Interaction of *Escherichia coli* with opportunistic pathogens during biofilm formation

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Ph.D. Thesis submitted

by

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GRADUATE SCHOOL OF MEDICINE, DENTISTRY AND PHARMACEUTICAL SCIENCES

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Interaction of *Escherichia coli* with opportunistic pathogens during biofilm formation

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by

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GRADUATE SCHOOL OF MEDICINE, DENTISTRY AND PHARMACEUTICAL SCIENCES

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To Whom It May Concern

We hereby certify that this is a typical copy of the original Doctor Thesis of Mr. Han Min Ohn. This Doctor Thesis was successfully defended and officially accepted for the degree of Doctor of Philosophy in Pharmaceutical Sciences (Microbiology).

Title of the Thesis: Interaction of *Escherichia coli* with opportunistic pathogens during biofilm formation

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List of abbreviations

A570	Absorbance at 570nm
B. subtilis	*Bacillus subtilis*
CFU	Colony forming unit
DW	Deionized water
E. coli	*Escherichia coli*
GI	Gastrointestinal
L. rhamnosus	*Lactobacillus rhamnosus*
MA	MacConkey agar
MSA	Mannitol salt agar
NaCl	Sodium chloride
PBS	Phosphate buffered saline
P. aeruginosa	*Pseudomonas aeruginosa*
SCFAs	Short chain fatty acids
S. Agona	*Staphylococcus* Agona
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
TSA	Trypticase soy agar
TSA-NaCl	Trypticase soy agar supplemented with 0.5% NaCl
TSB	Trypticase soy broth
TSB-NaCl	Trypticase soy broth supplemented with 0.5% NaCl
USA	United States of America
V. cholerae	*Vibrio cholerae*
V. vulnificus	*Vibrio vulnificus*

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Abstract

1. Abstract:

In the first part of the study, I explored how *Escherichia coli* ATCC 35218 or its culture supernatant interacts with *Vibrio vulnificus* L-180 during its biofilm formation. *V. vulnificus* is a foodborne pathogen causing septicemia with high mortality rate while *E. coli* is a commensal bacterium commonly present in the gastrointestinal tract of mammals including humans. My study showed that, the amount of biofilm produced by *V. vulnificus* L-180 was reduced in the presence of *E. coli* ATCC 35218, although the growth of *V. vulnificus* L-180 remains unaffected. I also found that even a minute amount of *E. coli* ATCC 35218 culture supernatant could interfere with the biofilm formation of *V. vulnificus* L-180. *E. coli* ATCC 35218 culture supernatant could also reduce the amount of preformed *V. vulnificus* biofilm. In addition, I found that antibiofilm effect of *E. coli* ATCC 35218 culture supernatant against *V. vulnificus* L-180 did not get reduced even after heat treatment. These findings indicate that *E. coli* and its culture supernatant may be suitable to prevent the biofilm formation by *V. vulnificus*. On the other hand, *V. vulnificus* L-180 living cells could reduce the amount of preformed *E. coli* ATCC 35218 biofilm, but culture supernatant could not. This suggests that the cell-associated factors contribute towards reduction in the *E. coli* ATCC 35218 biofilm. Therefore, I speculate that ingestion of infectious dose of *V. vulnificus* might induce dislodging of the commensal bacteria from the intestine and thus can colonize to initiate the infection.

Second part of the study demonstrated that a commensal bacterium *E. coli* might prevent the biofilm formation of *Staphylococcus epidermidis*, a skin commensal bacterium which is also a nosocomial pathogen. Staphylococci, including *S. epidermidis*, are also regularly isolated from the GI tract of infants and small children. When co-cultured with *S. epidermidis* ATCC 35984, the cells of *E. coli* ATCC 35218 dominated in both culture fluid and biofilm. In addition, *E. coli* ATCC 35218 significantly incorporated into and grew in a niche preoccupied by *S. epidermidis* biofilm. However, *S. epidermidis* ATCC 35984 could not incorporate well into a niche preoccupied by *E. coli* ATCC 35218 biofilm. Although far greater amount was required and less efficient, the culture supernatant from *E. coli* ATCC 35218 also showed to reduce the amount of biofilm formed by *S. epidermidis* ATCC 35984 and the component(s) of the culture supernatant that exhibit antibiofilm activity were also found to be heat-stable. *E. coli* culture supernatant, however, did not have any effect on preformed *S. epidermidis* biofilm. Two other *E. coli* strains (strain K-12 and B) were also able to interfere with the formation of *S. epidermidis* ATCC 35984 biofilm. These findings suggest that, through inhibition of the biofilm development and growth, *E. coli* and its culture supernatant may take part in preventing

colonization of *S. epidermidis* in the adult gastrointestinal tract. In addition, my findings also suggest that *E. coli* may also destabilize *S. epidermidis* colonizing the GI tract of infants and small children and may be useful in removing potentially pathogenic *S. epidermidis* colonizing the GI tract of infants and small children.

Introduction

2. Introduction:

2.1. Background:

2.1.1. Colonization resistance in the human gastrointestinal tract

About 60 tonnes of food pass through human gastrointestinal (GI) tract through an average lifetime together with many microorganisms from the environment that contaminate the food. These microorganisms might pose threat on GI tract integrity (1) or may colonize the GI tract and serve as a source for spreading perilous microorganisms, such as antibiotic resistant bacteria, to other individuals or to the environment (2). The gut microbiota is the term used to refer to all bacteria, archaea and eukarya, living in the GI tract. Balance between these GI tract microbiota is important for maintaining host health and distortion of this balance has been hypothesized to be involved in various diseases such as inflammatory bowel diseases, diabetes and others (3).

Colonization resistance is the mechanism whereby the intestinal microbiota protects itself against invasion by new and potentially harmful microorganisms. Colonization resistance can occur through direct or indirect manner (4). In the first form, the commensals compete with invading microorganisms for available nutrient and niche establishment or through production of antimicrobial peptides and toxins against the invaders. Indirect colonization resistance occurs due to stimulation of host innate and adaptive immune responses against the invading microorganisms by the commensals (5).

2.1.2. Distribution of normal bacterial flora in the GI tract

GI tract constitutes the second largest body surface area with approximately 250-400m^2 and more than 500 species of bacteria are thought to inhabit the GI tract (6). More than 10^{14} microorganisms are estimated to be inhabiting the GI tract and this number is more than 10 times the number of human cells. (7,8). Normal GI tract flora varies with the age of human. *Bifidobacterium* dominated the gut microbiota of a newborn and during lactation. With the introduction of solid food and continuation of breast feeding during weaning period, Bacteroidetes and Firmicutes start to dominate the GI tract of children (9,10,11). In adult small intestine, with its fast transit time, rapidly dividing facultative anaerobes such as Proteobacteria and Lactobacillales dominate. Simple sugar and amino acid metabolisms are favored in this part of the intestine (12). However, in the large intestine where the flow is slower, Bacteroidiales and Clostridiales dictate and metabolism favors fermentation of complex polysaccharides derived from host mucus or undigested plant fibers (13,14,15).

GI tract microbiota and their numbers also differs depending on the site. In the esophagus, only facultative anaerobes originating from oral cavity such as streptococci and lactobacilli are seen with a number of 10^2-10^3 colony forming unit (CFU)/cm^2. In healthy people, stomach is relatively free from microorganisms containing only small number of lactobacilli at concentration of 10^1-10^2 CFU per ml of content (16). Duodenum and jejunum principally contain lactobacilli and streptococci at population of 10^2-10^3 CFU per ml of aspirate (17). As intestinal content moves further down the GI tract, bacterial number increase and at ileo-caecal valve and below, the number reach to around 10^8-10^9 CFU per gram of gut content (18).

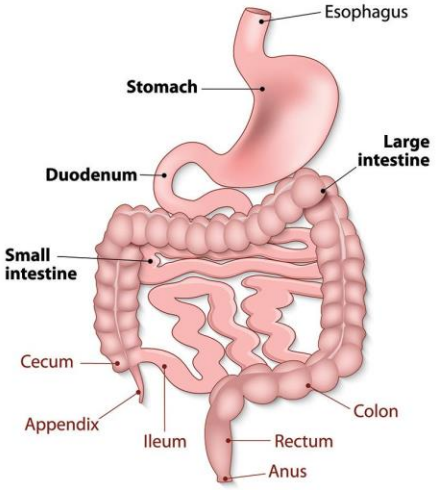

Figure 2.1. Schematic diagram of human GI tract

According to Freter (19), these bacteria may inhabit in any of the four sections of intestine, namely:

1.The surface of the epithelial cells,

2. Deep mucus layer of the crypts in the ileum, caecum and colon

3. Mucus layer that covers the epithelial cells throughout the GI tract and

4. The lumen of the intestine

Many commensal and pathogenic bacteria can attach to the surface of epithelial cells through specific receptors (20,21,22,23). Spiral shaped motile bacteria such as *Borrelia* and *Treponema* were recovered from the second microhabitat. Many commensal bacteria, including the *Escherichia coli* live in the third microhabitat, i.e. the mucus layer covering the epithelial cells (24). Microbiota in the lumen are a result of sloughing of these three microhabitats.

The intestinal microbiota, in addition to protecting host against invading pathogens together with host defense mechanisms, also performs essential metabolic function by acting as a source of essential nutrients and vitamins and aid in extracting energy and nutrient such as

short chain fatty acids (SCFAs) and amino acids from food (25). For example, up to 95% of SCFAs butyrate, propionate, and acetate, which are mainly produced by bacterial fermentation of undigested carbohydrates, are absorbed by colonocytes for use as energy substrates (26). These SCFAs also suppress inflammation in GI tract through stimulating the production of interleukin 10 by regulatory T cells (27).

2.1.3. Formation of biofilm by bacteria

Although he did not mention the term, Anthonie van Leuwenhoek may be the first person to have observed biofilm when he examined the scraping from his teeth with his primitive microscope more than 300 years ago (28). In 1978, Costerton and colleagues postulated that under nutrient-sufficient condition, most bacteria will form and grow in biofilms (29). Biofilm formation can be considered as a life-style change of bacteria from free living unicellular state to sedentary multicellular state which leads to formation of structured communities (30). For bacteria to form biofilm they must first attach to a surface. For non-motile bacteria, when conditions are favorable for biofilm formation, they increase the expression of adhesins on their outer surface for cell-cell and cell surface adherence (31). In case of motile bacteria, flagella may or may not take part in biofilm formation depending on the bacterial species. Some motile bacteria lose their motility once they encounter a surface under favorable condition and produce extracellular matrix that holds the cells together. For other motile bacteria species, flagella driven motility is important for biofilm formation as it helps the bacteria overcomes the repulsive force of the surface to be attached (32).

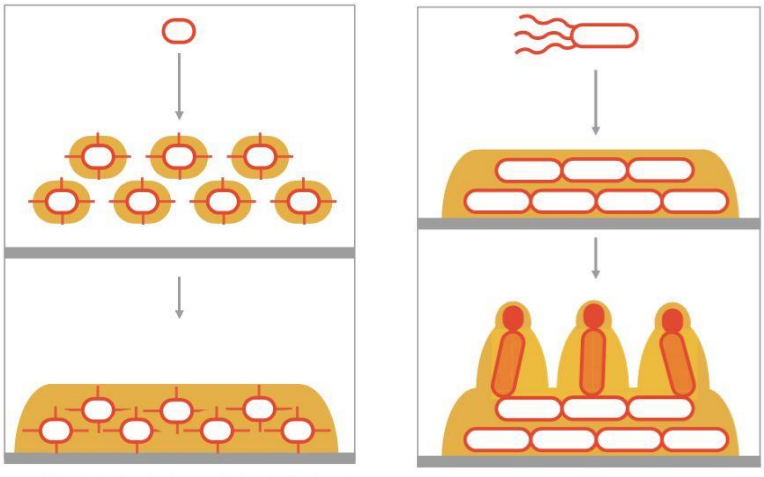

Figure 2.2. Basic model of biofilm formation by non-motile bacteria (left) and motile bacteria (right) as proposed by Lemon *et al.* (30). Thick grey line represents surface to which bacteria adhere.

There are five stages involved in development of bacterial biofilm (32). These include

1. Initial surface attachment
2. Formation of monolayer of bacteria
3. Stacking up of bacterial cells to form multiple layers
4. Production of extracellular matrix
5. Maturation of biofilm.

All major classes of macromolecules such a polysaccharides, proteins, nucleic acids, peptidoglycans and lipids can be present in mature biofilm with polysaccharides constituting the major part of it (33). A mature biofilm can be classified into three different zones, namely, adhesion area, core of the biofilm and detachment area. Water channels that carry ions and nutrients can be found in the core of the biofilm (34).

2.1.4. Bacterial biofilms in the GI tract and their interactions with exogenous microorganisms

In GI tract, although bacteria may exist as individual cells, many exists in microcolonies either alone or in combination with other species. Majority of biofilms associated with different regions of GI tract are usually found to be multispecies (16). These biofilm communities often show coordinated multicellular behaviors within and between species (35) and express phenotypes that are different from non-adherent cells (16). Epithelial cells of human GI tract are covered with a layer of mucus (36) and bacterial biofilms have been observed in both the epithelium and mucus layer of GI tract (37). These biofilms disperse the planktonic bacteria in the GI tract (38).

Entero-pathogens may cause abnormalities in the gut microbiota biofilms. Protozoan parasite *Giardia duodenalis* is found to alter the bacterial abundance and diversity of human gut microbiota biofilm by increasing the population of Clostridiales. It also disrupts the biofilm exopolysaccharides of human gut microbiota biofilm. These alterations were not observed when commensal bacteria *E. coli* is introduced (39). Reti *et al* (40) found that *Campylobacter jejuni* promotes translocation of commensal *E. coli* through intestinal epithelial cells by increasing the expression of fimbrial and flagellar genes. Disruption of gut microbiota biofilms may also lead to inflammatory bowel diseases and colorectal cancer (41).

On the other hand, the gut microbiota may also serve as a barrier against pathogenic bacteria invading the GI tract. *Barnesiella intestihominis*, an abundant colonic anaerobe, prevented the colonization of vancomycin resistant enterococci in the intestine in a study done by Ubeda and colleagues (42).

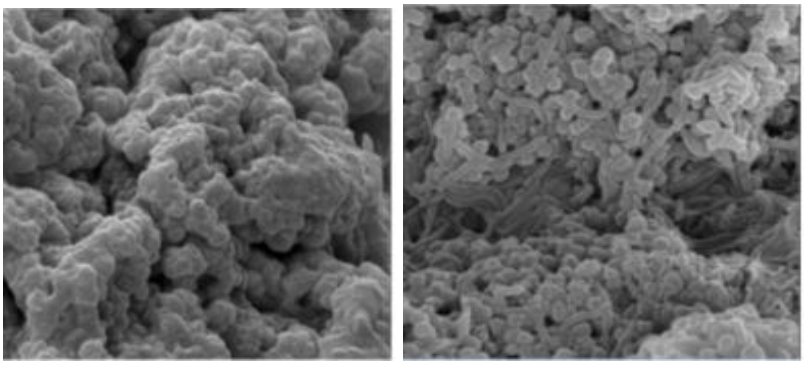

Figure 2.3. Human microbiota biofilms observed under scanning electron microscopy. The slimy exopolysaccharide coating of the biofilm hides underlying bacterial morphology in healthy conditions (left), and this exopolysaccharide can be lost upon exposure to an enteropathogen like *Giardia* sp. (right). Figures taken from Buret *et al*. (41)

Lu *et al.* (43) reported that commensal *E. coli* strain was able to prevent the mucosal colonization of *Salmonella* Typhimurium. These studies demonstrated that interactions of gut microbiota with invading microorganisms occurs in the GI tract and these interactions play an important role in determining the outcome of invasion.

2.1.5. Biogeography of commensal *E. coli* in the GI tract

E. coli is a Gram-negative, non-spore-forming facultative anaerobic bacillus. Although obligate anaerobes constitute more than 99.9% of culturable bacteria in adult human GI tract (44), *E. coli* is the predominant facultative anaerobe (45) and up to 90% of human population is colonized with this bacterium (46). It is also the first intestinal flora to colonize the GI tract of humans (47, 48, 49, 50). During the first year of life, *E. coli* reached to the density of 10^9 CFUs per gram of feces and the number stabilizes at around 10^7-10^8 CFUs/ml in colon and 10^3-10^5 CFUs/ml in ileum by the age of 2 years and above (46). Colonization of GI tract with *E. coli* during the early stage of life is important to create an anaerobic environment that is essential for growth and colonization of obligate anaerobes in the latter stage of life (51). The commensal *E. coli* lives in the mucus layer of the GI tract (24) and grows with nutrient acquired from the mono and disaccharides that are released from degradation of complex polysaccharides by obligate anaerobes (52). *E. coli* also contribute to human physiology by aiding in the digestion of food and production of vitamin B and K (53).

Commensal strains of *E. coli* are reported to be present in mixed biofilms in the large intestines of healthy humans, baboons, rats and mice (16, 54, 55, 56, 57) and Bollinger *et al* (58) found that presence of secreted immunoglobulin A and mucin stimulate biofilm formation

of environmental *E. coli* strains. So, it is likely that in addition to cell-cell interaction, these intestinal *E. coli* biofilms may play important role in microbe-microbe interactions of *E. coli* with other commensals or exogenous microorganisms that may gain access into the GI tract. Various factors such as fimbriae, curli play important role in formation of *E. coli* biofilm (59). Main components of mature *E. coli* biofilm consist of proteinaceous curli fibers, flagella, cellulose, β-1,6, N-acetyl-D-Glucosamine and colonic acid (60,61).

2.1.6. Biogeography of *Staphylococcus epidermidis*

Staphylococci are regarded as commensals of the skin and mucous membranes of human and other mammals. They are Gram-positive, non-spore-forming, facultative anaerobic cocci. *S. epidermidis*, is the most frequently isolated staphylococcus from the human epithelia and colonizes the head, nose and axilla (62). Although *S. epidermidis* usually has a benign relationship with the host, it is also an important nosocomial pathogen and it is the most frequent causative agent of indwelling medical device associated infections (63) and approximately 13% of prosthetic valve endocarditis infections are caused by *S. epidermidis* with mortality rate of 24% between 2000-2005 globally (64).

Although it is not considered as a normal GI tract flora in adults, staphylococci, including *S. epidermidis*, are regularly isolated from the GI tract of infants and small children and were found to be resistant to various antibiotics and contain genes related to biofilm formation (65, 66, 67). This suggests that GI tract flora may express colonization resistance against *S. epidermidis* which lead to its absence from human GI tract as a person gets older.

Moreover, since *S. epidermidis* is a skin flora, it is highly likely that it is being ingested into the GI tract together with food and drink on regular basis. However, it is not reported as a normal gut flora. This fact suggests that colonization resistance against *S. epidermidis* by GI tract microbiota exists. Although it is not clear if *S. epidermidis* forms biofilm or not in the GI tract, it frequently forms biofilms on indwelling medical devices (64). So, it is likely that *S. epidermidis* lives in sessile community in the GI tract. A mature *S. epidermidis* biofilm contains various adhesive molecules such as intercellular adhesin, proteinaceous factors (Bhp, Aap and Embp), teichoic acids and extracellular deoxyribonucleic acids (68).

2.1.7. *Vibrio vulnificus* and its clinical importance

V. vulnificus is a Gram-negative, halophilic, motile bacterium that is ubiquitous in marine and estuarine water. In addition, it has also been isolated from various sources including sediment, water, oysters, shrimp, clam and fish (69,70). It is an opportunistic pathogen and is the most virulent species of non-cholera vibrios (71). People with liver disorders such as

cirrhosis, hematological abnormalities such as hemochromatosis, and compromised immune status are susceptible to infections with *V. vulnificus* (72).

Clinical strains of *V. vulnificus* can be classified into two genotypes. For instance, based on the *vvh*A gene encoding the hemolysin, only human pathogenic strains are placed to the genotype 1, a major human pathogenic genotype, while human-eel pathogenic strains are in the genotype 2 (73). *V. vulnificus* can present with three distinct clinical syndromes, namely, septicemia, gastroenteritis and wound infections. Septicemia and gastroenteritis occurred following ingestion of food containing *V. vulnificus* with high mortality rate among patients with septicemia. Infective dose of *V. vulnificus* is relatively low for susceptible individuals ranging from 1 to 100 CFU or 100/1000 CFU/g of oyster meat (74,75).

If *V. vulnificus* survives the hostile environment in the stomach and reach the lower part of GI tract, it can penetrate the intestinal wall and invade into the blood stream (76). Before entering the bloodstream, *V. vulnificus* colonizes the small intestine and compete with GI tract microbiota for available nutrition (77, 78). Although it is not known if *V. vulnificus* forms biofilms in the GI tract or not, Paranjpye *et al.* (79) and Froelich *et al.* (80) reported that *V. vulnificus* embedded themselves in oyster tissues and form biofilms to colonize and persist in the oyster. In addition, formation of *V. vulnificus* biofilm is a well-documented phenomenon in vitro (81, 82). So, it is highly likely that *V. vulnificus* multiplies and forms biofilm before it invades the intestinal epithelial cells and it is important to understand how *V. vulnificus* interacts with GI tract microbiota. Exopolysaccharides are the major components of *V. vulnificus* biofilm while flagella and pili are important for initial adhesion of the bacterium to the surface (83).

2.1.8. Impact of interspecies interaction in biofilm formation

In nature, mixed species biofilms are undoubtedly the dominant form and they are also prominent in the human host (for example, in the oral cavity and the GI tract). Therefore, research directed at delineating interactions within multi-species biofilms and the effects of such interactions on the development of biofilm community is important (84). These interactions may have positive or negative impact on the biofilm formation of the microorganisms involved.

In a study of mixed culture of *E. coli* and *Salmonella* Typhimurium, it was found that biofilm negative *Salmonella* could utilize parts of curli from *E. coli* and form biofilm (85). Hancock *et al.* found that probiotic *E. coli* strain Nissle 1917 could inhibit the biofilm formation

of intestinal pathogens (86). *Pseudomonas aeruginosa* PAO1 strain was found to be able to promote biofilm formation of different *E. coli* strains (87).

Culture supernatants of several *Lactobaillus* species were found to be able to inhibit the biofilm formation of *Vibrio cholerea* and *V. parahemolyticus*. The culture supernatants were also found to be able to disperse the preformed *V. cholerae* biofilm (88). Yu *et al* (89) also reported that *Proteus mirabilis* could inhibit the biofilm formation of *Vibrio harveyi*.

Habimana *et al.* (90) reported that *Staphylococcus piscifermentans* and *Pseudomonas* species increased biofilm growth of *S.* Agona compared to what was found in single species biofilms. Iwase *et al.* (2010) showed a subset of commensal *S. epidermidis* inhibits biofilm formation and nasal colonization by *Staphylococcus aureus* and destroys pre-existing biofilms of *S. aureus* (91).

Although various studies have been done on the effect of interspecies interaction on biofilm formation in mixed culture, no report has been observed regarding interaction between *E. coli* and *V. vulnificus* or *S. epidermidis*. Therefore, this study was carried out to explore how *E. coli* affects the biofilm formation of *V. vulnificus* and *S. epidermidis*.

2.2. Objectives of the study and study design to achieve objectives

Since ingestion of *V. vulnificus* contaminated seafood could lead to fatal infections, it is important to understand how GI tract microbiota interacts with this bacterium. Therefore, as my first general objective, I explore how *E. coli* ATCC 35218 (a biosafety level 1 microorganism) (92) and its culture supernatant interact with *V. vulnificus* L-180 (a genotype 1 human pathogen) (93). The following specific objectives are set to fulfill the general objective:

1- To determine the cell population and total biofilm mass of mono and mixed cultures of *E. coli* and *V. vulnificus.*

2- To check if *E. coli* excreted antibacterial and antibiofilm substances against *V. vulnificus* or not and vice versa.

3- To explore the effect of addition of *E. coli* on preformed *V. vulnificus* biofilm.

4- To detect whether addition of *V. vulnificus* to *E. coli* precoated surface affects the total biofilm mass of *E. coli* biofilm or not.

The second general objective of this study was to investigate whether *E. coli*, a commensal bacterium in the GI tract, expresses colonization resistance against *S. epidermidis,* a skin commensal and a nosocomial pathogen, or not. Since *S. epidermidis* is a skin commensal, frequent exposure of this bacterium to GI tract microbiota is likely to occur thorough ingestion

of food and drink. However, although it can be recovered from GI tract of small children, this bacterium is not recognized as part of the GI tract microbiota in adult. These facts suggest that normal adult GI tract microbiota may be expressing colonization resistance against *S. epidermidis* introduced with food and drink so that it could not establish itself in the GI tract They may also be responsible for gradually dislodging the coexisting *S. epidermidis* in the GI tract of small children. Since *E. coli* is present in both small and large intestine and colonize majority of human population, I used this bacterium to check my hypothesis. To achieve the general objective, the following specific objectives were carried out:

1- To study how mixing small amount of *S. epidermidis* ATCC 35984 with large proportion of *E. coli* ATCC 35218 affects the cell population and total biofilm mass when compared to each monoculture.

2- To determine if *E. coli* produced antibiofilm and antibacterial substances against *S. epidermidis* or not.

3- To check whether *S. epidermidis* could attach and grow on a surface preoccupied by *E. coli*

4- To explore if *E. coli* could incorporate into and grow on a surface precoated with *S. epidermidis*.

5- To study whether *E. coli* K-12 and B strains (strains isolated from human faeces and non-pathogenic) (94, 95, 96, 97) could also express colonization resistance against *S. epidermidis* or not through checking the total biofilm mass. When compared to *E. coli* K-12 strain, *E. coli* B strain lacks flagella, has an additional type II secretion system, a different cell wall and outer membrane composition (98).

Materials And Methods

3. Materials and Methods

3.1. Materials:

3.1.1. Bacterial strains utilized in this study

Table 3.1. Bacterial strains

Strain	Origin and relevant features	Reference(s)
Staphylococcus epidermidis ATCC 35984	Isolate recovered from patient with catheter sepsis from Tennessee, United States of America (USA) in 1982 Forms biofilm and resistant to methicillin	(99), (100)
Escherichia coli ATCC 35218	Isolate recovered from canine source in Tennessee, USA Biosafety level 1 strain	(92)
Escherichia coli K-12	Most frequently used as host strain in gene cloning experiments and isolated from the feces of diphtheria convalescent patient in 1922 at Stanford University, California, USA Biofilm forming, non-pathogenic strain	(94), (95), (96)
Escherichia coli B	First described by Luria and Delbruck in 1943 and derived from *Bacillus coli* which was first isolated by Felix d` Herelle of Pasteur Institute, Paris from human feces. Biofilm forming, non-pathogenic strain Lacks flagella	(97), (98) (101), (102)
Vibrio vulnificus L-180	Clinical isolate recovered from septicemic patient in Japan Produces cyto-hemolysin and metalloproteases	(93)

3.1.2. Bacterial cultivation media

Unless otherwise specified, all media were prepared using deionized water (DW) and sterilized by autoclaving at 121ºC, 15 pound-force per square inch above atmosphere pressure for 15 minutes.

3.1.2.1. Trypticase soy broth and agar

Trypticase soy media (also known as soybean-casein digest media) was used for cultivation of *E. coli* strains and S. *epidermidis.* BD BBL™ Trypticase™ Soy Broth (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) was used to prepare the media. The composition of the medium is as follows:

Table 3.2. Composition of BD BBL™ Trypticase™ Soy Broth

BD BBL™ Trypticase™ Soy Broth	
Pancreatic digest of casein	17g/L (1.7%)
Papaic digest of soybean	3g/L (0.3%)
Sodium chloride (NaCl)	5g/L (0.5%)
Dipotassium phosphate	2.5g/L (0.25%)
Dextrose	2.5g/L (0.25%)
pH	7.3 ± 0.2

For preparation of solid medium, i.e., trypticase soy agar (TSA), 1.5% agar was added to the medium before autoclaving.

3.1.2.2. Trypticase soy broth and agar supplemented with 0.5% sodium chloride

These culture media were used for studies involving *E. coli* and *V. vulnificus.* To prepare these media, additional 0.5% sodium chloride (NaCl) was supplemented to either trypticase soy broth (TSB) or TSA mentioned above before autoclaving.

3.1.2.3. Mannitol salt agar

Mannitol salt agar (MSA) (Nissui, Ibaraki, Japan) was used to isolate *S. epidermidis* in mixed-culture studies where *E. coli* was also present. The composition is as follows:

Table 3.3. Composition of Nissui™ mannitol salt agar

Nissui™ mannitol salt agar	
Tryptone	5g/L (0.5%)
Peptic digest of meat	5g/L (0.5%)
Meat extract	1g/L (0.1%)
Mannitol	10g/L (1%)
NaCl	75g/L (7.5%)
Phenol red	0.025g/L (0.0025%)
Bacteriological agar	15g/L (1.5%)
pH	7.4 ± 0.2

This selective culture medium is normally used to differentiate staphylococci from other bacteria. High salt concentration inhibits the growth of most bacteria. Most strains of *S. epidermidis* grow as pink or red colonies on this agar due to their inability to ferment mannitol.

3.1.2.4. MacConkey Agar

MacConkey agar (Nissui, Ibaraki, Japan) was used to recover *E. coli* in mixed-culture studies with *S. epidermidis*. Bile salts and crystal violet in this medium inhibits the growth of Gram-positive cocci and this medium is commonly used to isolate *Enterobacteriaceae* from various samples. *E. coli* ferments lactose and forms red colonies on this agar. The composition of MacConkey agar (MA) is as follows:

Table 3.4. Composition of Nissui™ MacConkey agar

Nissui™ MacConkey agar	
Pancreatic digest of gelatin	17g/L (1.7%)
Tryptone	1.5g/L (0.15%)
Peptic digest of meat	1.5g/L (0.15%)
Lactose	10g/L (1%)
Bile salts	1.5g/L (0.15%)
NaCl	5g/L (0.5%)
Neutral red	0.03g/L (0.003%)
Crystal violet	0.001g/L (0.0001%)
Bacteriological agar	13.5g/L (1.35%)
pH	7.1 ± 0.2

3.1.2.5. Chromogenic agar for *E. coli*

To isolate *E. coli* in mixed-culture study with *V. vulnificus*, CHROMagar™ *E. coli* (Paris, France) was utilized. *E. coli* appeared as blue colonies due to the production of β-glucuronidase enzyme while other Gram-negative bacteria appear colorless. Growth of *V. vulnificus* was not observed on this agar even when 100µL of overnight culture was plated. This culture medium was prepared by boiling instead of autoclaving as per manufacturer`s instruction. The composition of CHROMagar™ *E. coli* is as follows:

Table 3.5. Composition of CHROMagar™ *E. coli*

CHROMagar™ *E. coli*	
Agar	15g/L (1.5%)
Peptone and yeast extracts	8.3g/L (8.3%)

Continue Table 3.5. Composition of CHROMagar™ *E. coli*

NaCl	5g/L (0.5%)
Chromogenic mix	9g/L (0.9%)
pH	6.0 ± 0.2

3.1.2.6. Chromogenic agar for *V. vulnificus*

CHROMagar™ *Vibrio* (Paris, France) was used to recover *V. vulnificus* in mixed-culture study involving *E. coli*. Growth of *E. coli* is inhibited in this medium while *V. vulnificus* appeared as green blue colonies. Instead of autoclaving, this agar was also prepared by boiling. The composition of CHROMagar™ *Vibrio* is as follows:

Table 3.6. Composition of CHROMagar™ *Vibrio*

CHROMagar™ *Vibrio*	
Agar	15g/L (1.5%)
Peptone and yeast extracts	8g/L (8%)
NaCl	51g/4 (5.1%)
Chromogenic mix	0.3g/L (0.03%)
pH	9.0 ± 0.2

3.1.3. Solutions and buffers

3.1.3.1. 0.5% NaCl solution

0.5% NaCl was used for serial dilution of *E. coli* and *S. epidermidis* before plating on culture media, to dilute *E. coli* culture supernatant and to wash microtiter plate wells in studies where *S. epidermidis* was involved. 0.5g of NaCl was dissolved in 100 ml of DW to make 0.5% NaCl and it was autoclaved after preparation.

3.1.3.2. 1.0 % NaCl solution

1.0 % NaCl was used for serial dilution of *E. coli* and *V. vulnificus* before plating on culture media, to dilute *E. coli* culture supernatant and to wash microtiter plate wells in interaction studies involving *V. vulnificus*. 1 g of NaCl was dissolved in 100ml of DW to make 1.0 % NaCl and it was autoclaved after preparation.

3.1.3.3. Phosphate buffered saline

Phosphate buffered saline (PBS) was used to wash microtiter plate wells to remove non-adherent cells from biofilm before staining it with crystal violet. The composition of PBS is as follows:

Table 3.7. Composition of phosphate buffered saline

Phosphate buffered saline	
NaCl	8g/L (0.8%)
Potassium chloride	0.2g/L (0.02%)
Disodium hydrogen phosphate	1.44g/L (0.144%)
Potassium dihydrogen phosphate	0.24g/L (0.024%)
pH	7.4 ± 2

3.1.3.4. 95% methanol solution

This solution was prepared by adding 5ml of DW to 95ml of 100% methanol. This solution was used to draw the remaining water out of the biofilm after washing with PBS and drying at 60ºC. It also fixes the biofilm to the microtiter plate.

3.1.3.5. 0.1% crystal violet

0.1g of crystal violet was dissolved in 100 ml DW to make 0.1% crystal violet solution. This solution was used to stain biofilm mass attached to the microtiter plate well.

3.2. Methods:

3.2.1. Bacterial cultures

3.2.1.1. For study involving *S. epidermidis* and *E. coli*

Bacterial strains were cultivated overnight into TSB at 37 ºC, and the cell density was adjusted to 0.5 McFarland Standard for *E. coli* ATCC 35218, *E. coli* K-12 and *S. epidermidis* and 1 McFarland Standard for *E. coli* B with fresh TSB by using a McFarland Densitometer DEN-1B (Biosan, Riga, Latvia). In 1.0 mL of the suspension, the cell numbers of *E. coli* ATCC 35218, K- 12 and B were $8.3 \pm 2.1 \times 10^7$ CFU, $5.5 \pm 0.9 \times 10^7$ CFU, $2.1 \pm 1 \times 10^7$ CFU and *S. epidermidis* ATCC 35984 was $7.1 \pm 4.3 \times 10^6$ CFU, respectively, on TSA plates. Namely, when 100 µL of each of the adjusted suspensions is mixed, an approximate percentages of *E. coli* ATCC 35218 cells and *S. epidermidis* cells are 92.1% and 7.9%, *E. coli* K-12 cells and *S. epidermidis* cells are 88.57% and 11.43% and *E. coli* B cells and *S. epidermidis* cells are 74.73%

and 25.27% respectively. These ratios may simulate an intestinal environment, in which *E. coli* apparently dominates *S. epidermidis* because the latter is an invader.

For biofilm formation by the single culture, 200 µL of the adjusted *E. coli* or *S. epidermidis* suspension was inoculated into a well of a Nunclon™ delta surface 96-welled microtiter plate (Thermo Fisher Scientific, Waltham, MA, USA). For biofilm formation by the mixed-culture, 100 µL of each of the bacterial suspension was mixed and inoculated into the well of the microtiter plate. Thereafter, the microtiter plate was incubated at 37 ºC for 24 ± 2 hours in static condition.

3.2.1.2. For study involving *V. vulnificus* and *E. coli*

E. coli was cultivated overnight into TSB supplemented with 0.5% NaCl (TSB-NaCl) at 37 ºC, and the cell density was adjusted to 0.5 McFarland Standard. As mentioned above, in 1.0 mL of the cell suspension, the number of *E. coli* cells were approximately 8.3 x 10^7 CFU. *V. vulnificus* was cultivated overnight into TSB-NaCl at 25 ºC, and the cell density was adjusted to 0.2 McFarland Standard (approximately 1.1 ± 0.91 x 10^6 CFU/ml). When 100 µL of each of the cell suspensions is mixed, an approximate percentages of *E. coli* cells and *V. vulnificus* cells were 98.7% and 1.3%. This ratio may simulate an intestinal environment, in which *E. coli* apparently dominates *V. vulnificus* because the latter is an invader. For biofilm formation by single and mixed cultures, similar protocol as mentioned above was followed.

3.2.2. Addition of *S. epidermidis* or *V. vulnificus* on *E. coli* precoated surface

The *E. coli* suspension (200 µL) was inoculated into the well of the microtiter plate and incubated at 37 ºC for 24 ± 2 hours. Then, the culture fluid was removed, and the well was washed three times with 0.5% NaCl. After that, the *S. epidermidis* suspension (200 µL) was added to each well, and the microtiter plate was incubated at 37 ºC for 24 ± 2 hours. To kill *E. coli* cells in the precoated surface, the well was treated with 300 µL of 70% ethanol (v/v) for 15 minutes. Then, it was washed with 0.5% NaCl and air dried for 30 minutes.

For *V. vulnificus, E. coli* was precoated on the microtiter plate as mentioned above and washed with 1.0% NaCl. After that, *V. vulnificus* suspension (200 µL) was added to each well, and the microtiter plate was incubated at 37 ºC for 24 ± 2 hours.

3.2.3. Introduction of *E. coli* into *S. epidermidis* or *V. vulnificus* pre-occupied niche

The *S. epidermidis* or *V. vulnificus* suspension (200 µL) was inoculated into the well of the microtiter plate and incubated at 37 ºC for 24 ± 2 hours. Then, the culture fluid was removed, and the well was washed three times with 0.5% NaCl or 1.0% NaCl. After that, the *E. coli*

suspension (200 µL) was added to the well, and the microtiter plate was incubated at 37 ºC for 24 ± 2 hours.

3.2.4. Crystal violet method to measure the biofilm amount

The biofilm amount was measured according to the method of Extremina *et al* (103) and Baldassarri *et al* (104) by using 0.1 % crystal violet. After incubation at 37 ºC for 24 ± 2 hours, the microtiter plate was gently washed three times with PBS and dried at 60 ºC for 1 hour. Thereafter, the biofilm was fixed with 95 % methanol and stained with 0.1% crystal violet for 5 minutes. Free crystal violet was removed by washing the wells with distilled water, and then, crystal violet bound to the biofilm was extracted by incubation with 80 % ethanol-20 % acetone for 5 minutes and quantified by measuring the absorbance at 570 nm (A570) with iMark^{TM} Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA).

3.2.5. Counting of the bacterial cell numbers

For counting of the planktonic cells, an aliquot of the bacterial culture fluid was withdrawn and serially 10-fold diluted with 0.5% NaCl (for studies involving *S. epidermidis* and *E. coli*) or with 1.0% NaCl (for studies involving *V. vulnificus* and *E. coli*) , and 100 µL of each diluted sample was inoculated on three agar plates. Then, the plates were incubated at 37 ºC for 24-48 hours and the colonies formed were counted.

For counting of the biofilm-associated cells, the biofilm formed was washed three times with either 0.5 % NaCl or 1.0% NaCl, then scraped free from the well and resuspended into 0.5 % NaCl or 1.0% NaCl by using a pipette tip as described by Leuck *et al* (105) and Lopes *et al* (106). The bacterial suspension was serially 10-fold diluted with respective NaCl solution, and appropriate dilutions were plated. MSA was used for *S. epidermidis* but MA was used for *E. coli* in experiments that involved mixing two bacteria. For other experiments, TSA was used. For studies involving *V. vulnificus* and *E. coli*, TSA-NaCl plates were used in the experiments of the single culture, but CHROMagar for *Vibrio* and CHROMagar for *E. coli* were used in the experiments of the mixed culture.

3.2.6. Cross-streak plate assay

S. epidermidis, cultivated overnight at 37º C in TSB, was horizontally streaked on TSA plate with intervals at least 1 cm. Then, the overnight culture of *E. coli* was streaked vertically across the middle of *S. epidermidis* streaks. Thereafter, the plates were incubated at 37º C for 24 hours, and the presence of the growth-inhibiting zone at the intersection points was observed. For *V. vulnificus*, similar procedure was utilized with the exception that culture was done in TSB-NaCl and streaking was done on TSA-NaCl plate.

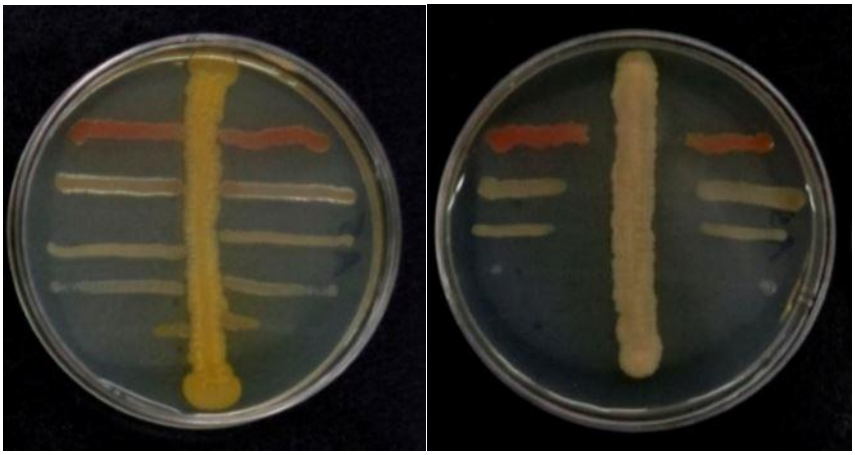

Figure 3.1. Representative photos of negative cross streak plate assay (left) with no clear zone of inhibition at the intersection points and positive cross streak plate assay (right) with distinct zones of inhibition at intersection points. Figures taken from Skowronek *et al.* (107)

3.2.7. Assay for the extracellular substance(s) degrading or dispersing the preformed biofilm

3.2.7.1. For study involving *S. epidermidis* and *E. coli*

Biofilm of *S. epidermidis* was formed in the wells of the microtiter plate by incubation of 200 µL of *S. epidermidis* suspension at 37 ºC for 24 ± 2 hours. Thereafter, the culture fluid was removed, and 200 µL of fresh TSB, the mixture of 100 µL of fresh TSB and 100 µL of 0.5% NaCl, or the mixture of 100 µL of fresh TSB and 100 µL of *E. coli* culture supernatant were added to the respective well, and then, the microtiter plate was incubated at 37 ºC for 24 ± 2 hours and total biofilm amount was measured colorimetrically by crystal violet staining method.

To prepare the culture supernatant used for the experiments, *E. coli* ATCC 35218 was cultivated overnight into TSB at 37 ºC, and the culture supernatant was collected and filtrated with a Millex GV 0.22 µm filter (Merck Millipore, Burlington, MA, USA).

3.2.7.2. For study involving *V. vulnificus* and *E. coli*

The biofilm of *V. vulnificus* was formed by the cultivation of 200 µL of inoculum in a microtiter plate at 37 ºC for 24 ± 2 hours. Thereafter, the planktonic phase was removed, and the wells were washed with 1.0 % NaCl. Then, I added a mixture of *E. coli* culture supernatant (100 µL) and TSB-NaCl (100 µL) over the preformed *V. vulnificus* biofilm and as controls of this experimental setup, I used 200 µL of TSB-NaCl and the mixture of 100 µL TSB-NaCl and 100 µL 1.0 % NaCl. The microtiter plate was incubated at 37 ºC for 24 ± 2 hours and the

amount of biofilm was colorimetrically measured by crystal violet staining. In another set of experiment, I examined the dose-dependent effect of the *E. coli* culture supernatant on the preformed biofilm of *V. vulnificus*. I used 20-100 µL of *E. coli* culture supernatant and the total volume was adjusted to 200µL with TSB-NaCl (100µL) and 1.0 % NaCl (0-80 µL).

Similarly, I studied the effect of *V. vulnificus* culture supernatant on the preformed biofilm of *E. coli*. For *E. coli,* the biofilm was formed in wells of the microtiter plate. Thereafter, the culture fluid was removed, and the wells were washed with 1.0 % NaCl. Then, TSB-NaCl (200 µL), the mixture of TSB-NaCl (100 µL) and 1.0 % NaCl (100 µL), or the mixture of TSB (100 µL) and *V. vulnificus* culture supernatant (100 µL) was added to the respective well, and then, the microtiter plate was incubated at 37 ºC for 24 ± 2 hours.

Culture supernatant was prepared as described previously with the exception that TSB-NaCl was used to culture the bacteria. In some experiments, the culture supernatant obtained was heat-treated at 60º C for 10 or 30 minutes, or at 100 º C for 10 minutes and utilized in the study to check the heat-stability of its action.

3.2.8. Assay for the extracellular substance(s) interfering the biofilm formation

3.2.8.1. For study involving *S. epidermidis* and *E. coli*

S. epidermidis suspension (200 µL) was inoculated into the wells of the microtiter plate, and an appropriate volume (0 to 100 µL) of *E. coli* culture supernatant prepared as described above was added to each well. Then, the total volume was adjusted to 300 µL with 0.5 % NaCl, and the microtiter plate was incubated at 37 ºC for 24 ± 2 hours. As the controls, 100 µL of fresh TSB or 100 µL of 0.5% NaCl was added to *S. epidermidis* suspension. To check the heat stability of the extracellular *E. coli* substance(s) that interfere with biofilm formation, the culture supernatant was heat- treated at 60º C for 10 minutes, 60º C at 30 minutes, or at 100º C for 10 minutes.

3.2.8.2. For study involving *V. vulnificus* and *E. coli*

For *V. vulnificus,* the bacterial suspension (200 µL) and the *E. coli* culture supernatant (0-5 µL) was added to wells of the microtiter plate, and the total volume was adjusted to 205 µL with 1.0 % NaCl, and then, the microtiter plate was incubated at 37 ºC for 24 ± 2 hours. As the controls, TSB-NaCl or 1.0 % NaCl (5 µL) was added to the *V. vulnificus* suspensions.
For *E. coli,* the bacterial suspension (200 µL) and the *V. vulnificus* culture supernatant (100 µL) was added to wells of the microtiter plate, the microtiter plate was incubated at 37 ºC for 24 ± 2 hours. As controls, TSB-NaCl or 1.0 % NaCl (100 µL) were added to the *E. coli*

3.2.9. Statistical analysis

All experiments were performed with three biological replicates. For the crystal violet assay, three technical replicates were included in each biological replicate test. Each data presented as the mean ± SD was analyzed by Student's t test (two-tailed analysis), and the *p* value less than 0.05 was determined to be significantly different (*).

Results

4. Results

The result section is divided into two parts. The first part explores whether *E. coli* ATCC 35218 or its culture supernatant affects the *V. vulnificus* L-180 biofilm or not. Second part describes the interaction of *E. coli* ATCC 35218, K-12, or B strain with *S. epidermidis* ATCC 35984 biofilm.

4.1. Interaction of *Escherichia coli* ATCC 35218 and its culture supernatant with *Vibrio vulnificus* L-180 during biofilm formation

Since *E. coli* is a commensal bacterium in the GI tract of mammals, I hypothesized that it might express colonization resistance against *V. vulnificus*, an intestinal pathogen that may also cause systemic illness. Therefore, in this study I explored how *E. coli* ATCC 35218 or its culture supernatant interacts with *V. vulnificus* L-180, a human pathogen.

4.1.1. Biofilm formation by the single or mixed culture of *E. coli* and *V. vulnificus*

First, I determined the total amount of biofilm formed by the single and mixed culture of *E. coli* and *V. vulnificus*. The obtained results showed overall reduction in biofilm formation where *E. coli* and *V. vulnificus* were co-cultured (Table 4.1). In the mixed culture, the amount of the biofilm (A570) was reduced to 33% for *V. vulnificus* and to 43% for *E. coli* when compared with their respective single culture.

Table 4.1. Biofilm formation by the single culture and mixed culture of *E. coli* ATCC 35218 and *V. vulnificus* L-180

Culture	Biofilm amount (A_{570})	Bacterial cell numbers (CFU/mL)	
		Biofilm-associated	Planktonic
Single culture *V. vulnificus*	0.70 ± 0.18	$2.4 \pm 0.5 \times 10^5$	$1.5 \pm 1.3 \times 10^7$
Single culture *E. coli*	0.54 ± 0.12	$2.3 \pm 2.0 \times 10^9$	$9.8 \pm 6.8 \times 10^9$
Mixed culture *V. vulnificus* *E. coli*	0.23 ± 0.07	*V. vulnificus* $1.4 \pm 1.0 \times 10^5$ *E. coli* $1.9 \pm 1.5 \times 10^9$	$4.9 \pm 5.4 \times 10^7$ $5.4 \pm 3.3 \times 10^9$

Next, I determined the viable cell count of *E. coli* and *V. vulnificus* present in the planktonic and adherent condition after 24 hours of biofilm formation at 37ºC. As shown in

Table 4.1, the bacterial count in planktonic state and adherent state were found to be similar for *E. coli* and *V. vulnificus* whether I used single or mixed culture for biofilm cultivation. This finding indicated that even when 98.7% of bacterial population consists of *E. coli* in the mixed culture condition, mere 1.3% of *V. vulnificus* still could grow to attain the viable count similar to single culture condition. This also suggests that none of the bacteria produced either bactericidal or bacteriostatic agents which could inhibit the growth of other.

I further confirmed this by cross-streak plate assay which also showed no production of any antibacterial substance(s) by either of the test organism (Figure 4.1).

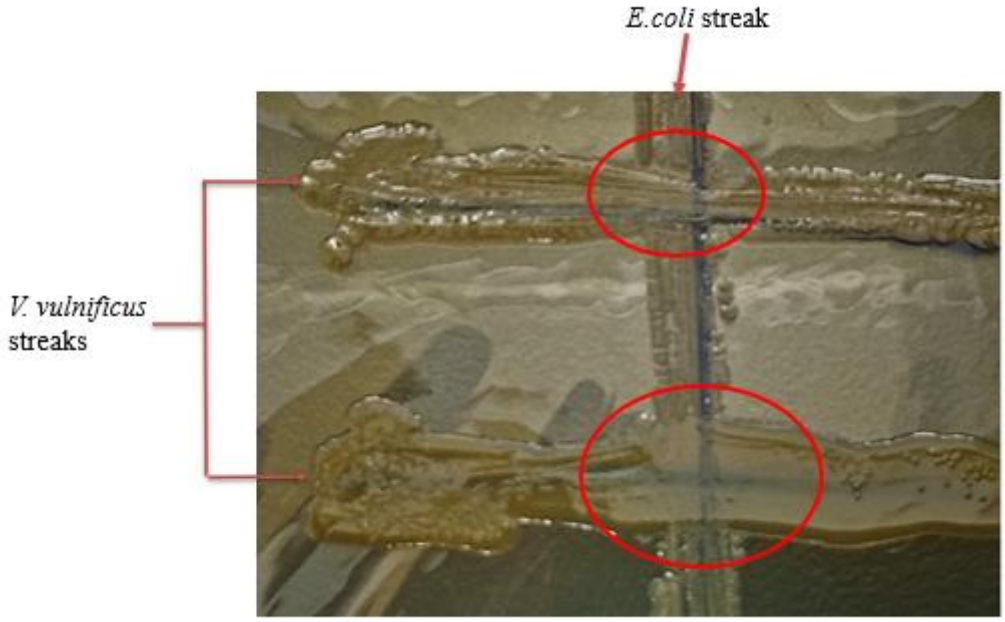

Figure 4.1. Cross-streak plate assay of *E. coli* ATCC 35218 and *V. vulnificus* L-180. *V. vulnificus* was cultivated overnight at 37º C in TSB-NaCl and horizontally streaked on TSA-NaCl plate with intervals at least 1 cm. Then, the overnight culture of *E. coli* was streaked vertically across the middle of *V. vulnificus* streaks. Thereafter, the plate was incubated at 37º C for 24 hours, and the presence of the growth-inhibiting zone at the intersection points was observed (n=3).

These results suggested that either *E. coli* or *V. vulnificus* produces the antibiofilm agent(s) which might act on biofilm. Therefore, next I examined whether the culture supernatant from each bacterium could either interfere with the biofilm formation or disperse preformed biofilm.

4.1.2. Negative impact of the *E. coli* culture supernatant on the *V. vulnificus* biofilm

First, I tested if *E. coli* culture supernatant could inhibit the biofilm formation by *V. vulnificus*. The *V. vulnificus* cell suspension (200 µL) was mixed with the *E. coli* culture supernatant (1-5 µL) and cultivated in microtiter plate at 37 ºC for 24 ± 2 hours (Figure 4.2). The amount of the biofilm formed on the surface of wells was reduced by approximately 57% with the addition of as small as 4 µL of the culture supernatant. These findings suggest that *E. coli* secretes component(s) into the culture medium that could interfere with the biofilm formation of *V. vulnificus*.

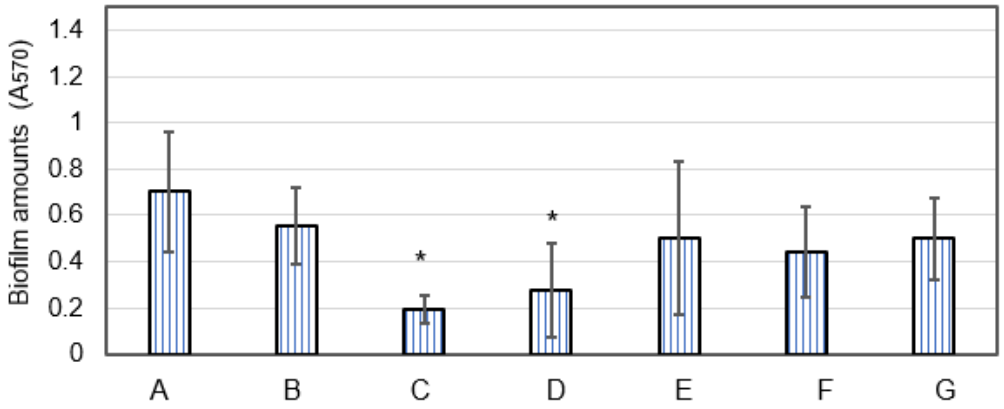

Figure 4.2. Negative impact of the *E. coli* ATCC 35218 culture supernatant on the biofilm formation by *V. vulnificus* L-180. The cell suspension of *V. vulnificus* (200 µL) was mixed with *E. coli* culture supernatant (1-5 µL) and cultivated into the wells of a microtiter plate at 37 ºC for 24 ± 2 hours, and then, the amounts of the biofilm formed were measured (n = 3). A: TSB-NaCl 5 µL, B: 1.0 % NaCl 5 µL, C: the culture supernatant 5 µL, D: the culture supernatant 4 µL and 1.0 % NaCl 1 µL, E: the culture supernatant 3 µL and 1.0 % NaCl 2 µL, F: the culture supernatant 2 µL and 1.0 % NaCl 3 µL, and G: the culture supernatant 1 µL and 1.0 % NaCl 4 µL. The asterisks (*) indicate the *p* value was less than 0.05 when compared to B.

Next, I checked whether the component(s) present in *E. coli* culture supernatant were responsible for the dispersal of preformed *V. vulnificus* biofilm by addition of the culture supernatant in different volumes (20-100 µL). As shown in Figure 4.3, the addition of 100 µL *E. coli* culture supernatant could reduce the amount of *V. vulnificus* biofilm by 47 %. This negative impact was also observed even when 40 µL of the culture supernatant was added to the preformed biofilm. These findings suggest that *E. coli* might secrete some unknown components into the growth medium that eventually responsible either for the dispersal or degradation of the preformed *V. vulnificus* biofilm.

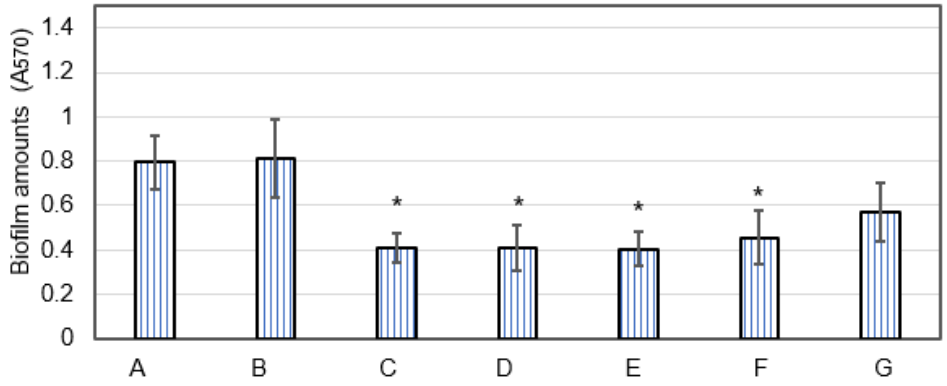

Figure 4.3. Negative impact of the *E. coli* culture ATCC 35218 supernatant on preformed *V. vulnificus* L-180 biofilm. The wells of a microtiter plate were coated with *V. vulnificus* biofilm by cultivation of the bacterial cells at 37 ºC for 24 ± 2 hours. Thereafter, the culture supernatant from *E. coli* ATCC 35218 (20-100 µL) was added to each well, and a total volume was adjusted to 200 µL with TSB-NaCl and 1.0 % NaCl. Then, the microtiter plate was incubated at 37 ºC for 24 ± 2 hours, the amounts of the biofilm were measured (n = 3). A: TSB-NaCl 200 µL, B: TSB-NaCl 100 µL and 1.0 % NaCl 100 µL, C: TSB-NaCl 100 µL and the culture supernatant 100 µL, D: TSB-NaCl 100 µL, 1.0 % NaCl 20 µL and the culture supernatant 80 µL, E: TSB-NaCl 100 µL, 1.0 % NaCl 40 µL and the culture supernatant 60 µL, F: TSB-NaCl 100 µL, 1.0 % NaCl 60 µL and the culture supernatant 40 µL, and G: TSB-NaCl 100 µL, 1.0 % NaCl 80 µL and the culture supernatant 20 µL. The asterisks (*) indicate the *p* value was less than 0.05 when compared to B.

To confirm whether *E. coli* culture supernatant contain any antimicrobial substance(s), I added either 100 µL of *E. coli* culture supernatant or 100 µL of TSB-NaCl (as control) to the preformed *V. vulnificus* biofilm and incubated at 37 ºC for 24 ± 2 hours. Thereafter, the viable cell numbers were determined by plate count method. Interestingly, I found that viable cell count of *V. vulnificus* in the biofilm was found to be approximately 1.0 x 10^6 CFU/mL in both

cases. Therefore, it is confirmed that the *E. coli* culture supernatant used did not contain any antimicrobial substance(s).

4.1.3. Heat stability of the antibiofilm substance(s) present in the *E. coli* culture supernatant

Next, I checked whether the component(s) of the *E. coli* culture supernatant that confers antibiofilm activity were heat-stable or not by heating the *E. coli* culture supernatant at 60 °C for either 10 minutes or 30 minutes and at 100 °C for 10 minutes. I found that the heat-treatment did not reduce the antibiofilm activity of the culture supernatant. The amount of biofilm formed was reduced to 0.15 ± 0.01 when non-treated culture supernatant (5 µL) was added to the *V. vulnificus* cell suspension at the time of inoculation in microtiter plate. On the other hand, that amounts were 0.17 ± 0.04, 0.15 ± 0.01 and 0.16 ± 0.03, when 5 µL of the *E. coli* culture supernatant heat-treated at 60 °C for 10 minutes, 60 °C for 30 minutes and 100 °C for 10 minutes, respectively was added along with the cell suspension (Figure 4.4).

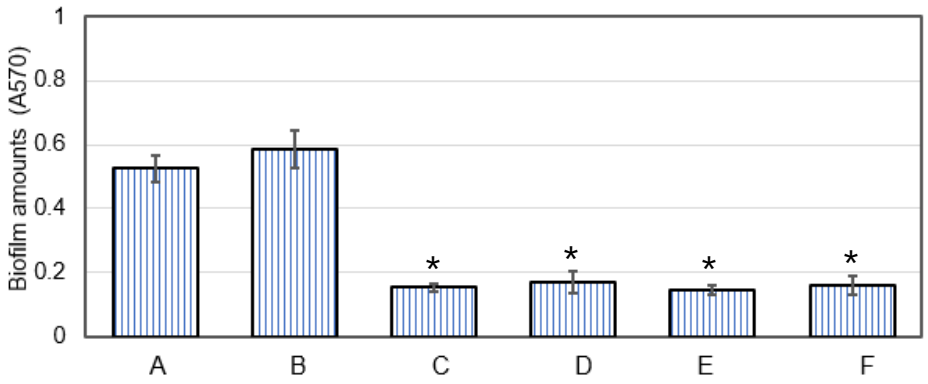

Figure 4.4. Negative impact of the heat-treated *E. coli* ATCC 35218 culture supernatant on the biofilm formation by *V. vulnificus* L-180.The cell suspension of *V. vulnificus* (200 µL) was mixed with non-treated or heat-treated culture supernatant from *E. coli* ATCC 35218 (5 µL) and cultivated into the wells of a plastic plate at 37 ºC for 24 ± 2 hours, and then, the amounts of the biofilm formed were measured (n = 3). A: TSB-NaCl 5 µL, B: 1.0 % NaCl 5 µL, C: non-treated culture supernatant 5 µL, D: the culture supernatant heat-treated at 60 ºC for 10 minutes 5 µL, E: the culture supernatant heat-treated at 60 ºC for 30 minutes 5 µL, and F: the culture supernatant heat-treated at 100 ºC for 10 minutes 5 µL. The asterisks (*) indicate the *p* value was less than 0.05 when compared to B.

In addition, I also found that the heat-treatment could not abolish the antibiofilm activity of the *E. coli* culture supernatant on the preformed biofilm of *V. vulnificus*. As shown in Figure 4.5, the amount of biofilm was equally decreased even when heat-treated culture supernatant

was added to the preformed *V. vulnificus* biofilm. These findings suggest that component(s) of *E. coli* culture supernatant that confer antibiofilm activity are heat-stable and might not be protein in nature.

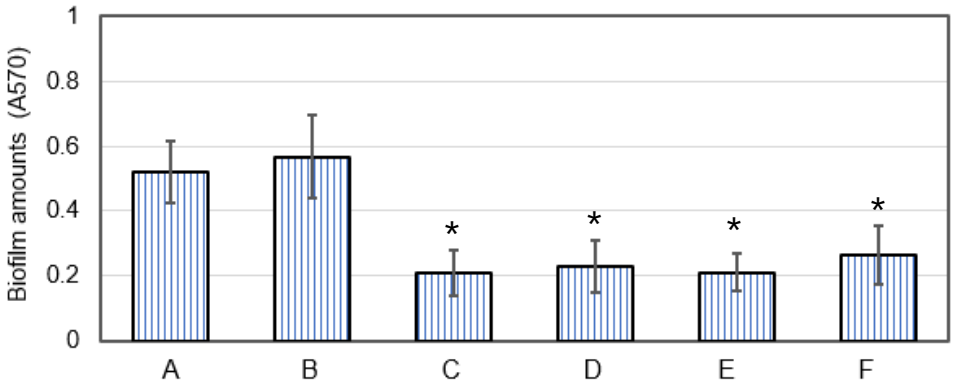

Figure 4.5. Negative impact of the heat-treated *E. coli* ATCC 35218 culture supernatant on preformed *V. vulnificus* L-180 biofilm. The wells of a microtiter plate were coated with *V. vulnificus* biofilm by cultivation of the bacterial cells at 37 ºC for 24 ± 2 hours. Thereafter, non-treated or heat-treated culture supernatant from *E. coli* ATCC 35218 (100 µL) was added to each well, and a total volume was adjusted to 200 µL with TSB-NaCl. Then, the microtiter plate was incubated at 37 ºC for 24 ± 2 hours, the amounts of the biofilm were measured (n = 3). A: TSB-NaCl 200 µL, B: TSB-NaCl 100 µL and 1.0 % NaCl 100 µL, C: TSB-NaCl 100 µL and non-treated culture supernatant 100 µL, D: TSB-NaCl 100 µL and the culture supernatant heat-treated at 60ºC for 10 minutes 100 µL, E: TSB-NaCl 100 µL and the culture supernatant heat-treated at 60ºC for 30 minutes 100 µL, and F: TSB-NaCl 100 µL and the culture supernatant heat-treated at 100ºC for 10 minutes 100 µL. The asterisks (*) indicate the *p* value was less than 0.05 when compared to B.

4.1.4. No Impact of *V. vulnificus* culture supernatant on *E. coli* biofilm

I had observed reduction in *V. vulnificus* biofilm production in presence of *E. coli* supernatant. However, I found that the vice-versa was not true in this study. The addition of even 100 µL of the *V. vulnificus* culture supernatant into 200 µL of *E. coli* cell suspension could not affect the amount of *E. coli* biofilm after cultivation for 24 hours. The biofilm amounts were, 0.66 ± 0.21, 0.60 ± 0.15 and 0.63 ± 0.17 when *E. coli* was cultivated in the presence of TSB-NaCl, 1.0 % NaCl, and the *V. vulnificus* culture supernatant respectively (Figure 4.6). Similarly, addition of 100 µL of the *V. vulnificus* culture supernatant to the preformed *E. coli* biofilm did not show any significant impact on the biofilm amount. The biofilm amount was 1.02 ± 0.38 when the culture supernatant was allowed to act on the preformed *E. coli* biofilm, and it was 1.21 ± 0.33 when TSB-NaCl was allowed to act on the

preformed biofilm (Figure 4.7). These findings suggest that *V. vulnificus* does not secret the extracellular substance(s), which either inhibits the biofilm formation or degrades the preformed biofilm of *E. coli*.

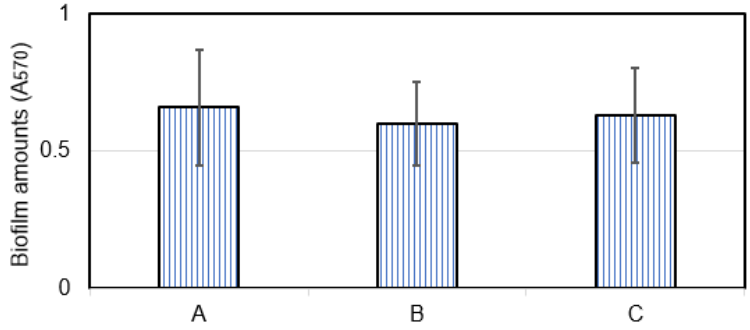

Figure 4.6. *V. vulnificus* L-180 culture supernatant had no impact on *E. coli* ATCC 35218 biofilm formation. The cell suspension of *E. coli* (200 µL) was mixed with culture supernatant from *V. vulnificus* (100 µL) and cultivated into the wells of a microtiter plate at 37 ºC for 24 ± 2 hours, and then, the amounts of the biofilm formed were measured (n = 3). A: TSB-NaCl 100 µL, B: 1.0 % NaCl 100 µL, C: *V. vulnificus* culture supernatant 100 µL.

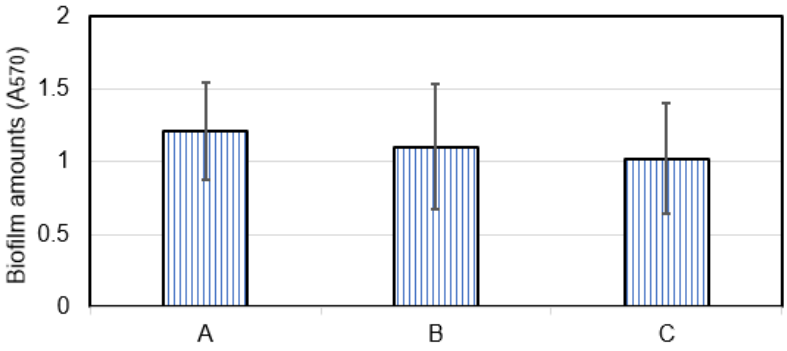

Figure 4.7. No effect of *V. vulnificus* L-180 culture supernatant on preformed *E. coli* ATCC 35218 biofilm. The wells of a microtiter plate were coated with *E. coli* biofilm by cultivation of the bacterial cells at 37 ºC for 24 ± 2 hours. Thereafter, *V. vulnificus* culture supernatant (100 µL) was added to each well, and a total volume was adjusted to 200 µL with TSB-NaCl. Then, the microtiter plate was incubated at 37 ºC for 24 ± 2 hours, the amounts of the biofilm were measured (n = 3). A: TSB-NaCl 200 µL, B: TSB-NaCl 100 µL and 1.0 % NaCl 100 µL, C: TSB-NaCl 100 µL and *V. vulnificus* culture supernatant 100 µL.

4.1.5. Reduction in preformed biofilm produced by single culture due to addition of fresh inoculum

I also examined the effect of addition of the living cells on preformed single culture biofilm. When 200 µL of *E. coli* culture was applied over preformed *V. vulnificus* biofilm, I observed apparent reduction in the amount of preformed *V. vulnificus* biofilm (Figure 4.8). Interestingly, *V. vulnificus* living cells also showed similar kind of negative impact on the amount of preformed *E. coli* biofilm (Figure 4.9).

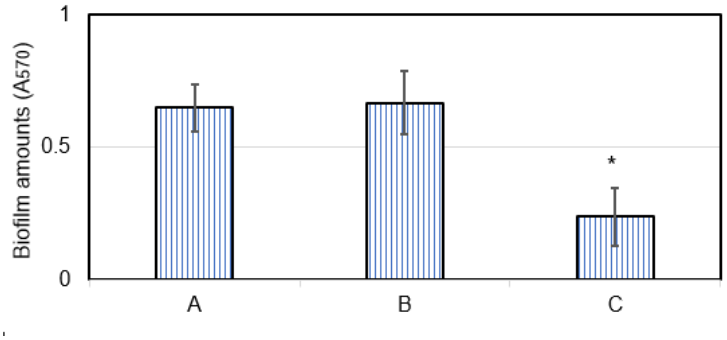

Figure 4.8. Reduction of the amount of *V. vulnificus* L-180 biofilm by the addition of *E. coli* ATCC 35218 cells. The wells of a microtiter plate were coated with *V. vulnificus* biofilm by cultivation of the bacterial cells at 37 ºC for 24 ± 2 hours. Thereafter, TSB-NaCl (A), *V. vulnificus* (B), or *E. coli* (C) was added to the wells, and the plate was incubated at 37 ºC for 24 ± 2 hours. Thereafter, the amount of the biofilm was measured (n=3). The asterisk (*) indicates the *p* value was less than 0.05 when compared to B.

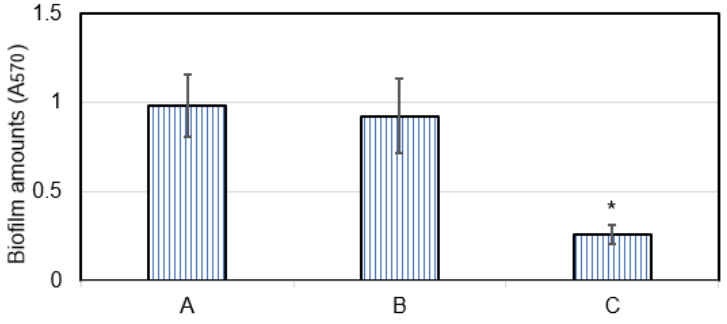

Figure 4.9. Reduction of the amount of *E. coli* ATCC 35218 biofilm by the addition of *V. vulnificus* L-180 cells. The wells of a microtiter plate were coated with *E. coli* biofilm by cultivation of the bacterial cells at 37 ºC for 24 ± 2 hours. Thereafter, TSB-NaCl (A), *E. coli* (B), or *V. vulnificus* (C) was added to the wells, and the plate was inoculated at 37 ºC for 24 ± 2 hours. Thereafter, the amounts of the biofilm were measured (n=3). The asterisks (*) indicate the *p* value was less than 0.05 when compared to B.

4.2. Study on interactions of *E. coli* ATCC 35218 with *S. epidermidis* ATCC 35984 during biofilm formation

This study was done with the assumption that *E. coli*, as a normal GI tract flora, could prevent the growth and colonization of *S. epidermidis*, a commensal bacterium and nosocomial pathogen from another part of the body. In addition, I also hypothesized that *E. coli* may be able to remove *S. epidermidis* from its niche.

4.2.1. Biofilm formation in single and mixed culture

First, I quantified the total amount of biofilm formed by either of the single culture and mixed culture of *E. coli* and *S. epidermidis*, using crystal violet staining method. In mixed culture, total biofilm amount (A570) was reduced by about 72% when compared to *S. epidermidis* single culture (Table 4.2). This finding suggests that mixing with *E. coli* had negative impact on the biofilm forming capacity of *S. epidermidis*.

Table 4.2. Biofilm formation by the single culture and mixed culture of *E. coli* ATCC 35218 and *S. epidermidis* ATCC 35984

Culture	Biofilm amount (A570)	Bacterial cell numbers (CFU/mL)	
		Biofilm -associated	Planktonic
Single culture *S. epidermidis*	2.64 ± 0.55	$1.4 \pm 0.5 \times 10^8$	$9.5 \pm 4.2 \times 10^8$
Single culture *E. coli*	0.47 ± 0.17	$1.1 \pm 0.9 \times 10^8$	$1.8 \pm 0.2 \times 10^9$
Mixed culture *S. epidermidis* *E. coli*	0.73 ± 0.20	*S. epidermidis* $6.1 \pm 3.3 \times 10^5$ *E. coli* $1.1 \pm 2.5 \times 10^8$	$1.5 \pm 1.7 \times 10^6$ $1.1 \pm 0.5 \times 10^9$

Next, I checked the number of *E. coli* and *S. epidermidis* cells in the culture supernatant and biofilm of single and mixed culture. I found that although the number of *E. coli* cells in the culture fluid and biofilm of the mixed culture was not affected by mixing with *S. epidermidis*, significant reduction in *S. epidermidis* cells, both in the culture supernatant and biofilm, was observed in the mixed culture when compared to *S. epidermidis* single culture. In other words, in the mixed culture, the percentage of *S. epidermidis* cells in the culture fluid and biofilm constituted only 0.14 % and 0.55% of total cells respectively. In contrast, the proportion of *E. coli* cells in mixed culture was 99.86% (in the culture fluid) and 99.45% (in the biofilm)

(Table 4.2) These findings suggest that *E. coli* may be producing antibacterial agent(s) that inhibit the growth of *S. epidermidis* or antibiofilm agent(s) that reduce the amount of *S. epidermidis* biofilm.

Therefore, I also screened if *E. coli* produced antibacterial agent(s) against *S. epidermidis* or not by using cross streak plate assay and found that neither of the bacteria secreted antibacterial agent(s) against each other (Figure 4.10).

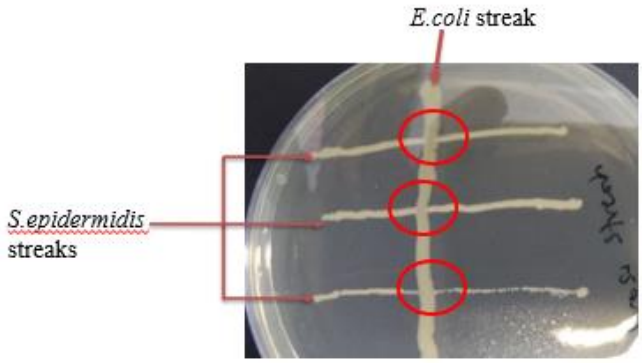

Figure 4.10. Cross-streak plate assay of *E. coli* ATCC 35218 and *S. epidermidis* ATCC 35984**.** *S. epidermidis* was cultivated overnight at 37º C in TSB and horizontally streaked on TSA plate with intervals of at least 1 cm. Then, the overnight culture of *E. coli* was streaked vertically across the middle of *S. epidermidis* streaks. Thereafter, the plate was incubated at 37º C for 24 hours, and the presence of the growth-inhibiting zone at the intersection points was observed (n=3).

4.2.2. Effect of *E. coli* culture supernatant on biofilm formation of *S. epidermidis*

Since *E. coli* does not produce antibacterial agents against *S. epidermidis*, I next tested if it excretes agents that interfere with biofilm formation of *S. epidermidis* by incubating 200 µL of *S. epidermidis* cell suspensions with (25-100 µL) of *E. coli* culture supernatant and incubating at 37ºC for 24 ± 2 hours in a microtiter plate. About 20% reduction in total biofilm amount (A570) was observed in the presence of (75-100 µL) of *E. coli* culture supernatant (Figure 4.11). These findings suggest that *E. coli* culture supernatant may be one of the factors the cause the reduction of total biofilm amount in mixed culture.

Although *E. coli* culture supernatant could also interfere with the biofilm formation of *S. epidermidis*, its inhibitory activity against *S. epidermidis* biofilm might be lower when compared to its activity against *V. vulnificus* biofilm.

Cell numbers of *S. epidermidis* in the culture supernatant and biofilm of well incubated with 100 µL of 0.5% NaCl or 100 µL of *E. coli* culture supernatant were also checked. The numbers of *S. epidermidis* cells in the culture fluid and the biofilm were 4.60 ± 2.70 x 10^8 CFU/mL and 10.0 ± 3.00 x 10^7 CFU/mL, respectively, when incubated with 0.5 % NaCl, and they were 5.10 ± 2.50 x 10^8 CFU/mL (the culture fluid) and 9.10 ± 3.60 x 10^7 CFU/mL (the biofilm) when incubated with *E. coli* culture supernatant. This finding further confirms the fact that *E. coli* does not excrete antibiotic against *S. epidermidis*.

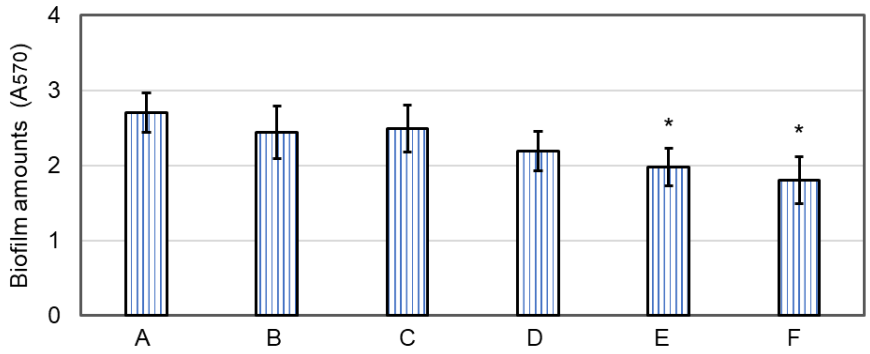

Figure 4.11. Effect of *E. coli* ATCC 35218 culture supernatant on the amount of *S. epidermidis* ATCC 35984 biofilm. The cell suspension of *S. epidermidis* (200 µL) was inoculated into wells of the microtiter plate, and an appropriate volume (25 to 100 µL) of *E. coli* culture supernatant was added to each well. Then, a total volume was adjusted to 300 µL with 0.5 % NaCl, and the microtiter plate was incubated at 37 ºC for 24 ± 2 hours. Thereafter, the amounts of *S. epidermidis* biofilm was measured. As the controls, 100 µL of TSB or 0.5% NaCl was added (n = 3). A: TSB 100 µL, B: 0.5 % NaCl 100 µL, C: 0.5 % NaCl 75 µL + the culture supernatant 25 µL, D: 0.5 % NaCl 50 µL + the culture supernatant 50 µL, E: 0.5 % NaCl 25 µL + the culture supernatant 75 µL, F: the culture supernatant 100 µL. The asterisks (*) indicate the *p* value was less than 0.05 when compared to B.

4.2.3. *E. coli* culture supernatant has no effect on preformed *S. epidermidis* biofilm

I next determined if *E. coli* culture supernatant has any effect on *S. epidermidis* biofilm already formed on the surface of microtiter plate by adding 100 µL of *E. coli* culture supernatant to preformed *S. epidermidis* biofilm and making up the total volume to 200 µL with TSB. Then, the plate was incubated at 37 ºC for 24 ± 2 hours. As control wells, 200 µL of TSB or 100 µL of 0.5% NaCl + 100 µL of TSB were added to preformed *S. epidermidis* biofilm. No significant difference in the total biofilm amount was observed in the wells supplemented with either 0.5% NaCl or *E. coli* culture supernatant. These findings suggest that,

although *E. coli* culture supernatant could degrade or disperse preformed *V. vulnificus* biofilm, it has no effect on preformed *S. epidermidis* biofilm (Table 4.3)

Table 4.3. Addition of *E. coli* ATCC 35218 culture supernatant (100 µL) to preformed *S. epidermidis* ATCC 35984 biofilm

Cultivation condition	Biofilm amount (A570)
Preformed *S. epidermidis* biofilm with 200 µL of TSB	2.66 ± 0.24
Preformed *S. epidermidis* biofilm with 100 µL of 0.5% NaCl + 100 µL of TSB	2.86 ± 0.18
Preformed *S. epidermidis* biofilm with 100 µL of *E. coli* culture supernatant + 100 µL of TSB	2.75 ± 0.35

4.2.4. Substance(s) in *E. coli* culture supernatant that interfere with *S. epidermidis* biofilm formation are also heat stable

My previous findings suggested that the antibiofilm effect of *E. coli* culture supernatant against *V. vulnificus* was due to heat-stable component(s) of *E. coli* culture supernatant. Therefore, I explored if similar phenomenon was observed or not for *S. epidermidis* by testing the activity of *E. coli* culture supernatant after heating it at 60º C for 10 minutes, 60º C at 30 minutes, or 100º C for 10 minutes. This time also, I found that the heat treatment did not reduce the antibiofilm activity of the culture supernatant. The amount (A570) of *S. epidermidis* biofilm was 1.63 ± 0.33 when the non-treated *E. coli* culture supernatant was added to *S. epidermidis* suspension. On the other hand, the amounts were 1.52 ± 0.25, 1.57 ± 0.17 and 1.60 ± 0.37 when culture supernatants heated at 60°C for 10 minutes, 60°C for 30 minutes and 100°C for 10 minutes were added (Figure 4.12). Therefore, the extracellular *E. coli* substance(s) causing the antibiofilm action are also considered to be heat stable.

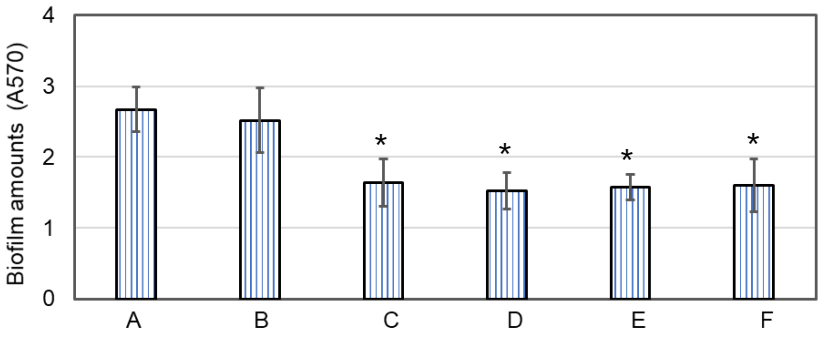

Figure 4.12. Negative impact of the heat-treated *E. coli* ATCC 35218 culture supernatant on the biofilm formation by *S. epidermidis* ATCC 35984. The cell suspension of *S. epidermidis* (200 µL) was mixed with non-heat-treated or heat-treated culture supernatant from *E. coli* ATCC 35218 (100 µL) and cultivated into the wells of a microtiter plate at 37 ºC for 24 ± 2 hours, and then, the amounts of the biofilm formed were measured (n = 3). A: TSB 100 µL, B: 0.5 % NaCl 100 µL, C: non-heat-treated culture supernatant 100 µL, D: the culture supernatant heat-treated at 60 ºC for 10 minutes 100 µL, E: the culture supernatant heat-treated at 60 ºC for 30 minutes 100 µL, and F: the culture supernatant heat-treated at 100 ºC for 10 minutes 100 µL. The asterisks (*) indicate the *p* value was less than 0.05 when compared to B.

4.2.5. *S. epidermidis* has difficulty in growing and forming biofilm on *E. coli* precoated surface

Since I hypothesized that *E. coli* expresses colonization resistance against *S. epidermidis*, I next introduced *S. epidermidis* suspension into a surface precoated with *E. coli*. No significant difference in the total biofilm amount (A570) was observed in well to which 200 µL of *S. epidermidis* suspension or TSB or *E. coli* suspension was added (Figure 4.13). However, when *E. coli* ATCC 35218 cells in the biofilm were killed by treatment with 70 % ethanol before the addition of *S. epidermidis* cells, the amount of the biofilm (A570) was increased to 2.16 ± 0.36. The cell numbers of *S. epidermidis* in the biofilm formed on the surface of a well occupied with ethanol-killed *E. coli* ATCC 35218 cells were $3.4 \pm 2.4 \times 10^7$ CFU/mL. In contrast, those in the biofilm formed on the surface of a well occupied with living *E. coli* ATCC 35218 cells were as low as $1.5 \pm 0.6 \times 10^4$ CFU/mL. In addition, the total biofilm amount (A570) formed by *S. epidermidis* was 2.64 ± 0.55 and biofilm associated *S. epidermidis* cells were $1.4 \pm 0.5 \times 10^8$ CFU/ml when it was inoculated into an uncoated well (*S. epidermidis* single culture as shown in Table 4.2). These findings suggest that *S. epidermidis* has difficulty in growing on *E. coli* precoated surface.

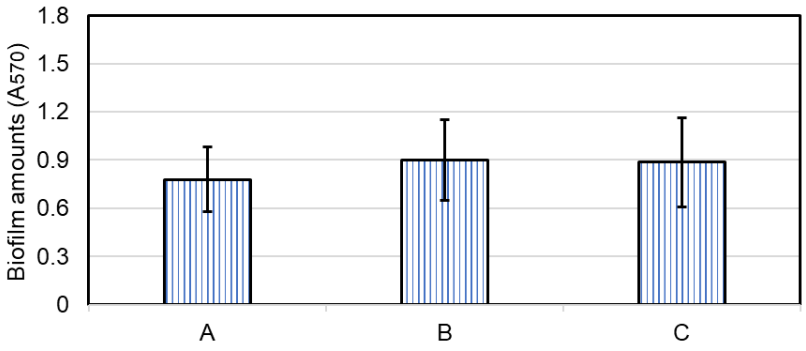

Figure 4.13. Effect of *S. epidermidis* ATCC 35984 on the development of *E. coli* ATCC 35218 biofilm. The well of the microtiter plate was coated with the biofilm of *E. coli* ATCC 35218 by preincubation at 37 ºC for 24 ± 2 hours. Thereafter, each well was washed with 0.5 % NaCl, and then, 200 µL of TSB (A), *S. epidermidis* cell suspension (B) the cell suspension of *E. coli* (C) was added into the well. Then, the microtiter plate was incubated at 37 ºC for 24 ± 2 hours, and the amount of the biofilm was measured (n = 3).

4.2.6. *E. coli* destabilizes *S. epidermidis* from its established niche

I then checked if *E. coli* could disperse *S. epidermidis* biofilm from the surface of the microtiter plate or not. *S. epidermidis* biofilm was pre-formed in the well of the microtiter plate by preincubation with 200 µL of the *S. epidermidis* suspension at 37 ºC for 24 ± 2 hours. When *E. coli* suspension was added into the well, the amounts of the total biofilm amount was 1.01 ± 0.37 (C in Figure 4.14) which was lower than the total biofilm amount in well to which either TSB (200 µL) or *S. epidermidis* (200 µL) was added. Additionally, the numbers of *S. epidermidis* cells in the biofilm were 3.4 ± 2.7 x 10^5 CFU/mL in the wells into which *E. coli* was added (C in Figure 4.14), but in the wells into which *S. epidermidis* suspension was added (B in Figure 4.14), those were 5.5 ± 3.7 x 10^7 CFU/mL. These findings suggest that *E. coli* may destabilize *S. epidermidis* from its well-established niche.

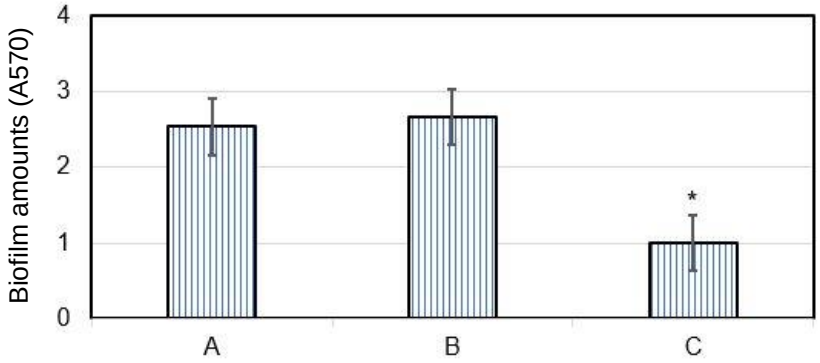

Figure 4.14. Biofilm amount of preformed *S. epidermidis* ATCC 35984 biofilms incubated with TSB, suspension of *S. epidermidis* ATCC 35984 or *E. coli* ATCC 35218. Wells of the microtiter plate were inoculated with 200 µL of *S. epidermidis* suspension and the microtiter plate was incubated at 37 ºC for 24 ± 2 hours. Thereafter, the wells were washed with 0.5% NaCl and 200 µL of TSB, *S. epidermidis* and *E. coli* suspensions were inoculated into separate wells. Then, the microtiter plate was incubated at 37 ºC for 24 ± 2 hours, followed by measurement of biofilm amounts (n=3). A: TSB 200 µL, B: *S. epidermidis* suspension 200 µL, C: *E. coli* suspension 200 µL. The asterisks (*) indicate the *p* value was less than 0.05 when compared to well to which *S. epidermidis* suspension was added as shown in B.

4.3. *E. coli* K-12 and B strains inhibit biofilm formation of *S. epidermidis* and reduce preformed *S. epidermidis* biofilm amount

Since *E. coli* ATCC 35218 was able to exhibit colonization resistance against *S. epidermidis*, I checked if other strains of *E. coli* could express similar response or not. When *E. coli* K-12 was mixed with *S. epidermidis* suspension, biofilm amount (A570) in mixed culture was found to be about 70% less than that of *S. epidermidis* monoculture. Similarly, biofilm amount (A570) in mixed culture of *E. coli* B and *S. epidermidis* was found to be 80% less than that of the *S. epidermidis* monoculture (Table 4.4). No significant difference in biofilm amount (A570) was observed when *S. epidermidis* was introduced into a well precoated with *E. coli* strain K-12 (0.34 ± 0.11) or B (0.41 ± 0.07) when compared to wells to which TSB or *E. coli* suspension was added (Figure 4.15). However, as shown in Table 4.4, when *S. epidermidis* was introduced into an uncoated well, i.e., *S. epidermidis* single culture, it could efficiently form biofilm. On the other hand, addition of *E. coli* K-12 or B suspensions to *S. epidermidis* precoated surface reduced the biofilm amount of *S. epidermidis* to 33.93% and 43.28% respectively when compared to well incubated with *S. epidermidis* suspension (Figure

4.16). These findings suggest that the ability to inhibit the growth and biofilm formation of *S. epidermidis* may be widespread among different *E. coli* strains.

Table 4.4. Biofilm formation by the single culture and mixed culture of *E. coli* K-12, *E. coli* B or *E. coli* ATCC 35218 and *S. epidermidis* ATCC 35984

Bacteria and cultivation	With *E. coli* K-12	With *E. coli* B	With *E. coli* ATCC 35218
	Biofilm amounts (A570)	Biofilm amounts (A570)	Biofilm amounts (A570)
S. epidermidis single culture	2.73 ± 0.55	2.68 ± 0.32	2.64 ± 0.55
E. coli single culture	0.59 ± 0.14	0.24 ± 0.05	0.47 ± 0.17
Mixed culture	0.82 ± 0.30	0.53 ± 0.14	0.73 ± 0.20

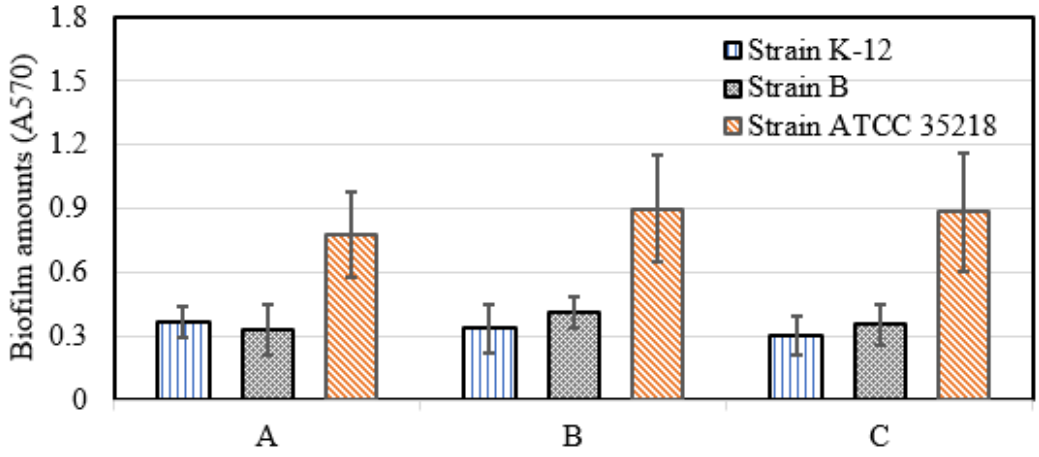

Figure 4.15. Effect of *S. epidermidis* ATCC 35984 on the development of *E. coli* K-12, *E. coli* B and *E. coli* ATCC 35218 biofilms. The well of the microtiter plate was coated with the biofilm of *E. coli* K-12, B or ATCC 35218 by preincubation at 37 ºC for 24 ± 2 hours. Thereafter, each well was washed with 0.5 % NaCl, and then, 200 µL of TSB (A), *S. epidermidis* cell suspension (B) or the cell suspension of the corresponding *E. coli* strain (C) was added into the well. Then, the microtiter plate was incubated at 37 ºC for 24 ± 2 hours, and the amounts of the biofilm were measured with crystal violet staining method (n = 3).

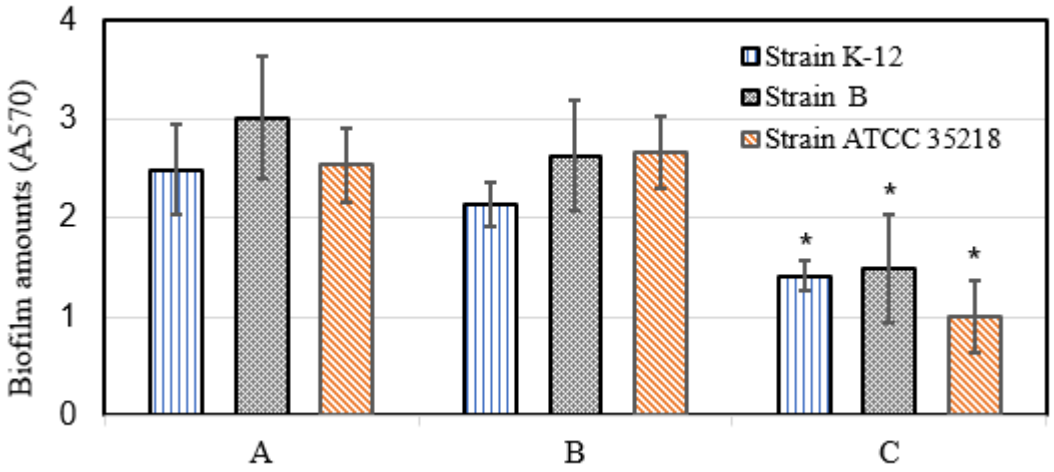

Figure 4.16. Biofilm amount of preformed *S. epidermidis* ATCC 35984 biofilm cultured with TSB, suspension of *S. epidermidis* ATCC 35984, *E. coli* K-12, B or ATCC 35218 strain. Wells of the microtiter plate were inoculated with 200 µL of *S. epidermidis* suspension and the microtiter plate was incubated at 37 ºC for 24 ± 2 hours. Thereafter, the wells were washed with 0.5% NaCl and 200 µL of TSB, *S. epidermidis* and corresponding *E. coli* strain suspensions were inoculated into separate wells. Then, the microtiter plate was incubated at 37 ºC for 24 ± 2 hours, followed by measurement of biofilm amounts (n=3). A: TSB 200 µL, B: *S. epidermidis* suspension 200 µL, C: *E. coli* suspension 200 µL. The asterisks (*) indicate the *p* value was less than 0.05 when compared to well to which *S. epidermidis* suspension was added as shown in B.

Discussion

5. Discussion:

Human beings are born into, and develop in, a microbial world. Although completely sterile at birth, we are immediately colonized by microbes from the local environment (108). As we age, the microbial communities that inhabit us become more complex and stable in the absence of external disturbances such as antibiotic treatment (109). Gut is a complex organ composed of multilayer of tissues, which acts as the frontline in response to the direct and indirect contact of luminal microbes (110).

From a bacterial point of view, successful colonization of the intestinal tract is a difficult task. First, a bacterium must survive in an environmental reservoir before finding its way to the oral cavity. Then it must pass through the esophagus, survive the low pH of the stomach, locate to a suitable niche within the intestine and ultimately gain access to nutrients to begin replication (111). For successful colonization, replication to sufficient numbers is required for the invading bacteria to resist peristalsis and washout from the intestine. During this journey, the invading bacterium must compete with the resident microbiota for niches and nutrients (112).

The mammalian intestinal microbiota plays a central role in host development and basic physiology, including immune system development, tissue integrity, digestion, vitamin and nutrient production and colonization resistance (111). Therefore, it is important to understand how these GI tract microbiotas interact with both commensal and pathogenic microorganisms.

My study explored how *E. coli* and its culture supernatant affects the biofilm formation of two opportunistic pathogens, *V. vulnificus* and *S. epidermidis,* in static, in vitro setting. However, human GI tract is in constant motion and further researches should be conducted to explore how interaction of these microorganisms in non-static condition affect the biofilm formation. In addition, in vivo conditions vary greatly with the experimental settings employed in this study. Therefore, it is also necessary to explore whether similar patterns of interaction were observed in in vivo setting or not. Structural analysis of the biofilms and transcriptomic, proteomic and metabolomic analyses of the biofilm associated cells may also be done to get better insight into the interaction between these microorganisms. In addition, it will also be interesting to explore how *E. coli* affects the biofilm formation of microorganisms other than *V. vulnificus* and *S. epidermidis*.

5.1. Interaction between *E. coli* ATCC 35218 and its culture supernatant with *V. vulnificus* L-180 during biofilm formation

I found that the amount of biofilm formed by *V. vulnificus* L-180 was apparently decreased when co-cultured with *E. coli* ATCC 35218. It has been reported by Nag *et al*. (113) that two of the *E. coli* strains could inhibit colonization of *V. cholerae* in zebra fish. So, it might be possible that *E. coli* ATCC 35218 also interferes with the surface attachment of *V. vulnificus* L-180 in a microtiter plate. However, I found that the adhered cell number of strain L-180 in the biofilm remain unaffected even when strain ATCC 35218 was present in a huge excess.

Chen *et al.* (114) reported that the amount of the biofilm formed by *Vibrio parahaemolyticus* was reduced when mixed with *Listeria monocytogenes*. They mentioned that this reduction might be due to downregulation of the biofilm-regulated genes and due to decrease in the metabolic activity of the bacterial cells in the biofilm. Induction of the similar phenomenon might be possible by the interaction between *E. coli* ATCC 35218 and *V. vulnificus* L-180, which results in reduction of the biofilm amount in mixed culture.

In the present study, total amount of biofilm was reduced greatly in the mixed culture. However, the number of cells recovered from the mixed culture biofilm for both *E. coli* and *V. vulnificus* did not significantly differ from their respective single culture biofilm. This suggests that although the cell-cell or cell-surface attachment and multiplication of each bacterium remain unaffected, the mixing of two bacterial species showed a negative impact on the accumulation of the extracellular polymeric matrix substances in the biofilm.

In general, the microbial cells account for only 2-5% in the biofilm matrix (115). Since crystal violet can stain bacteria cells as well as the extracellular matrix (116), it may lead to discrepancies between the biofilm amount and cell numbers in the biofilm. Kuehl and colleagues (117) reported that amount of the biofilm measured by crystal violet-staining method was significantly reduced by the addition of the furanone compound 2 to *S. aureus* or *B. subtilis*, but the cell numbers were not affected. Similar result was also observed when the furanone compound 3 was added to *S. epidermidis*. In this case, approximately 50% reduction in the biofilm amount was observed without affecting the bacterial cell numbers. Also, Haney *et al* (118) found that, when *P. aeruginosa* was grown in the presence of 0.1% glucose, the biofilm amounts quantified by using crystal violet were remarkably decreased but sufficient bacterial attachment to the surface of microtiter plate was observed.

The amount of biofilm formed by *V. vulnificus* L-180 was apparently reduced when incubated with the *E. coli* ATCC 35218 culture supernatant. The antibiofilm substance(s) in the *E. coli* ATCC 35218 culture supernatant was unlikely a protein factor because the factor

was found to be resistant to even the heat-treatment at 100°C for 10 minutes. Capsular polysaccharide production inhibited the attachment and biofilm formation of *V. vulnificus* (119, 120). *E. coli* capsular polysaccharides present in the culture supernatant may reduce the amount of *V. vulnificus* L-180 biofilm through similar action.

I also observed *E. coli* ATCC 35218 culture supernatant could also reduce the amount of preformed *V. vulnificus* biofilm. *V. vulnificus* capsular polysaccharides facilitate the dispersal of bacterial cells from biofilm structures after the maturation stage by providing the hydrophilic basis of biofilm matrix due to the presence of a negatively charged sugar (121). So, it may be possible that polysaccharides in *E. coli* ATCC 35218 culture supernatant may act through similar mechanism. However, in my study, the cell number of *V. vulnificus* L-180 recovered from preformed biofilm which was treated with *E. coli* culture supernatant did not differ from control well which was treated with TSB-NaCl. This suggests that *E. coli* ATCC 35218 culture supernatant may act in a different way.

The biological role of the biofilm is to withstand the fluid flow or other mechanical forces, which work to wash out or sweep microorganisms (122, 123) and the formation of extracellular matrix is important to cement the entire bacterial population to the solid surface (124). The reduced formation of the extracellular matrix will make the biofilm more susceptible to the external forces because of impaired cohesion of the biofilm. Moreover, the extracellular matrix functions to protect bacterial cells from both non-specific and specific host defense systems and to confer tolerance to various antimicrobial agents (125). Therefore, the decrease in the extracellular matrix will make bacterial cells more sensitive to hostile environments.

Exopolysaccharides in the culture supernatant from *Vibrio* species QY101 was reported to have the antibiofilm effect against *P. aeruginosa* and *S. aureus* (126). However, the culture supernatant from *V. vulnificus* L-180 did not show any antibiofilm activity against *E. coli* ATCC 35218. On the other hand, the addition of live *V. vulnificus* L-180 cells to the preformed *E. coli* biofilm leads to huge reduction in the amount of biofilm. These results suggest that the cell-cell interaction between *E. coli* and *V. vulnificus* is required for the antibiofilm action of *V. vulnificus* L-180. To clarify how *V. vulnificus* reduces the amount of *E. coli* biofilm, some additional experiments using both living and dead *V. vulnificus* cells are necessary.

If dead *V. vulnificus* L-180 cells could reduce the amount of preformed *E. coli* ATCC 35218 biofilm, this suggests that component(s) of the *V. vulnificus* cells, that are already present before *V. vulnificus* L-180 cells encounter *E. coli* ATCC 35218 cells, may be responsible for the reduction of preformed *E. coli* ATCC 35218 biofilm amount. If dead *V. vulnificus* L-180 cells could not reduce the amount of preformed *E. coli* ATCC 35218 biofilm,

it may be due to living *V. vulnificus* L-180 cells producing some component(s), which may or may not be secreted, in response to the presence of *E. coli* ATCC 35218 cells. In this case, the culture supernatant that was obtained by incubating *V. vulnificus* with preformed *E. coli* may be tested against preformed *E. coli* ATCC 35218 biofilm to determine if the antibiofilm component(s) are secreted or not. If the component(s) are unlikely to be secreted, comparative analysis of *V. vulnificus* L-180 cells that are exposed to preformed *E. coli* ATCC 35218 biofilm and *V. vulnificus* L-180 cells that are not exposed to preformed *E. coli* ATCC 35218 biofilm may be done to screen which component might be responsible for the antibiofilm effect.

5.2. Interaction between *E. coli* ATCC 35218 and *S. epidermidis* ATCC 35984 during biofilm formation

The present study indicated that *E. coli* ATCC 35218 could readily outcompete *S. epidermidis* ATCC 35984. I observed significant reduction in the amount of the biofilm and the number of *S. epidermidis* cells in mixed culture with *E. coli* ATCC 35218 when compared to *S. epidermidis* single culture. Similarly, Millezi *et al* (127) reported that total biofilm amount of the mixed culture of *S. aureus* and *E. coli* were lower than either of the single culture. Millezi *et al* (127) and Makovcova *et al* (128) also documented significant reduction in the cell numbers of *S. aureus* in the biofilm via mixed cultivation with *E. coli*.

E. coli has faster growth rate than *S. epidermidis*. The generation times of *E. coli* (17.9 ± 0.9 minutes) is shorter than that of *S. epidermidis* (27.5 ± 10.5 minutes) when grown in TSB at 36-37 ºC (129, 130). So, it is possible that due to its faster growth rate and overwhelming population, *E. coli* ATCC 35218 consumes all available nutrient in the culture medium, leaving very few nutrients for *S. epidermidis* to utilize. This may lead to reduced cell growth and biofilm formation of *S. epidermidis* in mixed culture.

As previously described (114), this reduction might also be due to downregulation of the biofilm-regulated genes due to interaction *E. coli* ATCC 35218 and *S. epidermidis* 35984, which results in reduction of the biofilm amount in mixed culture.

Colicins and microcins are two bacteriocins produced by *E. coli* or other species in the *Enterobacteriaceae*, but their action spectrum is limited to closely related bacterial species. Staphylococcins are bacteriocins produced by staphylococcal species. Duarte *et al* (131) and Carson *et al* (132) reported bacteriocin production by *S. epidermidis*. However, the result of the cross-streak plate assay in this study indicated neither *E. coli* ATCC 35218 nor *S. epidermidis* produced active bacteriocin.

Total amount of the biofilm formed by *S. epidermidis* was apparently reduced by incubation with *E. coli* ATCC 35218 culture supernatant. This finding suggests that production

of a substance, which interferes the biofilm formation by invading microorganisms, might be one of the colonization resistant mechanisms employed by *E. coli* or other commensal bacteria. Valle *et al* (133) reported that the culture supernatant of uropathogenic *E. coli* inhibited the biofilm formation in a wide variety of microorganisms including *S. epidermidis* without affecting cell viability, and they also mentioned that this inhibition might be due to the impairing interaction of the bacterial cell to the solid surface and/or to another bacterial cell. However, the present study demonstrated that the *E. coli* culture supernatant did not affect the numbers of *S. epidermidis* cells in the biofilm. Therefore, it is unlikely that the *E. coli* culture supernatant caused reduction of the amount of *S. epidermidis* biofilm by impairing the cell-cell or cell-surface interactions of *S. epidermidis*.

Ma *et al* (134) showed that BdcA protein produced by *E. coli* could increase dispersal of the biofilms formed by gram-negative bacteria including *P. aeruginosa* through binding to the ubiquitous bacterial signal molecule, cyclic diguanylate (c-di-GMP). However, the present study showed that *E. coli* ATCC 35218 did not excrete agent(s) that might degrade or disperse the *S. epidermidis* biofilm.

First antibiofilm activity of *E. coli* culture supernatant was reported by Valle and colleagues in 2006 (133). They also found that the antibiofilm activity was due to group 2 capsular polysaccharide secreted into the culture supernatant. On the other hand, Fang *et al* (135) reported that *E. coli* Nissle 1917 secreted DegP, a bifunctional (protease and chaperone) protein which suppress the biofilm of enterohemorrhagic *E. coli.* In my study, heat treatment of *E. coli* culture supernatant did not alter its antibiofilm activity. So, it is likely that polysaccharide component is responsible for the antibiofilm activity of *E. coli* culture supernatant.

There are more than 80 *E. coli* capsular polysaccharides or K antigens. These capsular polysaccharides are differentiated into 4 groups based on genetic and biosynthetic criteria (136). Group 2 and 3 capsular polysaccharides are low-molecular-weight, high-charge-density molecules while group 1 capsular polysaccharides are high-molecular-weight structures with low charge density (137, 138). The molecular weight of capsular polysaccharides ranges from 20 kDa to 3000 kDa (139, 140).

There are three possible modes of action of these non-biocidal, antibiofilm polysaccharides:

1. The polysaccharides may act as surfactant molecules that modify the physical characteristics of bacterial cells and abiotic surfaces (133).

2. They might also act as signaling molecules that modulate gene expression of recipient bacteria (141).

3. Another possible mode of action is competitive inhibition of multivalent carbohydrate–protein interactions. For example, antibiofilm polysaccharides might block sugar binding proteins present on the surface of bacteria (142).

Adhesion is of paramount importance in the life of bacteria and provides two vital roles; it allows targeting of a given bacterium to a specific surface (e.g., a particular epithelial surface in a mammalian host), and in flow environments, it enables bacteria to resist physical removal by hydrodynamic shear forces (143). Bacterial adhesion is also the first crucial step in biofilm formation (30). Sherman and colleagues (144) reported that *Lactobacillus acidophilus* R0052 and *L. rhamnosus* R0011 inhibited the adherence of *E. coli* 0157:H7 to epithelial cells in dose dependent manner. Similar, *E. coli* Nissle was found to be able to inhibit the adhesion of adhesive-invasive *E. coli* strain LF82 (145). In my study, it is likely that *E. coli* ATCC 35218 reduced the growth and biofilm formation of *S. epidermidis* ATCC 35984 when surface of microtiter plate was pre-coated with *E. coli* ATCC 35218. This finding suggests that commensal *E. coli* may limit the adhesion of *S. epidermidis* ingested with food and drink to the intestinal surface.

I also found that *E. coli* ATCC 35218 could incorporate into preformed *S. epidermidis* ATCC 35984 biofilm. At the same time, it could also reduce the number of *S. epidermidis* cells and total biofilm amount of preformed biofilm. Hourya *et al* (146) reported that *Bacillus thuringiensis* 407 could infiltrate and form pores in biofilm matrix due to its motile nature. Preformed *S. aureus* biofilms were found to be eradicated by *Streptococcus pneumoniae* in contact dependent manner (147). Similar mechanisms may be employed by *E. coli* ATCC 35218 to reduce the amount of preformed *S. epidermidis* biofilm. My finding suggests that although *S. epidermidis* establishes itself as one of the GI tract commensals in younger children, increasing exposure to adult GI tract commensals including *E. coli* as they become older could lead to gradual loss of *S. epidermidis* from their GI tract.

5.3. Interaction between *E. coli* K-12 or B strain and *S. epidermidis* ATCC 35984 during biofilm formation

Both *E. coli* K-12 and B strains are non-pathogenic strains recovered from human faeces (94, 97). Yoon *et al.* (98) reported that growth rate of *E. coli* K-12 and B strains were similar in nutrient rich condition. He also reported that genes involved in cell motility, transcription, carbohydrate transport, or energy production are highly expressed in *E. coli* K-12 strain while genes that are highly expressed in the *E. coli* B strain are involved in transport and metabolism

of various amino acids and carbohydrates. In addition, he reported that *E. coli* B strain lacks flagella and is more susceptible than the *E. coli* K-12 strain to a variety of stressful conditions caused by osmolarity, pH, or exposure to inhibitory compounds such as salicylate and b-lactam antibiotics. . However, my findings suggest that, these differences do not have any significant impact on how *E. coli* K-12 or B interacts with *S. epidermidis* ATCC 35984 during biofilm formation as both *E. coli* K-12 and B strains were able to interfere with biofilm formation by *S. epidermidis* ATCC 35984 in mixed culture. In addition, both strains could also reduce the amount of total biofilm of preformed *S. epidermidis* ATCC 35984 biofilm. This suggests that the ability to resist colonization of *S. epidermidis* may be widespread among different *E. coli* strains.

My findings suggest that there is no significant difference in the way *E. coli* K-12, B or ATCC 35218 interacts with *S. epidermidis* ATCC 35984 during its biofilm formation. This means that differences that are present between these strains may not significantly affect how these three strains interact with other bacteria during the biofilm formation process. Therefore, from these findings, it is suggested that *E. coli* K-12 and B strains may also interact with *V. vulnificus* L-180 in similar way as *E. coli* ATCC 35218 during biofilm formation.

Conclusion

6. Conclusion:

My findings suggest that *E. coli* may not be a good candidate to prevent the colonization of *V. vulnificus* in GI tract. It could not prevent the growth of *V. vulnificus* in the culture supernatant and biofilm of mixed culture. In addition, I found that *V. vulnificus* cells could reduce the amount of preformed *E. coli* biofilm. This suggests that *V. vulnificus* may destabilize *E. coli* from its niche inside GI tract and take its place. However, my findings suggested that *E. coli* culture supernatant may be useful in combating *V. vulnificus* infections since it could effectively reduce the amount of *V. vulnificus* biofilm.

The biofilm lifestyle of bacterium is associated with a high tolerance to exogenous stress. Since less biofilm was formed by *S. epidermidis* in the presence of *E. coli* or its culture supernatant, it is likely that *S. epidermidis* will be more vulnerable to hostile GI tract environment. In addition, growth of *S. epidermidis* was also reduced in the presence of *E. coli*. These findings suggest that *E. coli* may express colonization resistance against *S. epidermidis* and helps in removing *S. epidermidis* from GI tract of adults through suppression of growth and biofilm formation. In addition, I found that *E. coli* was able to destabilize the established *S. epidermidis* biofilm, suggesting that *E. coli* may be involved in removal of *S. epidermidis* from the GI tract of infants and small children.

My in vitro studies suggest that *E. coli* and its culture supernatant may have potential as an antibiofilm agent against *V. vulnificus* and removing potentially harmful pathogenic *S. epidermidis*. Therefore, in vivo studies should be done to explore whether similar phenomena are observed or not. Moreover, further studies should also be done to identify the component(s) in the *E. coli* ATCC 35218 culture supernatant that exhibit antibiofilm effect so that these component(s) may be utilized to control the biofilm formation of *V. vulnificus*. In addition, mechanisms of how *E. coli* interferes with the biofilm formation of *V. vulnificus* and *S. epidermidis* is not known and should also be examined for better understanding of their interactions.

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List of publication from this thesis

8. Scientific publication from this thesis:

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