

**PREVENTION OF BIOFILM FORMATION ON FOOD CONTACT
SURFACES BY NANOSCALE PLASMA COATINGS**

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**PREVENTION OF BIOFILM FORMATION ON FOOD CONTACT
SURFACES BY NANOSCALE PLASMA COATING**

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Table of Contents

Acknowledgements.....	ii
List of Figures	vi
List of Tables	vii
Abstract.....	viii
Chapters.....	1
1. Introduction	1
2. Literature review.....	4
2.1 Biofilm	4
2.1.1 Biofilm formation.....	5
2.1.2 Existing methods to control the growth of biofilms.....	6
a) Antimicrobial chemicals	7
b) Antimicrobial coating	10
c) Surface modification	12
2.2 Pathogens in the food industry.....	15
2.2.1 <i>Escherichia coli</i> O157:H7	16
2.2.2 <i>Staphylococcus aureus</i>	17
2.2.3 <i>Listeria monocytogenes</i>	18
2.2.4 <i>Salmonella</i>	19
2.3 Nanoscale plasma coating.....	21
2.4 Trimethylsilane (TMS)	23
2.4.1 Application of TMS	24
2.4.2 Safety tests of TMS coating	25
2.4.3 Anti-biofilm effects of TMS coating.....	26
3. Materials and Methods.....	27
3.1 Coating of wafers with trimethylsilane	27
3.2 Contact angle test	29
3.3 Selection of pure cultures	29
3.4 Optimization of ultrasonication treatment time	30
3.5 Anti-biofilm efficacy on single strains on stainless steel wafers.....	30
3.6 Anti-biofilm efficacy on strain cocktails on stainless steel and HDPE wafers....	31

3.7 Bacterial suspension concentration study	32
3.8 Effect of sanitizer addition	32
3.9 Anti-biofilm efficacy of TMS and TMS+O ₂ on <i>L. monocytogenes</i> in beef juice..	33
3.10 Effect of cultivation broth (BHI and TSB)	34
3.11 Fluorescence staining and laser confocal microscopic scanning	34
3.12 Scanning electron microscopy (SEM) and energy dispersive x-ray spectroscopy (EDS)	35
3.13 Zeta potential tests on bacteria suspensions.....	37
3.14 Gene expression	37
4. Results and Discussion	42
4.1 Contact angle test	42
4.2 Optimization of ultrasonication treatment time	42
4.3 Anti-biofilm efficacy on stainless steel wafers - single strains.....	44
4.4 Anti-biofilm efficacy on stainless steel and high-density polyethylene (HDPE) – strain cocktails.....	48
4.5 Bacterial suspension concentration study	53
4.6 Hurdle concept of coatings with sanitizer.....	54
4.7 Trial in beef juice	55
4.8 Effects of cultivation broth (BHI and TSB).....	58
4.9 Fluorescent staining of adherent bacterial - Laser Confocal Scanning	60
4.10 Scanning electron microscopy (SEM) and Energy Dispersive X-Ray Spectroscopy (EDS).....	64
4.11 EDS (Energy-dispersive X-ray spectroscopy)	72
4.12 Zeta potential tests on bacterial suspensions.....	73
4.13 Gene expression studies	75
5. Conclusions	79
References	81

List of Figures

Figure	Page
1. Structure of Biofilm.....	4
2. Formation steps of biofilm.....	6
3. Trimethylsilane.....	23
4. Cell viability on TMS coated SS and silicone.....	25
5. Bell jar-type plasma reactor powered by direct current (DC) power supply for coating deposition on SS wafers.....	28
6. Main experimental steps.....	31
7. Contact angle change on SS wafer before and after TMS coating.....	42
8. Relationship between wash time and bacterial count.....	43
9. Efficacies of TMS and TMS+O ₂ on single pathogenic strains of <i>E. coli</i> O157:H7, <i>S. aureus</i> and <i>L. monocytogenes</i>	46
10. Efficacies of TMS and TMS+O ₂ on single non-pathogenic strains of <i>E. coli</i> , <i>Listeria</i> and <i>Salmonella</i>	47
11. Efficacies of TMS and TMS+O ₂ on SS surfaces on bacterial cocktails.....	50
12. Efficacies of TMS and TMS+O ₂ on HDPE surfaces on bacterial cocktails.....	51
13. Bacterial suspension concentration study on TMS-coated SS.....	54
14. Anti-biofilm effects of TMS coating with the application of Quat sanitizer on <i>L. monocytogenes</i> SCOTT A.....	55
15. Anti-biofilm effects of TMS coating with the application of Quat sanitizer on <i>L. monocytogenes</i> SCOTT A in beef juice.....	57
16. Effects of changing cultivation medium on SS.....	60
17. Live/Dead laser confocal images of <i>L. monocytogenes</i> biofilm.....	61
18. Live/Dead laser confocal images of <i>S. aureus</i> biofilm.....	62
19. Live/Dead laser confocal images of <i>E. coli</i> O157:H7 biofilm.....	63
20. SEM images of <i>E. coli</i> O157:H7 biofilm on SS.....	65
21. SEM images of <i>S. aureus</i> biofilm on SS.....	67
22. SEM images of <i>Salmonella</i> biofilm on SS.....	69
23. SEM images of <i>L. monocytogenes</i> biofilm on SS.....	71
24. EDS elements scanning results of uncoated, TMS coated, and TMS+O ₂ coated on SS.....	73
25. Gene expression results of <i>E. coli</i> O157:H7 <i>arcA</i> , <i>eaeA</i> , <i>stx1</i> , and <i>stx2</i> gene on SS.....	76
26. Gene expression results of <i>L. monocytogenes</i> <i>ActA</i> , <i>rpoB</i> , <i>hly</i> , <i>inlA</i> , and <i>inlB</i> gene on SS.....	77

List of Tables

Table	Page
1. Target genes and primers used for gene expression.....	40
2. Pathogenic bacterial zeta potential results.....	74

Abstract

Microbial biofilm formation can lead to a series of important safety problems in food industry because, once formed, the microbial cells are much harder to remove compared to plankton cells and can lead to circulating contamination. Low temperature plasma coating technology is a novel and effective method to prevent biofilm formation. However, previously it was only applied on medical devices.

In this study, the anti-biofilm activity of trimethylsilane (TMS) coating and its adapted recipe TMS+O₂ (1:4) on common food contact surfaces: stainless steel (SS) and high-density polyethylene (HDPE) against biofilms formed by common foodborne pathogens. The factors which will affect the anti-biofilm efficacy of this TMS coating was also studied and combined to form a basic theory.

SS or HDPE wafers (1 cm × 1 cm) were coated with TMS or TMS+O₂(1:4) plasma to an approximate thickness of 30 nm using direct current power supply. The surface contact angle to water was tested, and then *Escherichia coli* O157:H7, non-O157 *E.coli*, *Salmonella*, *Staphylococcus aureus*, and *Listeria monocytogenes* each was allowed to form biofilms on the wafer surfaces for 48h. The biofilms cells were then be removed by rinsing and ultrasonicing each wafer four times for 30s each time and the pour-plate method was conducted to determine bacterial counts. In addition, the efficacy of TMS-coated wafers in combination with a sanitizer was also tested by dipping the wafers in a Quat solution for 15s and rinsing before sonicating and plating. Besides to that, scanning microscope, Live/Dead laser confocal scanning and gene expression were applied to help study the anti-biofilm mechanism of TMS

coating.

Results showed that TMS coating on SS succeed in reducing the cell count of *S. aureus* cocktail biofilm by around 2 log CFU/wafer (99% reduction) and *L. monocytogenes* by 1.5 log CFU/wafer. TMS+O₂ coating on SS also achieved a 2 log CFU/wafer reduction in *S. aureus* cocktail biofilm, and a 2 log CFU/wafer reduction in *L. monocytogenes* cocktail biofilm. On HDPE surfaces, TMS+O₂ decreased the cell count by around 1 log CFU/wafer in *E. coli* O157:H7 cocktail. For different single strains, their efficacy fluctuated and sometimes the total count even increased slightly. However, most of the time, these coatings worked well or at least did not increase the cell count in our study. Factors like bacterial strain (*E. coli* O157:H7, *S. aureus* or *L. monocytogenes*), coating surface (SS or HDPE), coating composition (TMS or TMS+O₂), as well as the medium (Tryptic Soy Broth (TSB) or Brain Heart Infusion (BHI)) can all affect the anti-biofilm efficacy of this TMS coating. The mechanism was thought to be related to surface energy and surface contact angle change which can help reduce the initial attachment of bacterial. No change of relative gene expression level was detected which confirmed the theory that this method would not lead to resistant strains. Moreover, when combined with sanitizers, TMS coating shows greater promise to remove and prevent biofilm from forming on stainless steel surfaces. The result showed that TMS plasma coating is a promising method to solve the biofilm issue in food manufacturing lines. However, the theory of anti-biofilm TMS coating was still on basic level and more factors needs to be studied in order to form a well fitted model which can be applied in food industry in the future.

Chapter 1. Introduction

Biofilm is any group of microorganisms that stick to one another and to a surface in high numbers, forming a highly efficient micro-colony which is extremely more difficult to remove as compared to unattached cells (Kumar and Anand 1998; Stewart and Costerton 2001). Biofilm could lead to serious problems in both the clinical aspect and food science area because of its resistance to the environmental changes (Blackman and Frank 1996; Donlan and Costerton 2002). What is more, most of the time, biofilm can form quickly in several days, and thus, the prevention of the biofilm is sometimes difficult.

Proper removal of biofilms on food contact surfaces plays a vital role on the microbial status of food, since once a biofilm is formed, it can release free cells back to the surrounding areas which will result in continuous contamination or even get into foods and lead to foodborne illness when consumed (Myszka and Czaczyk 2011). In 2015 alone, there was a total 150 cases of food recalls in the U.S. These recalls lead to more than 21 million pounds of food waste in this country. Among them, more than 5 million pounds of recalls were related to foodborne pathogens (USDA, 2015). These facts reminded us that even for well processed products, sometimes pathogens can still be problem. Further, there is no mention of the importance of removing biofilms at decreasing the risks of foodborne pathogens.

Biocides and disinfectants are the common ways to control biofilms in water pipelines and food processing plants (Stewart and Costerton 2001). However, the relatively short application time and concentration of common sanitizers are not

sufficient to remove or kill all bacterial in a biofilm. For example, active chlorine concentrations of at least 1000 ppm are needed to achieve a significant reduction of biofilm cells which is much higher than the suggested 200 ppm in 1 to 2 min (Meyer 2003; McGlynn 2015). Under this condition, anti-biofilm coatings were developed to help fight against biofilm. Among different anti-biofilm coatings, trimethylsilane (TMS) plasma coating showed strong activity at reducing the cell count of *Staphylococcus epidermidis* biofilm by more than 2 log CFU/wafer (99%). The mechanism of the anti-biofilm coating was considered to be related to the anti-adhesion surface created by TMS which helped reduce the attachment of cells and slows down the forming process of biofilm (Ma and others 2012a; Chen and others 2013). Safety studies were done on this novel plasma coating. In an accelerated adhesion test, after immersion in a water bath set at 60 °C for 10 days, a rating of 99 was achieved for TMS-coated SS which indicates that the stainless steel (SS) surface modified by this TMS plasma is long lasting and that the coating will not transfer easily to food. Further, cytotoxicity studies on TMS-coated surfaces showed little to no negative effect on cell viability, indicating its non-toxic nature to mammalian cells. However, a lot of factors and details still need to be studied if the TMS coating is to be applied in the food industry.

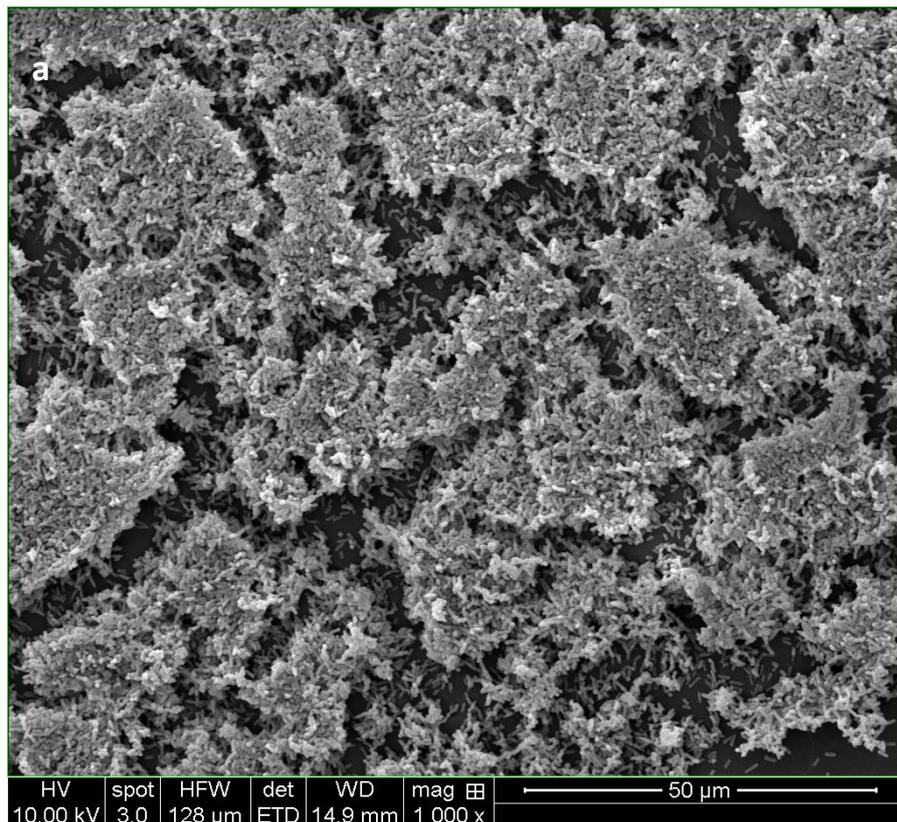
Therefore, the objective of this research was to test the anti-biofilm efficacy of TMS and TMS+O₂ (1:4) on common food contact surfaces, *viz.* SS and high-density polyethylene (HDPE) against biofilms formed by the common foodborne pathogens, *Escherichia coli* O157:H7, non-O157 *E. coli*, *Salmonella*, *Staphylococcus*

aureus, and *Listeria monocytogenes*. Factors that affect the anti-biofilm efficacy of this TMS coating were also studied and combined to form a basic theory according to this and previous studies. In addition, practical tests were also conducted to test anti-biofilm effects of the coatings when applied together with sanitizers.

Chapter 2. Literature Review

2.1 Biofilm

Biofilm is any group of microorganisms that stick to one another and to a surface in high numbers, forming a highly efficient micro-colony (Figure 1). Biofilm formation is a problem of great importance in the food industry, because once formed, the attached cells are extremely more difficult to remove as compared to unattached cells (Kumar and Anand 1998). Biofilms can easily form in any environment, if proper cleaning or sanitation procedures are not effectively and strictly maintained.



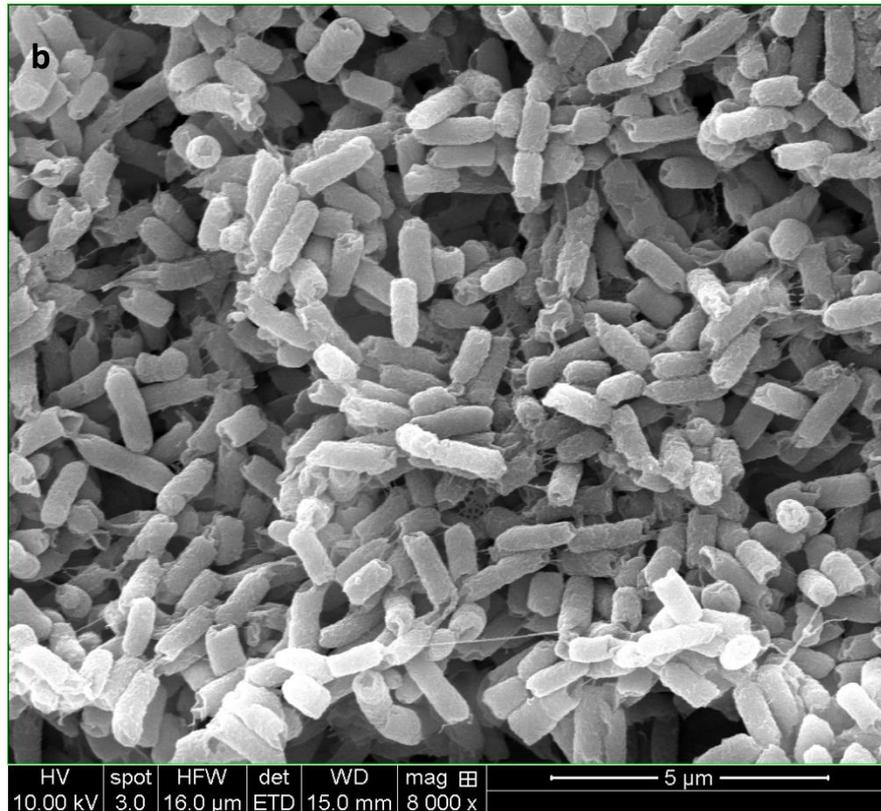


Figure 1 Structure of Biofilm (general) (a) and (detail) (b).

2.1.1 Biofilm formation

The formation of biofilms involves several steps. As the bacteria reach the surface of an object, their movement speed will slow down and they will interact with other microbes previously attached to the surface and join the group to form a more complexing biofilm. Then, a stable micro-colony will form and the bacteria inside the biofilm will reproduce and finally end up with a three-dimensional biofilm structure. Sometimes, the bacteria in a biofilm will be detached from the biofilm and undergo another cycle of forming a new biofilm which can lead to cycling contamination (Watnick and Kolter 2000). During the formation of biofilm, extracellular polymeric substance (EPS) which contains DNA, proteins, polysaccharides and other chemicals

will be produced. The development of a biofilm also involves gene expression as the main method of communication between cells (Pratt and Kolter 1998; Stretton and others 1998).

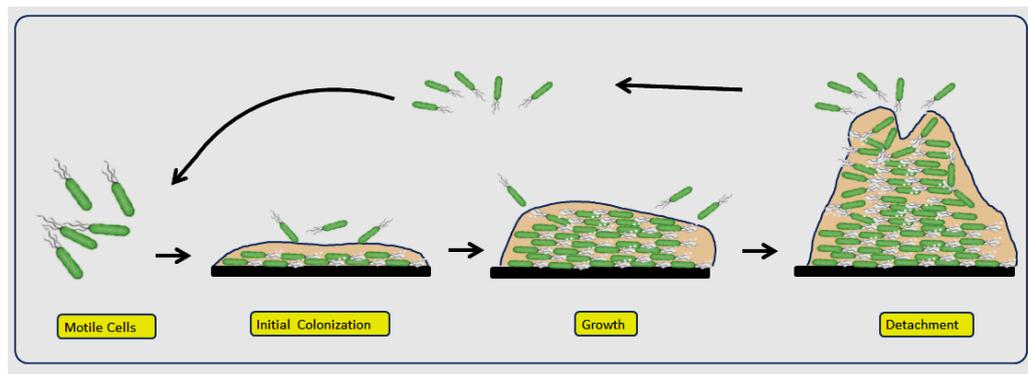


Figure 2 Formation steps of biofilm. (Adapted from Hera Vlamakis 2013)

2.1.2 Existing methods to control the growth of biofilms

Once a biofilm is formed, the cells in the biofilm are extremely more difficult to remove as compared to unattached cells. It is becoming a focus of food manufacturers for food processing plants. Because the prerequisite of biofilm formation is having enough live bacteria in the environment, controlling the live cell number becomes a common way to control biofilm formation. Biocides and disinfectants are common ways to control biofilms in water pipelines and food processing lines (Chen and Stewart 2000). Other common methods to clean processing equipment in the food industry also produce sanitizing effects, such as hot water or steam, lactic acid, or bleach wash. However, the relatively short application time of common sanitizers is not sufficient to remove or kill all bacteria in a biofilm. For example, active chlorine concentrations of at least 1000 ppm are needed to

achieve a significant reduction of biofilm cell numbers while only 10 ppm in 1 minutes is sufficient for planktonic cells (Meyer 2003). Because the limitation of traditional anti-biofilm methods, alternative methods were developed by researchers, such as antimicrobial coating, modifying the surface chemistry, or changing the characteristics of food contact surfaces. Most of these methods can be divided into three areas: Frequent applying of antibacterial chemicals, antimicrobial surface coating, or modification of the surface characters to reduce the initial attachment of bacteria (Wang and others 2014).

a) Antimicrobial chemicals

Antimicrobial chemicals have been studied intensively as a way to control biofilms. A common way to apply them is by adding them into foods as food preservatives. Many chemicals are allowed in foods, including sodium chloride; organic acids like acetic, benzoic sorbic acids; nitrates and nitrites in cured meat products; sulfur dioxide and sulfites for dried fruits and wines; and nisin and natamycin from microorganisms for processed meats (Stevens and others 1991; Prajna and others 2010). The application areas of these chemicals are limited to some extent (certain preservatives only well fit in certain foods) and some customers are quite sensitive and may not accept some of them. Despite this, preservatives are widely used in food industry now as a simple way to extend the expiration date of foods.

Antimicrobial chemicals are also applied to help the environmental

sanitization of food manufacturing lines. Hypochlorites, chlorine dioxide, iodophors, peroxyacetic acid, and quaternary ammonium compounds are all commonly used sanitizers in food manufacturing plants. Their use is strictly controlled with maximum concentrations allowed during sanitizing process (FDA, 1999). The application of these sanitizers is quite easy and feasible which makes this method the most widely used by food manufacturers and processors. However, the limitations of sanitizing chemicals are also quite obvious. Among others, are that they are relatively not environmentally friendly, need to be applied every time when there is a contamination or before and after production, require a relatively long time of treatment, less effective on bacteria in biofilms than planktonic cells, and promote the formation of resistant strains after numerous repeated applications. Thus, more explorations were done in other areas to cover the shortage of the common sanitizers.

Balaban and others (2003) applied a quorum-sensing inhibitor, an RNAi-inhibiting peptide, to successfully prevent biofilm formation by a drug-resistant *Staphylococcus epidermidis* on a plastic surface. However, the efficacy of the compound was limited, resulting in only an 80% reduction of the biofilm (Balaban and others 2003). Earlier in 2002, Singh and others found that lactoferrin has a better antibacterial activity. At a concentration of 10 µg/mL, lactoferrin can significantly kill more than 99.99% *Pseudomonas aeruginosa* free cells in suspensions. However, the effect of this compound was smaller (about 90% reduction) for the cells in biofilms (Singh and others 2002). Aranicu and others (2014) also found some 4,2 and 5,2 bithiazole derivatives with moderate anti-biofilm activities against *Escherichia coli*,

although these compounds are quite effective, they are not easy to make as the chemical formula is not simple (Araniciu and others 2014). Later in an evaluation of the anti-biofilm activity of *Streptococcus mutans* adhesion to fluoride varnishes, Chau and others (2014) achieved about a 2 log CFU (99%) reduction in cell numbers after a 94 h growth treatment. However, fluoride, in high levels, is not only bad for human health but also for the environment which limited the application of this compound (Chau and others 2014). Other researchers started to put their sight on natural derivatives; they discovered that cinchona alkaloid derivatives and *Lavandula angustifolia* (LEO), *Melaleuca alternifolia* (TTO), *Melissa officinalis* (MEO) essential oils possess some anti-biofilm functions. Unfortunately, these compounds need to be frequently applied to the targeted surfaces and, are thus, not well fitted for industry application (Budzyńska and others 2011; Skogman and others 2012). Some commercial products like: Easyclean (an alkaline detergent) and Ambersan (an acidic cleaner) were also tried by researchers against biofilm. However, only 1-log reduction was observed on *P. aeruginosa* and *S. aureus* biofilms on stainless steel surfaces (Pan and others 2006).

Although different trails has been applied to find out a better antimicrobial chemical, there always seems to be some limitations to this method, especially the relatively short gaps of applying. Thus, antimicrobial coating was developed to help improve bacterial contamination.

b) Antimicrobial coating

Antimicrobial coating is quite an efficient way to control biofilms and most of them are long-lasting and stable. Silver nanoparticle is a common material used as an antimicrobial coating as it is quite effective against bacteria and is used as a bacterial growth control reagent in a variety of aspects (Jun Sung Kim and others, 2007). On a Titania and silver-titania composite films on glass surfaces, the coating by itself was able to reduce the bacterial count by 50% to 69% depending on the bacterial strains (*Staphylococcus aureus* (NCTC 6571) for 50% and *E. coli* (NCTC 10418) for 69%) (Page and others 2007). In another experiment on plasma enhanced silver nanoparticle coating, the bacterial numbers on silver coated surfaces were reduced by more than 2 log CFU/chip after 12 h. Further, no viable *L. monocytogenes* cells were detected on the surfaces after 18 h (Jiang and others 2004). In another silver-montmorillonite (Ag-MMT) nanoparticles coated carrot experiment, the total *Enterobacteriaceae* count was reduced by 2 log/g compared to untreated control (Costa and others 2012). For low level usage, such as in antibacterial medication or gels, nanosilver works well. However, for large-scale industry applications, it is not that practical because of its relatively high cost and safety concern (Gottenbos and others 2002). Except for silver nano-particles, other antimicrobial coatings were also studied. Nowatzki and others (2012) found salicylic acid (SA) releasing polyurethane acrylate polymers were tested as anti-biofilm urological catheter coatings (Nowatzki and others 2012). They found that the SA release vastly inhibited the growth of *E. coli* biofilm and even killed the cells during

the adhesion step. The disadvantage of this method is that the coating relies so heavily on high SA concentrations that once SA release from the coating ceases, the inhibition effect immediately ceases as well. Other materials, such as N-halamine epoxide precursors, 3-glycidyl-5,5-dialkylhydantoin, O-acrylamidomethyl-N-[(2-hydroxy-3-trimethylammonium)propyl] chitosan chloride were also applied to fiber surfaces as an antimicrobial treatment (Lim and Hudson 2004). There are also edible antimicrobial coatings, like starch–chitosan matrix coating. Experiments showed that the starch–chitosan coating on carrots was able to reduce the mesophilic aerobes, molds, yeasts and psychotropic count by as much as 2.5 log CFU/cm² (Durango and others 2006). Carlson and others (2008) also found that chitosan carries a strong anti-biofilm activity, reducing the numbers of *Staphylococcus epidermidis*, *S. aureus*, *Klebsiella pneumoniae*, *P. aeruginosa* and *Candida albicans* in biofilms by 95% to 99.9997% (Carlson and others 2008). Even though this is one of the highest reported reductions of an anti-biofilm coating thus far, the stability of a chitosan coating is relatively low because chitosan is water soluble and hence can be destroyed by water. More explorations have been done in the medical area as biofilm is a problem on implantation devices. For example, polylactide add description on degradable medical devices which can reduce the bacteria adhesion number by 99.999% (El Habnoui and others 2013). Studies also showed that gold and amalgam surfaces can help kill cells in biofilms. But as the high cost of gold and the fragile feature of amalgam, their applications are also limited (Auschill and others 2002; Schmidlin and others 2013).

Antimicrobial coating is a promising way to solve the bacterial and biofilm problem. But more studies are still needed and not all of them can be applied to the food sector because of safety concern. Most of these afore-mentioned methods are high technology techniques, not cost-effective, and often not long-lasting, thus necessitating frequent applications, all of which drastically limit their practical applications. More safety related work needs to be done on them and the costs need to be controlled as well. Moreover, the edible coating is mostly applied to root vegetables, but for meat products, coating is not a feasible way.

c) Surface modification

Surface modification is also a quite promising way to fight biofilms. As mentioned, the formation of a biofilm involves the attachment of planktonic cells. The development of the biofilm is controlled by expression of certain genes or chemicals excreted by cells. Thus, if the surface property of biofilm forming surfaces can be changed on certain level such as the contact angles (more hydrophilic or hydrophobic), or the surface energies, then the biofilm problem can be controlled to some extent. Variety kinds of surface modifications were done by various researcher, which proved the possibility of solving biofilm problem in this way.

Lindel and others found ceramic brackets are less vulnerable to biofilm accumulations than metal orthodontic brackets which indicated that under certain conditions, ceramic can be used as a substitute for metal to avoid the accumulation of biofilm (Lindel and others 2011). In another study, an 1800 kDa polysaccharide

extract from *Bacillus licheniformis*, with monomeric units of α -D-galactopyranosyl-(1 \rightarrow 2)-glycerol-phosphate showed strong anti-biofilm activity against *E. coli* PHL628 and *P. fluorescens* bacterial biofilms by reducing the number around 90%. At the same time, no bactericide effect was detected. The mechanism seems to be that the extracellular polysaccharides of *B. licheniformis* can interfere with the cell-to-cell interactions which result in less growth of the biofilm (Sayem and others 2011). In another experiment done on indwelling medical devices like denture acrylic or intravenous catheters, Jyotsna and others (2005) found modified polyetherurethane surfaces (hydrophilic, hydrophobic, cationic, or anionic) can vastly affect the formation of *Candida albicans* biofilm. Under different modified surfaces, the total weight of the biofilm can be reduced significantly by at most 78%. The mechanism of this reduction still needs more study and the author considered it is multifactorial and may be mainly related to the prevention of initial adhesion (Chandra and others 2005). Another bacterial adhesion assay done by Gadenne proved that ulvans polysaccharides was able to decrease 1 log (90%) of the total cell count of *Pseudomonas aeruginosa* on certain indwelling medical devices titanium surfaces. It was proved that the antibiofilm effect of the coating can be maintained for more than 24 h (Gadenne and others 2013). Another experiment done by Li and others indicated that polymerizable methacryloxyethyl cetyl dimethyl ammonium chloride (DMAE-CB) monomer can also influence biofilm formation by reducing the count of *Streptococcus mutans* by 1 log. The mechanism was discussed and the author considered the DMAE-CB coating may affect biofilm formation by down-regulating

the expression of *gtf* genes in *S. mutans* (Li and others 2009). In 2013, Juanzhong and others found that 1% nafion coated on the surface on stainless steel can significantly reduce *E. coli* DH5 α adhesion by 70% in 24 h. Also, they performed some contact angle tests and found that before and after the 1% nafion coating, the surface contact angle changed from around 85° to 100°. Since bacterial cells are negatively charged and nafion coated stainless steel surface is also negatively charged, this provided a theory that the contact angle or the surface charge can greatly influence bacterial adhesion step due to the strong electrostatic repulsion force between them (Zhong and others 2013).

The surface modification method is quite promising to some extent. However, because some of them are quite complicated, more explorations are needed. Most of these surface modifications do not kill bacteria, which means they are less prone to create resistant strains which is a great advantage of this method. But at the same time, compared to antimicrobial chemicals and antimicrobial coatings, the reduction levels of surface modifications are not as competitive. Most of them can only reduce the total cell number by around 1 log which is 90% reduction. But in the food industry, a 90% reduction is still not good enough as there is still a high possibility that the food or food manufacturing surfaces would be contaminated by bacteria and pathogens which is unwanted by manufacturers and consumers. What is more, most of the anti-biofilm studies were done in the medical area, while little of them were applied to the food industry, because of the high cost for the coating material or more safety studies were needed before application in food areas. So,

more related studies are being done to find out a better way to solve the biofilm problem in the food industry.

2.2 Pathogens in the food industry

A pathogen is an infectious agent such as a virus, bacterium, or parasite that can lead to a disease in the host. Food borne pathogens include bacteria, such as *Escherichia coli* O157:H7, *Salmonella*, *Listeria monocytogenes* and others. Most are widely spread in the environment such as in soil or water, and can contaminate human food. In 2006 alone, 37.2 million people were sickened by pathogens, and among them 9.4 million were food related (Scallan and others 2011). Because most pathogens are heat sensitive, with proper handling, most food-borne illnesses can be avoided by proper cooking, drying, and pasteurization processes (Rosenberg and Bogl 1987). However, there are still conditions where these control methods cannot work well, such as for raw fruits and salads or raw meat products. In an research done in Edmonton, Alberta, Canada, 800 meat samples including raw ground beef, chicken legs, pork chops, sausages, roast beef, processed turkey breast, chicken wieners, and beef wieners were collected. It was found that 30% and 62% of raw chicken legs contain *Salmonella* and *Campylobacter*. In 52% of the ground beef products, 34% of raw chicken legs, 24% of raw pork chops, 4% of fermented sausages, 3% of processed turkey breast, 5% of beef wieners, and 3% of chicken wieners *L. monocytogenes* was found (Bohaychuk and others 2006). In another research done on 2013 on raw milk and cheese, the authors found *E. coli* O157 in 11% raw milk and

6% Herby cheese samples (Sancak and others 2015). In 2013 alone, more than 13 million pounds of meat products were recalled (USDA, 2013), and Shiga toxin producing *E. coli* (STEC), *L. monocytogenes* and *Salmonella* were the top 3 reasons. More than 10 million pounds were recalled because of STEC contamination and around 1 million pounds were recalled because of *L. monocytogenes* contamination. The total quantity recalled from beef and mix meats was more than 12 million pounds (USDA, 2013). These facts remind us that even for well processed products, sometimes the pathogen is still a problem. Therefore, how to fight and control these pathogens has become a long term challenge for the food industry.

2.2.1 *Escherichia coli* O157:H7

Escherichia coli, Domain: Bacteria, Phylum: Proteobacteria, Class: Gammaproteobacteria, Family: *Enterobacteriaceae*, Genus: *Escherichia*, Species: *E. coli*. *E. coli* O157:H7 is an enterohemorrhagic serotype strain of *E. coli*. It is gram negative, facultative anaerobic, rod-shaped, mesophilic bacteria. Once infected, *E. coli* O157:H7 can cause bloody diarrhea, severe abdominal cramps, nausea, vomiting, kidney damage and other symptoms (James M. Jay and others, 2005). *E. coli* O157:H7 is often carried by contaminated raw milk, leaf green vegetables, and sometimes ground beef as well. It is not resistant to most of the antimicrobial treatments, but its wide prevalence makes it easy to get into foods, and the possibility of getting a foodborne illness will be high if the contaminated foods are not properly handled. From 2006 to 2016, *E. coli* O157:H7 was responsible for 17 foodborne

illness outbreaks among all the 25 cases. The most recent ones (from 2013) are ready to eat salad, ground beef, and Costco rotisserie chicken salad. Although there was no death in these outbreaks due to the advances of medical technology, it still drew attention from consumers about the potential occurrence of foodborne illnesses. Several recalls were attributed to *E. coli* O157:H7 contamination in as recent as December, 2015, when several celery products were recalled. In June 4, 2015, Ground elk meat products were also recalled because of this pathogen (Centers for Disease Control and Prevention, 2016; U.S. Food and Drug Administration, 2016).

2.2.2 *Staphylococcus aureus*

Staphylococcus aureus, Domain: Bacteria, Kingdom: Eubacteria, Phylum: Firmicutes, Class: Coccus, Order: Bacillales, Family: *Staphylococcaceae*, Genus: *Staphylococcus*, Species: *S. aureus*. *S. aureus* is a gram positive, grape-like, non-motile, non-capsular, non-sporulating, mesophilic, facultatively anaerobic bacteria. It is known for its enterotoxin and toxic shock syndrome toxin-1 (TSST-1) which can lead to nausea, vomiting, severe abdominal cramps, sweating, headache, diarrhea symptoms (Thomas and others 2007). Although most of the symptoms are relieved in around 48 h, it is still a miserable experience for victims once the enterotoxin was ingested. Enterotoxin is produced by *S. aureus* when they are alive. And even after bacterial death, the toxin can still last. Luckily, the enterotoxin can be deactivated with more than 30 min 100 °C heating process, but the long period of heating will affect the structure of food vastly. *S. aureus* cannot grow as fast as most

gram negative bacteria, but they can grow at low water activity (about 0.86), high salt and sugar concentrations (up to 10%), and even in the presence of NO₂ (James M. Jay and others, 2005). Thus, *S. aureus* are often carried by dried food or candies and sometimes cheese. In July 2015, an outbreak of *S. aureus* linked to candy products affected around 2000 people in the Phillipines. In April 2015, a recall of La Clarita Queseria Cheese was announced because of *S. aureus* contamination. In December 2013, another dried sausage product from Lee Bros. Foodservice was recalled because of *S. aureus* contamination (Food Safety News, 2016). Although outbreaks and recalls of *S. aureus* are not as often as other lethal pathogens, *S. aureus* always causes unpleasant symptoms, and large populations were involved in each time (Centers for Disease Control and Prevention, 2016; Food Safety News, 2016).

2.2.3 *Listeria monocytogenes*

Listeria monocytogenes, Kingdom: Bacteria, Division: Firmicutes, Class: Bacilli, Order: Bacillales, Family: *Listeriaceae*, Genus: *Listeria*, Species: *L. monocytogenes*. *L. monocytogenes* is gram positive, non-sporing short rod shaped, psychrotrophic, facultative anaerobic bacteria. Because it is psychrotrophic, it can grow under refrigeration temperature (4 °C). This feature can lead to great problems as most of the fresh food or ready-to-eat meat products are kept under this temperature. Once food is contaminated with *L. monocytogenes*, this pathogen will start to reproduce in the food under refrigeration conditions. More importantly, the required number of *L. monocytogenes* that can cause the illness, listeriosis is quite low,

at only about 100-1000 cells. Thus, *L. monocytogenes* is a zero tolerance pathogen in ready-to-eat meat products. The symptoms of listeriosis are: meningitis, meningoencephalitis, encephalitis, sepsis, abortion, premature or stillbirths (James M. Jay and others, 2005). *L. monocytogenes*, a pathogen of great concern in the meat industry, can be introduced into foods from numerous routes because of its ubiquitous nature. Common sources of *L. monocytogenes* in processing facilities include processing equipment, conveyors, other product contact surfaces, floors, drains, and condensate (Zottola and Sasahara 1994). Like many bacteria, *L. monocytogenes* can form biofilms, when the conditions are suitable, and in a biofilm, the cells are protected against many different microbial control steps, including cleaning and sanitizing. Cells in a biofilm can persist for a long time in a food processing environment which provides a moist, nutrient-rich environment. Further, *L. monocytogenes* can adhere to materials commonly used in the food industry, including metals and plastics (Mørretrø and Langsrud 2004). In 2013 alone, around 1 million pounds of meat products was recalled because of *L. monocytogenes* and 6 foodborne outbreaks were found related to it. Just in the first season of 2016, 11 cases of food recalls were announced because of *L. monocytogenes* contamination. (Centers for Disease Control and Prevention, 2016; U.S. Food and Drug Administration, 2016)

2.2.4 *Salmonella*

Salmonella, Kingdom: Bacteria, Phylum: Proteobacteria, Class: Gammaproteobacteria, Order: Enterobacteriales, Family: *Enterobacteriaceae*, Genus:

Salmonella. *Salmonella* is a gram negative, non-spore forming, rod shape, mesophilic, facultative anaerobic bacterium. The most common strains of *Salmonella* are *S. enterica*, *S. bongori*, *S. subterranean*, *S. Typhi*, *S. Paratyphi A*, and *S. Paratyphi C*. They are all human pathogens. Symptoms of *Salmonella* infections are nausea, vomiting, abdominal pain, headache, fever, chills and diarrhea that result from the invasion of *Salmonella* cells into our body (James M. Jay and others, 2005). It is also one of the top pathogens that lead to foodborne diseases. From 2013 till March 2016, 29 cases of *Salmonella* outbreaks were recorded. They are from nuts, cucumber, meat, fish, and bean sprouts. In the first three months of 2016, there were already 11 cases of food recall because of *Salmonella* contamination (Centers for Disease Control and Prevention, 2016; U.S. Food and Drug Administration, 2016) As one of the most lethal pathogens, *Salmonella* is receiving more attention from customers.

All the pathogens above have different characteristics and many food illness outbreaks and recalls are related to them. Also, they can all form biofilms which makes them even more difficult to deal with. Foodborne illness is a serious issue related to the health of the public. With the help of antibacterial and anti-biofilm methods afore-mentioned, the biofilm forming pathogens were already controlled to some extent as the total number of food illness outbreaks and recalls were reduced compared to the last decade. However, more efforts are still needed as there are still large numbers of recalls and outbreaks every year and room for development still exists.

2.3 Nanoscale plasma coating

Plasma coating is a covering that is applied to the surface of an object by the use of electricity or high radio frequency-generated plasma to attain/improve specific surface functions, such as adhesion, wettability, sealability, printability, corrosion resistance, anti-bacterial properties and surface cleaning of packaging materials like plastics and metals. Nanoscale indicates that the coating amount is quite small and the thickness of the coating is also quite thin. The coating process changes the surface energy and contact angle of the coated surface, preventing bacterial cells from attaching, and even killing them. It is a sound and currently available processing technique. Plasma coating process is a feasible approach in industry with coating companies such as Flame Spray Coating Company (Fraser, MI), Plasma Treat Solution Company (Elgin, IL), Plasma Coatings Company (Middlebury, CT), and others. The plasma coating functions vary from corrosion protection to wear resistance to heat and oxidation resistance to electrical resistivity and conductivity. Depending on the target feature, different materials can be applied into making the plasma coating (Plasma Spray Coating Services, 2016). Plasma coating, as an industrialized and commercialized protocol, is not far and the application of plasma coating is quite versatile.

Early in the 1980, Schreiber and others started to study the anti-corrosion effects of plasma-polymerized coatings and found that metal plasma combinations work better than “conventional” polymeric coatings. Organosilicon films can withstand high temperatures while still hold mechanical properties well (Schreiber

and others 1980). Plasma treatment can also help at optimizing fiber composition. In an experiment done by Zheng and others (2008), they applied 5-15 nm of acrylic acid/1,7-octadiene and allylamine/1,7-octadiene plasma coating on the surface of e-glass fibers and successfully optimized the glass fiber composition condition (Liu and others 2008). Plasma coating can also give or strengthen special features of materials as needed. Denes and others (1990) applied Hexamethyldisiloxane to the surface of wood and succeed in giving the wood water repellent characteristic (Denes and others 1999). Researchers also found that only 3-5 nm thick coating of oxide can improve the polar fluid infiltration, and surface bond strength. Also they found that the firmness of carbon polymer composites was increased after being coated (Pulikollu and Mukhopadhyay 2007). Except for those applications in engineering aspects above, plasma coating can also be applied to fight against bacterial growth. Plasma coating hydroxyapatite (HA) on metal surfaces has been shown to kill 95% of *E. coli*, *P. aeruginosa*, and *S. aureus* in bacterial suspension (Chen and others 2008). Fluorine-based plasma is known as a method to improve surface cleaning of packaging materials with sustained antimicrobial properties, and can reduce the number of *S. mutans* in BHI broth by 2 to 3 log CFU (Quintavalla and Vicini 2002). In the food industry, nanoscale plasma coating is widely used on beverage cans to prevent corrosions, and the plasma treatment itself has also been proven to exhibit anti-bacterial effects (Kelly-Wintenberg and others 1998). Plasmatreat Corporation has developed a nano-coating using its PlasmaPlus® process that can be applied on both the inside and outside of soft drink packaging, which is transparent yet flexible

and significantly reduces diffusion and extends the shelf life of the soft drink (Plasma Spray Coating Services, 2016). Polyethylene glycol (PEG) plasma coating on stainless steel used in the food industry resulted in >95% decrease in *L. monocytogenes* biofilm cell numbers as compared with unmodified stainless steel (Wang and others 2003). These afore-mentioned information indicates that plasma coating is a high-quality, eco-friendly and cost-effective method that can prevent the growth of bacteria which implies us they may also function well in biofilm prevention (Lee 2010).

2.4 Trimethylsilane (TMS)

TMS, which is short for trimethylsilane, is a compound with the formula $C_3H_{10}Si$. It has a structure as shown in Figure 3, and is very flammable. TMS is used as an etchant in the plasma phase for semi-conductor industry. TMS has been approved for use in the drugs and cosmetics industry. Studio fix fluid, skinning foundation mineral makeup, matchmaster foundation are all approved products. (Drug Library trimethylsilane, 2016). Further, TMS is also applied to other areas such as anti-corrosive and anti-bacterial area.

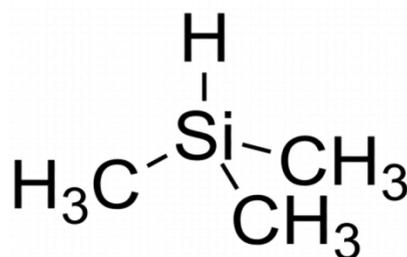


Figure 3 Trimethylsilane

2.4.1 Application of TMS

TMS have been applied to a variety of areas, Most of which are chemical and engineering areas. They have been applied as methylthiolation reagent to help produce aryl methyl sulfide derivatives (Qiao and others 2010). TMS can also be used in nuclear magnetic resonance (NMR) spectrum as an internal reference (Zimmerman and others 1979). Early in 1999, Weikart and others found TMS and TMS+O₂ plasma deposited films have different wetting properties on the surface of polymers. They found TMS coating tend to be little bit more hydrophobic but TMS+O₂ is more prone to be hydrophilic compared to the original polymers (Weikart and others 1999). This result implies provides a way to modify different surfaces via TMS or TMS+O₂ which can result in certain hydrophobicity or hydrophilicity as the composition is changed. TMS plasma coating was also found to have the anti-corrosion activity as normal plasma coating when coated on the surfaces of cold-rolled steel (Sabata and others 1993; Wang and others 1996). TMS was also combined with chloromethyl, acetoxyethyl, acryloxy and other groups to meet more complicated requirements like enhancing the surface adhesion, or improving mechanical properties and other aspects (Sekiguchi and Ando 1979). Yasuda and others also found that post-plasma treatment with TMS plasma with argon gas was able to clean out the surface-contamination effect which can make other coating material easier to adhere to the surface of the aluminum alloy (Yasuda and others 2002).

2.4.2 Safety tests of TMS coating

Studies conducted previously on the TMS coating included an accelerated adhesion test. After immersion in a water bath set at 60 °C for 10 days, a rating of 99 was achieved for TMS-coated SS. This indicates that the SS surface modified by this TMS-coated plasma is long lasting and that the coating will not transfer easily to food. Further, cytotoxicity studies on TMS-coated surfaces was conducted to evaluate the effect of TMS coating on the viability of L-929 mouse fibroblast cells using a protocol based on ISO 10993-5 and the MTT assay (Chen M, 2010). The TMS coating on either SS wafers or silicone rubber wafers had little to no negative effect on cell viability (Fig. 4), indicating its non-toxic nature to mammalian cells. However still more food safety tests are needed before TMS coating was applied in food area.

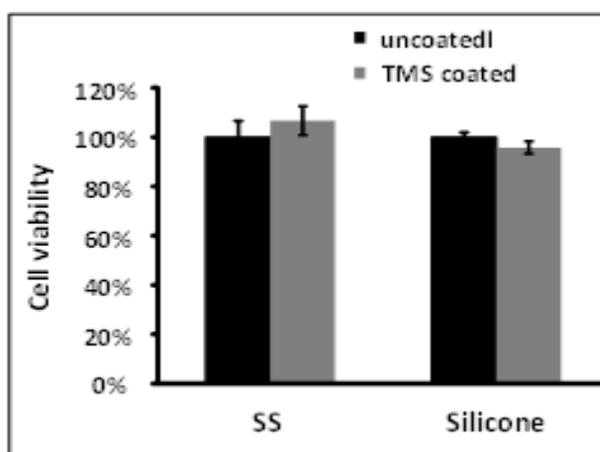


Figure 4 Mouse fibroblast cell viability on TMS coated SS and silicone (Ma and others 2012)

2.4.3 Anti-biofilm effects of TMS coating

Except for the most commonly used applications (anti-corrosion and cleaning), TMS was also found to have anti-biofilm activity. In a study done by Ma and others (2012), they found that with nano-scale TMS plasma coating on stainless steel and titanium surfaces, the count of *S. epidermidis* was reduced by both by around 2 log/chip (1cm*1cm). The anti-biofilm mechanism may relate to chemical inertness, low surface free energy, reduction of roughness, and surface-bound CH₃ groups. With the surface TMS coating and charge, less protein will be attached to the surfaces, as well as to *S. epidermidis* and *S. aureus*. This opens a new area of the application of TMS plasma coating as previously the TMS coating was only applied to engineering aspects while little research was done on the microbiological side (Ma and others 2012; Chen and others 2013). However, the anti-biofilm mechanism of this plasma coating still needs further studies. Also, more strains of bacteria needed to be examined with this coating to learn more about this coating and the principle. Last but not least, TMS coating was only applied to the engineering and medical areas. However, little safety information has been acquired. Thus, more studies are needed to be done on this novel TMS plasma coating to improve its application to the food area.

Chapter 3. Materials and Methods

3.1 Coating of wafers with trimethylsilane

Stainless steel (SS) wafers type 316L (1 mm thick) or high-density polyethylene (HDPE) wafers (2 mm thick) were first cut into 1 cm × 1 cm size, suspended in 3% detergent 8 (Alconox, Inc., White Plains, NY, USA) solution, and ultra-sonicated for 3 h at 50 °C for cleaning. After cleaning, the SS wafers were blotted dry with Kimwipes and held by an aluminum sample holder, which was then placed vertically in the middle of a coating chamber (Figure 5). The pressure of the chamber was decreased to 1.8 mTorr (760 mTorr is atmospheric pressure) before coating. The chamber was then filled with oxygen until the pressure reached 50 mTorr. Then, a direct current power supply was turned on to 20 W, the pressure maintained, and the wafers were treated for 2 min with the oxygen plasma pre-treatment that both cleans the SS as well as improves the adhesion of the subsequent trimethylsilane (TMS) plasma polymer. After the pre-treatment of oxygen plasma, the pressure inside the chamber was reduced to 1.8 mTorr again. The reactor was filled with TMS gas until the pressure reached 50 mTorr. The direct current power supply was turned to 5 W, the pressure was maintained, and the wafers were treated for 15 s to coating the TMS onto the surface of the SS. A silicon wafer was included in each batch to ensure that the coating thickness is correct, as verified with an ellipsometer. In this coating processing, MDX-1K magnetron drive (Advanced Energy Industries, Fort Collins, CO, USA) was used as the DC power source. After the coating process, the coating thickness and contact angle tests were conducted to control the quality of the wafers.

For TMS+O₂ (1:4) coating, the pre-treatment was similar while the deposition condition changed to 5 W with 1 standard cubic centimeters per minute (sccm) flow rate of TMS and 4 sccm O₂, under 50 mTorr pressure, and a total deposition time of 90 s.

For 1 cm × 1 cm (thickness 2 mm) HDPE wafers, a high radio frequency coating method was applied. The preparation steps were similar to that for SS wafers. The HDPE wafers were coated with the help of a radio frequency glow discharger in the same chamber where SS wafers were coated. Pre-treatment was 1 sccm Argon under 50 mTorr pressure and 20 W radio frequency (RF) for 5 min. For deposition, 30 W RF was applied to initiate breakdown of the gases at a pressure of 50 mTorr. 1 sccm TMS flow rate was used for TMS and for TMS+O₂, 1 sccm TMS and 4 sccm oxygen flow rate was applied. The coating pressure was 50 mTorr and the coating time was 10 min for TMS and 20 min for TMS+O₂.



Figure 5 Bell jar-type plasma reactor powered by direct current (DC) power supply for coating deposition on SS wafers.

3.2 Contact angle test

The contact angle refers to an angle formed between a limited amount of liquid and the solids at the liquid-solid interface. The contact angle of TMS plasma coating on SS wafers was measured by a computer-aided VCA-2500XE video contact angle system (AST Products Inc., Billerica, MA, USA). A droplet of 1 μ L distilled water was dropped on the surface of a wafer. A picture was recorded by a high resolution camera and the angle was read directly by the video system.

3.3 Selection of pure cultures

Escherichia coli O157:H7 (505b, 3178-85, 43894, MF1847, C7927), non-O157 *E. coli* (*E. coli* O103:H2 MT80, O111:H8 3215-99, O26:H111 DEC10B), *Salmonella* (I4-9, I4-10, I4-11, B&B3, 788), *Staphylococcus aureus* (β -hemolytic, 183B, FRI, DLV1), and *Listeria monocytogenes* (Scott A, Brie, V37CE, Murray, 7644) were from the culture collection of the Food Microbiology laboratory of the Food Science Department, University of Missouri, Columbia, MO. Strains, except for *L. monocytogenes*, were first activated in Tryptic Soy Broth (TSB). *L. monocytogenes* was activated in Brain-Heart Infusion (BHI). Then, *E. coli* and *S. aureus* strains were streaked for isolation on plate count agar (PCA) (Becton and Dickinson Company, Sparks, MD, USA). *L. monocytogenes* strains were streaked for isolation first on BHI agar, then on modified oxford (MOX agar, Becton and Dickinson Company, Sparks, MD, USA). Plates were kept in a 4 °C refrigerator for use before each experiment.

3.4 Optimization of ultrasonication treatment time

Wafers were separated into two groups: (1) uncoated SS control group, and (2) TMS-coated SS group. As shown on Figure 6, the wafers were sanitized under ultra violet (UV) light for 15 min per side before use, and then placed in sterilized 24-well plates. *L. monocytogenes* SCOTT A was pre-enriched in BHI for 24 h. The bacterial suspension was diluted in BHI at a ratio of 1:100, transferred to the wells that contain one wafer per well, and incubated for 48 h at 37 °C. The BHI medium was changed every 12 h. After 48 h, all the wafers were removed by clean forceps and rinsed gently for four times with peptone water to remove non-adherent bacterial cells. After rinsing, the wafers were placed in tubes containing 9 mL peptone water. The biofilms on the wafers were detached 2, 4, 6, and 8 times by ultrasonication (120 V, 50 kHz) for 30 s each time and vortexing for 30 s after each sonication treatment. The number of bacteria in peptone water was quantified by using the pour plate method on BHI agar. Each dilution was duplicated twice. The whole experiment was performed three times to enhance the reliability of the data achieved.

3.5 Anti-biofilm efficacy on single strains on stainless steel wafers

In SS tests, wafers were separated to three groups: uncoated control SS group, TMS-coated SS group, and TMS+O₂-coated SS group. Wafers were sanitized under UV light for 15 min per side before use, and then transferred into sterilized 24-well plates. *E. coli* O157:H7 505b, *S. aureus* FRI, *L. monocytogenes* (Scott A, V37CE, 7644), *E.coli* P24, *Listeria innocua*, and *Salmonella enterica* ATCC 53648

were pre-enriched in TSB (*Listeria* in BHI) for 24 h. Bacterial suspensions were diluted in the same respective broths at a ratio of 1:100 before use. The biofilms on the wafers were detached by sonicating 4 times in an ultrasonicator (120 V, 50 kHz) for 30 s each time and vortexed for 30 s after each sonicating treatment. The number of bacteria in peptone water was quantified by using the pour plate method on TSA (*L. monocytogenes* on BHI agar). Each dilution was duplicated and each group was triplicated. For each single strain, the whole experiment was performed three times.

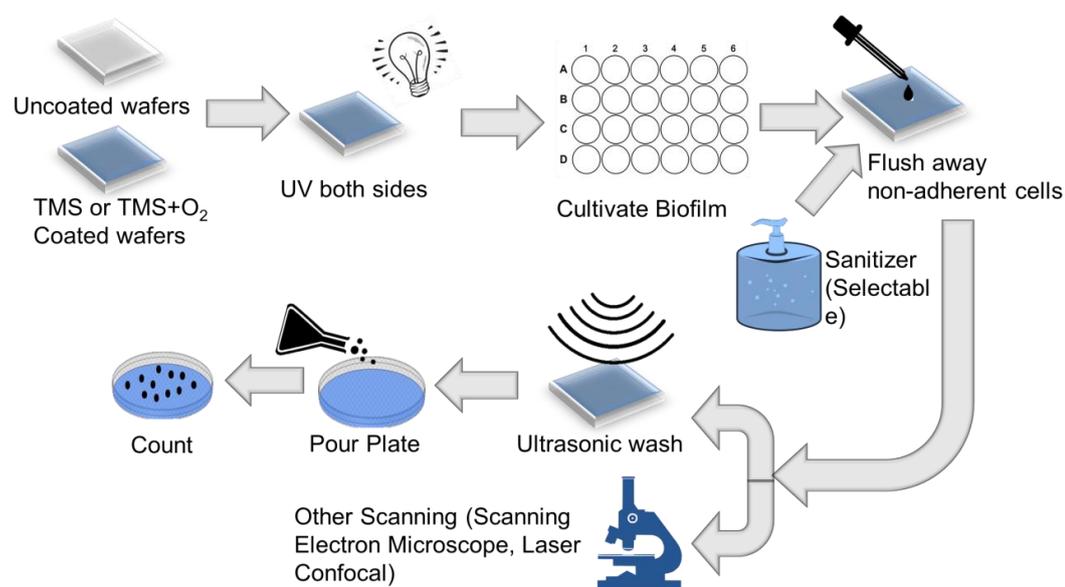


Figure 6 Main experimental steps.

3.6 Anti-biofilm efficacy on strain cocktails on stainless steel and HDPE wafers

SS and HDPE wafers were divided into: (1) uncoated control group, (2) TMS-coated group, and (3) TMS+O₂ (1:4)-coated group separately as described above.

E. coli O157:H7 (505b, 3178-85, 43894, MF1847, C7927), non-O157 *E. coli* (*E. coli* O103:H2 MT80, O111:H8 3215-99, O26:H111 DEC10B), *S. aureus* (β -hemolytic, 183B, FRI, DLV1), and *L. monocytogenes* (Scott A, Brie, V37CE, Murray, 7644) were pre-enriched in TSB (*L. monocytogenes* in BHI) for 24 h and then stored at 4 °C. Then, each single bacterial strain was enumerated by the pour-plate method. After the count of each strain was determined, cocktails of *E. coli* O157:H7, non-O157 *E. coli*, *S. aureus*, and *L. monocytogenes* were made separately using all the sub-species mentioned above for each strain. Then the tests steps according to those performed for single strains, as described above were conducted. Each dilution was duplicated and each group was triplicated. For each strain, the whole experiment was performed three times to enhance the reliability of the data.

3.7 Bacterial suspension concentration study

Wafers were separated into two groups: (1) uncoated SS and (2) TMS-coated SS. Biofilms of *L. monocytogenes* SCOTT A were cultivated as described above. After a 48-h cultivation, the bacterial suspension was pour-plated on BHI agar for incubation and enumeration. Each dilution was duplicated twice. The entire experiment was performed three times.

3.8 Effect of sanitizer addition

Wafers were divided into three groups: (1) uncoated control SS, TMS-coated SS, and TMS+O₂-coated SS. The biofilm of *L. monocytogenes* SCOTT

A, *S. aureus* FRI and *E. coli* O157:H7 505B were cultivated as described above. After 48 h, the wafers were removed by sterile forceps and dipped into a Quat Sanitizer (300 ppm) (Ecolab Inc., Saint Paul, MN, USA), or 200 ppm Bleach (Ecolab Inc., Saint Paul, MN, USA) or 100ppm Hypochlorite (Ecolab Inc., Saint Paul, MN, USA) for 15 s. Then, they were rinsed thoroughly and gently with peptone water for 4 times before they were treated by the ultrasonicator. All subsequent steps were conducted as described above. Each dilution was duplicated twice and each group was triplicated. The whole experiment was performed three times to enhance the reliability.

3.9 Anti-biofilm efficacy of TMS and TMS+O₂ on *L. monocytogenes* in beef juice

One hundred fifty milliliters of water were added to 1.5 lbs. of ground beef and the suspension stomached for 2 min. The beef broth (100 mL) was collected and autoclaved for 15 mins at 121 °C. *L. monocytogenes* cocktail (1 mL) suspension was added to 10 mL of the beef broth and incubated for 24 h. Then, three groups of wafers: (1) uncoated control SS, (2) TMS-coated SS, and (3) TMS+O₂ coated SS were immersed in the beef juice suspension. The wafers were placed into the beef juice bacterial suspension and incubated for 48 h at 37 °C. After that, they were thoroughly and gently rinsed with peptone water for 4 times before they were treated by ultrasonication or with sanitization step. All the steps for sonicator wash and pour plate steps were the same as described above. Each dilution was duplicated twice. The whole experiment was performed three times to enhance the reliability of the data.

3.10 Effect of cultivation broth (BHI and TSB)

Wafers were separated into two groups: (1) uncoated control SS, and (2) TMS-coated SS. The biofilm of *E. coli* O157:H7 505b and *S. aureus* FRI was cultivated on the surfaces as described above in BHI medium instead of TSB medium. After 48 h, the wafers were removed by clean forceps and rinsed thoroughly and gently with peptone water for 4 times before ultrasonication. All the steps for sonicator wash and pour plate method were conducted as described above. Each dilution was duplicated twice and each group was triplicated. The whole experiment was performed three times to enhance the reliability.

3.11 Fluorescence staining and laser confocal microscopic scanning

Both SS and HDPE wafers were divided into: (1) uncoated control group, and (2) TMS-coated group as described above. Biofilms of *E. coli* O157:H7 cocktail (on HDPE wafers, with TMS+O₂ group), *S. aureus* cocktail and *L. monocytogenes* cocktail (on SS surfaces) were cultivated as described above. After the biofilm was formed on the surface of SS wafers, the wafers were rinsed with peptone water to wash down the unattached cells. Then an appropriate volume of propidium monoazide (PMA) (Biotium, Hayward, CA, USA) stock was added to the culture medium to a final concentration of 50 μ M. Cells were incubated in the dark for 10 min at room temperature. The incubation vessel was gently shaken occasionally. Then, samples were exposed to a 1300W halogen lamp (Interfit, Atlanta, GA, USA). Culture vessels

with cells were laid on ice and set 20 cm from the light source with the exposure time set to 10 min. The cells were then fixed with fixative (2% paraformaldehyde, 2% glutaraldehyde in 100 mM sodium cacodylate buffer, pH=7.35, provided by the Molecular Cytology Core Facility, University of Missouri, Columbia, MO) for 10 min to ensure that all the pathogens were killed. After that, the wafers were rinsed with peptone water. An appropriate volume of Hoechst 33342 (Life Technologies, Eugene, OR, USA) was added again for a final concentration of 1 μ M. Cells were stained for 30 min in the dark at room temperature. After staining, the incubation chamber was placed on the microscope stage and cells were imaged on a confocal microscope (Leica TCP SP8 MP, Buffalo Grove, IL, USA) using 405 nm (Hoechst 33342) and 510 nm (PMA) excitation laser lines. Several random areas were selected to ensure the consistency of the result. The whole experiment was repeated three times to ensure the accuracy.

3.12 Scanning electron microscopy (SEM) and energy dispersive x-ray spectroscopy (EDS)

SS wafers were divided into: (1) uncoated control group, and (2) TMS-coated group as described above. Biofilms of *E. coli* O157:H7 cocktail, *Salmonella* cocktail, *S. aureus* cocktail and *L. monocytogenes* cocktail (one more TMS+O₂ (1:4)-coated group was added) were cultivated in the same method as in the cocktail biofilm section. After the biofilm was formed on the surface of SS wafers, they were rinsed with peptone water to wash down the unattached cells. Cells were

then fixed in 2% paraformaldehyde, 2% glutaraldehyde in 100 mM sodium cacodylate buffer pH=7.35. Then, the fixed specimens were rinsed three times in 100 mM sodium cacodylate buffer pH=7.35 (Electron Microscopy Core, University of Missouri, Columbia, MO) for 20 min each and incubated in the dark at 4 °C in a secondary fixative solution of 2% osmium tetroxide in 100 mM sodium cacodylate buffer pH=7.35 for 1 h (Electron Microscopy Core, University of Missouri, Columbia, MO). Three more rinses (5 min each) were performed with 100 mM sodium cacodylate buffer pH=7.35 followed by three rinses with distilled water for 20 min each. Biofilms on wafers were then dehydrated in a graded ethanol series (10 minutes per exchange) and critical point dried with an AutoSamdri-815B (Tousimis, Rockville, MD, USA). For SEM imaging, specimens were attached to 12 mm stubs on carbon adhesive and sputter-coated (Emitech K575x Turbo Sputter Coater, Kent, UK) with platinum. Secondary electron imaging was performed on an FEI Quanta 600F environmental scanning electron microscope operated at 10 kV and spot size 6. For EDS examination, 3 wafers were used: control (uncoated chips wafer), TMS coated wafer and TMS+O₂ coated wafer. All the wafers were UV sanitized before use and placed in the same chamber that was used to run the SEM experiment. The data was analyzed by the ESPRIT 2 software. For comparison, all the other factors were similarly set (3keV, 2000X magnification). Several random areas were selected to ensure the consistency of both the SEM and EDS results. The whole experiment was repeated three times to ensure the accuracy.

3.13 Zeta potential tests on bacteria suspensions

Cultures of *E. coli* O157:H7 505b, *S. enterica* Typhimurium 788, *S. aureus* FRI, and *L. monocytogenes* 7644 were pre-enriched for 24 h before the test. An appropriate dilution of suspension in peptone water was done to adjust the final concentration to around 10^6 CFU/mL. The tests were all carried out in a zetasizer (Malvern, Worcestershire, UK) with zeta selection. Every sample was tested 5 times and an average number was taken. The whole experiment was repeated three times.

3.14 Gene expression studies

Cultures of *E. coli* O157:H7 505b and *L. monocytogenes* 7644 were used in this test. Bacteria were first cultivated under 37 °C for 24 h before the experiment. Then, 0.5 mL of bacterial culture was added to 1 mL of RNA protect reagent (), vortexed for 5 s, and incubated for 5 min at room temperature. The whole mixture was then centrifuged for 5 min at 11,000 ×g (Eppendorf Centrifuge 5415 D, Hamburg, Germany). The supernatant was discarded and residual supernatant was removed. Then, 0.5 mL of TE buffer with lysozyme was added (Qiagen RNeasy Kit, Hilden, Germany), and the mixture was vortexed for 10 s and incubated at room temperature for 30 min in a shaker incubator. Buffer RLT (Qiagen RNeasy Kit, Hilden, Germany) (0.25 mL) was added after incubation followed by vigorous vortexing. Seven hundred microliters of lysate were then transferred to an RNeasy Mini spin column placed in a 2 mL collection tube, and centrifuged for 15 s at 12,000 ×g. The precipitate was

mixed with 600 μ L Buffer RW1 (Qiagen RNeasy Kit, Hilden, Germany), transferred to the RNeasy Mini spin column, and centrifuged again for 15 s at 12,000 $\times g$ to wash the spin column membrane. Then, 600 μ L Buffer RPE (Qiagen RNeasy Kit, Hilden, Germany) was used to wash the precipitate again and the sample centrifuged for 15 s at 12,000 $\times g$ to discard the flow through. The RNA was then dissolved in 50 μ L of RNase-free water (Qiagen RNeasy Kit, Hilden, Germany).

A following step of DNA decomposition was applied by adding 5 μ L 10 \times TURBO DNase Buffer and 1 μ L TURBO DNase (Ambion, Carlsbad, CA, USA) to the RNA followed by incubation at 37 $^{\circ}$ C for 25 min. Then, re-suspended DNase inactivation reagent was added and the DNase was inactivated. RNase-free water (Qiagen RNeasy Kit, Hilden, Germany) (400 μ L) was added and mixed in. Then, 450 μ L of the mixture was transferred to a new tube and 450 μ L phenol-chloroform-isoamyl alcohol (25:24:1, pH 4.5) (Sigma Aldrich, St. Louis, MO, USA) was added and the mixture vortexed for 1 min. The mixture was then centrifuged at 16,000 $\times g$ for 2 min. The upper aqueous phase was kept and 2 μ L glycogen (Qiagen RNeasy Kit, Hilden, Germany) and 1 mL 100% ethanol (Aaper, Shelbyville, KY, USA) was added. The solution was mixed well and stored at -20 $^{\circ}$ C for 10 min to precipitate out the RNA. After that, the suspension was centrifuged at 16,000 $\times g$ for 2 min and the pellet was kept and placed in a hood until totally air dried. DNase-free water (Qiagen RNeasy Kit, Hilden, Germany) (10 μ L) was then added to suspend the RNA in. The concentration and quality of RNA were tested by a Nanodrop Lite (Thermo Scientific, Waltham, MA, USA).

The RNA suspension was then used in the cDNA synthesis step with 4 μ L 5 \times iScript reaction mix (Bio-Rad, Hercules, CA, USA), 1 μ L iScript reverse transcriptase (Bio-Rad, Hercules, CA, USA), 5 μ L Nuclease-free (Bio-Rad, Hercules, CA, USA) water and 10 μ L RNA template. The reaction temperature was set as: 25 $^{\circ}$ C, 5 min; 42 $^{\circ}$ C, 30 min; 85 $^{\circ}$ C, 5 min; and then hold at 4 $^{\circ}$ C. The cDNA synthesis was performed in a 96-well plate in a Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA).

Polymerase chain reaction (PCR) was conducted in a Light Cycler 96 (Roche, Basel, Switzerland). EvaGreen qPCR Mix (Bullseye, St. Louis, MO, USA) was used and Sybr Green I detection method was applied. For *E. coli* O157:H7 505b, primers of *gapA* (reference), *arcA* (housekeeping), *stx1*, *stx2*, and *eaeA* (virulence) were tested. For *L. monocytogenes* 7644, 16s RNA (reference), *ropB* (housekeeping), *inlA*, *inlB*, *actA*, and *hly* (virulence) were tested. The details of the gene primers are shown in Table 1. The reaction temperature was set as: 10 min at 95 $^{\circ}$ C for 1 cycle as pre-incubation, followed by 45 cycles of 95 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 1 min and 72 $^{\circ}$ C for 1 min as amplification, and finally with a melting curve (65-97 $^{\circ}$ C at 2.2 $^{\circ}$ C/s). Each gene primer test was triplicated and the average number was used. The whole experiment was performed three times to enhance the reliability.

Table 1 Target genes and primers used for gene expression.

Target		Sequence	Reference
16s RNA	Forward	TTAGCTAGTTGGTAGGGT	(Fraser and others 2003)
	Reverse	AATCCGGACAACGCTTGC	
<i>ropB</i>	Forward	TGTAATAATATGGACGGCATCGT	(Sue and others 2003)
	Reverse	GCTGTTTGAATCTCAATTAAGTTTGG	
<i>inlA</i>	Forward	GAACCAGCTAAGCCIGTAAAAG	(Werbrouck and others 2009)
	Reverse	CGCCIGTTTGGGCATCA	
<i>inlB</i>	Forward	GCAAATTTTTCCAGATGATGCTTT	(Kim and others 2005)
	Reverse	TGTCACTGCATCTGTCACACTTTT	
<i>actA</i>	Forward	TAGCGTATCACGAGGAGG	(Wiedmann and others 1997)
	Reverse	TTTTGAATTTTCATATCATTCACC	
<i>hly</i>	Forward	CATGGCACCACCAGCATC	(Köppel and others 2013)
	Reverse	ATCCGCGTGTTTCTTTTCGA	
<i>gapA</i>	Forward	ACTTCCGTGTTTCGATGCTAAA	This study
	Reverse	GGTCCAGAACTTTGTTGGAGTA	
<i>arcA</i>	Forward	GAAGCGGAAGGCTATGATGT	This study

	Reverse	GTTCTTACCCGGCAGATTGATA	
<i>stx1</i>	Forward	GTGGCATTAAATACTGAATTGTCATCA	This study
	Reverse	GCGTAATCCCACGGACTCTTC	
<i>stx2</i>	Forward	TACCACTCTGCAACGTGTCG	This study
	Reverse	AGGCTTCTGCTGTGACAGTG	
<i>eaeA</i>	Forward	GGCATGAGTCATACAATAAGAAAG	This study
	Reverse	ATCGTCACCAGAGGAATCG	

For data analyzing, the $2^{-\Delta\Delta Cq}$ calculation method was applied by comparing to the reference gene as shown below:

$$\Delta Cq (\text{treatment}) = Cq (\text{treatment}) - Cq (\text{reference gene})$$

$$\Delta Cq (\text{control}) = Cq (\text{control}) - Cq (\text{reference gene})$$

$$\Delta \Delta Cq = \Delta Cq(\text{treatment}) - \Delta Cq(\text{control})$$

$2^{-\Delta\Delta Cq}$ = the final data used

Chapter 4. Results and Discussion

4.1 Contact angle test

The water droplet contact angle on uncoated stainless steel (SS) was about 68° (Fig 7 a), whereas that for trimethylsilane (TMS) coated SS was around 106° (Fig 7b). A contact angle of less than 90° is defined as hydrophilic and that of more than 90° is defined as hydrophobic. This change in surface contact angle between the coated and uncoated SS was quite dramatic. There was also a high possibility that the surface energy was changed because there was another layer of material (TMS) on the surface that contains carbon, silicon, oxygen which will affect the surface energy.

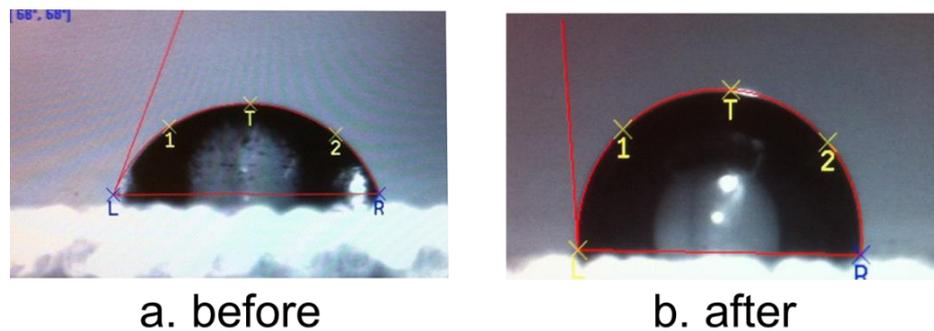


Figure 7 Contact angle change on SS wafer before and after TMS coating.

4.2 Optimization of ultrasonication treatment time

There was a clear increasing trend in *Listeria monocytogenes* count when the ultrasonication washing time was increased from 2 to 4 times (Figure 8a). But as the wash time increased from 4 to 8, the final count fluctuated slightly but not significantly ($P>0.05$). In Figure 8b, the final total count of *L. monocytogenes* on the TMS coated SS wafers dropped as the ultrasonication washing time increased from 2

to 8 times. However, no significant difference between the 2, 4, and 6 wash times ($P>0.05$) was observed. The count on the uncoated control group was around 10^7 CFU/wafer, which was significantly higher ($P\leq 0.05$) than that in the TMS coated SS group count (10^5 CFU/wafer). This provided some preliminary sense of the anti-biofilm efficacy of the TMS coating on *L. monocytogenes*.

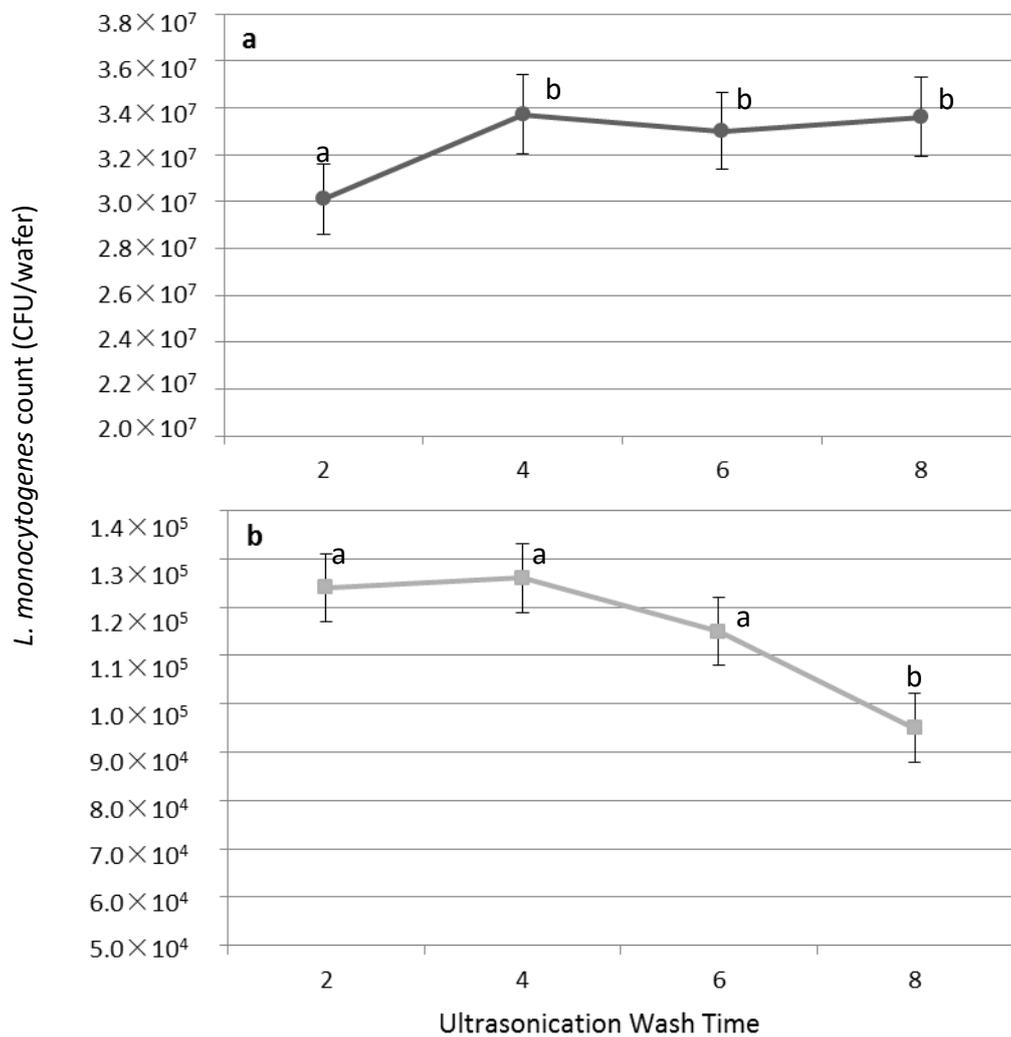


Figure 8 Relationship between wash time and bacterial count on untreated (a) and TMS treated (b) SS wafers (different letters indicate significant differences ($P=0.05$) from the control)

The reason why the two groups showed such different trends in counts can be

explained by their different initial counts. In the control group, the bacterial count was much higher than that in the TMS group, thus, during the first two times of the ultrasonication washes, not all the cells could be washed off thoroughly since the biofilm layer was so thick. As the wash time increased, more cells were washed off, but at the same time, some of the previously washed off planktonic cells or even the newly washed off cells were more susceptible to the ultrasound wash damage, since ultrasound can do damage to and even kill microbes them (Piyasena and others 2003). Because some of the cells were being killed by the ultrasonication treatment at the same time as when some cells were washed off to make up the dead cells, this made the total count constant and as the wash time increased. But in the TMS group, the initial count was so low that most could be washed off within two ultrasonication washes. With the increase in wash time, more cells were killed, hence the continued decreasing trend in count in the TMS group. In this research, the same number of wash time was needed so parallel experimental conditions could be maintained for the control and treatment groups since the ultrasonication could also affect the results either by washing off more cells or killing them (Piyasena and others 2003). For the control group, 4, 6, and 8 ultrasonication wash times were the optimal, while for the TMS group, 2, 4, and 6 were the optimal count time. When combining the two results, either 4 or 6 ultra-sonicate wash time was acceptable, thus, the 4 ultrasonication wash time was applied in all following experiments.

4.3 Anti-biofilm efficacy on stainless steel wafers - single strains

There was a significant difference in counts between the control group, TMS+O₂ (1:4) group and TMS group for *L. monocytogenes* SCOTT A on SS surfaces. As compared to the control uncoated group, counts of this pathogenic biofilm for the

TMS+O₂ group decreased by more than 1 log CFU/wafer (Figure 9). Moreover, the TMS group count decreased to around 2 log CFU/wafer, indicating that the cells in the biofilm was reduced by 99% just with the TMS coating. However, in the *Escherichia coli* O157:H7 505B and *Staphylococcus aureus* FRI groups, the reductions were not as great as that for the *L. monocytogenes* SCOTT A groups. In *S. aureus* FRI groups, compared to the control uncoated group, counts on the TMS+O₂ group decreased by only 0.5 log CFU/wafer and that of the TMS group decreased by around 1 log CFU/wafer. In the *E. coli* O157:H7 505B groups, as compared to the control uncoated group, counts for the TMS+O₂ and TMS groups barely went down. But there was still a significant difference between the control and the two treatment groups ($P \leq 0.05$). The result suggested that although the TMS+O₂ and TMS coatings could significantly reduce the total *L. monocytogenes* SCOTT A count in the biofilm, the efficacy was mild for *S. aureus* FRI or even less for *E. coli* O157:H7 505B. Also, in the species tested, the different plasma coating (TMS+O₂ group and TMS group) also functioned differently. For the most part, there were quite big differences in counts between the TMS+O₂ and TMS groups (except for *E. coli* O157:H7 505B).

Several strains of *L. monocytogenes* (SCOTT A, V373E, 7644) were tested and significant differences ($P \leq 0.05$) among their reductions were found. As shown in Figure 9, there was only around 1 log CFU/wafer reduction of strain V373E while at the same conditions, strains SCOTT A and 7644 demonstrated around a 2 log CFU/wafer reductions. Also, the TMS+O₂ coating appeared to be more efficient than the TMS coating at reducing counts of V373E. In the SCOTT A group, there was around a 1 log CFU/wafer difference between the TMS+O₂ and TMS groups, but when it came to 7644, the change became less significant. This indicated that even in the same strain, the anti-biofilm efficacy of the coatings varied.

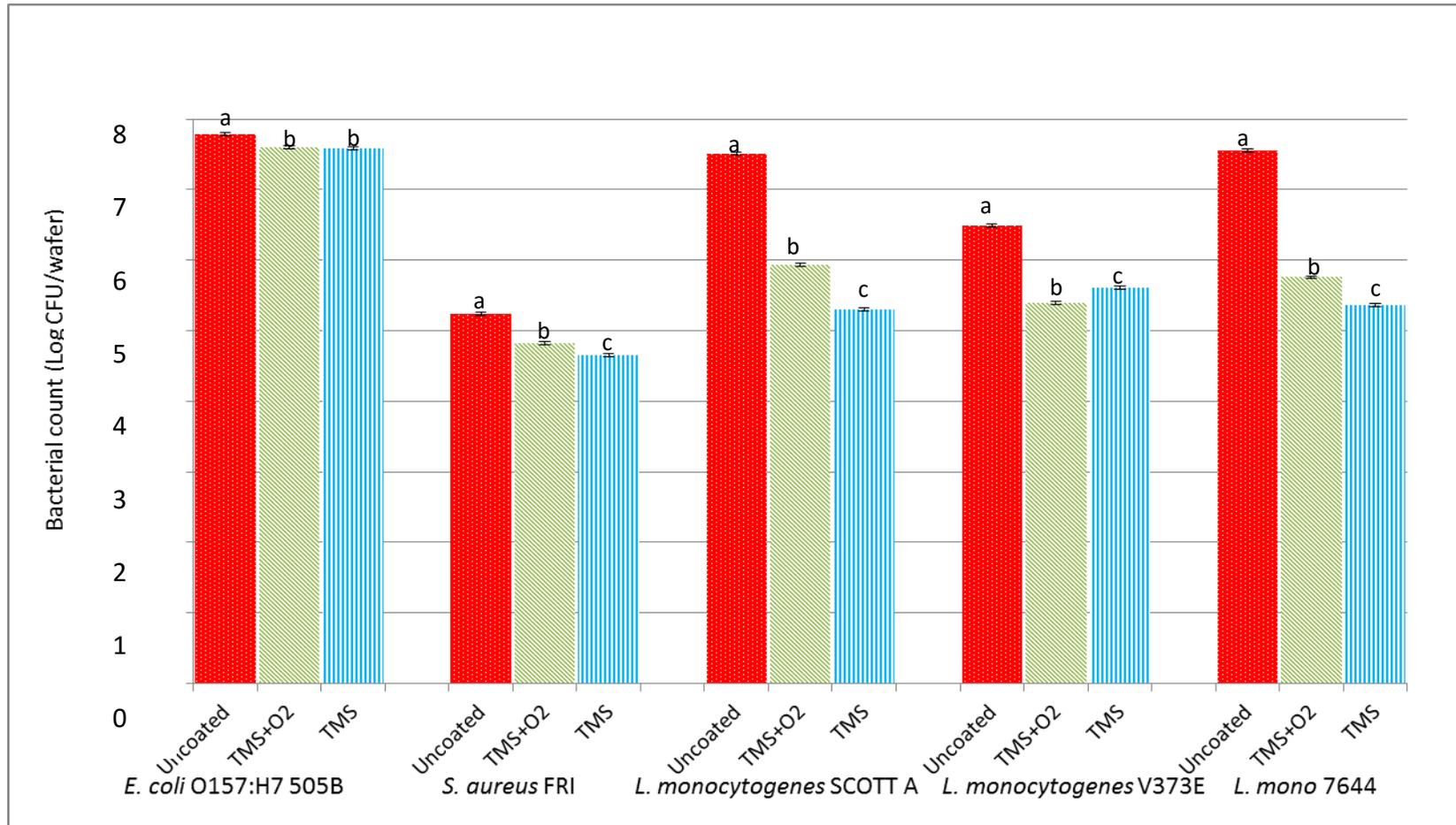


Figure 9 Efficacies of TMS and TMS+O₂ on single pathogenic strains of *E. coli* O157:H7, *S. aureus* and *L. monocytogenes*.

(different letters indicate significant differences ($P=0.05$) from the control)

Other than the pathogenic strains, several non-pathogenic single strains (*E. coli* P24, *Listeria innocua* and *Salmonella enterica* ATCC 53648) were also examined (Figure 10). There were no big reductions in counts among these strains and there was even an insignificant increase ($P>0.05$) in *E. coli* P24 TMS+O₂ group. In the *S. enterica* ATCC 53648 groups, around a 0.5 log CFU/wafer reduction was found in the TMS+O₂ group, and an even lower reduction in the TMS group was found for this strain. These results verified our findings above on the variable reductions in biofilm cell counts conferred by the coatings that are species and strain dependent.

Our results implied that the anti-biofilm efficacy of the TMS coating varies between bacterial strains as well as between coating methods (TMS or TMS+O₂). As part of the preliminary tests, the result showed some potential. However, more tests on different stains are needed and thus more cocktails were tested in the following tests to mimic real life conditions.

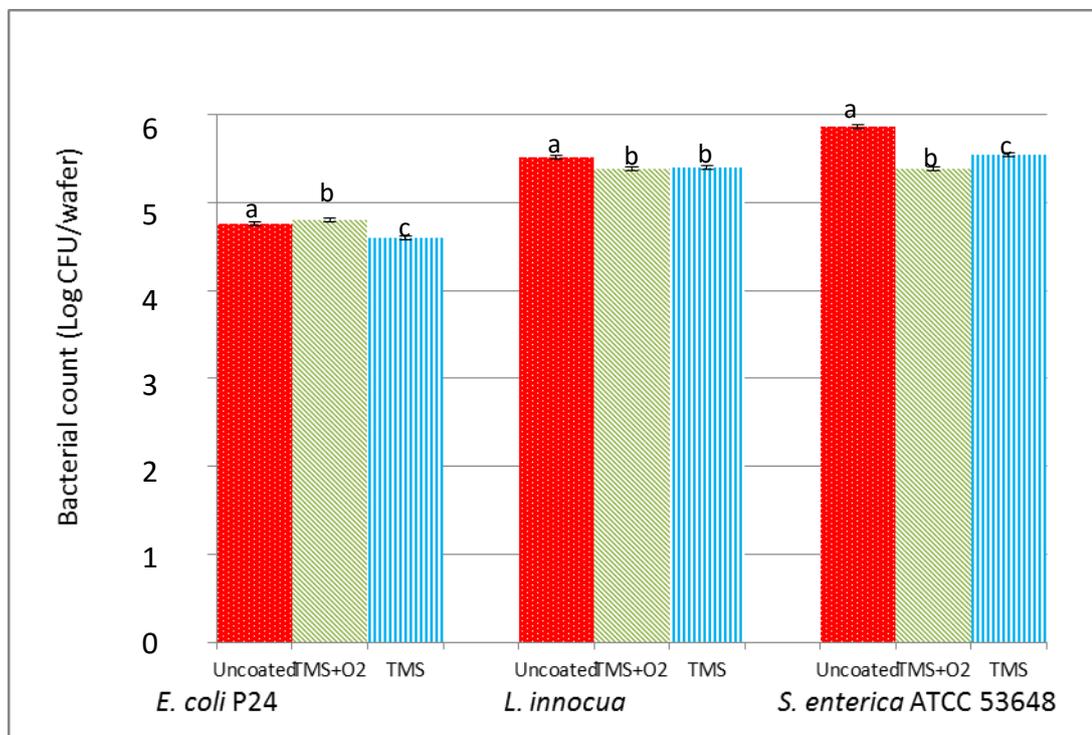


Figure 10 Efficacies of TMS and TMS+O₂ on single non-pathogenic strains of *E. coli*, *Listeria* and *Salmonella* (different letters indicate significant differences ($P=0.05$) from the control).

4.4 Anti-biofilm efficacy on stainless steel and high-density polyethylene (HDPE) – strain cocktails

No significant reduction in counts of *E. coli* O157:H7 cocktail biofilm in the TMS+O₂ (1:4) group was observed when compared to the uncoated control group on SS surfaces (Figure 11). However, there was around a 1 log CFU/wafer reduction of counts in the TMS group as compared to the control. The reduction increased when it came to *S. aureus* cocktail biofilms, whereby the counts in the TMS+O₂ and TMS's group were reduced by 2 log CFU/wafer compared to the control. For *L. monocytogenes*, both TMS+O₂ and TMS group counts were reduced by 2 log CFU/wafer compared to the uncoated group count. However, in the cocktail, the anti-biofilm efficacy of TMS+O₂ coating appeared to be higher as compared to the TMS group. This result can be explained by the single strain results. For single strain *L. monocytogenes*, part of the TMS+O₂ coated surfaces achieved lower counts, such as *L. monocytogenes* V373E. Because a cocktail is the combination of all the mixed cells together, the condition is more complex. In a cocktail, all strains are living together, which could result in synergistic or antagonistic interactions among the cells (Hibbing and others 2010). As a result, the final counts would not be an easy sum of all the single strain results. Some species can affect more of the final count than others. Thus, as a consequence, the cocktail results were slightly different from those of the single strains, even though most of the time, they are still parallel.

In the results of non-O157 *E. coli* cocktails, the count in the TMS group increased slightly compared to the uncoated control groups. However, the increment was not very high, at only around 0.5 log CFU/wafer. From this perspective, TMS and TMS+O₂ plasma coating was not effective on non-O157 *E. coli* cocktails. This result demonstrated that even though TMS and TMS+O₂ plasma coating were quite

effective on *S. aureus* and *L. monocytogenes* strain cocktails, there are shortcomings of these plasma coatings that need to be addressed if the coatings are going to be applied in real life.

On HDPE surfaces, the results showed significant reductions ($P \leq 0.05$) (Figure 12). Although in *S. aureus* and *L. monocytogenes* cocktails, barely any significant reduction ($P \leq 0.05$) could be observed (the most significant reduction was for TMS+O₂ in *L. monocytogenes* cocktails.). For *E. coli* O157:H7, the reduction became quite clear in both TMS and TMS+O₂ groups. The reduction was around 2.5 log CFU/wafer compared to control. This result was totally different from those obtained for SS surfaces (Figure 11).

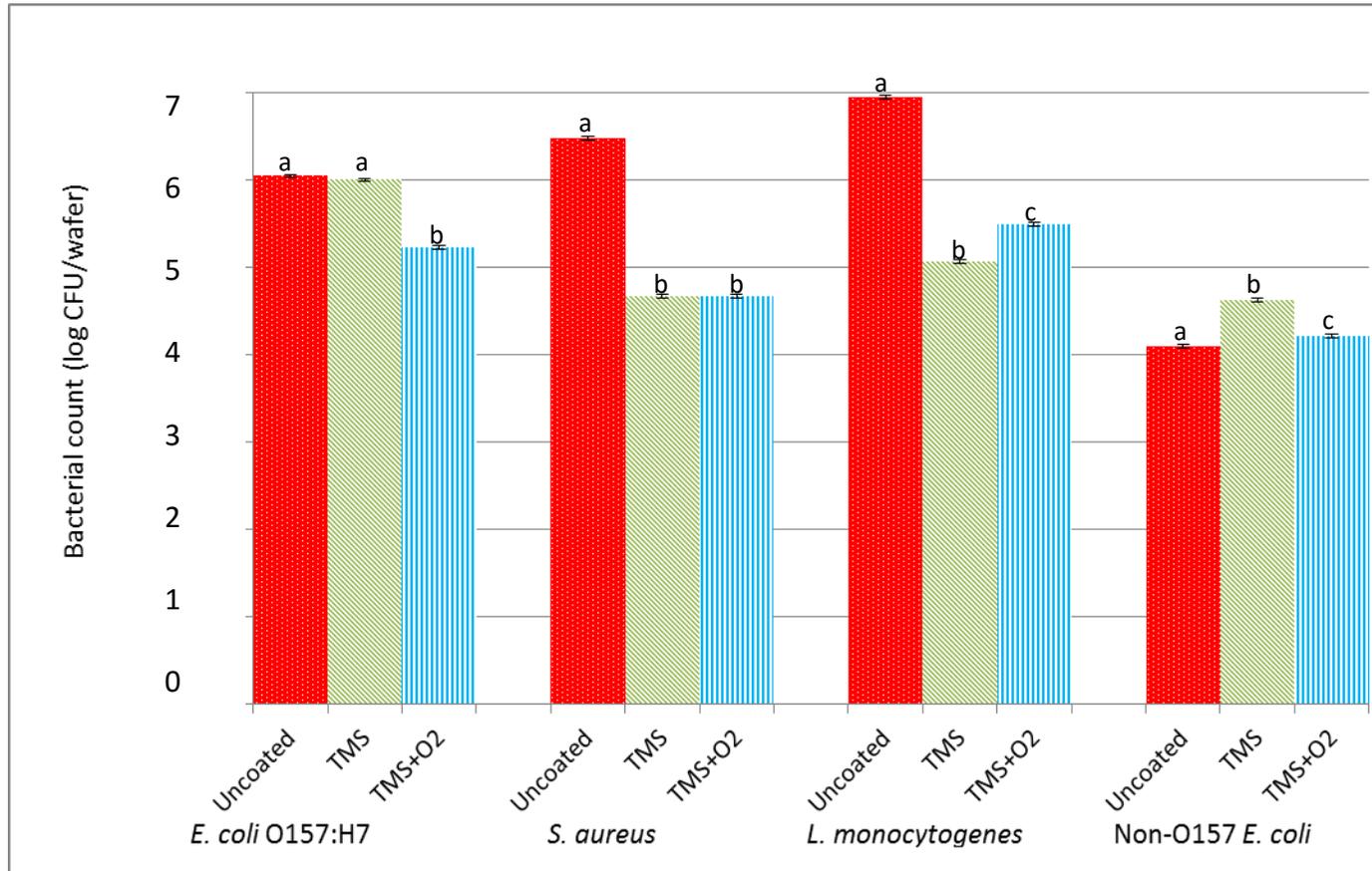


Figure 11 Efficacies of TMS and TMS+O2 on SS surfaces on bacterial cocktails (different letters indicate significant differences (P=0.05) from the control).

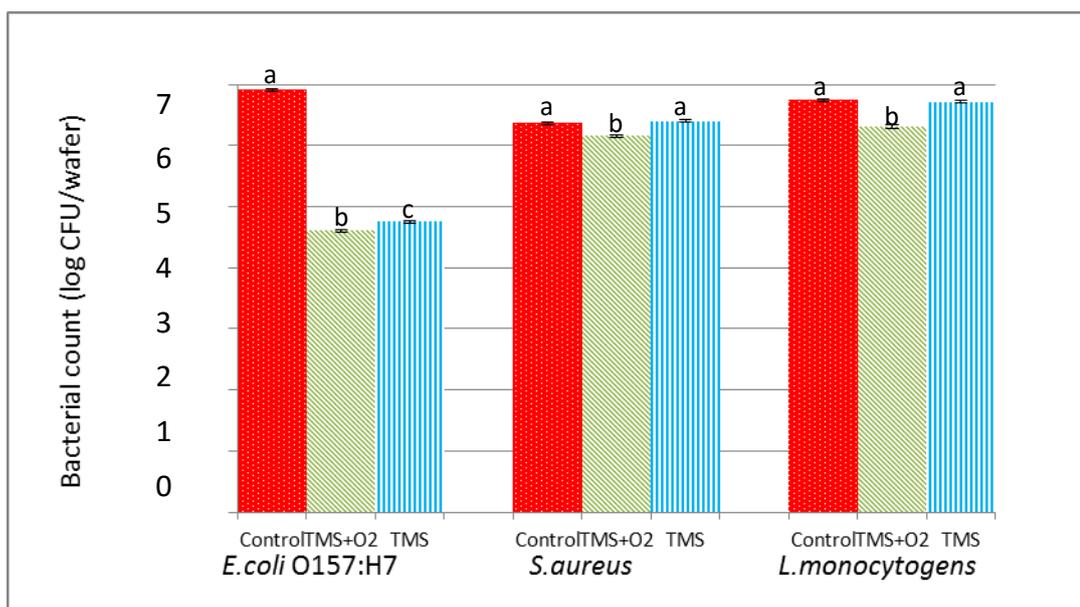


Figure 12 Efficacies of TMS and TMS+O₂ on bacterial cocktails on HDPE surfaces (different letters indicate significant differences (P=0.05) from the control).

These results led to the questions of why different strains acts differently and why different plasma coated surfaces resulted in different levels of bacterial reductions. As reported by other researchers, bacterial attachment to a surface is related to surface factors, such as roughness, chemical composition, absolute values of zeta potential, and reactivity (Tsuneda and others 2003; Wu and others 2011; Ma and others 2012a). For example, a previous study on *Staphylococcus epidermidis* showed that the cells are prone to attach to less hydrophobic acrylic surfaces than the more hydrophobic silicone surfaces, which means that if the surface of a silicone was changed to acrylic, the attachment of *S. epidermidis* will be reduced (Sousa and others 2009). In our research, SS or HDPE was coated with TMS or TMS+O₂ which contains carbon, silicon, and oxygen, and a change of hydrophobicity was detected. This change, as previous studies showed, could lead to the differences in initial bacterial attachment and affect the final biofilm formation. What is more, the surface

property changes can also lead to the differences in protein attachment which will also affect bacterial attachment (Roach and others 2005). As a result, the change of certain surface properties will affect the attachment of bacteria to a surface.

It was also found that even for the same surface, different bacterial strain acts differently. On the same surface of polydimethylsiloxane (PDMS), *S. aureus* can attach well, but *S. epidermidis* cannot attach to it easily. Even for the same species but different strains, bacterial attachment varies (Jiang and others 2004; Girshevitz and others 2008). The reason for why different bacteria act differently is still unclear. It is reported that pH of the environment and surface charges can also affect the adhesion process significantly (Stenström 1989).

The results of our experiments and that of previous studies are supportive. The starting surface contact angle and surface energy of SS surface were different from those of the HDPE surfaces. Hence, the change of the surface contact angle can lead to a change in bacterial attachment, which in turn, affects the formation of biofilms (Ma and others 2012a; Chen and others 2013).

Different bacterial strains have different preferred contact angles or surface energy. Thus, if the surface contact angle and surface energy (surface charge) were closer to the cells' preferred conditions, more bacteria will adhere to the surfaces. On SS surfaces, the initial contact angle and surface energy were more preferred by *S. aureus* and *L. monocytogenes*, thus more of these bacterial cells attached to the surfaces of SS. However, as soon as the surface contact angle and surface energy were changed by TMS and TMS+O₂ coating process, less bacteria attached to the surfaces since the after coating surfaces were less preferred by *S. aureus* and *L. monocytogenes* (Figure 11). With HDPE surfaces, over the results were different. The original contact angle and surface energy of HDPE were more preferred by *E.coli* O157:H7, but after

coating, the surface contact angle and surface energy were changed and thus fewer bacterial attached to the surfaces. For *S. aureus* and *L. monocytogenes*, HDPE surfaces were not preferred by them at the beginning, thus even after TMS coating, their counts did not change significantly (Figure 12). The increase in numbers of non-O157:H7 *E. coli* could also be explained. In the beginning, SS surfaces were not preferred by non-O157:H7 *E. coli* cells, but after TMS coating, the surface contact angle and surface energy became more conducive for non-O157:H7 *E. coli* cells to attach, thus there was an increasing on the total count (Figure 10).

Overall speaking, although there were times when increasing counts were observed, TMS and TMS+O₂ nanoplasma coating were quite effective against some pathogenic strains and species. However, the technique could not work equally well on all species and all surfaces. The final efficacy of the coating depends on the bacterial stain and the coating surface, as well as the coating material (TMS or TMS+O₂).

4.5 Bacterial suspension concentration study

In the bacterial suspension study, total bacterial concentration in a suspension was studied to determine if the plasma coating can affect the counts in a bacterial suspension. The result showed no reduction in counts for the TMS coated treatment group as compared to the control group (Figure 13). This result implied that TMS plasma does not significantly kill the bacteria in the suspension, which points to the fact that the TMS coating is likely not an antibacterial surface coating. It is a non-selective coating method which does not kill bacterial cells and lead to selective results while at the same time reducing the biofilm cell count for certain surfaces. Conversely, this result also supported our theory that the change of surface contact

angle and surface energy would not result in bactericidal effects.

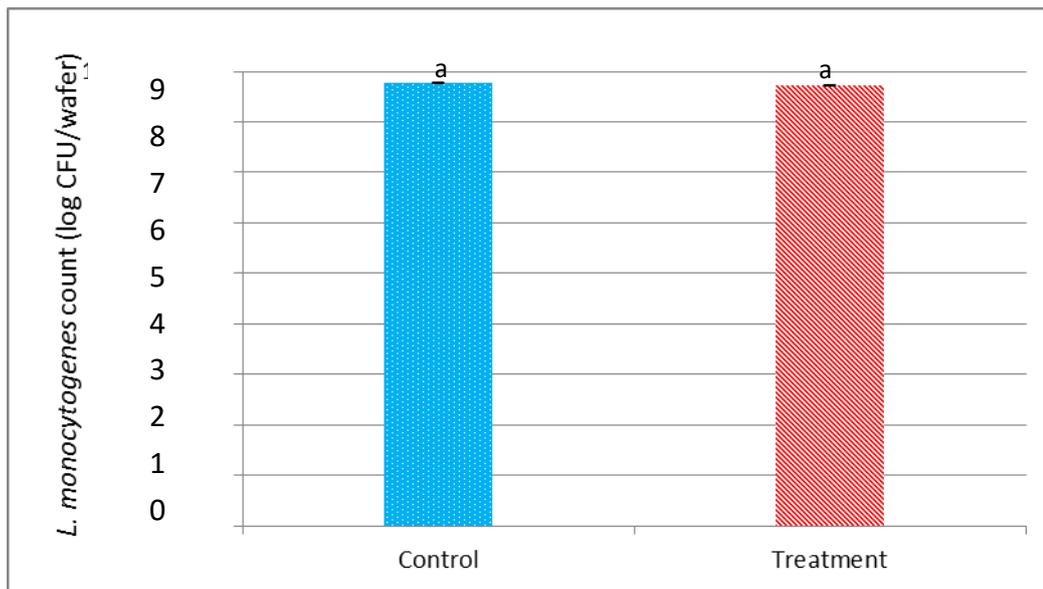


Figure 13 Bacterial suspension concentration study on TMS-coated SS.

4.6 Hurdle concept of coatings with sanitizer

In the hurdle concept test (Figure 14), a Quat sanitizer was combined with the coating method to evaluate any synergistic effects between the two on SS surfaces against *L. monocytogenes*, *S. aureus* and *E.coli* O157:H7. In *L. monocytogenes*, when Quat was applied alone, the control count fluctuated slightly, depending on the cleaning process. But all the counts were between 10^4 to 10^6 CFU/wafer. On the other hand, less than 10 CFU/wafer was obtained in the TMS+Quat group. This made the final reduction around 10^3 to 10^5 (as compared to Quat only control). At the same time, when TMS coating were applied alone, around 2 log CFU/wafer reductions were observed (Figure 7). But when combined with Quat sanitizer, the reduction reached around 10^3 to 10^5 (Figure 14) which is a significant difference ($P \leq 0.05$) from the Quat on uncoated SS. This indicated that there were synergistic effects when these two methods were applied together and they can be combined as a hurdle technique when applied to the food industry (JH Han and others 2003). However, the synergistic

effects were not clear in *S. aureus* and *E. coli*, where the reduction is almost similar to the single strain result in Figure 7. This might be related to the initial reduction and bacterial characteristic. *S. aureus* and *E.coli* showed a 1 log/wafer or less reduction but *L. monocytogenes* started from 2 log/wafer reduction which has more potential to be enhanced when combined with sanitizer. We can also observe the increasing trend when comparing *S. aureus* and *E.coli* in Figure 7 and 14. However, more following tests will be needed to testify the anti-biofilm efficacy in real food processing conditions.

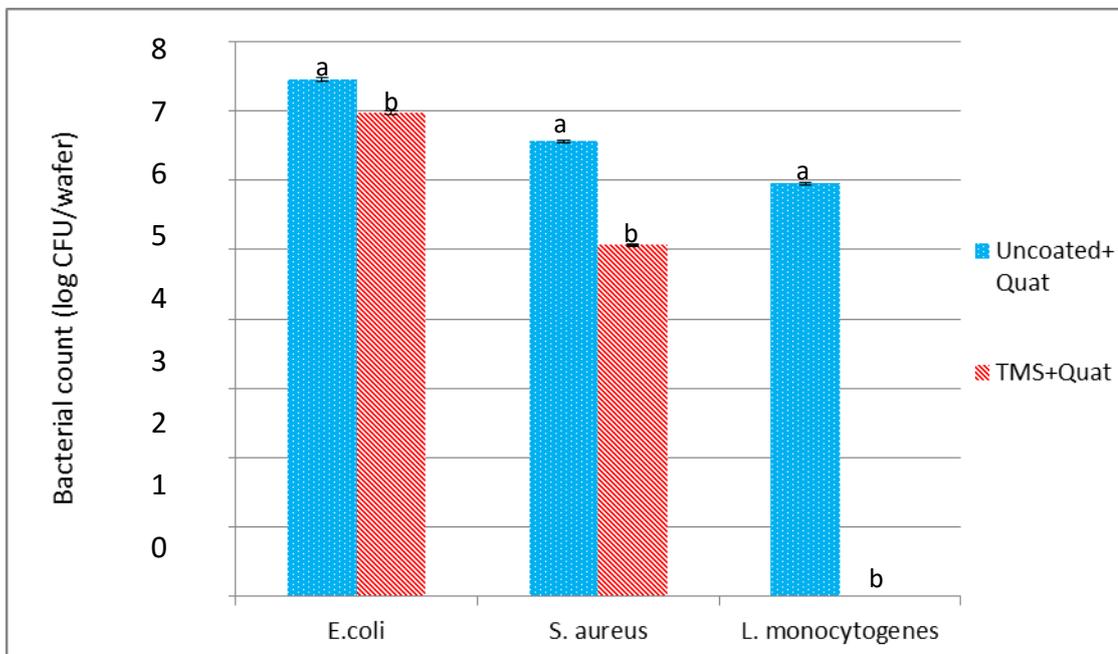


Figure 14 Anti-biofilm effects of TMS coating (on SS) with the application of Quat sanitizer on different species (different letters indicate significant differences ($P=0.05$) from the control).

4.7 Trial in beef juice

In a beef juice test (Figure 15a), the combination of Quat and TMS coating on SS were applied to *L. monocytogenes* or *E. coli* O157:H7 biofilms in beef juice to

mimic the real environment in meat processing plants and Quat with uncoated surfaces were used as control. From Figure 15, a significant reduction ($P \leq 0.05$) could be observed in the uncoated+Quat group and TMS+Quat group when compared to the control (uncoated without Quat) group. The result indicated that the Quat sanitizer could kill *L. monocytogenes* in the biofilm although the effect was quite limited partly because of the limited time and mild sanitizing effects of Quat. When TMS coating was incorporated, more reductions could be observed as the final count reached less than 10 CFU/wafer. When compared to the Quat treatment, the TMS+Quat combination worked better, implying the synergistic effect of a hurdle technique. More practical tests will be needed before TMS coating can be applied in the food industry.

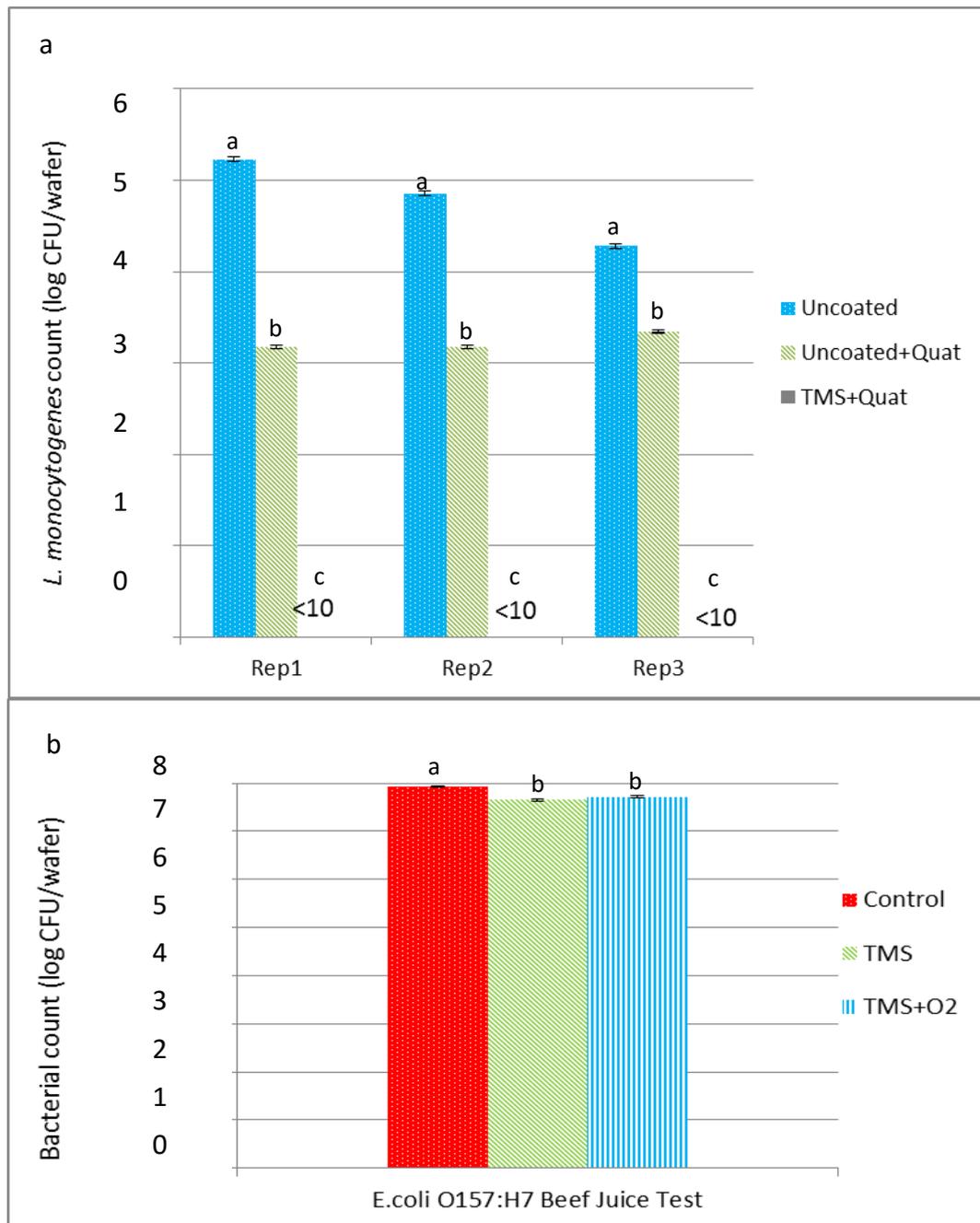


Figure 15 Anti-biofilm effects of TMS coating with the application of Quat sanitizer on *L. monocytogenes* SCOTT A biofilm in beef juice on SS (a), and anti-biofilm effects of TMS coating on *E. coli* O157:H7 on HDPE surface without Quat (b)(different letters indicate significant differences (P=0.05) from the control).

On HDPE surfaces, a 2 log CFU reduction in medium level test was achieved, but when it came to the beef juice, the reduction became 50% (data not shown). Multiple reasons can lead to this huge change. According to our following tests, we found the cultivation medium can also affect the antibiofilm efficacy quite a bit (Figure 15), which might be the major reason why HDPE worked quite well on medium but just so-so when it was in beef juice.

At the same time, no significant reduction ($P>0.05$) was found between the uncoated+Quat and TMS+Quat for *E. coli* O157:H7 or *S. aureus* biofilms in beef juice when the Quat sanitizer was incorporated (all round 10^5 log CFU/wafer). For the synergistic effects, only tried two kinds of sanitizers were tested, bleach and Quat, and only Quat showed synergistic effects with the coating on *L. monocytogenes* here. Bleach (200 ppm) was so strong that it killed all the bacterial cells within the *L. monocytogenes* biofilms (data not shown). The reduction in *L. monocytogenes* might be related to the original biofilm cell reduction. Without sanitizer, the TMS coating succeeded in reducing the *L. monocytogenes* count by 2 log CFU/wafer. The sanitizer further also killed the bacteria at the same time, hence the synergistic effects. For future studies, other commercial sanitizers could be tested. There might be some other sanitizers like phenolics, alcohols, that might have synergistic effects when applied to certain species. For example, phenolics sanitizer with TMS might have a synergistic effect that can reduce *S. aureus* biofilm count greatly, or there might be other combinations, that can help increase the antibiofilm efficacy and render surfaces in better hygiene condition.

4.8 Effects of cultivation broth (BHI and TSB)

In Figure 16, the TMS anti-biofilm efficacy seems to be weakened when *S.*

aureus were cultivated in BHI compared to plasma coating anti-biofilm efficacy of *E. coli* O157:H7 and *S. aureus* in TSB medium. This indicated that another factor (growth medium or matrix) may also affect the anti-biofilm efficacy of TMS coating.

In the previous tests, it was found that the final efficacy of the plasma coating depends on the bacterial stain, the features of the initial surface, and the coating method (TMS or TMS+O₂). Here, we figured out another parameter which may affect the efficacy of TMS plasma coating method: the environment (or the chemicals in the environment). As the medium was changed, the anti-biofilm efficacy of TMS coating on *S. aureus* was reduced from 1 log CFU/wafer to nearly no reduction (Figure 9 and 16). Even though everything was kept the same, even the cultivating time and strain, just the change of TSB medium to BHI medium resulted in a change in the final reduction. Researchers found environmental factors like pH, temperature, nutrient composition and population characteristics of bacteria can affect the formation of biofilm. For *L. monocytogenes*, the best adhesion environment to stainless steel is 30 °C and pH 7, but for *Yersinia enterocolitica* it became pH 8-9 (Herald and others 1988a; Herald and others 1988b). Different media have different nutritional compositions and might lead to minor changes of bacterial metabolic properties. Also, the contact angles of BHI and TSB on the same surfaces are different (data not shown). As discussed above, the change in bacterial characteristics and the surface contact angles could lead to a change of the anti-biofilm efficacy of the coatings. Further, the proteins in a medium can also affect the formation of biofilms (Fletcher 1976; Roach and others 2005), which was demonstrated with this experiment. The antibiofilm efficacy of the TMS plasma coating is more complicated than predicted and more detailed studies will be needed to understand more about the mechanism of its anti-biofilm activity.

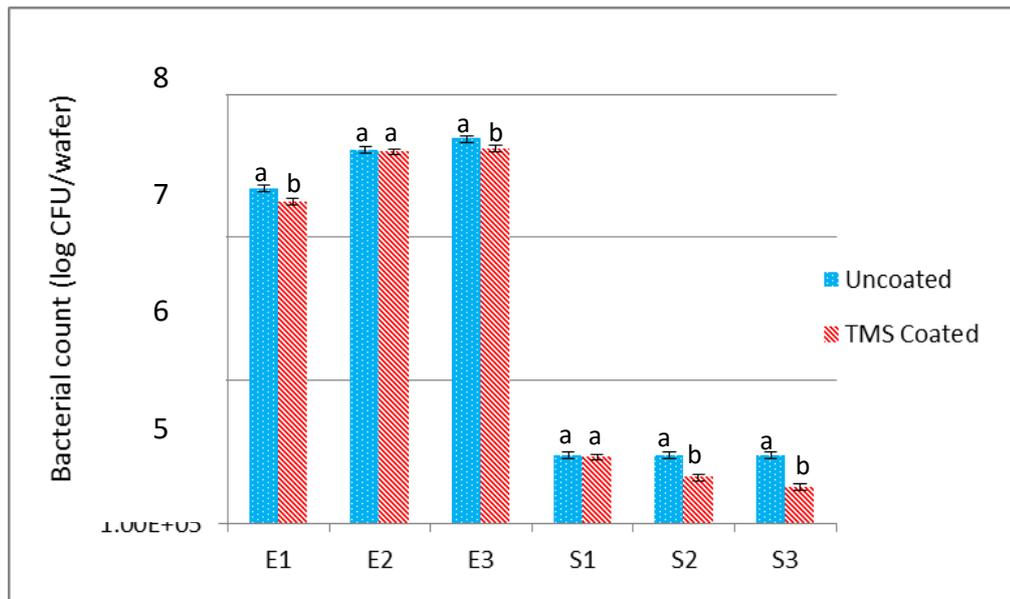


Figure 16 Effects of changing cultivation medium on SS (E=*E. coli* O157:H7 and S=*S. aureus* in BHI) (different letters indicate significant differences (P=0.05) from the control).

4.9 Fluorescent staining of adherent bacterial - laser confocal microscopic scanning

From Figure 17 it is observed that the bacterial cell numbers decreased significantly in the TMS group when compared to the control group. This result is parallel to the pour plate count for *L. monocytogenes*. The live and dead ratios were quite close in both the control and TMS groups as few dead cells (red) could be observed. The image implied that the TMS coating is not likely to kill bacteria on SS surface while at the same time, it can affect the attachment of *L. monocytogenes* and reduce the final count of biofilm bacteria. Thus, it has a potential to be a non-selective method to prevent the growth of biofilm.

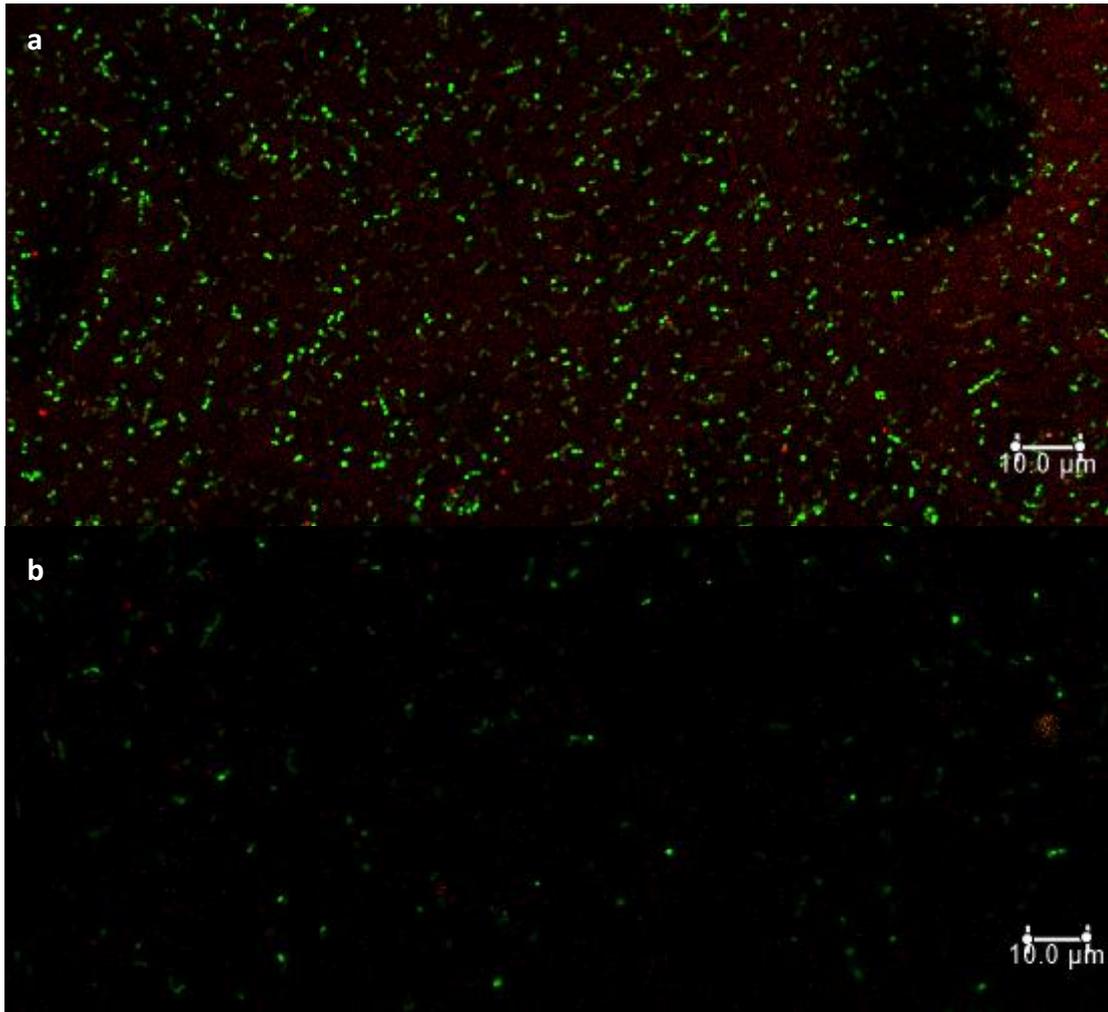


Figure 17 Live/Dead laser confocal images of *L. monocytogenes* biofilm on uncoated (a) and TMS coated SS (b). (Live cells are green and dead cells are red).

As shown in Figure 18, the TMS coated group also had less *S. aureus* count compared to the control group, which was parallel to the pour plate results. The cells in the control group that were about to form a layer of biofilm on the surface were so thick that there was hardly any effect of the TMS coating on their survival. The live and dead ratio also did not change much. Combined with the result for *L. monocytogenes*, the TMS coating may not affect the live and dead ratio significantly.

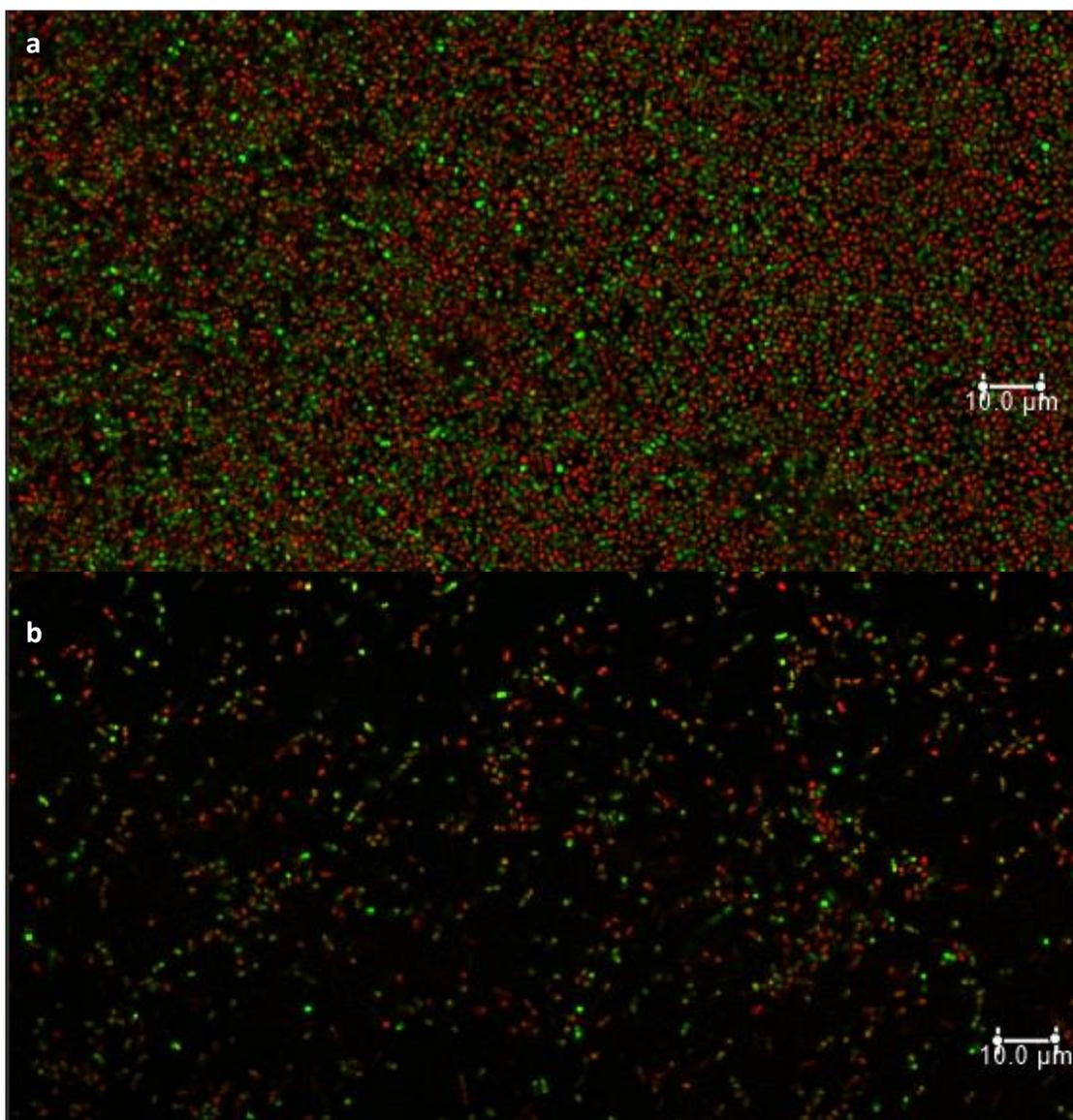


Figure 18 Live/Dead laser confocal images of *S. aureus* biofilm on uncoated (a) and TMS coated SS (b). (Live cells are green and dead cells are red).

On HDPE surfaces, it was still clear that the TMS coating reduced the numbers of *E. coli* O157:H7 505B significantly compared to the untreated group which were parallel to the pour plate results (Figure 19). Most of the cells were live cells in both images and this might support the theory that TMS coating might only reduce the adhesion of cells instead of killing them.

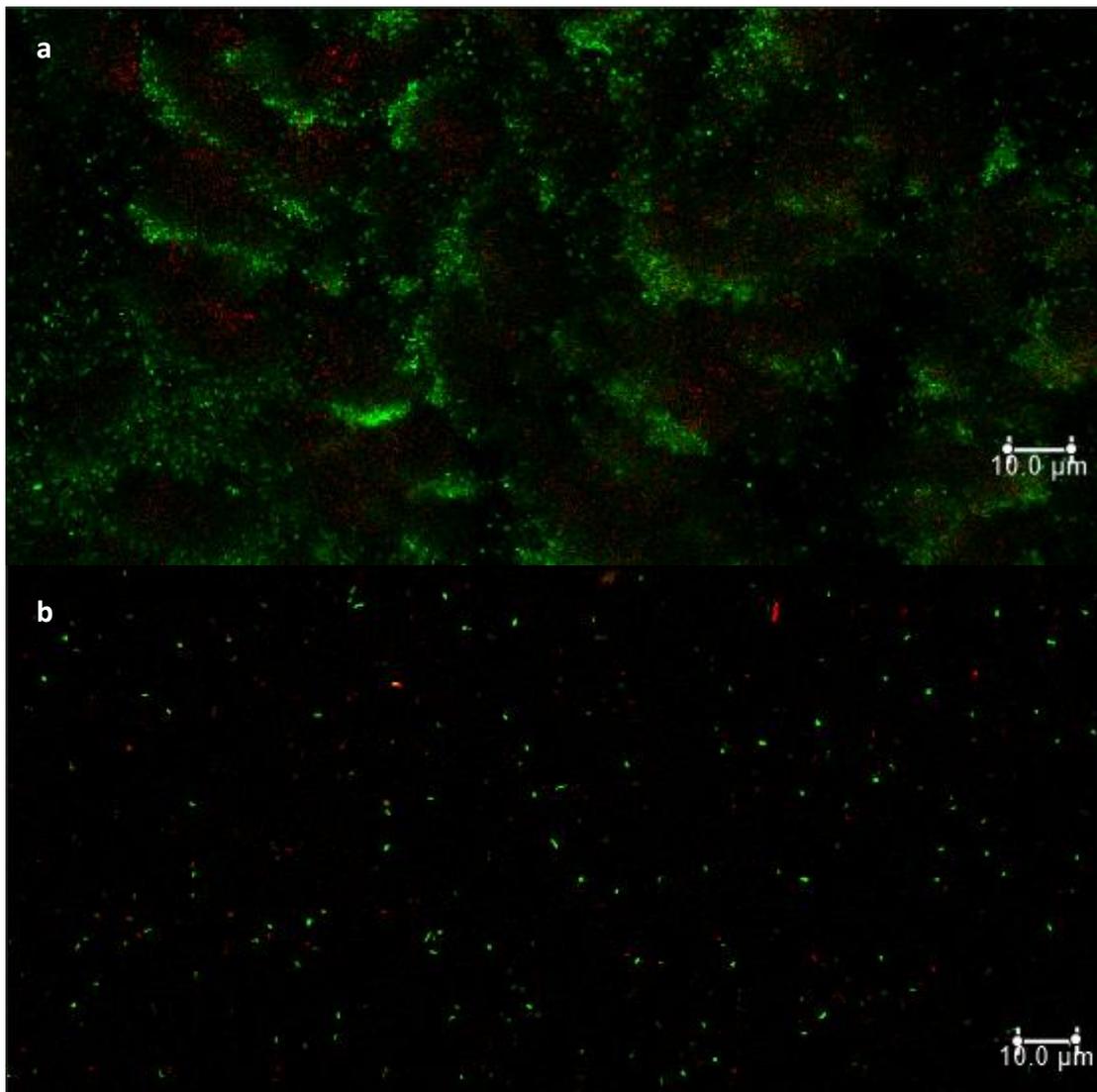


Figure 19 Live/Dead laser confocal images of *E. coli* O157:H7 biofilm on uncoated (a) and TMS coated HDPE (b). (Live cells are green and dead cells are red).

All laser confocal results were parallel with the pour plate results, as well as our previous theory that the TMS coating has anti-biofilm activity but it will not cause bacterial death and will not lead to resistant strains. However, live and dead cell ratios are only an indirect support of our theory and more researches are needed to confirm the actual anti-biofilm activity of this coating.

4.10 Scanning electron microscopy (SEM) and energy dispersive x-ray spectroscopy (EDS)

Compared to laser confocal microscopic images, SEM images are clearer and more details can be observed and studied. Among all the figures in SEM, little distortions and broken cells were found, and most of the cells looked normal (Figure 20). A significant difference in bacterial number was seen in the figures which supports the pour plate results and the theory that the TMS coating will not kill cells on coated surfaces.

As seen in Figure 20, *E. coli* O157:H7 biofilms on both control and TMS coated SS surfaces were quite thick. This finding supported the pour plate results for SS surfaces which showed only a slight reduction in *E. coli* O157:H7 on TMS coated surfaces (Figure 9). However, a trend of less thick biofilm structure can be observed. Although the count in the TMS group did not decrease significantly ($P>0.05$, Figure 9), the biofilm structure was thinner when compared to control group which showed a biofilm that is thicker, firmer and tighter (Figure 20). This might be the reason of the slight reduction in the cell count (Figure 9).

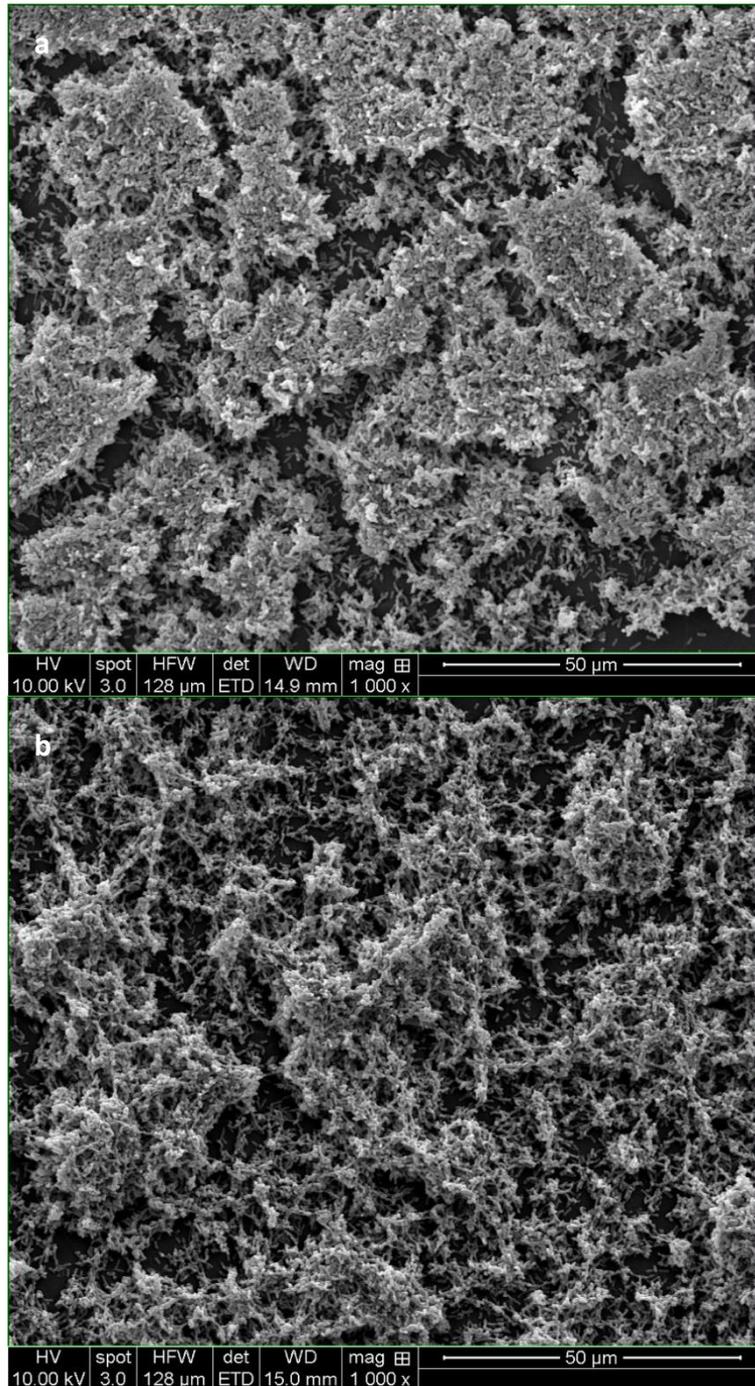


Figure 20 SEM images of *E. coli* O157:H7 biofilm on uncoated (a) and TMS coated SS (b).

For the *S. aureus* experiment (Figure 21), it was quite clear that only a small number of single cells were detected on TMS coated SS surface. On the untreated surfaces, a thin layer of *S. aureus* on the surface could be observed, and a significant reduction can be observed between the two. At the same time, no distorted shaped cells were seen. This provided us a more direct sense of how effective the TMS coating is at fighting against *S. aureus* biofilms without affecting its cell viability. Besides that, it also confirmed that compared to *E. coli* O157:H7, *S. aureus* took a longer time to form a biofilm since for the same amount of time, *E. coli* O157:H7 had already formed a thick layer of biofilm on the surfaces. This indicated that the bacterial type (Gram positive or Gram negative) should also be seriously considered as the factors that can affect the anti-biofilm efficacy of TMS plasma coating.

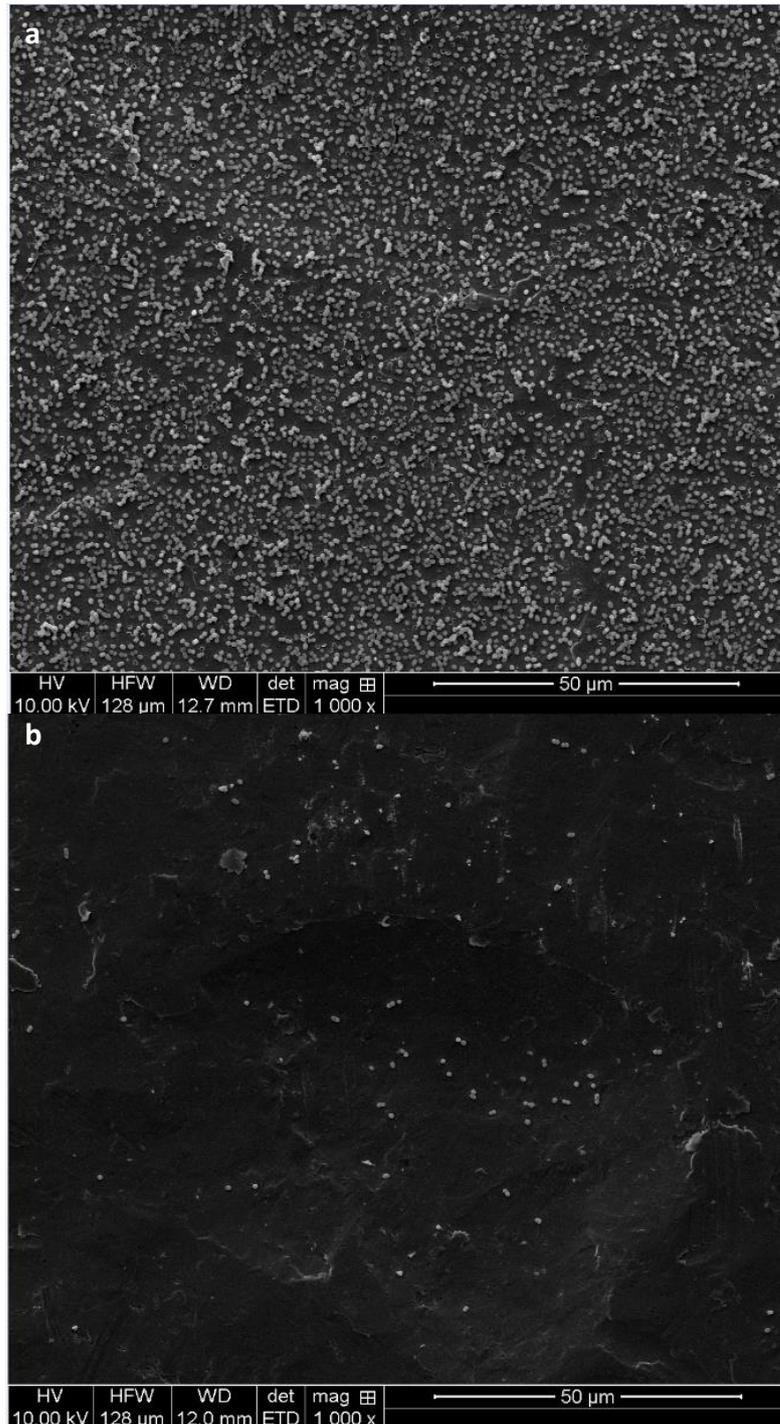


Figure 21 SEM images of *S. aureus* biofilm on uncoated (a) and TMS coated (b) SS.

In the SEM images of *Salmonella* (Figure 22), both the control and treatment groups showed high numbers of cells but their forms were different. In the control group, cells tended to stick together while in the TMS group, they were more widely

spread. However, even though the cell growing form was different, the total count was quite similar (Figure 10). Further, small numbers of distorted cells can be found in both groups. This might be related to the sample preparation reagent which dehydrated the cells slightly. Cells on the untreated control was considered to be more of a concern than those in the TMS group because cells that stick together easily will form biofilms better and once the biofilm is formed, it would be much more difficult to remove them than just a layer of evenly distributed cells.

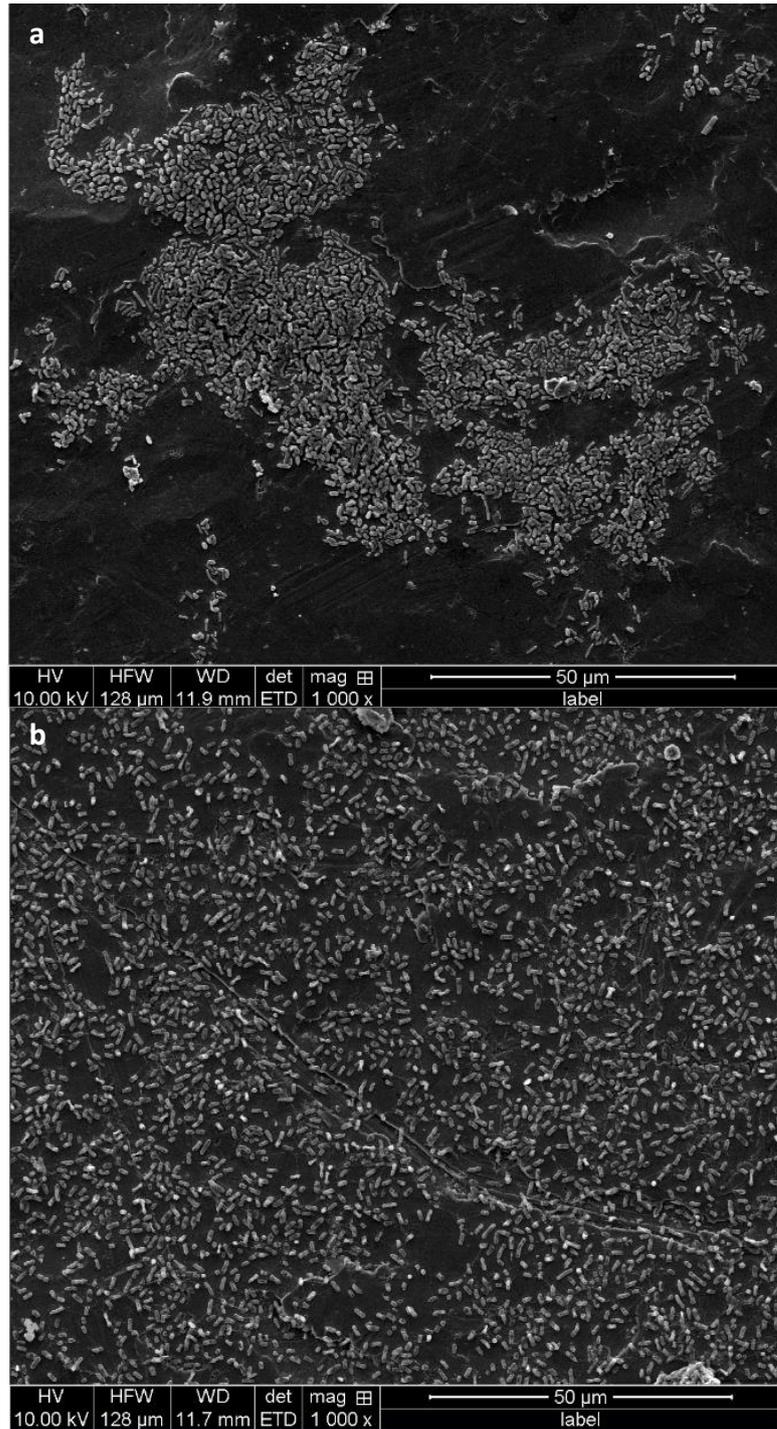
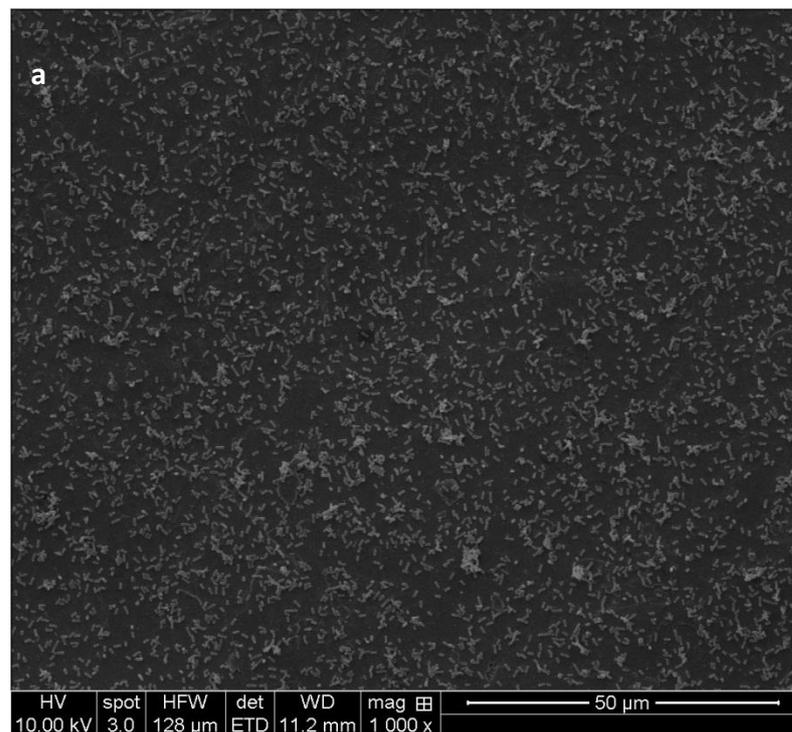


Figure 22 SEM images of *Salmonella* biofilm on uncoated (a) and TMS coated (b) SS.

In the SEM images of *L. monocytogenes* (Figure 23), the differences between the cells in the control, TMS and TMS+O₂ groups were obvious. As showed in Figure

23, some cells had already stuck together and formed small groups of cell “towers”. These tiny groups of cells are the prototype of biofilms. However, in the TMS and TMS+O₂ group, no clear signals of biofilm formation are observed, although small amount of cells can be observed. These images also supported the pour plate results. What is more, few distorted cells were observed in all groups, indicating no significant bactericidal effects of TMS and TMS+O₂ coating on this organism on coated SS surfaces.

SEM images clearly show how the bacterial biofilms look like and provided us sound evidence of the anti-biofilm activity of the TMS plasma coating. However, figure out the actual anti-biofilm mechanism, more works are still needed.



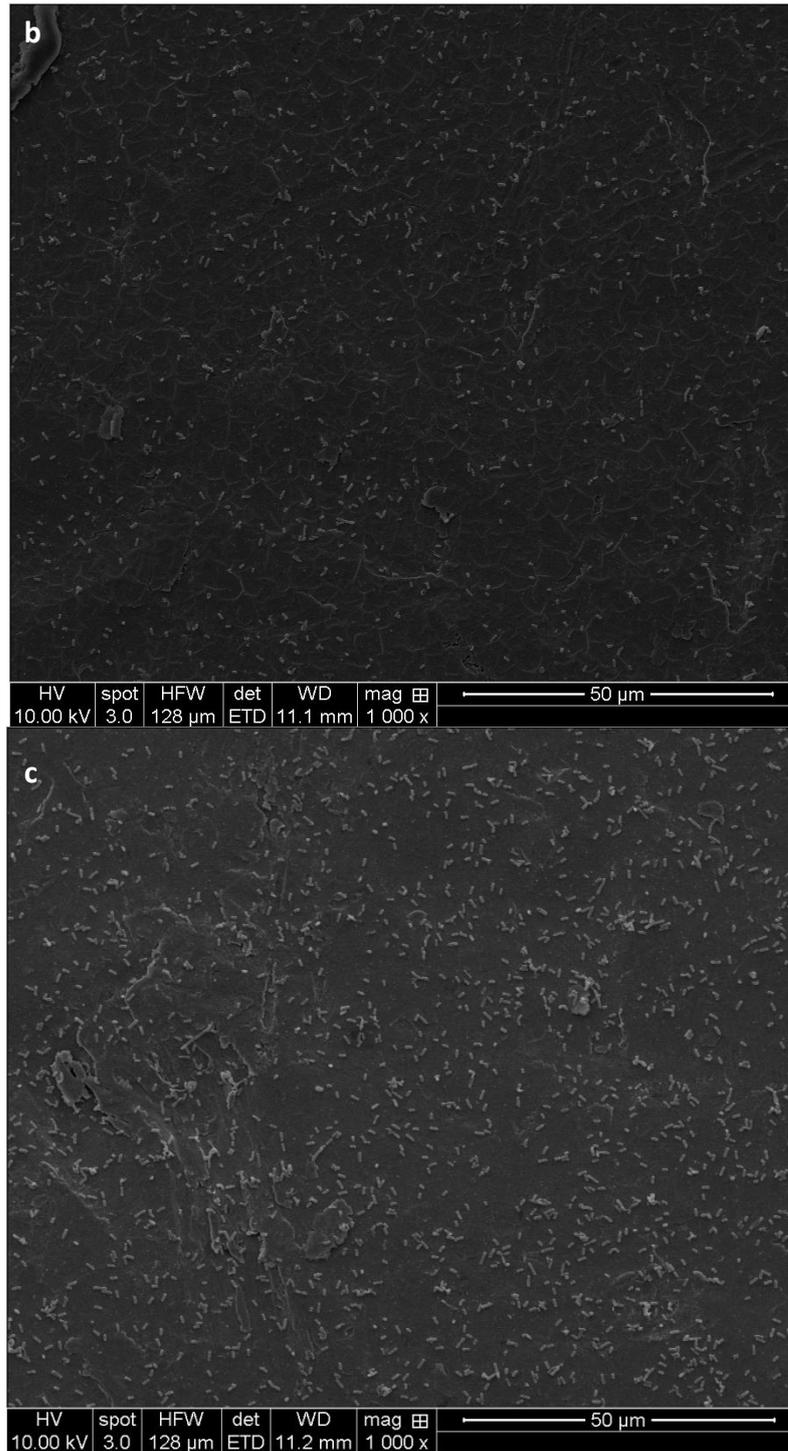


Figure 23 SEM images of *L. monocytogenes* biofilm on uncoated (a), TMS coated (b) SS and TMS+O₂ coated (c).

4.11 EDS (Energy-dispersive X-ray spectroscopy)

As shown in Figure 24, significant changes of carbon, oxygen, silicon, and iron were observed on TMS and TMS+O₂ coating wafer surfaces. Compared to the control group, carbon, silicon and oxygen content increased while the iron content decreased as a result of TMS or TMS+O₂ coating on SS surfaces. There are also significant differences in the elements between TMS or TMS+O₂ groups. These results provided more evidences and proved that the surface characters were changed after the coating. The change in the composition of the surface elements is the main reason for the surface energy change and surface contact angle change that led to the decrease of bacterial initial attachment and helped reduce biofilm formation to some extent. As we modified the surface with TMS, the CH₃ groups and carbon groups will stay on the surface as a layer of coating. This layer will make it more difficult for cells or proteins to attach to the surface, which helps bring down the final count of the biofilms.

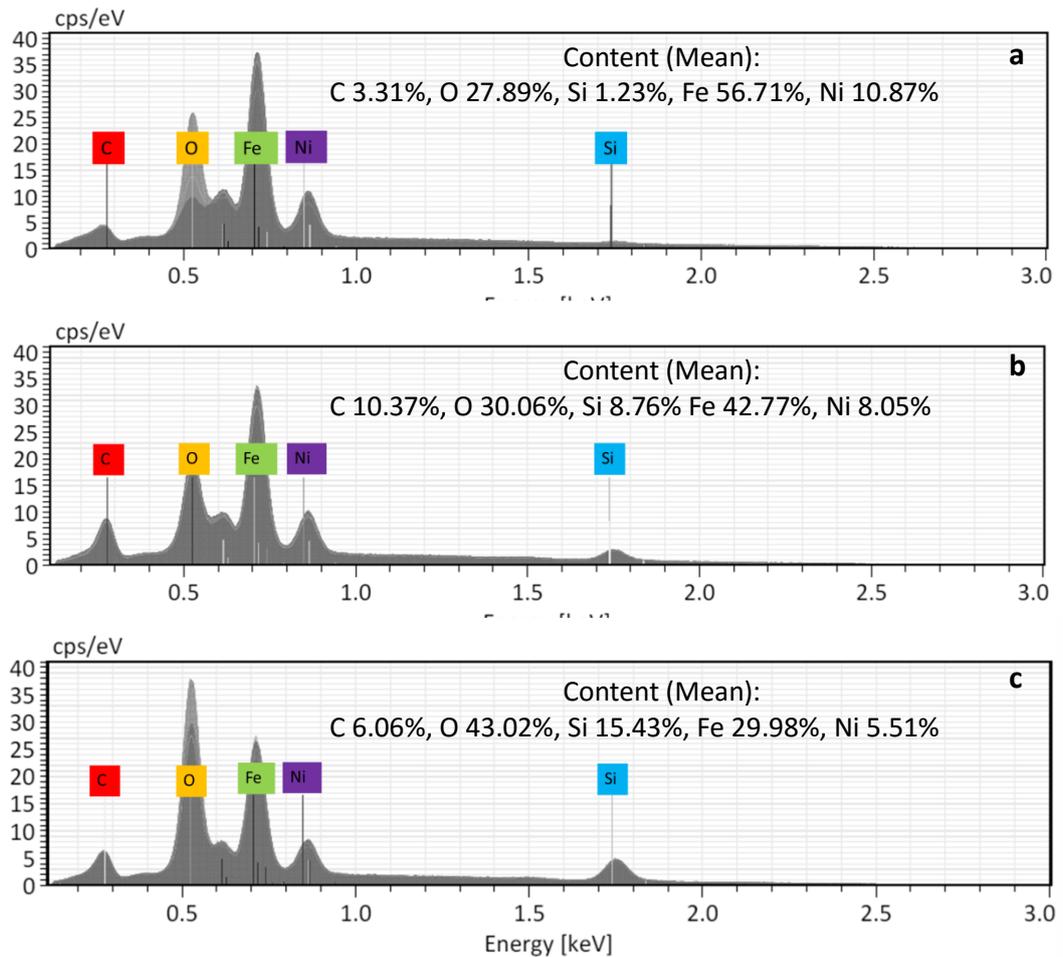


Figure 24 EDS elements scanning results of uncoated (a) TMS coated (b) and TMS+O₂ coated (c) on SS.

4.12 Zeta potential tests on bacterial suspensions

Zeta potential in a bacterial suspension is an indirect indicator of the surface energy of the suspension. The larger the absolute number of the zeta potential is, the more stable the solution will be because there are more repulsive forces between the cells. Significant differences in zeta potential of *Salmonella enterica* Typhimurium 788, *L. monocytogenes* Brie, *E. coli* O157:H7 505B and *S. aureus* FRI were observed ($P \leq 0.05$). Zeta potential of *S. Typhimurium* 788, *L. monocytogenes* Brie were significantly lower than the other two ($P \leq 0.05$), which implies that they are more

likely to coagulate or flocculate. Because *E. coli* O157:H7 505B and *S. aureus* FRI have a lower (more negative) zeta potential than *S. Typhimurium* 788 and *L. monocytogenes* Brie, which means they are more likely to stay in the suspension than precipitate out. However, the lower negative zeta potential means that the cells have more negative electrons on their surface which indicates that if the surface of a material has a lot of positive electrons, they are more likely to attach to that while if the surface of a material is negatively charged, the cells are less likely to attach, resulting in less biofilm formation. Researchers have found that the surface charges of bacterial cells can affect the adhesion process (Stenström 1989). The attachment process is a complex process that combines both natural cell coagulation and the electrical attractive forces. The zeta potential of bacterial is also related to the pH of the ambient medium which can affect the adhesion process (Stenström 1989). From the results alone, it was difficult to tell much about the mechanisms of the coatings. In fact, using just the zeta potential data alone is insufficient to understanding the mechanisms of the coatings. The results indicated us the anti-biofilm efficacy of TMS plasma coating is related to more than just surface energy and electrons. More things are involved or else we would be able to build a model to predict the anti-biofilm efficacy of this coating. Therefore, more studies are needed to understand the anti-biofilm mechanism of TMS plasma coating.

Table 2 Pathogenic bacterial zeta potential results.

Strains	<i>E. coli</i> O157:H7 505B	<i>S. aureus</i> FRI	<i>L. monocytogenes</i> Brie	<i>Salmonella</i> <i>enterica</i> Typhimurium 788
Zeta potential	-10.4 mv	-10.7 mv	-7.8 mv	-7.1 mv

4.13 Gene expression studies

The main purpose of the gene expression study was to study if the TMS coating will lead to resistant strains and if virulence genes are affected. When a bacterial cell is seriously injured or threatened, some metabolic actions will be taken by those cells, which could result in a change in the expression of genes controlling the cells' metabolism (Fraser and others 2003; Sue and others 2003). If a change in gene expression level of certain genes was found, there is a possibility that new metabolic features can be found in the cells. Among the entire gene expression levels tested, *E. coli* O157:H7 505b: *arcA* (housekeeping), *stx1*, *stx2*, and *eaeA* (virulence); *L. monocytogenes* 7644, *ropB* (housekeeping), *inlA*, *inlB*, *actA*, and *hly* (virulence), no significant differences ($P>0.05$) in gene expression level was detected. Even though the expression level of some of the genes fluctuated slightly, all of them remain within the statistical error range, thus no significant difference was observed ($P>0.05$). Without affecting the virulence gene expression level, no selective effect was showed by this plasma coating method. This implied that the plasma coating will not promote dangerous virulence genes in treated strains. The results added more support to our previous theory that TMS coating can reduce the cell count in biofilms by changing the surface contact angle and surface energy which will lead to non-selective effects of reductions on biofilm forming cell numbers.

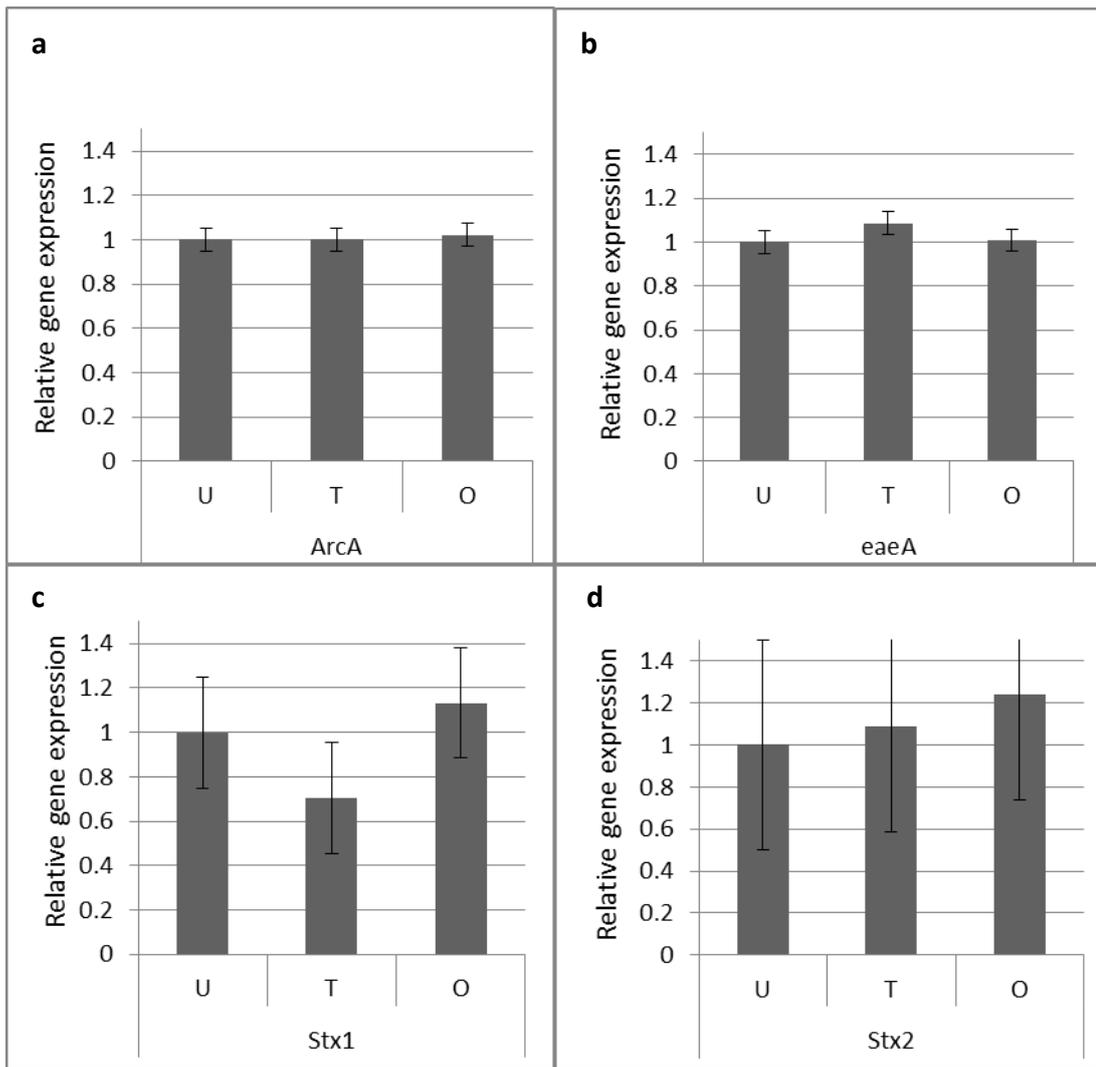


Figure 25 Gene expression results of *E. coli* O157:H7 *arcA* gene (a), *eaeA* gene (b), *stx1* gene (c), and *stx2* gene (d) on SS. (U:Control, T:TMS, O:TMS+O₂).

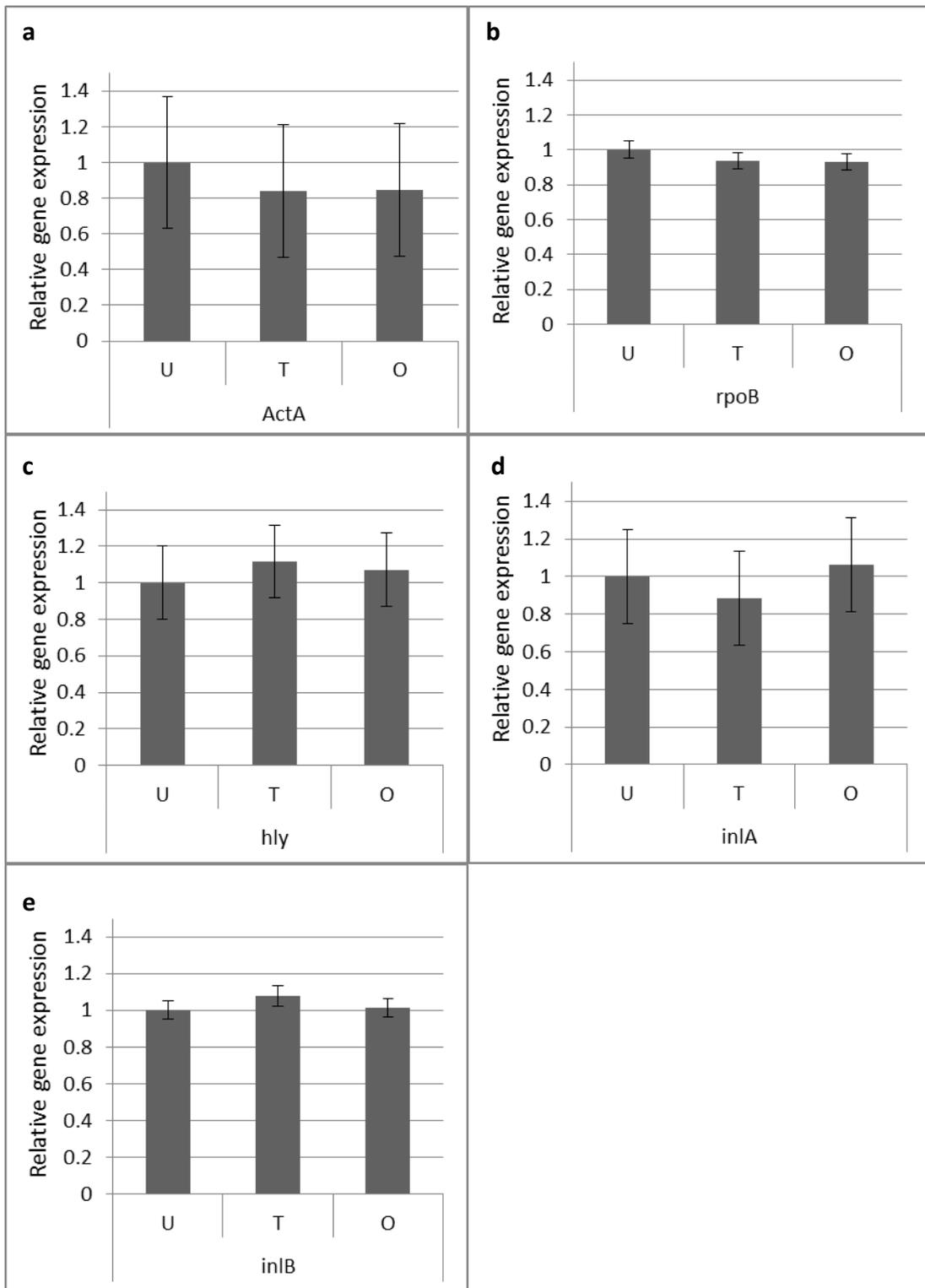


Figure 26 Gene expression results of *L. monocytogenes* ActA gene (a) *rpoB* gene (b) *hly* gene (c) *inlA* gene (d) and *inlB* gene (e) on SS (U:Control, T:TMS, O:TMS+O₂).

In this research, a series of direct or indirect effects of the antibiofilm properties of TMS coatings was found. Based on plate count and microscopic data, a fairly constructive picture of how the TMS or TMS+O₂ coating took effect against different bacterial biofilms could be made. At the same time, more additional tests were done to support these data and to help form basic hypotheses and build the basic model for the coating effects on the biofilms. However, most of the tests only focused on the 48 h pre-formed formed biofilms, which makes it difficult to determine the actual mechanisms of action of the coatings. In futures studies, studies could concentrate on the effects of the coatings on the ability of these biofilms to form. A Fourier transform infrared spectroscopy (FTIR) would be helpful to study the early stages of the biofilm formation process and could add more information to explain the mechanisms (Humbert and Quilès 2011). FTIR tests can give a direct sense of the biofilms during the formation steps. For example, after the wafers are mixed with bacterial suspension, an FTIR image can be captured every 2 h and a curve of how the biofilms in the control, TMS or TMS+O₂ groups can be drawn to demonstrate in which stage, the TMS or TMS+O₂ coat is most efficient. In addition, with the help of FTIR, maybe a different result could be observed especially in those slightly reduced strains like *E. coli* in SS and *L. monocytogens* in HDPE. In the early stages, they might be effective against the attachment but as the time increased, their efficacies might be reduced, and when by 48 h, the antibiofilm efficacy became what we observed (slight reduction) in this study. Last but not least, with the help of FTIR test, a range of the effective time zones can be set, which can also provide information of how long the coatings will last on the materials.

Chapter 5. Conclusions

In this study, the chemical material trimethylsilane (TMS) was used to form a nanoplasma coating on the surface of stainless steel (SS) and high-density polyethylene (HDPE). Both materials are widely used in the food industry for food processing and food storage. Two different coating formulae was tested: TMS alone and TMS+O₂ (1:4). Single strains of foodborne pathogens and some non-pathogenic bacterial: *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella* were used to test the anti-biofilm efficacy of TMS alone and TMS+O₂ coating. Among them, TMS coating on SS succeeded in reducing the cell count of *S. aureus* cocktail biofilm by around 2 log CFU/wafer (99% reduction). It also reduced the count of *L. monocytogenes* in biofilm by 1.5 log CFU/wafer. TMS+O₂ coating on SS also achieved a 2 log CFU/wafer reduction in *S. aureus* cocktail biofilm, and a 2 log CFU/wafer reduction on *L. monocytogenes* cocktail biofilm. On HDPE surfaces, TMS+O₂ decreased the cell count of *E. coli* O157:H7 cocktail by around 1 log CFU/wafer. For different single strains, their effects fluctuated, and occasionally, the total count even increased slightly like for non-O157 *E. coli* cocktails. However, mostly, these coatings worked well or at least did not increase the cell counts.

The mechanism of the anti-biofilm activity of the plasma coating was also studied. According to the results, the anti-biofilm efficacy of the plasma coating was related to bacterial strain (*E. coli* O157:H7, *S. aureus* or *L. monocytogenes*), coating surface (SS or HDPE), coating composition (TMS or TMS+O₂), as well as the medium (TSB or BHI). A change in the surface contact angle and surface elements was detected. With the help of pour plating results, laser confocal live/dead cell microscopy and scanning electron microscopy, a basic theory on the mechanism of

action of the coatings was formed. With the TMS or TMS+O₂ coating, the surface contact angle and surface energy were changed, and for different bacterial, different contact angle or surface energy were preferred. Thus, if the surface contact angle and surface energy were closer to the preferred conditions of the cells, more bacterial will adhere to the surfaces and vice versa. TMS or TMS+O₂ could reduce the biofilm cell count by changing the surface contact angle and surface energy to less preferred conditions for most of the bacterial, thus, reducing biofilm formation. The advantage of this coating was quite obvious. It will not likely to lead formation of resistant strains because it was not bactericidal and does not affect major virulence gene expression. It was also tested in real beef juice and found to have a synergistic effect when combined with Quat sanitizer in *L. monocytogenes*. A decreasing trend could also be observed in *E. coli* O157:H7 and *S. aureus*. This would allow for designing an effective, yet simple hurdle technique in food plants to fight against pathogenic biofilms.

More tests are still needed to complete the study of this plasma coating as it has only been tested on limited numbers of bacterial as well as surfaces (to avoid application conditions where the total bacterial count can grow and to maximize its anti-biofilm efficacy). There is still a long way to go before the application of this plasma coating technology in food industry. Also, only the TMS alone and TMS+O₂ (1:4) ratio was tested, more ratios or even more materials can be tried in subsequent tests to improve this coating method. Last but not least, although the test results in this study enabled us to form a preliminary theory about the mechanistic actions of the coatings, it is still a basic theory and more evidences are needed to prove the theory or more factors needed to be considered in order to form a well fitted model which can be applied in the food industry in the future.

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