobacterium ulcerans* as well as in Gram-negative bacteria like *Helicobacter pylori,* and *Pseudomonas aeruginosa* (108-

112). Enterococci are the major organism causing nosocomial infection and production of biofilm is important in the enterococci pathogenesis (113).

2. Membrane vesicles carry toxins.

The bacterial MVs are associated with toxins which are important in pathogenesis and helping the bacteria for their survival. Most of the Gram-positive bacteria produce toxins and this toxin helps the bacteria in forming the pores in the membrane facilitating in the entry of other virulence factors (47). *Bacillus anthracis* produces the anthrax toxin containing the three major proteins; the protective antigen, the lethal factor and the edema factor (114). *S. aureus* produces different toxins like α-haemolysins, and leukotoxins which are responsible for eliminating the host defences (115). *Clostridium perfringenes* produce the toxins like necrotic enteritis toxin B which is responsible in forming the pores in the membranes (116). Apart from this, in Gram-negative bacteria *E. coli,* produces the pore forming toxin ClyA (117).

3. Membrane vesicles are associated in defence and resistance.

MVs play an important role in defence as well as in resistance ultimately helping the bacteria in their survival. Membrane vesicles contains proteins, for example penicillin binding proteins (PBP): PBP1, PBP2 and PBP3 in *S. aureus* which binds to the β-lactam antibiotics and inactivate them by enzymatic degradation (96). Apart from this, MVs in *S. aureus* also contains membrane associated global regulators, MsrR which are involved in methicillin resistance (118). MVs in Staphylococcus aureus contains superantigens and immunoglobulin G binding proteins that help the bacteria to escape the immune system (96, 119). As a defence mechanism during the time of stress, Outer-membrane vesicles by Gram-negative bacteria helps in the removal of misfolded proteins and toxins (94). MVs produced by Gram-negative bacterium *Porphyromonas gingivalis* contains the enzyme peptidyl arginine deiminase (PPDA) which inactivates the host complement factor C5a and helps to escape the host immune system (120, 121).

4. Competing with the microbial cells.

Bacteria compete with the commensal microbes and invading pathogenic organism for their survival and establishing themselves within the host. *Pseudomonas aeruginosa* releases the MVs containing the peptidoglycan hydrolase which lyse the surrounding dissimilar bacterium

within the host environment. It has been shown that Pseudomonas aeruginosa lyses the *S. aureus* and *E.coli* (122). *Lysobacter spp* XL1, a Gram-negative bacteria releases MVs containing different proteins including the bacteriolytic enzymes that are capable of degrading the Gram-positive bacteria *S. aureus* 209-P and Gram-negative bacteria *Erwinia marcescens* EC-1 (123). The MVs produced by Gram-positive bacteria *S. aureus* contains N-acetylmuramyl-L-alanine amidase enzyme which kills the neighbouring cells by degrading their peptidoglycan that results in the membrane disruption (96).

5. Membrane vesicles in horizontal gene transfer.

MVs produced by bacteria contain different genetic materials like chromosome, plasmid DNA as well as different types of RNA. The genes coding for antibiotic resistance, virulence and metabolic traits has been transferred in both Gram-positive and Gram-negative bacteria via the MVs (97, 124-126). It has been shown that the enteropathogenic *E. coli* that releases the MVs containing DNA which are transferred to other strain of *E.coli* (126). In *Acinetobacter baylyi*, the membrane vesicles help in the transfer of plasmid born β-lactamase gene to *A. baylyi* and *E. coli* population (124).

6. Membrane vesicles in pathogenesis.

Several pathogenic bacteria produce different types of tools for colonizing and causing infections within the host tissues. It has been reported that there is the presence of MVs within the infected tissues or samples. In the Gram-positive bacteria *S. aureus* it was demonstrated the budding from the bacterial surface during the infection showing the presence of MVs during the infection (129). Adhesins produced by *S. aureus* are transported by the membrane vesicles to the site which are required for the colonization of host tissues (90). The Gram-negative bacteria *Helicobacter pylori* that colonizes the stomach of human secret VacA containing vesicles which are responsible to the attachment of epithelial cells (130).

2. OBJECTIVE

The main aim of this master project was to describe the host response to *Enterococcus faecium* E1007 membrane vesicles.

Specific Objective

I. To isolate the membrane vesicles from commensal *E. faecium* E1007 in exponential growth phase and stationary growth phase.
II. To study the morphological characteristics of MVs released from *E. faecium* E1007 in exponential phase growth and stationary growth phase.
III. To measure the cytotoxicity of *E. faecium* E1007 membrane vesicles in exponential growth phase and stationary growth phase to different human cells (HaCaT, CaCo-2 and neutrophil cells).

3. Materials and Methods

3.1. Bacterial culture

E. faecium strain E1007 which is a human commensal from the clade B structure, was used in this experiment. It was first isolated in New Zealand in 1998 from faeces of healthy human (131).

For long term storage this strain was stored at -80 °C. For culture, the strain was grown on blood agar (BA) plate and incubated at 37°C for overnight. Bacteria on BA plates were kept for a maximum of one week at 4°C.

To test bacterial growth on different types of growth media, bacteria from the BA plate were inoculated in Brain Heart Infusion (BHI), Chocolate Agar, Cysteine Lactose Electrolyte Deficient (CLED) Agar, Luria-Bertani agar (LB), Tryptic Soy Agar (TSA), Mueller-Hinton (MH) with 5% blood and Mueller-Hinton-Fastidious (MH-F) with 5% blood and NAD.

Bacteria on blood agar plates were used to inoculate the broth culture. As a standard growth medium for *E. faecium* BHI was used. The bacteria from freezing stock were streaked on the BA plates and incubated overnight. The colonies from BA plates were inoculated into 10 ml BHI in triplicates and incubated overnight. In a three 500 ml flask, 200 ml of BHI along with 2 ml overnight culture were poured and incubated in a shaker. After each 30 minutes Optical Density (OD) was measured. When the OD reaches 1 or above it was diluted, and true OD was calculated. The overall steps for the growth curve are shown in figure 2.

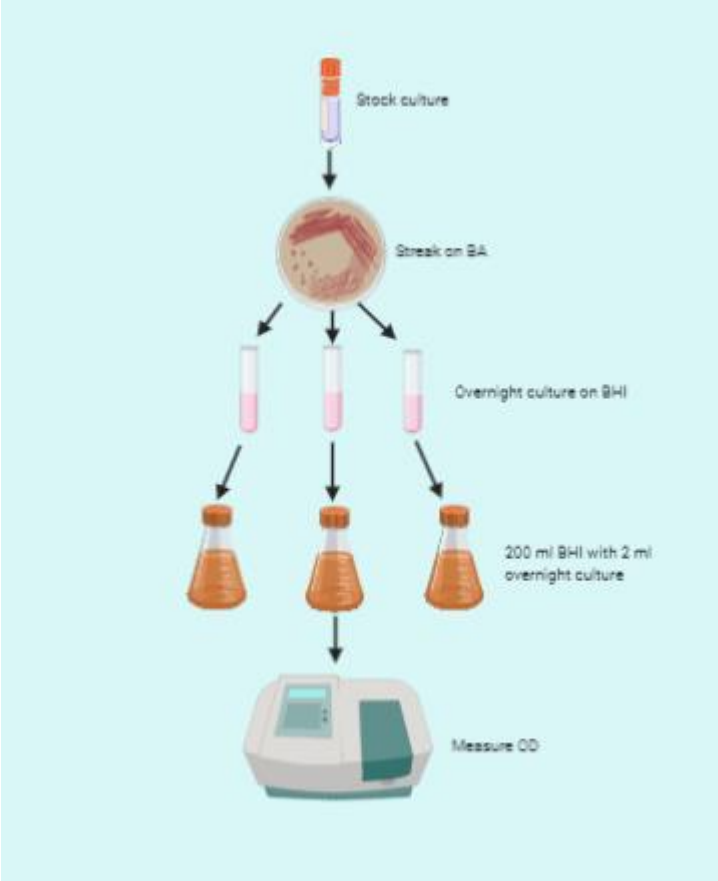


Figure 2: Diagrammatic representation of steps involved in obtaining growth curve. The figure was created by using BioRender tool.

3.2. Gram staining

Gram staining is a method to distinguish the bacterial species into two groups that is Gram-positive and Gram-negative (132). It was first discovered by Hans Christian Gram in 1884 when he was working with the section of lungs tissues to make the bacteria more visible (133, 134). The Gram staining technique is based on the differential structure of the cellular membranes and the type of cell wall present on the bacteria (135).

Initially, a smear is stained with a basic dye which is crystal violet followed by the iodine solution which act as a mordant and finally smear is washed with 95% ethanol which is a decolorizing agent (136). The cell wall of Gram-positive bacteria has thick peptidoglycan layer which is highly cross-linked that retains the primary dye that is crystal violet after the application of iodine (mordant). The iodine and crystal violet form a Crystal Violet-Iodine (CV-

I) complex within the peptidoglycan layer. When decolorizing agent (95% ethanol) is added to the cells, the CV-I complex is retained with in the peptidoglycan layer making the cells to appear dark purple or red. Whereas in Gram-negative bacteria they do not have thick peptidoglycan layer so that the CV-I complex does not remain in the layer. When the decolorizing agent is used it appears colourless. When the secondary stain, safranin is used it gives the colour to the cells and appear to be pink (136, 137). The Gram-staining was done as follows:

i. A drop of saline was added to three slides and a single colony from *E. faecium* E1007, *Streptococcus pyogenes* ATCC 19615, and *Pseudomonas aeruginosa* ATCC 27853 strains were mixed respectively in the saline. It was then air-dried.
ii. The slide was passed through flame three times to fix smear.
iii. The smear was flooded with crystal violet for 30 s - 1 minute.
iv. The slide was rinsed with tap water for 5 seconds.
v. Gram's iodine solution was added to the smear and incubate for 30 seconds to 1 minutes.
vi. The slide was rinsed with water and decolorize rapidly with 95% ethanol until no more colour is released.
vii. It was then rinse briefly in tap water.
viii. The smear was counter-stained with safranine for 1 minute and the smear was rinsed in tap water.
ix. The smear was allowed to dry, and a drop of immersion oil was added on the slide and examined with 100x objective.

3.3 Membrane vesicle isolation

Bacteria are able to produce the MVs and is a physiological process (47). For the production of pure membrane vesicles, there is not standard protocol and the protocol needs to be optimized according to the bacterial strain (138). A diagrammatic figure for the isolation of membrane vesicle is shown in the figure 3. The isolation of MVs was done according to the method described previously for Gram-positive *E. faecium* (101). First, the choice of bacteria (*E. faecium* E1007 in this study) is grown in the suitable culture medium (BHI in this study) until the required growth phase (Exponential or Stationary). Second, the supernatant is sterilized through filtration and third the concentrate is ultracentrifuged to obtain the membrane vesicles.

After the crude membrane vesicles are obtained, they are further purified using density gradient centrifugation process using the OptiPrep as a medium. This may help to remove the contamination like proteins, pili or phages. The OptiPrep solution is an iodixanol based solution which has advantage over other density gradient solutions as it prevents the damage of membrane structures as well as has the ability to form the self-generated gradients (139). During the centrifugation layers are formed according to the density and the fraction of membrane vesicles are collected which are later analysed for shape and sizes by transmission electron microscopy and dynamic light scattering methods.

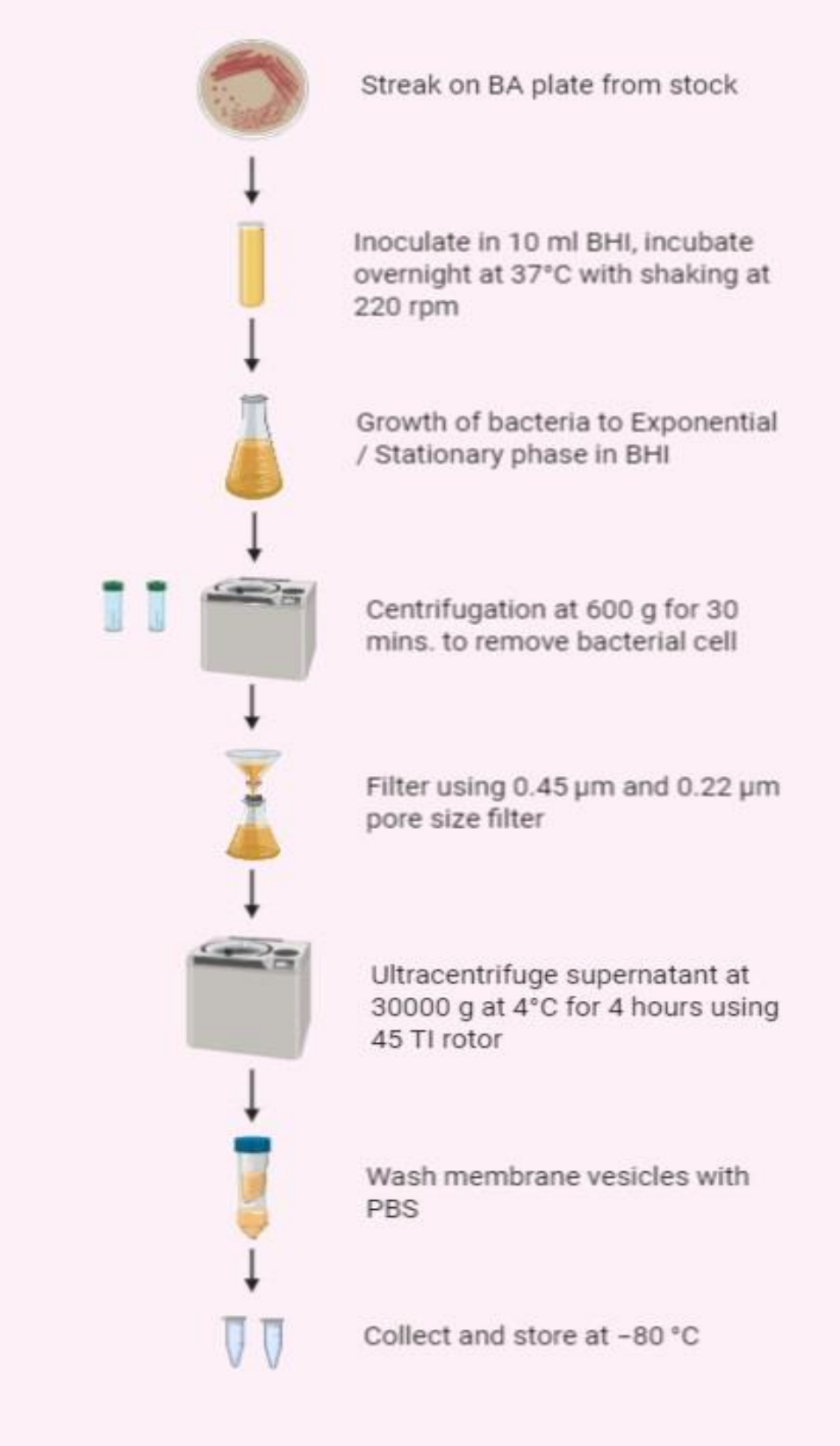


Figure 3: Diagrammatic representation of isolation of membrane vesicles. Figure was created using BioRender tool.

3.3.1 Isolation of MV from stationary growth phase

i. Colony of *E. faecium* strain E1007 from blood agar plate was picked and inoculated in 10 ml BHI medium and shaken vigorously at 220 rpm at 37°C for 16 hours to obtain the stationary growth phase.
ii. Five hundred millilitre of BHI was taken and 10 ml of overnight culture was added, and it was incubated with shaking at 220 rpm at 37°C for overnight to obtain the stationary growth phase. The OD at wavelength of 600 nm (OD_{600}) of the bacterial suspension was measured using spectrophotometer.
iii. The bacterial cells were removed by centrifugation at 6000 g for 30 minutes. The bacterial suspension was poured in one centrifuge tube and other with water for balance. Then it was centrifuged using JLA 9.1000 rotor (Beckman Instruments Inc., USA).
iv. The supernatant was transferred to a clean Erlenmeyer flask. It was then filtered through a 0.45 µm pore size filter and again using 0.22 µm pore size filter (Merck Millipore, USA).
v. The supernatant was used for ultracentrifugation. The supernatant was poured in six centrifuge tubes and the tubes were measured to make the balance. It was then centrifuged at 30000 g at 4°C for 4 hours using 45 TI rotor. To start the centrifuge the variables like speed, time and temperature were entered, then start the vacuum and start the centrifuge machine using slow acceleration and maximum deceleration.
vi. The pellet was washed with phosphate buffer saline (PBS) and ultracentrifuge the solution in a SW 50.1 rotor at 30000 rpm at 4°C for 3 hours using slow acceleration and deceleration.
vii. The purified MVs were stored at −80 °C until further analysis.

3.3.2 Isolation of MV from exponential growth phase

i. *E. faecium* strain E1007 colony from blood agar plate was picked and inoculated in 10 ml Brain Heart Infusion (BHI) medium and shaken vigorously at 220 rpm at 37°C for 16 hours.
ii. Five hundred millilitre of BHI was taken and 10 ml of overnight culture was added, and it was incubated with shaking at 220 rpm at 37°C to obtain the mid exponential

phase. The OD_{600} of the bacterial culture was measured approximately 1.5 using the spectrophotometer.

iii. The bacterial cells were removed by centrifugation at 6000 g for 30 minutes using JLA 9.1000 rotor (Beckman Instruments Inc., USA).

iv. The supernatant was transferred to a clean Erlenmeyer flask and filtered through a 0.45 μm pore size filter and again using 0.22 μm pore size filter (Merck Millipore, USA).

v. The supernatant was used for ultracentrifugation using 45 TI rotor for 30000 g at 4°C for 4 hours using slow acceleration and maximum deceleration.

vi. The pellet was washed with PBS and ultracentrifuge the solution in a SW 50.1 rotor at 30000 rpm at 4°C for 3 hours using slow acceleration and deceleration.

vii. The purified MVs were stored at −80 °C until further analysis.

3.4. Density gradient centrifugation

After ultracentrifugation the vesicular pellet may be contaminated with proteins, pili or phages, so it needs to be purified. Therefore, an additional purification step was done which was based on density gradient centrifugation (DGC). Centrifugation is a technique that is widely used for the separation of particles in a solution according to shape, size, density, viscosity of the medium and the speed of the rotor and is effective method for separation (140). Among many centrifugation method, DGC is highly efficient method for the separation of suspended particles (141).

DGC is a technique that are used for the separation of sub-cellular organelles, exosomes, and protein complexes on the basis of buoyant density differences (142). The samples are added in the tubes with liquid mixture in such a way that it forms a spatially varying density profile. When the samples are run through the centrifugation then they travel according to their individual density (143). The larger particles (size) and heavier particles (density) will travel faster and further down in gradient (144). The medium that can be used for density gradient is OptiPrep (in this study). It is a density gradient medium with 60% iodixanol in water with the density of 1.32 g/ml and have low viscosity and low osmolarity. It was developed initially for the X-ray contrast imaging as a medium (145, 146). The protocol for the DGC using OptiPrep medium was done as previously described for *E. faecium* (101) and was done as follows:

i. The MVs samples were mixed with an equal volume of 60% OptiPrep solution (D1556 Sigma; 60% stock) to form 30% solution (500 µl MV sample + 500 µl 60% OptiPrep solution). Make another 2 ml of 25% (w/v) and 1 ml 5% (w/v) of OptiPrep solution.

ii. The samples (500 µl MV sample + 500 µl 60% OptiPrep solution) were added on the bottom of an ultracentrifuge tube (Thin wall, Ultra-Clear^{TM}, 5 ml, 13 x 51 mm Beckman Coulter Centrifuge Tube), followed by 2 ml 25% and 1 ml 5% OptiPrep respectively as shown in figure 4.

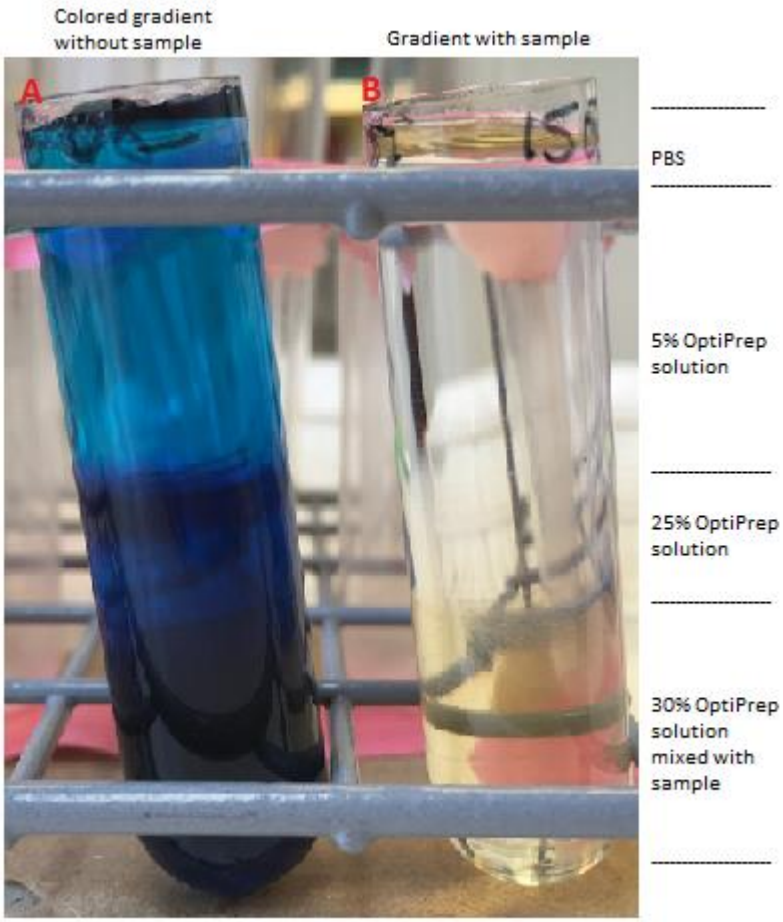


Figure 4: Samples prepared for OptiPrep showing different gradient A: Coloured gradient without sample B: Gradient with sample.

iii. The tubes were balanced using the PBS and centrifuged the solution in a SW 50.1 rotor at 30000 rpm at 4°C for 3 hours using slow acceleration and deceleration to prevent the disturbance of density.

iv. After the centrifugation transfer the PBS to one fraction from the top and 200 µl from each other fraction into eppendorf tubes.

v. The fractions that contain MVs were concentrated by centrifugation (10 kDa molecular weight cut-off, Vivaspin) at 4000 g for 10 minutes at 4°C, resuspended in PBS and stored them in the fridge.

3.5. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) is a method that is used in biochemistry for the separation of proteins by their molecular weight in an electric field. This technique was first described by Laemmli in 1970 (147). The technique is based upon the principle that a charged molecule will migrate in an electric field towards an electrode with opposite charge. The general electrophoresis technique cannot be used to determine the molecular weight of proteins because the mobility of a substance in the gel depends on both charge and size (148). Sodium dodecyl sulfate (SDS) is an anionic detergent with the chemical formula CH3(CH2)11OSO3Na, which solubilizes all proteins. SDS denatures proteins by denaturing their hydrogen bonds (147, 149). The SDS and proteins forms the complex and these complexes are cleaved by using the reducing agent like Dithiothreitol (DTT). All the polypeptides are completely unfolded, and the quaternary structures are completely dissolved (149). When the samples are loaded in an electric field, the negatively charged proteins start to move towards the positive charged electrodes (148). As the samples reach the end of the gel the current is stopped, and the gel is taken out and further analysed.

i. Sample preparation

   Ten microliters of each fraction obtained from OptiPrep were added in an eppendorf tube. Then add 1.5 µl of 1M DTT and 3.75 µl of NuPage LDS sample loading buffer (4X) (NuPage Novex 4–12%; Invitrogen, Life Technologies, USA) to make the final volume 15.25 µl.

ii. The samples were heated to 70°C for 10 minutes.

iii. The gel (12% NuPage Bis-Tris SDS-PAGE Invitrogen, Life Technologies, USA) was removed from the plastic bag and rinsed with MilliQ water.
iv. The white tape and comb were removed from the gel and the gel was placed in the inner chamber.
v. The chambers were filled with the 1X Running buffer (Novex, Life Technologies, USA).
vi. Samples were loaded in the gel. In the first well ladder (Novex, Life Technologies, USA) was added followed by samples.
vii. The gel was run at 170 Volts, 170 mA for about 30 minutes or the first band is about to leave the gel.

3.6 Coomassie staining

The Coomassie Brilliant Blue (CBB) assay is an important technique that is used in the analysis of proteins after the SDS gel electrophoresis (150). CBB is a name of a dye that was originally used in the textile industry in 19^{th} century however now it is used as staining of proteins in analytical biochemistry (151). This dye is mainly found in two forms, CBB R-250 and CBB G-250 whereas R stands for red tint, G stands for greenish tint and 250 stands for purity of the dye. Out of these two, the most commonly used is CBB R-250 and is more sensitive (152). The CBB dye works by binding the proteins with the Van der Waals force and with the ionic interactions between the dye sulfonic acid groups and amine group (153, 154).

i. The polyacrylamide gel was placed in a fixation solution (50% methanol, 10% acetic acid, Thermo Fisher Scientific USA). The solution should cover the whole gel and it was left at room temperature with careful shaking.
ii. The fixation solution was removed and Coomassie blue solution (50% methanol, 10% acetic acid, 0.05% Coomassie brilliant blue R-250, Thermo Fisher Scientific USA) was added. It was left at room temperature for 30 minutes with careful shaking.
iii. The Coomassie blue solution was removed and it was rinsed in fixation solution.
iv. The destaining solution (5% methanol, 7% acetic acid) was added and left at room temperature. The solution was changed regularly. A small piece of paper was placed at the side of gel so that it improves the destaining efficiency.
v. Then the picture of the gel was taken.

3.7 Measurement of protein concentration

Quantification of protein is important task for different research work. The protein concentration can be measured by different techniques. Some of them are fluorescence (Qubit) or absorbance-based assay such as Coomassie brilliant blue G-250 (CBBG) dye binding (Bradford) and bicinchoninic acid (BCA) (155).

The Bradford assay originally described by Marion M. Bradford in 1976, is a the simple, fast, and sensitive technique (156) which is broadly used technique for the measurement of protein concentration. In this assay, the Coomassie brilliant blue G-250 directly binds with protein at the arginine, tryptophan, tyrosine, histidine, and phenylalanine residues (157, 158). The CBBG dye mainly exists in three forms that is cationic (Red), Neutral (Green), and anionic (Blue). In acidic condition, the dye is in red cationic form and has the absorbance maximum at 470 nm. When the dye binds the protein, it is converted to blue anionic form and has the absorbance maximum at 595. Now the quantity of the protein can be measured by determining the amount of blue protein dye and it can be measured by using spectrophotometer at 595 nm absorbance (157).

NanoDrop technology that uses the spectrophotometer is another method by which the protein concentration can be measured. This is based on the innovative sample retention system and uses surface tension to hold and measure the absorbance of protein, DNA, RNA, and other biomolecules. Also, NanoDrop only uses a single 2µl drop of protein sample and doesn't require protein preparation time (159).

Qubit is another method for the measurement of the protein from the sample. It uses the fluorescence dye for the measurement of concentration either from nucleic acids or proteins in a sample. It uses different dyes, however there is specific dye for the specific molecule, DNA, RNA, or Protein. Initially these dyes are low fluorescence and when they binds their target molecule then they become intensively fluorescent (160).

3.7.1 NanoDrop

i. The measurement surfaces in the NanoDrop instrument (ThermoFisher Scientific) was cleaned with distilled water.

ii. One to two microliter of protein sample was pipetted out and was applied directly on the measurement pedestal.
iii. The sampling arm was lowered, and spectral measurement was done using the software in the computer. The software provides the protein concentration in mg/ml at 280 nm absorbance.
iv. After the measurement is complete the sampling arm was raised, and the sample was wiped from the pedestal using the dry lint free laboratory wipe.

3.7.2 Bradford assay

i. A series of protein standard (Bovine Serum Albumin) was diluted with 0.1 M NaCl in a test tube to make the final concentration of 0, 25,125,250,500,750,1000,1500, and 2000 µg/ml.
ii. Then, 0.05 ml of each standard or unknown sample was pipetted out in labelled test tubes.
iii. 1.5 ml of Coomassie Plus Reagent was added in each of the tubes and mixed well.
iv. The samples were incubated for 10 minutes at room temperature.
v. The absorbance of all samples was measured using the spectrophotometer at absorbance 595 nm.
vi. The absorbance of standard vs their concentration was plotted and using the graph the concentration of unknown samples was calculated.

3.7.3 Qubit assay

i. Three assay tubes (Qubit assay tubes) were prepared for the standards and one for each sample.
ii. Qubit working solution (Invitrogen, Life Technologies, USA) was prepared by diluting the Qubit reagent 1:200 in the Qubit buffer. 200 µl of Working solution for each standard and samples were prepared.
iii. One hundred and ninety µl of the Qubit working solution was added in 10 µl of standard from the kit to make the final volume 200 µl in the assay tubes.
iv. Then, 190 µl of the Qubit working solution was added in 10 µl of sample to make the final volume 200 µl in another assay tube.

v. The tubes were vortex for 2-3 seconds and incubated at room temperature for 15 minutes.
vi. The three standard tubes were inserted in the Qubit Fluorometer (Invitrogen life technologies) for calibration and then the sample tubes were inserted in the Qubit Fluorometer (Invitrogen, Life Technologies, USA) and final reading of the protein concentration were measured.

3.8 Transmission electron microscopy

Transmission electron microscopy (TEM) is a technique in which a beam of electrons is used to form an image so that the ultra-structure of the specimens can be observed. TEM uses an electron gun from where the beam of electrons is produced. The electron beams are focused onto the condenser lenses with high speed into the specimen. The beam of electrons emitted from the specimens are focused by objective lenses to form an image (161). TEM was performed at the Advanced Microscopy Core Facility (AMCF, IMB, Helsefak, UiT) by Augusta Hlin Aspar Sundbø.

i. Ten microliters of purified MVs were applied to Formvar coated mesh copper grids (Electron Microscopy Science, USA).
ii. The samples were incubated for 5 minutes.
iii. Then the samples were washed with double distilled water for four times and negatively stained with 9 parts of 2 % methylcellulose and 1 part of 3 % uranyl acetate for 2 minutes on ice.
iv. The excess stains were removed from the samples and the samples were left at room temperature for drying.
v. The samples were visualized with JOEL JEM 1010 transmission electron microscope (JEOL, Japan at 100 kV) at 30000X.
vi. The radius of the MVs were analysed using a software radius.

3.9 Dynamic light scattering

Dynamic light scattering (DLS) is a method that has been widely used for the measurement of size from the aqueous solution of vesicles or liposomes (162). DLS, also known as photon correlation spectroscopy (PCS) is an advanced technology that uses the scattered light to

characterize the nanoparticles (163). In this light scattering experiment generally samples are exposed to the monochromatic wave of light and the detectors detect the signal (164). John Tyndall describes one of the earliest light scattering experiment from the colloidal suspension (165). DLS mainly measures the Brownian motion of the macromolecules that is present in the sample solution which arise due to the bombardment of the molecules that are present in the solvent and relates this motion to form the size of nanoparticles (164).

Also depending upon the position of the detector in the DLS setup, the DLS system are categorized in two groups that is the homodyne and heterodyne DLS. In the homodyne DLS setup the laser and the detector are arranged perpendicularly. When the light from the laser strikes the sample then the light is scattered, and the intensity of the scattered light is measured by the detector. And in the heterodyne DLS setup, the light that are backscattered are measured. Only the scattered incident light that enter the optical fibre and reach the detector that is placed at 180º are measured (163).

DLS measurements were performed at the Nicomp Submicron Particle Sizer 370, (PSS Nicomp Particle Sizing Systems, USA) of the Drug Transport and Delivery Research Group (Institute for Pharmacy, UiT) by Prof. Natasa Skalko-Basnet.

i. The MV sample solution was diluted in PBS in cell free environment.
ii. The samples were measured and size of MVs were determined using DLS at an angle of 90º
iii. The analysis was run in vesicle mode and the data was calculated as intensity weighted distribution
iv. The analysis was run in two cycles and 20 minutes each cycle.

3.10 Protein analysis

MVs contains different types of components such as lipopolysaccharides, lipoproteins, peptidoglycan, phospholipids, periplasmic proteins, outer-proteins, RNA, DNA, different types of enzymes and toxins (90, 96, 101, 166, 167). In Gram-positive bacteria *S. aureus*, the proteomic analysis has shown 90 different types of proteins in the membrane vesicle. Some of the major proteins are the surface associated proteins including β-lactamase, coagulase,

haemolysin, and IgG binding proteins (96). Thirty five hundred different types of vesicular proteins have been identified from the spectrometry based proteomic study (168).

Different types of protein extraction methods can be used for the biological samples. One of the methods is the Trichloroacetic acid (TCA)-Acetone precipitation method which is used to identify the total proteins in membrane vesicles. The TCA-acetone precipitation method is much superior than the other methods for the extraction of proteins as it minimizes the protein degradation as well as reduces the contaminants (169). During this method , proteins and other insoluble substance are left out in the precipitate and the proteins are extracted using a suitable buffer (170). After the extraction, the proteins are analysed using mass spectrometry. Mass spectrometry is an analytical technique that is used for peptide and protein analysis which helps to analyse thousands of proteins from complex samples (171).

Proteomics, mass spectrometry, protein identification and analysis, were performed by Dr. Jack-Ansgar Bruun and Toril Anne Grønset at the Tromsø University Proteomics Platform (TUPP, IMB, Helsefak, UiT).

3.10.1 TCA-Acetone precipitation

i. Fifteen percentage of TCA (-20°C) was added in acetone at the ratio of 1:4 and the mixture was added to the MV samples in a LowProBind Eppendorf tubes.
ii. It was then mixed well and incubated on ice at 4°C for overnight.
iii. It was centrifuged for 99 minutes at 4°C at 20600 g to pellet the proteins.
iv. The protein pellet was washed with 1 ml 70% ethanol and centrifuged for 15 minutes at 4°C at 20600 g.
v. Then the pellet was re-suspended in PBS and delivered to TUPP for further analysis.

3.11 Cell culture

Cells are the basic unit of life and they are used in different biological research serving as a model system. These cells can be obtained either from skin biopsy or from the diseased tissues from the surgery and these cells should be capable of survival and proliferation on controlled conditions (172). Cell culture is a technique in which cells are grown in an artificial favourable condition outside of their natural environment. The cells are grown in monolayer on a solid

substrate or in as suspension in culture media (173). These cells in cultural medium can have different morphology; fibroblastic which can be bipolar or multipolar with elongated shape, epithelial that are polygonal in shape with the regular dimensions and lymphoblast with the spherical shape (174).

The cell culture can be either primary cell culture or continuous cell lines (173, 175). The primary cell culture is cells obtained directly from the tissues either plant or animal. They can grow and proliferate and have the same features as in the original cells (175). The continuous cell culture is the culture of cells from the primary cells and passed or divided invitro. These types of cells can be divided indefinitely and can be transformed to the tumor cells (174).

Three different types of cells are used in this study; HaCaT cells, Caco-2 cells and neutrophil cells. HaCaT cells are the immortalized human keratinocytes which are obtained from the adult human skin (176). This cell line is extensively used in scientific research due to their ability to differentiate and proliferate *invitro* (177). The Caco-2 cells are the human epithelial colorectal adenocarcinoma cells. These cells are used as a model of human intestine in different research study. Also, they resembles the enterocytes lining in the small intestine when grown on plastic dishes or nitrocellulose filters (178). Neutrophils are present in the blood and are the most abundant type of granulocyte. These are generated from the stem cells and plays an important role during the inflammation (179).

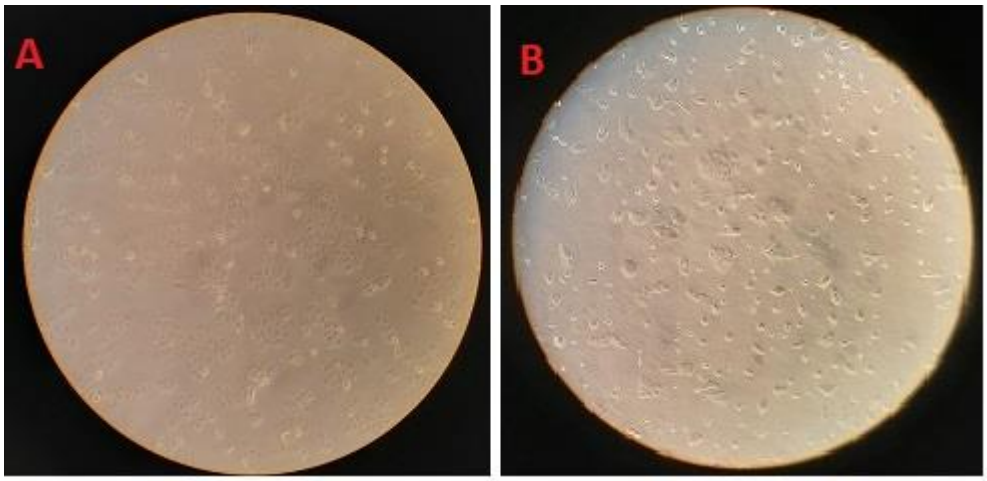


Figure 5: Different type of cells observed under the microscope using the 10x magnification. A: HaCaT cells, B: Caco-2 cells. First these cells are seeded into the cell culture flasks provided with the cultural medium and allowed them to grow until 80-90% confluent before splitting.

3.11.1 Freezing the cells and storage in liquid Nitrogen.

i. The medium in the flask was sucked up and washed with 10 ml of preheated PBS (37°C).
ii. Two millilitres of 5X Trypsin were added to the flask and incubated for 2-3 minutes until the cells were detached from the flask surface.
iii. Five millilitres of the medium were added in the cell culture and transferred to the 15 ml sterile centrifuge tube.
iv. It was then centrifuged at 200g for 5 minutes.
v. The supernatant was removed and 900 µl fetal bovine serum (FBS) was added in for each cryo tube.
vi. Then, 900 µl of cell culture and 100 µl of DMSO was added in the cryo tube with serum respectively.
vii. The suspension was mixed well, and the tubes were stored in -20°C freezer for 1 hour.
viii. The tubes were transferred to -70°C and stored for overnight and finally it was stored in Nitrogen tank.

3.11.2 Defrosting of cells from liquid Nitrogen.

i. The cryo tubes were taken from the nitrogen tank.
ii. It was kept in sterile pre-warmed water at 37°C to defrost.
iii. To avoid contamination the outside of tube was wiped with the ethanol before placing the tubes in the biological safety cabinet.
iv. The cells from the cryo tubes were added to 9 ml of cell culture media in a 50 ml falcon tube.
v. The cells were then pelleted by centrifugation at 200g for 10 minutes.
vi. Supernatant was removed and cells were resuspended in 15 ml fresh media and transferred to cultivation flask and incubated in the cell incubator (37°C and 5% CO_2).

3.11.3 Seeding of HaCaT cells.

HaCaT cells are seeded into 96 well plates at the concentration of 2 x 10^5 cells/ml in Dulbecco's Modified Eagle's Medium (DMEM)-low glucose (Sigma-Aldrich, UK) supplemented with 10% heat inactivated FBS.

i. The medium from the cell culture flask was removed and washed with 10 ml 1x PBS (Sigma-Aldrich, UK).
ii. The surface of cell flask was covered with 4 ml PBS supplemented with 0.25 mM Ethylenediaminetetraacetic acid (EDTA) and incubated at 37°C for 10 minutes.
iii. The PBS with EDTA was removed and 1 ml 5x trypsin was added, moved around the cell flask to ensure the cell surface to be covered and incubated at 37°C for 2 minutes or until the cell are detached.
iv. Again, 9 ml DMEM with 10% FBS was added to the cell with trypsin solution and the cells were gently resuspended by pipetting up and down.
v. The cell suspension was transferred to a 50 ml falcon tube and centrifuged at 200g for 10 minutes.
vi. The supernatant was discarded, 5 ml of media was added, and the pellet were dissolved.
vii. In two Eppendorf tube, 90 µl of PBS was added with 10 µl cell suspension and the concentration of cell was measured using the cell counter and the cell concentration was determined.
viii. In a new 50 ml tube, the cell suspension was diluted in DMEM with 10% FBS to the final concentration of 2×10^5 cells/ml using the equation, $C_1 \times V_1 = C_2 \times V_2$. The cell suspension was added to 96 well plate with 200 µl per well.
ix. The wells were checked under the microscope to make sure that the cells were evenly distributed over the surface and place in the incubated at 37°C.

3.11.4 Seeding of CaCo-2 cells.

CaCo-2 cells are seeded into 96 well plates at concentration of 3.75×10^4 cells per ml in Minimum Essential Medium Eagle (EMEM)-high glucose (Sigma-Aldrich, UK) supplemented with 10% FBS and 1% Non-essential Amino acids (NEAA).

i. The medium was removed from the cell culture flask and washed with 10 ml 1x PBS.
ii. The PBS was removed and 1.5 ml 5x trypsin was added to wash the cells and the trypsin was removed.
iii. Again 1 ml 5x trypsin was added, moved around the cell flask to ensure the cell surface were covered and incubated at 37°C for 2 minutes or until the cell are detached.

iv. Now, 9 ml EMEM with 10% FBS and 1% NEAA was added to the cells to stop the trypsinization. The cells were gently resuspended by pipetting up and down.

v. The cell suspension was transferred to a 50 ml falcon tube and centrifuged at 200g for 10 minutes.

vi. The supernatant was discarded, 5 ml of media was added to the pellet and the pellet were dissolved.

vii. In two eppendorf tube, 90 µl of PBS was added with 10 µl cell suspension and the concentration of cell was measured using the cell counter.

viii. First in a new 50 ml tube, required amount of cell suspension was prepared in EMEM with 10% FBS and 1% NEAA with concentration of 3.75×10^4 cells/ml using the equation, $C_1 \times V_1 = C_2 \times V_2$ and the amount of cell suspension was seeded in 96 well plate. The cell suspension was added in 96 well plate in 200 µl per well.

ix. The wells were checked under the microscope to make sure that the cells are evenly distributed over the surface and place the plates in incubated at 37°C.

3.11.5 Isolation and seeding of neutrophil cells from human blood.

Neutrophil cells were seeded in 96 well plate at concentration of 2.5×10^4 cells per 200 µl in RPMI-1640 Medium (Sigma-Aldrich, UK) with 0.05% human serum albumin.

i. First human venous blood was collected in 8 tubes with EDTA.

ii. In a 15 ml tube, 5 ml of Polymorphprep was transferred with the pipette and 5 ml of blood in each tube was carefully layered over the Polymorphprep.

iii. The tube was then centrifuged at 500 g for 40 minutes at room temperature with acceleration 7 and deceleration 1 so that the layer after centrifugation remain undisturbed.

iv. The plasma and mononuclear cells (upper band of cells as shown in figure 6) were removed and the lower band were collected in a 50 ml falcon tube.

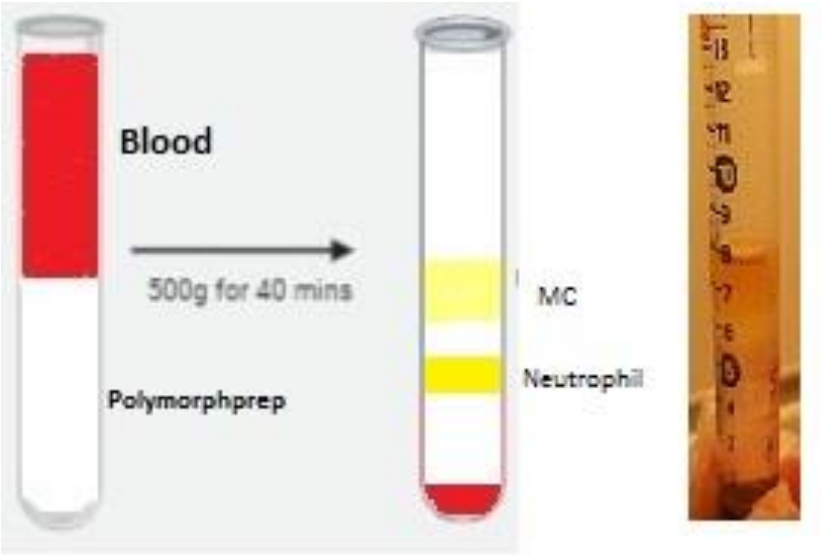


Figure 6: Isolation of neutrophil cells using Polymorphprep. The centrifugation of Polymorphprep with blood gives the different bands of mononuclear cells (MC), and Neutrophil cells in a centrifuge tube. At the bottom of the tube is residual erythrocyte cells. Right side of figure shows an actual separation in a 15 ml tube. The figure was created using BioRender tool.

v. First an aliquot of Hepes-buffered saline (0.85% NaCl w/v, 10mM Hepes-NaOH, pH 7.4) was diluted with an equal volume of water. This was mixed with 1 volume of to the cell suspension at ratio of 1:1.
vi. The tube was centrifuged at 400 g for 10 minutes and the supernatant was discarded.
vii. The pellet was resuspended in 3 ml ammonium chloride lysis buffer (0.83% w/v NH_4Cl, 10mM Hepes-NaOH, pH 7.4) and incubated at 37°C for 10 minutes. This was done in order to remove any residual erythrocyte contamination.
viii. It was then centrifuged at 400 g for 10 minutes and the supernatant was removed.
ix. The pellet was resuspended in 1 ml RPMI with 0.05% human serum albumin.
x. Now, in two eppendorf tube, 90 µl of medium was added with 10 µl cell suspension and the concentration of cell was measured using the cell counter.
xi. First in a new 50 ml tube, required amount of cell suspension was prepared in RPMI with 0.05% human serum albumin with concentration of 2.5 x 10^4 cells per 200 µl using the equation, $C_1 \times V_1 = C_2 \times V_2$ and the amount of cell suspension was seeded in 96 well plate. The cell suspension was added in 96 well plate in 200 µl per well.

xii. The wells were checked under the microscope to make sure that the cells are evenly distributed over the surface and place the plates in incubated at 37°C.

3.11.6 Addition of membrane vesicles to host cells in 96 well plates.

3.11.6.1 HaCaT cells and CaCo-2 cells

i. The membrane vesicle samples were diluted in MilliQ water to 4 different concentration; 1000 ng, 100 ng, 10 ng and 0 ng and it was added to HaCat cells, and CaCo-2 cells seeded in 96 well plates.
ii. Ten µl of the MV samples with different concentrations was added to each well as shown in figure 7.
iii. The high control (4 µl of lysis buffer) was added in the well 11 A, B and C marked as X in figure 7 and the concentration of 0 ng/ml was taken as the low control.
iv. The plates were incubated in incubator at 37°C and 5% CO_2.
v. After 1 hour 200 µl of sample from each well was collected from the two different cell types and transferred to a second 96 well plate.
vi. The plate was centrifuged at 250g for 10 minutes at 4°C.
vii. Then 180 µl of sample was transferred to third 96 well plate marked with 1 hour.
viii. After 3 hours 200 µl of sample from 3 hour marked from first plate incubated in incubator was transferred to second 96 well plate.
ix. The plate was centrifuged at 200g for 10 minutes at 4°C.
x. Then 180 µl of sample was transferred to third 96 well plate marked with 3 hours. Again, after 6 hours 200 µl of sample from 6 hour marked from first plate incubated in incubator was transferred to second 96 well plate.
xi. The plate was centrifuged at 200g for 10 minutes at 4°C.
xii. Then 180 µl of sample was transferred to third 96 well plate marked with 6 hours.

		Medium	1	1	1	3	3	3	6	6	6	High	/
		1	2	3	4	5	6	7	8	9	10	11	12
1000 ng	A											X	
	B											X	
100 ng	C											X	
	D												
10 ng	E												
	F												
0 ng	G												
	H												

Figure 7: Plate layout of 96 well plate showing the addition of membrane vesicles in different concentration of 1000 ng, 100 ng, 10 ng and 0 ng. 1, 3 and 6 are the times in hours indicating the transfer of samples in that time. High indicates high control added in X wells.

3.11.6.2 Neutrophil cells

i. The MV samples were diluted with MilliQ water in 4 different concentration; 1000 ng, 100 ng, 10 ng and 0 ng/ml and it was added to Neutrophil cells.
ii. Ten µl of MV samples in each well in 96 well plate was added with different concentration as shown in figure 7.
iii. The high control (4 µl of lysis buffer) was added in the well 11 A, B and C marked as X in figure 7 and the concentration of 0 ng/ml was taken as the low control.
iv. The plates were incubated in incubator at 37°C.
v. After 1 hour 200 µl of sample from 1 hour marked was transferred to second 96 well plate.
vi. The plate was centrifuged at 500g for 10 minutes at 4°C.

vii. Then 180 µl of the sample was transferred to Eppendorf tubes and centrifuged for 5 minutes and 180 µl of supernatant was transferred to 96 well plate marked 1 hour.

viii. After 3 hours 200 µl of sample from 3 hour marked from first plate incubated in incubator was transferred to second 96 well plate.

ix. The plate was centrifuged at 500g for 10 minutes at 4°C.

x. Then 180 µl of sample was transferred to the Eppendorf tube spin down for 5 minutes and 180 µl of supernatant was transferred to 96 well plate marked 3 hours.

xi. Again, after 6 hours 200 µl of sample from 6 hour marked from first plate incubated in incubator was transferred to second 96 well plate.

xii. The plate was centrifuged at 500g for 10 minutes at 4°C.

xiii. Then 180 µl of sample was transferred to the Eppendorf tube spin down for 5 minutes and 180 µl of supernatant was transferred to 96 well plate marked 6 hours.

3.12 Measurement of cell cytotoxicity using lactate dehydrogenase (LDH) assay.

LDH is an cytosolic enzyme present in the cytoplasm of a cell which converts the pyruvate into lactate in presence of nicotinamide adenine dinucleotide (NADH) (180) and are used to evaluate the damage of cells in different pathological states (181). Cells can be damaged due to various reasons and during these conditions there is swelling and rupture of intracellular organelles which cause the plasma membrane to break. This breakdown leads to the release of various intracellular contents of the cells (182).

The cell cytotoxicity can be measured using LDH enzyme that is released during this cell damage. It involves mainly two different steps to measure the cell death. At the first step, LDH converts lactate to the pyruvate by converting the NAD^+ to NADH. In second step, in presence of the electron acceptor, NADH reduces a tetrazolium salt (INT) which is converted to the red product formazan (183).This red product is proportional to the LDH released by the cell during the cell death and can be measured by measuring the absorbance by spectrophotometric microplate reader (ELISA reader). The cell cytotoxicity percentage can be calculated by using the formula.

$$Cytotoxicity\ (\%) = \frac{Experimental\ value - low\ control}{high\ control - low\ control}\ X\ 100$$

i. The different working solution for LDH assay were prepared. First 1 ml of distilled water was added to Catalyst (bottle 1 blue cap; Sigma-Aldrich, UK) and 45 ml of the dye solution (bottle 2 red cap; Sigma-Aldrich, UK) which is ready to use were mixed shortly before use.
ii. First, 180 µl of the different cell suspension (HaCaT cells, CaCo-2 cells and neutrophil cells) from the 96 well plates were transferred to new 96 well plate.
iii. Hundred µl of the working solution was added in each well of all plates.
iv. Two hundred µl of background control was added in 3 wells of 96 well plate and were incubated for 30 minutes in dark.
v. It was then added 50 µl of Stop solution (Triton X-100; Sigma-Aldrich, UK).
vi. The absorbance of the samples was measured at 490 nm using the ELISA reader.

4. RESULTS

4.1. Preliminary observations of *Enterococcus faecium* E1007

Different experimental methods were performed to analyse *E. faecium* E1007 growth in different cultural medium, and to make the growth curve in BHI medium and evaluate the morphology of bacteria by Gram staining method.

4.1.1 Growth of *Enterococcus faecium* E1007 on different cultural media

The *E. faecium* E1007 from the blood agar plate were used to inoculate in different cultural media as shown in figure 8: BHI, CA, CLED Agar, LA, TSA, MH with 5% blood and MH-F with 5% blood and NAD, and their colony structures were observed. The colonies on BHI were big and white in colour while appeared with silver colour on in BA media. On CA and TSA media the colonies were white in colour with medium size colonies. Colonies were small with pale colour in CLED media. On MH 5% blood agar the colonies were white and small in size whereas on MH-F 5% blood agar with NAD there were no growth of bacteria.

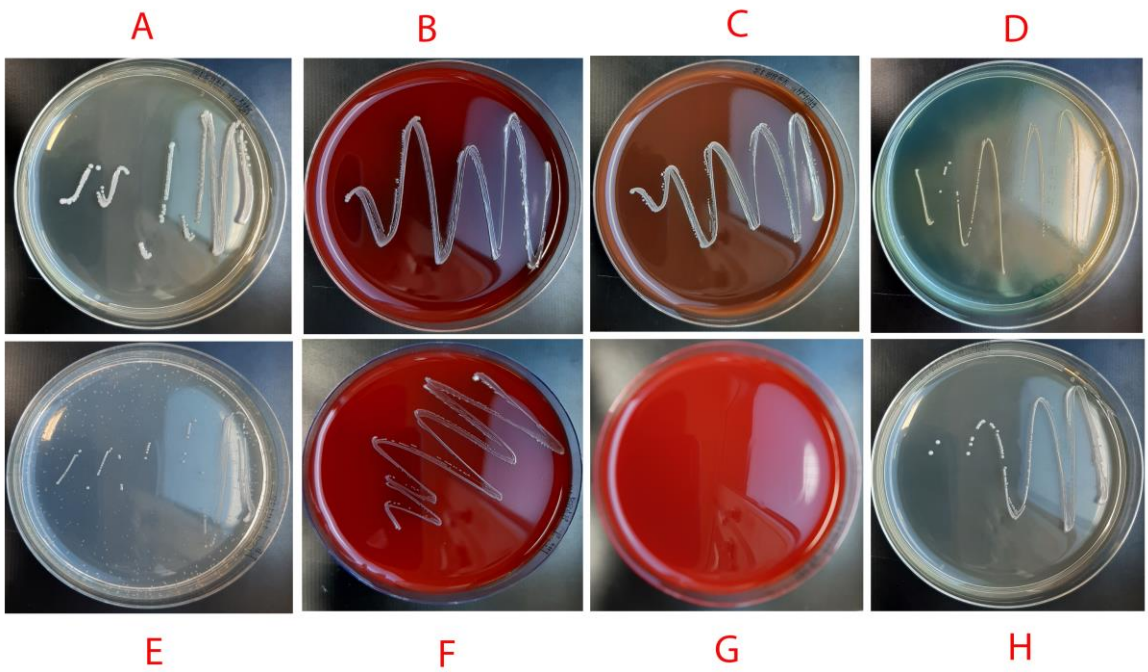


Figure 8: Colony isolation of *Enterococcus faecium* E1007 in different cultural medium. A: BHI, big colonies B: BA, colonies with silver colour C: CA, colonies with white colour D: CLED, small colonies

with pale colour E: TSA, medium sized colonies F: MH 5% blood agar, small white colonies G: MH-F 5% with NAD, no colonies H: LA, small white colonies.

4.1.2 Gram staining

Gram staining of three different bacteria, *Streptococcus pyogenes* ATCC 19615, *E. faecium* E1007 and *Pseudomonas aeruginosa* ATCC 27853 was performed. The two bacteria *Streptococcus pyogenes* and *Pseudomonas aeruginosa* were taken as reference for Gram-positive and Gram-negative bacteria respectively. *Streptococcus pyogenes* ATCC 19615 revels as purple coloured Gram-positive cocci single or in chains, *Enterococcus faecium* E1007 also shows Gram-positive purple cocci single or in clusters and *Pseudomonas aeruginosa* ATCC 27853 shows pink coloured rod-shaped Gram-negative bacteria as shown in figure 9.

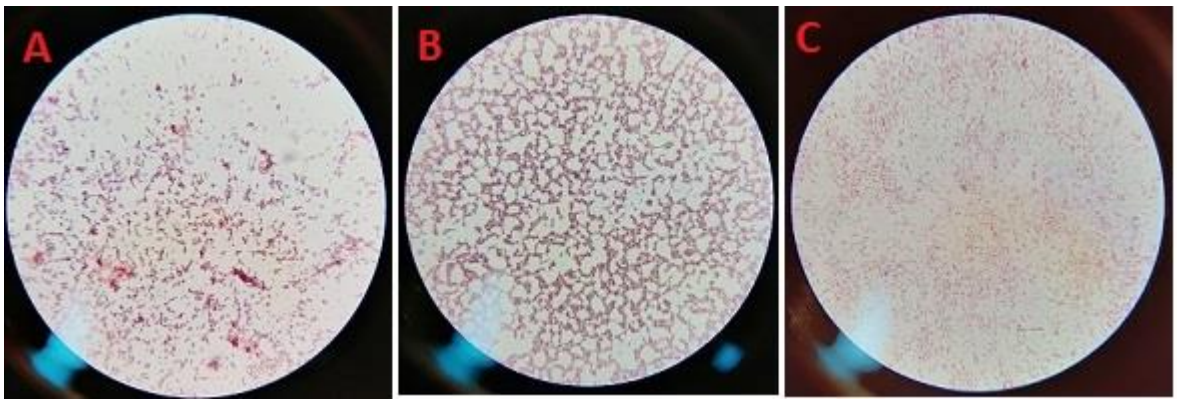


Figure 9: Gram staining from different bacteria observed at 100x magnification under microscope A: Gram-positive (Purple colour) cocci of *Streptococcus pyogenes* ATCC 19615 B: Gram-positive (Purple colour) cocci of *E. faecium* E1007 C: Gram-negative (Pink) rod shaped *Pseudomonas aeruginosa* ATCC 27853.

4.1.3 Growth curve of *Enterococcus faecium* E1007

The growth curve for *Enterococcus faecium* E1007 was observed by plotting the Optical density at 600 nm with time in minutes as shown in figure 10. It shows the three different phases that is the lag phase, exponential phase and the stationary growth phase.

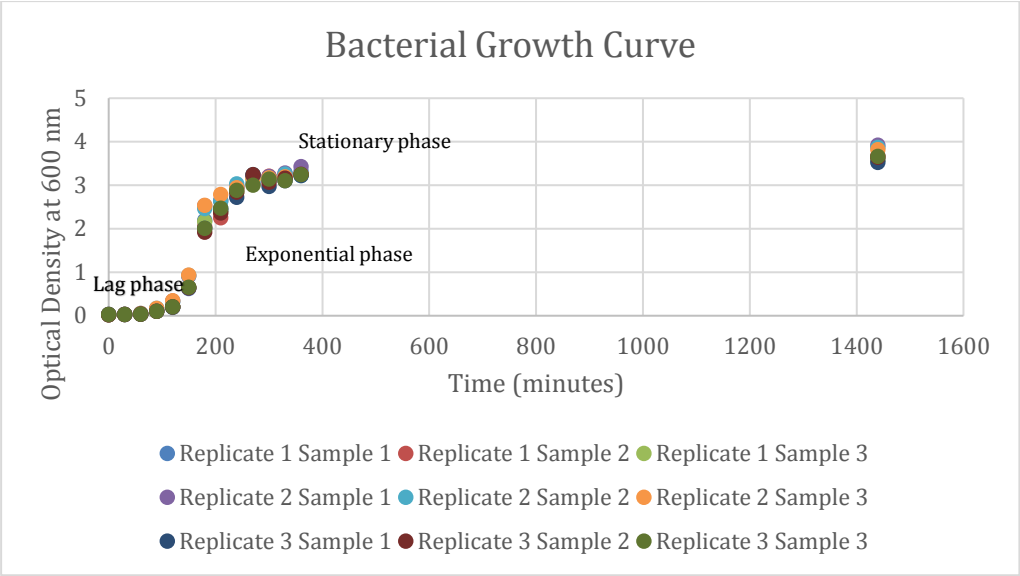


Figure 10: Growth curve of *Enterococcus faecium* E1007. The curve shows three phases; lag phase, exponential phase and stationary phase. The OD from three replicates with 3 samples from each replicate were measured every 30 minutes for 6 hours (360 minutes) and one after 24 hours.

4.2 *Enterococcus faecium* produces membrane vesicles in both exponential and stationary growth phase

E. faecium strain E1007 releases the membrane vesicles when observed in exponential as well as in stationary phase. For the exponential phase, *E. faecium* E1007 strains were grown in BHI medium till the mid-exponential phase (OD_{600} approximately 1.5) and for stationary phase, it was grown for 16 hours reaching the OD_{600} approximately to 2-3.5.

4.2.1 Membrane vesicles purification by DGC and SDS PAGE

The membrane vesicles were purified and fractionated by DGC process. The membrane vesicles were observed in a fraction in an OptiPrep solution shown in figure 11 A. These fractions obtained from DGC were further analysed by SDS PAGE as shown in the figure 11 B.

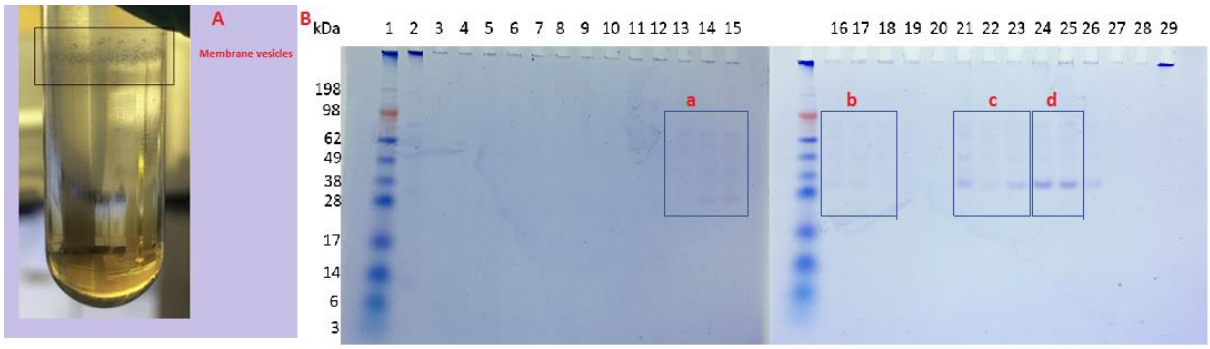


Figure 11: Purification of membrane vesicles obtained from the stationary growth phase. A: Showing the *E. faecium* E1007 membrane vesicles in OptiPrep solutions obtained after the density gradient centrifugation B: SDS PAGE for samples obtained from OptiPrep. 1: Ladder, 2: MV sample without OptiPrep, 3-26: MV Samples Obtained from OptiPrep, 27-28: Blank, 29: MV sample without OptiPrep, Fraction a: 13-15, Fraction b: 16-18, Fraction c: 21-24, Fraction d: 24-25. The fractions b, c, and d were used for the further analysis.

4.2.2 Transmission electron microscope (TEM) shows the membrane vesicles structure.

The fractions that are obtained from the purification were further analysed by using the TEM. The samples from both the exponential phase and stationary phase were observed and shows the small vesicles like circular structures as shown in figure 12 marked with small blue arrows.

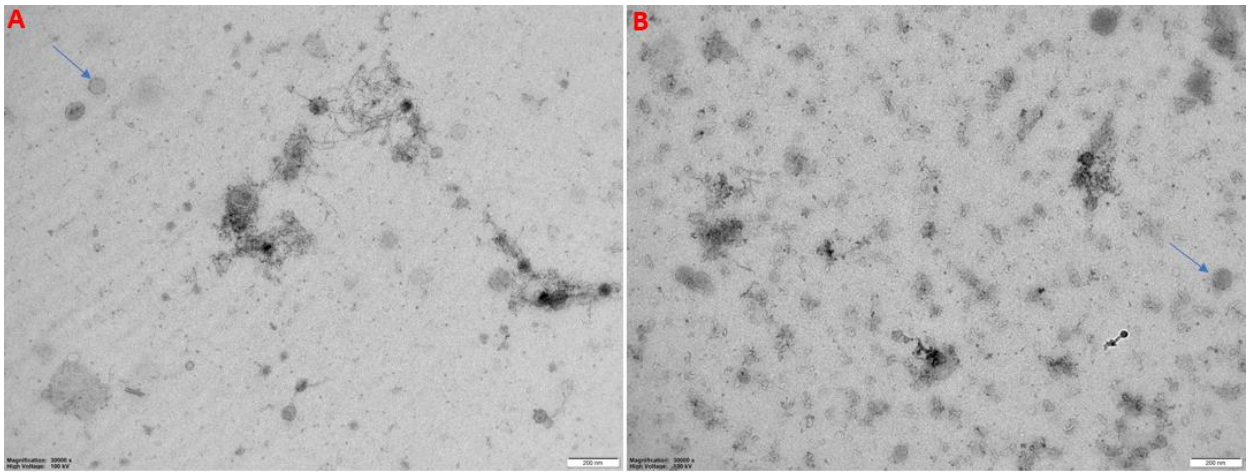


Figure 12: Images of membrane vesicles from *E. faecium* E1007 obtained by transmission electron microscopy A) MVs from stationary growth phase OptiPrep samples B) MVs from exponential growth phase OptiPrep samples. The blue arrow shows the membrane vesicles structures. The small hair like structures are the remaining from the OptiPrep.

Apart from this, the mean size of the membrane vesicles from exponential growth phase OptiPrep sample and stationary growth phase OptiPrep sample were found to 64.4 ± 29.64 nm and 63.1 ± 25.12 nm respectively which is shown graphically in figure 13.

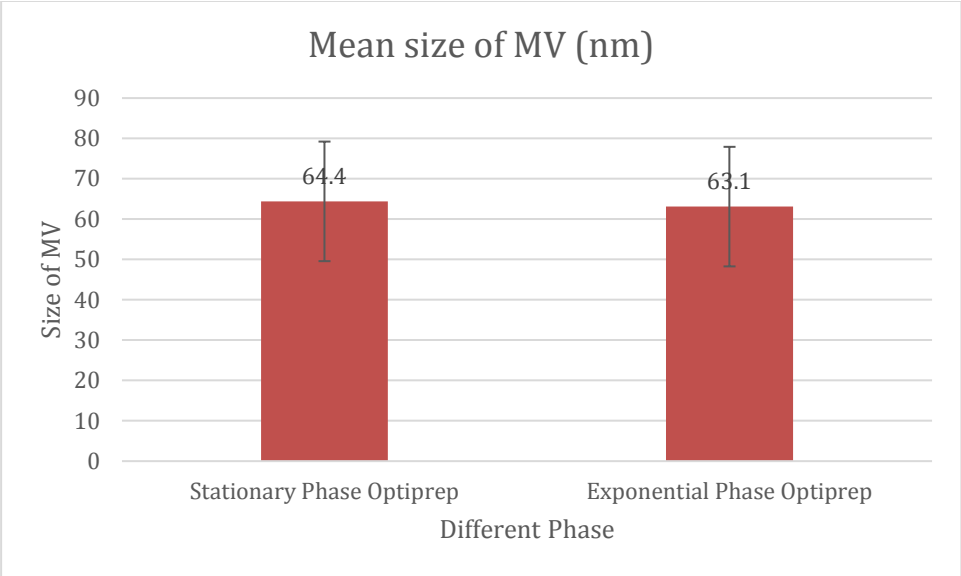


Figure 13: Comparison of mean size of membrane vesicles of *E. faecium* E1007 obtained from stationary growth phase OptiPrep samples and exponential growth phase OptiPrep samples.

4.2.3 Size measuring through dynamic light scattering (DLS) methods.

DLS method was employed for measuring the size of membrane vesicles from Exponential as well as Stationary growth phases. The sample taken were from crude as well as from the OptiPrep samples and were run for 2 cycles each. In the exponential growth phase, with the crude samples two peaks were observed in both the run and peak 2 showing the higher mean diameter in both runs as shown in figure 14 A. The mean diameter of membrane vesicles in peak 2 run 1 and run 2 were 55.1 ± 5.8 nm and 55.8 ± 6.9 nm respectively. The percentage distribution of mean diameter in Peak 2 run 1 and run 2 were also calculated and found to be 94.79% and 94.07% respectively as shown in figure 14 C. And for the OptiPrep samples, three peaks were observed as shown in figure 14 B. The first peak and third peak were ignored as the first peak was too small and third peak was too large indicating the agglomerates. The mean diameter of membrane vesicles in peak 2 run 1 and run 2 were 100.1 ± 14 nm and 48.5 ± 6.9

nm respectively. The percentage distribution of peak 2 in run 1 and run 2 were 42.48% and 11.27% respectively as shown in figure 14 D.

In the Stationary growth phase, for crude samples three peaks were obtained as shown in figure 15 A and the peak 1 and peak 3 were ignored. In peak 2 the mean diameter of membrane vesicles were 73.7 ± 9.6 nm and 58.8 ± 6.5 nm respectively. The percentage distribution of peak two in both runs were 58.28% and 46.51% respectively as shown in figure 15 C. For the OptiPrep samples two peaks were observed and peak 2 indicating the major peak and the mean diameter of membrane vesicles were 114 ± 14.8 nm and 123.9 ± 17.3 nm respectively as shown in figure 15 B. The percentage distribution of peak 2 run 1 and run 2 were 77.94% and 22.06% respectively as shown in figure 15 D.

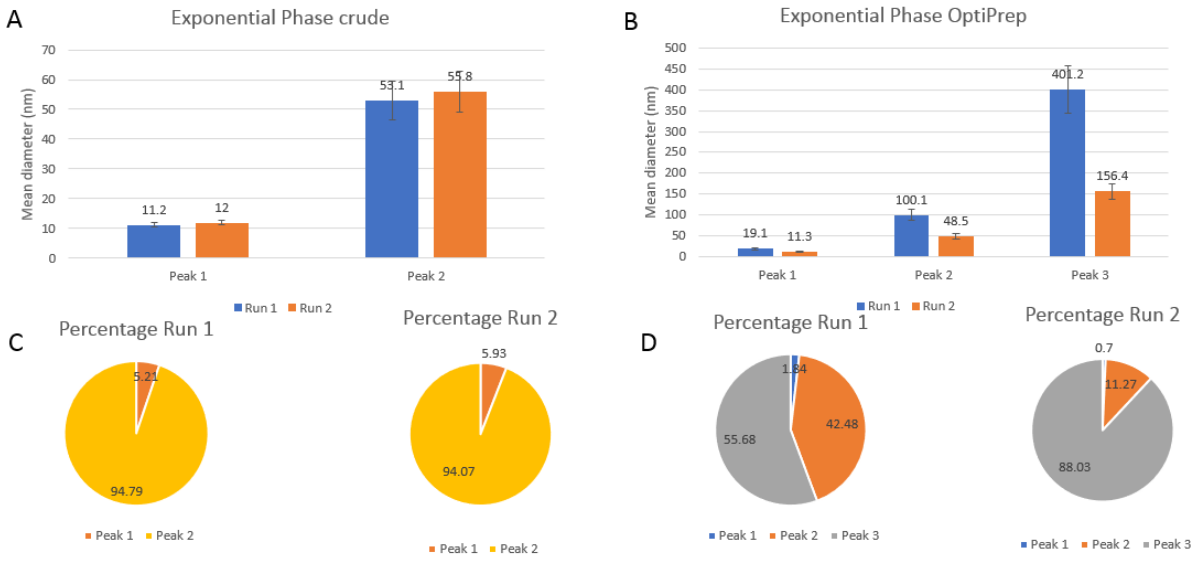


Figure 14: Comparison of mean diameter of membrane vesicles by dynamic light scattering method A) Exponential growth phase crude sample B) Exponential growth phase OptiPrep sample C) Percentage distribution of Exponential growth phase crude samples Run 1 and Run 2 D) Percentage distribution of Exponential growth phase OptiPrep samples Run 1 and Run 2.

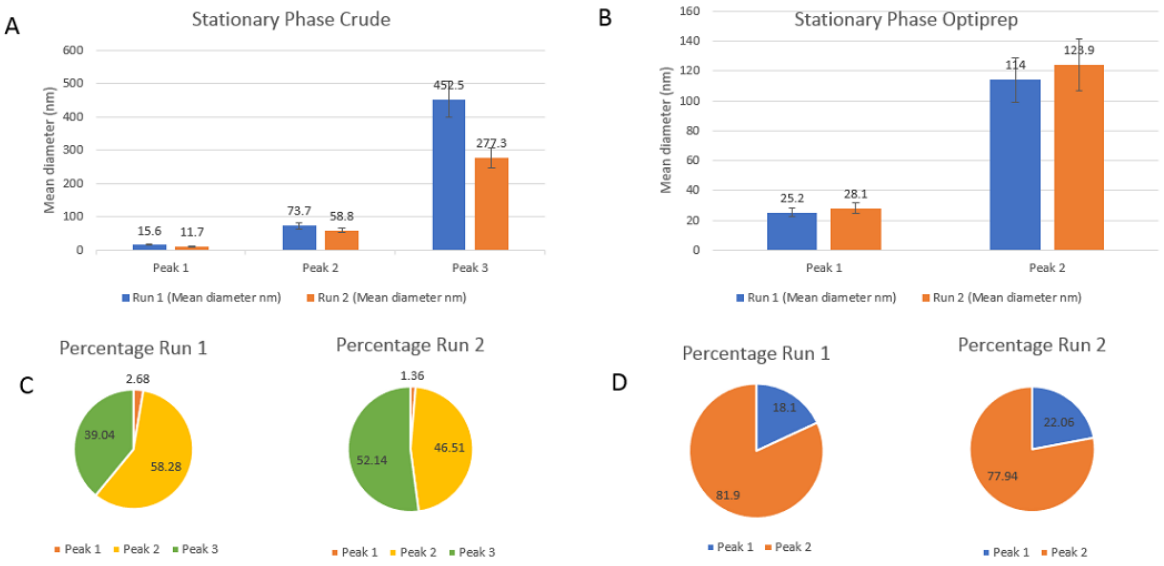


Figure 15: Comparison of mean diameter of membrane vesicles by dynamic light scattering method A) Stationary growth phase crude sample B) Stationary growth phase OptiPrep sample C) Percentage distribution of Stationary growth phase crude Run 1 and Run 2 D) Percentage distribution of Stationary growth phase OptiPrep Run 1 and Run 2.

4.3 Proteomic analysis shows total amount of proteins in membrane vesicles.

Proteomic was performed to evaluate the number of proteins in crude and purified vesicles isolated from the bacteria grown to exponential and stationary growth phases. The total number of proteins found within the membrane vesicles is shown in table 1. The total number of proteins from stationary phase OptiPrep fraction 1, stationary phase OptiPrep fraction 2, stationary phase OptiPrep Fraction 3, exponential phase OptiPrep, stationary phase crude, and exponential phase crude were 1160, 1012, 497, 362, 425, and 1044, respectively.

Table 1: Number of Protein obtain from membrane vesicle in different phases.

Samples	No. of proteins
Stationary growth phase OptiPrep Fraction 1	1060
Stationary growth phase OptiPrep Fraction 2	1012
Stationary growth phase OptiPrep Fraction 3	497
Exponential phase OptiPrep	362
Stationary growth phase crude	425
Exponential growth phase crude	1044

4.4 Membrane vesicles from *E. faecium* induces cytotoxicity to different cells.

The MVs from exponential and stationary growth phases were added to the three different cell types; HaCaT cells, CaCo-2 cells and neutrophil cells and their cytotoxicity to these cells were measured by using LDH assay. The MV samples from exponential and stationary growth phases were prepared separately in 4 different concentration; 1000 ng, 100 ng, 10 ng and 0 ng and the cell cytotoxicity was measured after 1 hour, 3 hours and 6 hours respectively.

4.4.1 Membrane vesicles shows cytotoxicity to HaCaT cells.

MVs from *E. faecium* E1007 exponential and stationary growth phases shows the cytotoxicity to HaCaT cells. The MVs (100 ng, 100 ng, 10 ng and 0 ng) from exponential growth phase were added to HaCaT cells and the cytotoxicity percentage was high when the HaCat cells were treated with 1000 ng/ml (3.10%, 29.65% and 48.21% at 1 hour, 3 hours and 6 hours respectively) as compared to the cells treated with 100 ng/ml and 10 ng/ml shown in figure 16. The HaCaT cells do not show any cytotoxicity when the cells were not treated with MVs (0 ng).

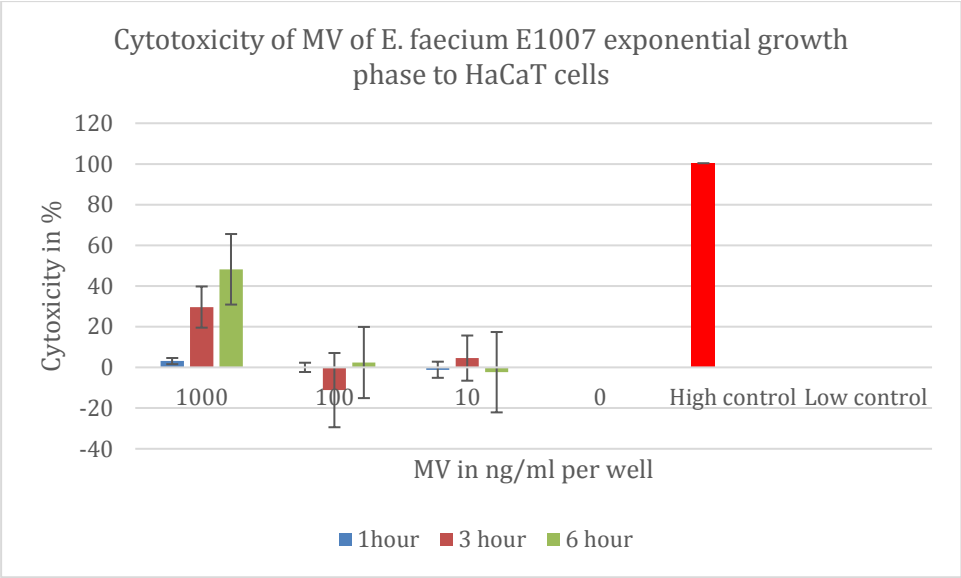


Figure 16: Measurement of cell cytotoxicity of membrane vesicles from the exponential growth phase to HaCaT cells. The HaCaT cells were treated with different concentrations (100 ng, 100 ng, 10 ng and 0 ng) of MVs from *E. faecium E1007* exponential phase and the cytotoxicity percentage were measured at different time interval (1 hour, 3 hours and 6 hours). The high control and low control were 100.2 and 0 percentage, respectively.

Similarly, during the stationary growth phase, the cytotoxicity percentage was increasing with the time when 1000 ng MVs were treated (6.30%, 29.72% and 40.56 % at 1 hour, 3 hours and 6 hours respectively as shown in figure 17. Also, the percentage of cytotoxicity was decreasing as the concentration of MVs decreases from 1000 ng to 0 ng at different time interval.

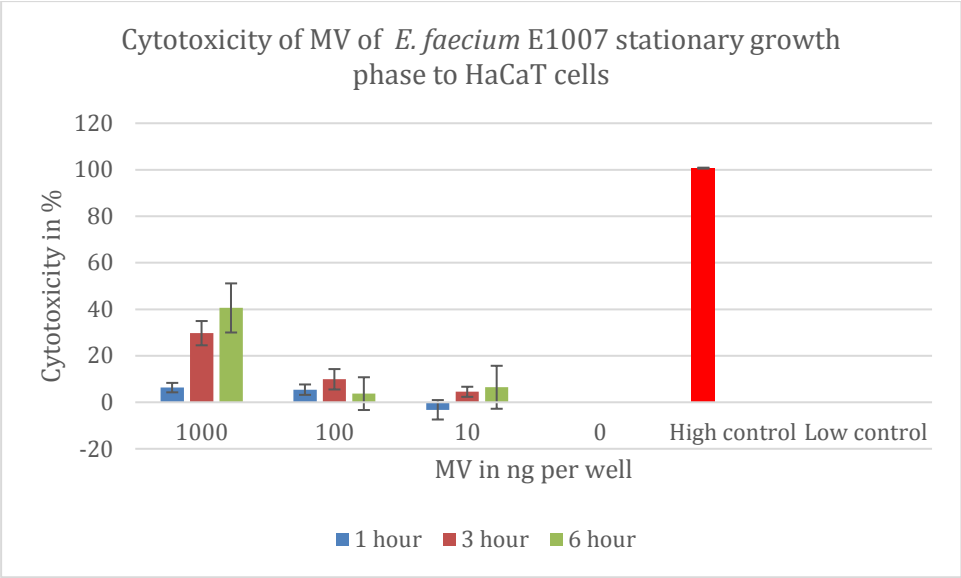
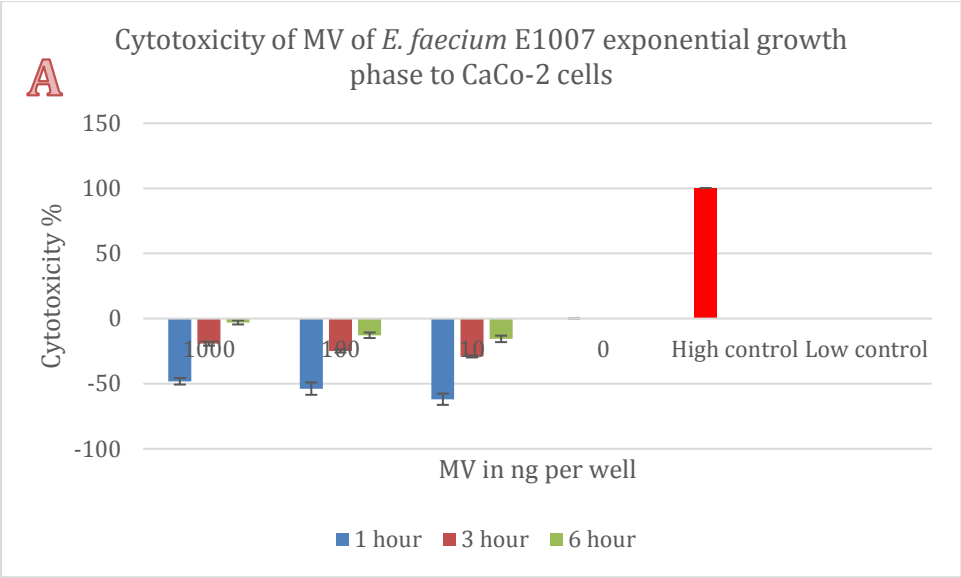


Figure 17: Measurement of cell cytotoxicity of membrane vesicles from the stationary growth phase to HaCaT cells. The HaCaT cells were treated with different concentrations (100 ng, 100 ng, 10 ng and 0 ng) of MVs from *E. faecium* E1007 stationary growth phase and the cytotoxicity percentage were measured at different time interval (1 hour, 3 hours and 6 hours). The high control and low control were 100.7 and 0 percentage, respectively.

4.4.2 Membrane vesicles show less cytotoxicity to CaCo-2 cells.

The MVs from exponential growth phase and stationary growth phase were added to CaCo-2 cells with 4 different concentrations (1000 ng, 100 ng, 10 ng and 0 ng). MVs isolated from both the phases show negative percentage of cytotoxicity with respect to the time;1 hour, 3 hours and 6 hours respectively as shown in figure 18 A and 18 B. MVs from *E. faecium* E1007 exponential and stationary growth phases are less cytotoxicity to CaCo-2 cells.



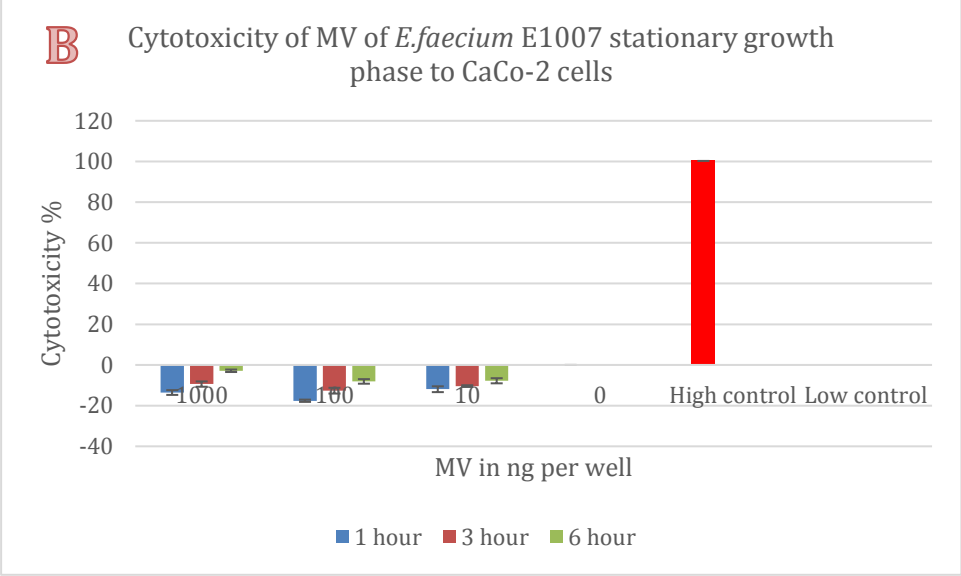


Figure 18: Measurement of cell cytotoxicity of membrane vesicles from *E. faecium* E1007 to CaCo-2 cells. A: MVs from exponential growth phase. The high control and low control were 100.3 and 0 percentage, respectively. B: MVs from stationary growth phase. The high control and low control were 100.3 and 0 percentage, respectively. The CaCo-2 cells were treated with different concentrations (100 ng, 100 ng, 10 ng and 0 ng) of MVs from *E. faecium* E1007 exponential and stationary growth phases and the cytotoxicity percentage were measured at different time interval (1 hour, 3 hours and 6 hours).

4.4.3 Membrane vesicles were highly cytotoxic to neutrophil cells.

The MVs from exponential growth phase and stationary growth phase were added to neutrophil cells with 4 different concentrations (100 ng, 100 ng, 10 ng and 0 ng). The percentage of

cytotoxicity in both phases is increasing when the concentration of MVs increases with time; 1 hour, 3 hours and 6 hours respectively as shown in figure 19 A and 19 B. MVs from *E. faecium* E1007 exponential and stationary growth phases are highly cytotoxic to neutrophil cells.

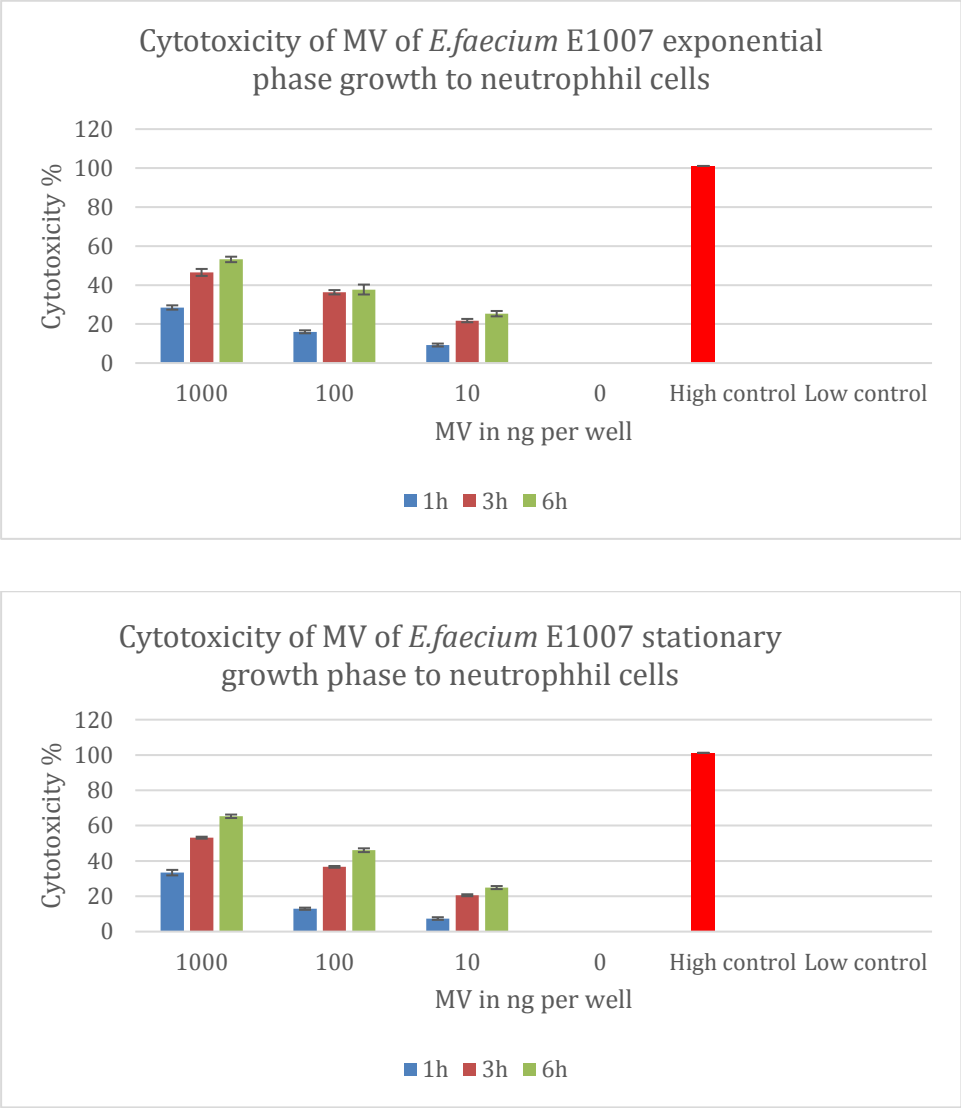


Figure 19: Measurement of cell cytotoxicity of membrane vesicles from *E. faecium* E1007 to neutrophil cells. A: Membrane vesicles from Exponential growth phase. The high control and low control were 101.2 and 0 percentage, respectively. B: Membrane vesicles from Stationary growth phase. The high control and low control were 101.1 and 0 percentage, respectively. The Neutrophil cells were treated with different concentrations (100 ng, 100 ng, 10 ng and 0 ng) of membrane vesicles from *E. faecium* E1007 Exponential growth phase and Stationary growth phase and the cytotoxicity percentage were measured at different time interval (1 hour, 3 hours and 6 hours).

5. DISCUSSION

In past few decades the study MVs were mainly confined to Gram-negative bacteria that produces the OMVs, however new studies have shown that Gram-positive bacteria were able to release the MVs. In fact MVs are produced by both Gram-positive and Gram-negative bacteria (184). Vesicles research in Gram-positive bacteria like *S. aureus* (96), *Bacillus anthracis* (*1*00), *Listeria monocytogenes* (98), *Streptococcus pneumoniae* (99) and *Clostridium perfringens* (105) has already been done proving the fact that Gram-positive bacterium also produces the MVs. This study was on the MVs of the Gram-positive bacteria *E. faecium* and MVs produced by this bacteria has already been shown in some previous studies (101). The size and morphology of MVs secreted by *E. faecium* E1007 was found to be similar to the MVs (20-400 nm) from *S. aureus* (96), *Bacillus anthracis* (*1*00), *Listeria monocytogenes* (98), *Streptococcus pneumoniae* (99) and *Clostridium perfringens* (105) . Also, these vesicular sizes resemble the size from some of the Gram-negative bacterial OMVs from *E. coli*, and *Pseudomonas aeruginosa* (185, 186). The goal of the present study was to detect the MVs from commensal *E. faecium* E1007, study their morphological structure and whether they were cytotoxic to human cells. This study suggests that the commensal *E. faecium* releases the MVs in both the exponential and stationary growth phases.

In the preliminary experiment prior to the MVs isolations, some of the properties of *E. faecium* E1007 were studied such as their growth in different cultural media, Gram staining and the growth curve. Different cultural media were used to observe the cultural growth in several media plates. Blood agar can be used as a standard laboratory media in this study as *E. faecium* E1007 grow easily with big colonies in this enriched medium. Gram staining is a differential technique to identify the bacteria on the basis of their cell wall structure and differentiate into Gram-positive and Gram-negative (132). During Gram staining of *E. faecium* E1007, it confirms that it is a Gram-positive bacterium as it appears purple in colour which is due to the presence of thick peptidoglycan layer entrapping the primary dye crystal violet. Gram staining of two other bacteria were also done in order to compare the appearances of Gram-positive and Gram-negative bacteria.

Apart from this, the growth curve of *E. faecium* E1007 was studied in BHI medium. In a batch culture under constant environmental condition, a sigmoid curve is obtained from the culture

of bacteria. Zwietering et al. (187) observed the modelling of bacterial growth curve using the data from *Lactobacillus plantarum* and showed the different growth phases that is lag phase, exponential phase, stationary phase and death phase. The growth curve obtained from *E. faecium E1007* was similar to the growth curve mentioned above. The growth curve (Figure 10) shows the log phase, exponential phase and the stationary phase over the 24 hours that was measured manually in every 30 minutes until 6 hours and one after 24 hours to make sure for the stationary phase. Exponential growth is reached after 2 hours and stationary growth is reached after 4.5 hours. Therefore, MVs were harvested from exponential growth phase after 2 hours and from stationary growth phase after 4.5 hours.

The isolation of MVs includes several steps involving growth of bacteria under the desired growth conditions, filtrations and ultracentrifugation to obtain the pellets of MV. These vesicular pellets may be contaminated with proteins, pili and phage so an additional purification step needed to be included and DGC using an OptiPrep as a density gradient medium was used for the purification of MV samples (188). Purification using OptiPrep medium were also used in most of the studies due to its superior nature than other density gradient solutions that prevents the damage of membrane structures. The purified MVs were used for the SDS-PAGE analysis which were stained with Coomassie staining and these vesicles showed a band with molecular mass of size approximately 38 kDa-98 kDa.

The TEM analysis of the MVs from exponential and stationary growth phases showed small vesicles like structures which were similar in size. The size distribution of MVs from TEM analysis showed the various size of MVs ranging from 20-182 nm (Figure S1). These sizes resemble the size obtained by TEM from *Mycobacterium* spp. (189). The MVs size were also analysed through the DLS which showed the heterogenous population of MVs with different peaks from both the exponential and stationary growth phases. The vesicular size obtained from DLS were bigger compared to the size from TEM analysis (Figure S2). Rivera et al. observed the vesicular size in *Bacillus anthracis* by both TEM and DLS and found to be similar in their size. However, in this study MV size varies when comparing to TEM and DLS methods. This may be due to that in TEM there is no distinction by peak, but all sizes are averaged and in DLS there are distinct peaks and the smaller and larger size of MVs peaks are excluded during the DLS analysis. The larger size of MVs peaks could be due to the vesicle agglomerates. The size of MV from the OptiPrep samples are found to be bigger compared to the crude samples. One

of the reasons may be because the OptiPrep samples are purified samples and it excludes the contaminations from other membrane proteins. However, Askarian et al. (190) has also shown that the size of MV in *S. aureus*, measured by DLS were smaller in size.

Protein measurement from the MV samples is an important part and, in this study, we use different methods for the measurement of proteins from the MV samples. Three different methods were used; Bradford assay, NanoDrop and Qubit methods (Table S1, Supplementary figure). The amount of protein obtained from Bradford assay and Qubit method were similar whereas NanoDrop gave very high measurement. Due to the high sensitivity and specificity nature of Qubit method, it was used for the measurement of protein from the MV samples where needed.

The MVs produced by both Gram-positive and Gram-negative bacteria contain large number of vesicular proteins that has been shown in different research (96, 166, 167, 190). In Gram-positive bacteria *S. aureus* Lee et al. (96) have shown 90 different vesicular proteins including major proteins like cytoplasmic proteins, transporter proteins, penicillin binding proteins and β-lactamase proteins. Also, Askarian et al. (190) have shown some high number of vesicular proteins in *S. aureus* grown in LB and BHI medium with 131 and 617 different vesicular proteins respectively among which the majority were cytoplasmic proteins (75.33%). In the Gram-negative bacteria *E. coli* Lee et al. (191) have identified 171 different vesicular proteins when grown on LB medium of which 46.1% were outer membrane protein. Also, Wagner et al. (101) has shown high number of proteins in four clinical *E. faecium* strains DO, E155, K59-68 and K60-39 grown in exponential growth phase in BHI medium and stationary growth phase in LB medium. These studies have shown that the number of vesicular proteins may differ according to the bacteria and also the cultural media. This may be due to the fact that the growth media may affect the expression of gene in bacteria which ultimately differs the amount and content of vesicles (191). In this present study, we observed high number of different proteins in MV from the *E. faecium* E1007 grown in BHI medium. The high number of proteins within the MV in our study may be due to different growth medium, different growth conditions, different bacterial strains than mentioned above and also the different bacterial growth phases during study.

Bacteria may carry a large quantity of virulence factors and toxins within their MVs (33, 90, 101, 130, 131). These virulence factors and toxins are responsible for the cytotoxicity to the host cells. Most Gram-positive bacteria and Gram-negative bacteria release the MVs that are cytotoxic to the host cells. Different types of human cell lines such as CaCo-2, HaCaT, Hep-2, HeLa are commonly used cell lines for the study of cytotoxicity. In Gram-positive bacteria *S. aureus*, Jeon et al. (192) have shown the cytotoxicity of MVs to the Hep-2 cells at the concentration of 50 µg/ml of MV. In similar study by Askarian et al. (190), they study the cytotoxicity in Neutrophil cells demonstrating the high cytotoxicity of *S. aureus* derived MVs. Also, in *Clostridium difficile* their MVs carry different cytotoxic factors which were responsible for the cytotoxicity in different cells like CaCo-2 and Hep-2 cells (193). Similarly, Ellis et al. (194) have studied the cytotoxicity in Gram-negative *Pseudomonas aeruginosa* which releases the OMVs and demonstrated that they are highly cytotoxic to the macrophages. In this present study, HaCaT cells, CaCo-2 cells and Neutrophil cells were used as a model for the study of host cell cytotoxicity from the MVs of *E. faecium*. MVs of *E. faecium* from both exponential and stationary growth phases were added to with these cells at different concentrations and their cell cytotoxic effect were observed over different time interval. HaCaT cells represent the human keratinocyte cells which are derived from the human skin (176). These cells confer high cell cytotoxicity when treated with MVs at different concentration. The cytotoxicity of MVs obtained from both exponential and stationary growth phases towards the HaCaT cells looks similar with minimum differences in their cytotoxicity.

The CaCo-2 cells are the epithelial cells representing the human intestine. The MVs showed no cytotoxicity towards the cells. The *E. faecium* is a commensal bacterium found in the human intestine so it may be due to this that CaCo-2 cells exhibit very good tolerance to MVs from *E. faecium* even with the exposure to very high concentration of MV that is 1000 ng per well. This could suggest that they may lack any major cytotoxicity on these intestinal cells. The neutrophils cells are the granulocyte cells and these cells represents the innate immune cells in the human. Bacterial vesicles may induce the immune responses towards these innate immune cells like neutrophils which are the blood cells where *E. faecium* are not found. When these cells are treated with the MVs in this present study, neutrophil cells show high cytotoxic response. This may be since Neutrophils are the major effector cells of innate immunity and in response to the MVs they may produce various cytokines, however due to different virulence factors within MVs this may damage the host cells.

This present study focuses on finding the cytotoxicity on different types of cells representing different parts of the human and suggests that the MVs from *E. faecium* plays a role in the cytotoxicity of cells. MVs can adhere and internalise to these types of cells easily causing a cytotoxic effect in the cells and its cytotoxicity depends on the type of cells. Although the different number of proteins were identified from the MVs of *E. faecium*, specific cytotoxic molecules were not identified which may be responsible for the cell cytotoxicity in different cell lines. Further studies identifying different virulence factors may provide more insights into the cytotoxicity of MVs to host cells.

Also, the MVs from *E. faecium* are an interesting vaccine candidate as they are stable, non-infectious and non-replicative. MVs carry a large amount of proteins as a cargo including immunogenic proteins and vaccine candidates like metal binding proteins, penicillin binding proteins, peptidoglycan binding proteins, D, D-carboxypeptidase (101). So, in the future studies regarding these vaccine candidates from *E. faecium* can help in finding a vaccine against enterococcal infections as no vaccine against enterococci is produced till date.

6. CONCLUSION

This study provides better understanding on MVs secreted by Gram-positive *E. faecium* E1007. In this study, MVs from *E. faecium* E1007 in exponential and stationary growth phases, were identified and further purified using the DGC using the OptiPrep as a density gradient medium. Apart from this, the morphology and size of MVs were studied using TEM and DLS. The main part of the study was to study the host cell responses in different types of human cell lines. Our result suggested that the MVs from *E. faecium* play a role in the cytotoxicity to different host cells and its cytotoxicity depends upon the type of cells. The cytotoxic activity of MVs towards the host cells may be due to the presence of different proteins and virulence factors with in the MVs themselves. And further precise study involving detailed proteogenomic analysis or mutagenesis of the specific genes may help in identifying the cytotoxic factors with in the MVs of *E. faecium*.

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Appendix

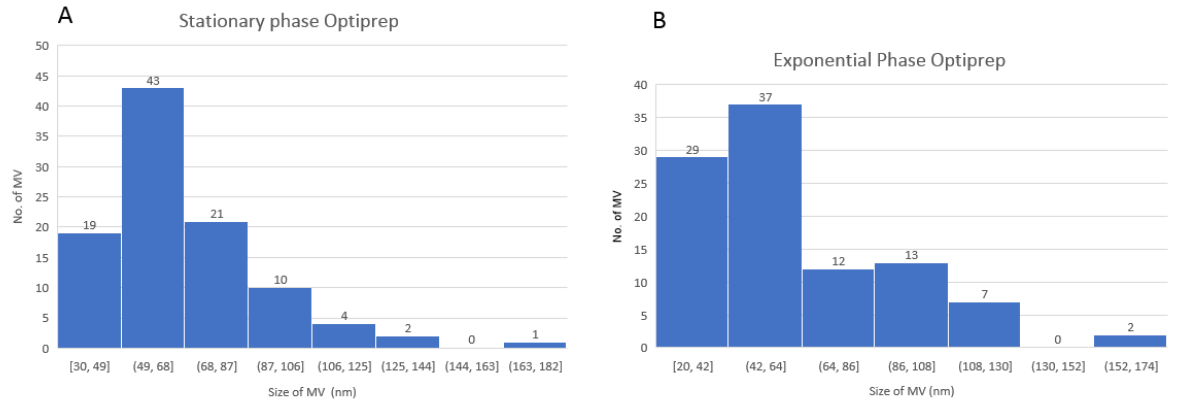

Figure S1: Size distribution of MVs obtained from Transmission Electron Microscopy A) Stationary growth phase OptiPrep samples B) Exponential growth phase OptiPrep samples

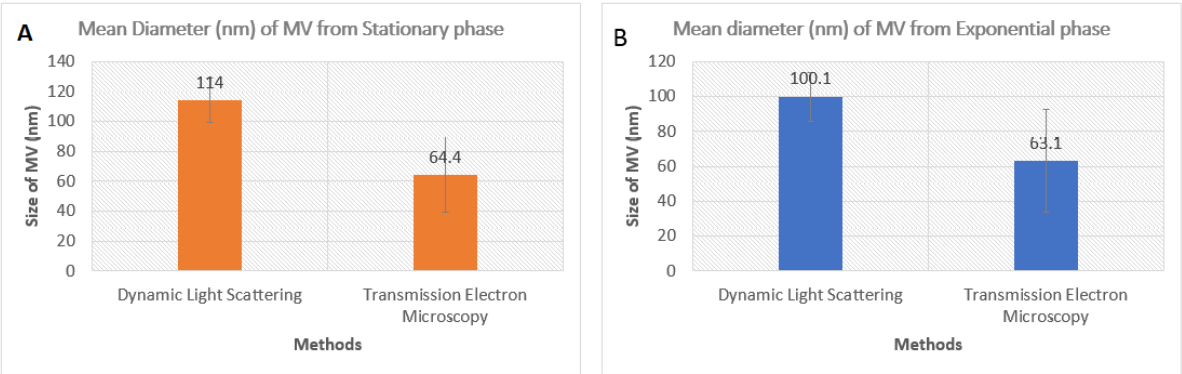

Figure S2: Comparison between the size of MVs obtained from Dynamic Light Scattering method and Transmission Electron Microscopy method. A) Stationary growth phase OptiPrep samples B) Exponential growth phase OptiPrep samples

Table S1: Measurement of protein concentration using different methods.

Date	**Methods**	**Protein concentration**	
		Sample 1	Sample 2
15/08	Nanodrop	10.945 mg/ml	10 mg/ml
	Qbit	498 µg/ml	500 µg/ml
	Bradford	169 µg/ml	261 µg/ml
16/08	Nanodrop	8.947 mg/ml	8.908 mg/ml
	Qbit	500 µg/ml	500 µg/ml
	Bradford	244.29 µg/ml	375.72 µg/ml