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United Arab Emirates University

College of Food and Agriculture

**BIOFILM PRODUCTION BY FOOD-TRANSMITTED BACTERIA AND
THEIR CONTROL BY SELECTED NANOPARTICLES**

Mariam Al Shamsi

This dissertation is submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Under the Supervision of Dr. Aisha Abushelaibi

May 2015

Declaration of Original Work

I, Mariam Al Shamsi, the undersigned, a graduate student at the United Arab Emirates University (UAEU) and the author of the dissertation entitled “*Biofilm production by food-transmitted bacteria and their control by selected nanoparticles.*”, hereby, solemnly declare that this dissertation is an original research work done and prepared by me under the supervision of Dr. Aisha Abushelaibi, in the College of Food and Agriculture, at UAEU. This work has not been previously formed as the basis for the award of any academic degree, diploma or similar title at this or any other university. The materials borrowed from other sources and included in this dissertation have been properly cited and acknowledged.

Student's Signature _____ Date _____

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Abstract

Biofilm is a population of bacteria attached to any types of surfaces and impeded in a self-produced matrix of extracellular polymeric substances. Biofilm exhibit up to 1000 fold antibiotic increased resistance to a broad range of antimicrobial agents. Several food-transmitted microorganisms are capable of forming biofilms and considered as a major source of contamination, transmission and infection. In the last few decades, nanoparticles has gained a great attention for their potential applications as antimicrobial agents. The aim of this work was to assess the biofilm formation capacity of food-transmitted bacteria under various environmental conditions and to investigate the efficacy of different nanoparticles (i.e. Ag-Cu-B, Ag-Na-B, and Ag-Mg-B) to kill microbial pathogens in biofilms. Nanoparticles were synthesized by using co-precipitation and microwave techniques and characterized for their physiochemical properties by transmission electron microscopy and light dynamic scattering. The antibiofilm and antimicrobial properties of the synthesized nanoparticles were investigated using *S. aureus* (10 strains), *P. aeruginosa* and *E. coli* (3strains). The findings revealed that all NPs significantly inhibited planktonic cells and biomass of the grown biofilms.

Moreover, the sanitization efficacy of nanoparticles were assessed on stainless steel surface that commonly come into contact with food. The surfaces were inoculated with strains of *S. aureus* and *Salmonella* and cleaned with NPs saturated sanitary wipes. A significant reduction was observed in viability of the cells on the stainless steel surfaces. The results demonstrated that the use of NPs incorporated into sanitary wipes is useful method to eliminate bacteria on food contact surfaces.

Keywords: Biofilm, food-transmitted bacteria, nanoparticles, co-precipitation, microwave technique, antimicrobial, antibiofilm, sanitation.

Title and Abstract (in Arabic)

العنوان : قدرة البكتيريا المنتقلة بواسطة الغذاء على إنتاج البيوفيلم واستخدام بعض المركبات النانوية للقضاء عليها.

الملخص

البيوفيلم هو تكس معقد للكائنات المجهرية، يتسم بإفراز نسيج خارج الخلية محصن ولاصق على أنواع مختلفة من الأسطح. يتميز البيوفيلم بقدرته على مقاومة فعالية المضادات الحيوية ما يصل إلى ألف ضعف مقاومة خلايا البكتيريا الحرة. تتميز العديد من البكتيريا المنتقلة بواسطة الغذاء بقدرتها على إفراز البيوفيلم ، وتعتبر مصدرا رئيسياً للتلوث ونقل العدوى.

في الأونة الأخيرة اكتسبت العناصر المحضرة بتقنية النانو اهتماما كبيرا، وخاصة في مجال استخدامها كمواد مضادة للبكتيريا. هدفت هذه الدراسة إلى قياس مقدرة بعض انواع البكتيريا منتقلة بواسطة الغذاء على إنتاج البيوفيلم تحت ظروف بيئية مختلفة، وأيضا إلى تحضير أنواع مختلفة من المركبات النانوية مكونه من (Ag-Cu-B, Ag-Na-B, and Ag-Mg-B) وتقييم مدى كفاءتها على محاربة البيوفيلم. تم تحضير هذه المواد النانوية باستخدام طريقتين (1) طريقة الترسيب الكيميائي (2) استخدام المايكرويف وقد قيمت خصائص هذه المركبات باستخدام جهاز المجهر الإلكتروني وجهاز ديناميكية تشتت الضوء. علاوة على ذلك تم تقييم قدرة هذه المركبات على عدة أنواع من البكتيريا المنتقلة بواسطة الغذاء (البكتيريا العنقودية الذهبية، بكتيريا القولون وبكتيريا السديموناس) والمقاومة لأنواع عدة من المضادات الحيوية على الخلايا الحرة والخلايا في طور البيوفيلم. اثبتت الدراسة بأن هذه العناصر تمتلك الكفاءة والقدرة على محاربة هذه البكتيريا الضارة.

بالإضافة لذلك تم دراسة امكانية استخدام هذه المركبات في مجال سلامة الغذاء وذلك عن طريق استخدامها كمناديل تستخدم للتنظيف والتطهير الصحي للأسطح الملامسة للأغذية. تم تقييم فعالية هذه المناديل بعد أن تم تشبعها بهذه المركبات، على بكتيريا السالمونيلا والمكورات العنقودية الذهبية على أسطح الستانلس ستيل ، وأظهرت النتائج كفاءة هذه المناديل في القضاء على هذه الميكروبات من على أسطح الستانلس ستيل.

مصطلحات البحث: البيوفيلم، البكتيريا المنتقلة بواسطة الغذاء، العناصر النانوية، طريقة الترسيب الكيميائي، طريقة تحضير المايكرويف، مضادات الميكروبات، مضادات البيوفيلم الميكروبي، صحي.

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Dedication

To my beloved parents and family

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List of Abbreviations

BHI	Brain heart infusion
CAA	Casamino acid
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
MLST	Multi-locus sequence type
SDS	Sodium dodecyl sulfate
<i>cont.</i>	Continued
DMSO	Dimethyl-sulfoxide
DNA	Deoxyribonucleic acid
EPS	Extracellular polymeric substance
ESBLs	Extended spectrum beta-lactamases
CTX-M	Cefotaximase
ETEC	Enterotoxin producing <i>Escherichia coli</i>
<i>L. monocytogenes</i>	<i>Listeria monocytogenes</i>
ExPEC	Extra-intestinal pathogenic <i>E. coli</i>
HUS	Hemolytic uremic syndrome
<i>E. coli</i>	<i>Escherichia coli</i>
LB	Luria broth
MHB	Mueller Hinton broth
MIC	<i>Minimum inhibitory concentration</i>
OD	Optical density
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NT	Nanotechnology
NP	Nanoparticles
NM	Nanomaterials
<i>P. putida</i>	<i>Pseudomonas putida</i>
PFGE	Pule field gel electrophoresis
QS	Quorum sensing
RNA	Ribonucleic acid
<i>S. epidermidis</i>	<i>Staphylococcus epidermidis</i>
<i>K. pneumoniae</i>	<i>Klebsiella pneumonia</i>
ROS	Reactive oxygen species
<i>S. typhi</i>	<i>Salmonella typhi</i>
spp	Species
<i>S. enterica</i>	<i>Salmonella enterica</i>
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
SEs	Staphylococcal enterotoxins
ST	Sequence type
QQ	Quorum quenching
STEC	Shiga toxin producing <i>E. coli</i>
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. typhimurium</i>	<i>Salmonella typhimurium</i>
TE	Tris/EDTA buffer
TEM	Transmission electron microscopy
TSA	Tryptic soy agar
TSB	Tryptic soy broth

UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UTI	Urinary tract infection
ZnO	Zinc Oxide

Foreword

Microorganisms, including but not restricted to those causing communicable diseases, spread between locations that are capable of accommodating and sustaining them. Depending on the microorganism, these niches could be inanimate objects, as well as living, susceptible hosts. It is important to note, that "transmissibility", i.e. the capacity to reach new niches, is an important feature of pathogens, as this secures that the microbes can continuously encounter new environments supporting its own life. This capacity is equally important for microorganisms which cannot, and hence do not establish themselves permanently in a living host, including human beings - they transmit between, and subsequently colonize inanimate objects. Transmissibility is also crucial for those organisms, which do colonize various body-parts of man, but in a fashion that is not harmful to the host. These microorganisms colonize the host for shorter or longer periods of time and thus become part of the very complex flora, *the microbiota*, exhibiting multiple interactions with the macroorganism accommodating it.

Members of this normal flora are often, not without unfounded generalization, considered non-pathogenic, as they usually do not cause perceptible pathological changes at the niche they colonize (e.g. the flora of the gut). However, this approach is misleading for several reasons. On one hand, members of the normal flora, once displaced within the host, may cause severe diseases as they may use different strategies to colonize different body parts. Furthermore, the host may also respond differently when encountering the same organism at different mucosal surfaces or in different organs. With other words, these organisms non-pathogenic at

one site could be involved in endogenous infections of other body parts. A typical example of these complex host-parasite interactions is urinary tract infections. In the overwhelming majority of these cases the source is the patient's own gut flora; i.e. what is in the gut, what kind of bacteria are there impacts the severity and outcome of some extra-intestinal infections.

Members of the normal flora, independently of their individual pathogenic potential, may contribute to the pathology of the host in another way, as well. In some body parts, particularly in the large intestine, the density of various microorganisms is enormous. This provides a unique chance for different strains and species to interact with each other and, importantly, to transfer genetic material. This phenomenon is a well-recognized driving force of microbial evolution as, by acquiring genes of virulence factors, it affects the pathogenic potential of the strains. Lately, however, an increasing attention is also being paid to the transfer of antibiotic resistant genes. By now it is clear that in the gut, a non-pathogenic, but highly resistant strain can easily transfer its resistant gene(s) to a susceptible, but highly pathogenic species creating, what the media like to describe as, a "superbug".

Consequently, when considering and discussing the possibilities of microbial transmission, modes of colonization and possible interference with them, one should not restrict attention to the "classical", "real" pathogens, only but, simultaneously, should also take non-pathogens, or organisms which may have a pathogenic potential in one, but not at another body part into consideration.

As my thesis work focuses on how to interfere with the spread of microorganisms via food, I intentionally followed this broader approach.

Chapter 1. Introduction

1.1 Source and transmission

Bacteria regularly establish more or less permanent contacts with humans. These encounters, depending on the microorganism and the host, result in a variety of outcomes. Beyond the microorganism and potential host, this complex and dynamic event, actually a sequence of events, requires a reservoir of the microbe, an immediate source, and a mechanism of transmission (Krämer et al., 2010). The *reservoir* is a living organism or material in or on which an infectious agent lives and usually multiplies. The *source* is the initial point from which the microbe passes to the new host. In case of directly transmitted infections the source is the person carrying (infected with) the microbe, while in case of indirectly transmitted microorganisms the source can be a variety of inanimate objects, food, water or vectors (Krämer et al., 2010).

Obviously, the source affects the possible mode of transmission. The most complex form of transmission takes place via living vectors, in which the microorganism may or may not go through a unique phase of its lifecycle (e.g. malaria, plague etc.). *Contact transmission* may take place by *direct contact* (i.e. by direct, physical contact with the infected or carrier host), or by *indirect contact* through objects (e.g. conjunctivitis, or the spread of a variety of nosocomial pathogens). *Droplet transmission* is mediated via droplets of usually airways excreta (e.g. influenza, measles). *Airborne transmission* is the result of the transfer of the organisms themselves (without a droplet coat) to the susceptible host (e.g. tuberculosis). During *common vehicle transmission* a shared inanimate object (e.g.

food, water, contaminated medicines) are carrying the infectious particle from the source to the new host (Beier and Pillai, 2007; Krämer et al., 2010).

The transfer of microorganism, however, is a more complex than just how they reach the host. This is particularly important in case of food-borne infections, i.e. the topic of the current theses. Food could be contaminated at any point in the food chain and food processing. Frequently, bacteria transfer to the food either by cross-contamination or re-contamination (Pérez-Rodríguez et al., 2008) or it may occur either indirectly through air-borne particles or by direct contact with contaminated surface (Kusumaningrum et al., 2003).

Airborne bacteria can be transferred through dust particles or aerosols (den Aantrekker et al., 2003). For example, in the meat and poultry industry the aerosols produced during dehidating, evisceration and carcass splitting are major routes of contamination (Mor-Mur and Yuste, 2010). This transmission (i.e. contamination of the "common vehicle of transmission") could happen through talking, coughing, sneezing or via a variety of activities such as sewage removal (Van Houdt et al., 2012)

Contamination often associated with unprocessed raw material, unclean food surfaces and personal handling (Reij and Den Aantrekker, 2004). Bad hygiene and improper sanitation of the food processing environment directly contribute in the food borne disease outbreaks and promote the development of biofilms which may contain pathogenic bacteria (Chmielewski and Frank, 2003).

1.2. Food-borne diseases

Food could transmit more than 200 known diseases (Oliver et al., 2005). The Centers for Disease Control and Prevention (CDC) estimates that every year 76 million foodborne illnesses occur in US and these illness costs the country \$10-83 billion (Nyachuba, 2010). Also, it is estimated that foodborne illness causes about 2.2 million deaths each year, mostly in the countries of the developing world (Tajkarimi et al., 2013).

Food borne diseases are defined as illness resulting from consumption of food contaminated with microbial pathogens and/or with their toxic materials (Unicomb, 2009). Microorganism-induced, food-related diseases can be intoxications and/or infections (Marriott and Gravani, 2006). During *intoxication* the pathological changes evoked are directly related to the microbial toxins consumed (e.g. staphylococcal intoxication, botulism). In these cases the microorganism contaminates the food and while multiplies in it, produces the toxic substance(s), which may not be destroyed by subsequent food processing. Once ingested, some of these toxins may have a very rapid (few hours) effect (particularly the emetic type of toxins, e.g. staphylococcal or *Bacillus cereus* toxins), while the neurotoxins (botulinus toxin) take a longer time. While in these cases the actual presence of the microorganism, at the time of inducing the pathological changes, are not needed anymore, from the point of view of food-hygiene these cases do not really form a separate entity, as the initial step here is also the microbial contamination of the food (Aytac and Taban, 2014).

During *toxico-infections* the pathological effect is also induced by a toxin, but the toxin is being produced within the host, and the food serves just as a vehicle to transfer the bacterium into the macroorganism. The clinical symptoms vary according to the type of toxin produced (most commonly diarrhea), but the incubation time is longer (often 12-36 hours), as the production of the toxin within the host takes time (e.g. Enterotoxin producing *Escherichia coli*, ETEC). Finally, a similar role is played by the food vehicle in "pure" *infections* where the pathological changes are (mostly) due to the interaction between the cells of the micro-, and macroorganism (e.g. shigellosis, salmonellosis) (Nantel, 1996). It should be noted, however that in some cases the clinical presentation is the result of the combination of both direct bacterial, as well as toxin effects (e.g. hemorrhagic colitis/hemolytic uremic syndrome (HUS) due to Shiga toxin producing *E. coli*, STEC) (Aytac and Taban, 2014).

Importantly, beyond the common diseases dominated by enteric symptoms, only, some of these pathogens cause primarily systemic or focal infections, like meningitis due to *L. monocytogenes*. In case of STEC dramatic enteric symptoms (bloody diarrhea, hemorrhagic colitis) may precede, or accompany toxin-induced systemic manifestations, such as hemolytic uremic syndrome (HUS). Infections due to others may induce auto-aggressive immune responses leading to a variety of manifestations from arthritis to nerve demyelination (e.g. reactive arthritis and Guillain-Barré syndrome seen after a variety of enteric infections, most commonly due to *Yersinia* and *Campylobacter*) (Israeli et al., 2012; Scallan and Mahon, 2012; Simonet, 1999).

Outbreaks have been frequently associated with the consumption of fresh and minimally processed product such as fruits and vegetables. For example, outbreaks caused by fruits, seeds and sprouts usually associated with *Salmonella enterica*, while outbreaks of *E. coli* O157:H7 have been linked, beyond beef, to leafy greens (Yaron and Römling, 2014).

1.3. Factors facilitating microbial transmission by food

Beyond technical issues related to food preparation and processing, several cultural, demographic, environmental and social factors, many of them changing rapidly, are playing roles in the transmission of the foodborne pathogens (Newell et al., 2010). The most important ones are

- The sharp increase in population number and a demographic shift towards an ageing population
- Remarkable increase in food globalization, particularly freshly produce food and farm animals
- Improved transport logistics and conditions, which enable bacteria to survive the short time needed to get transferred even between continents
- The enormously increased human travel and immigration with the consequent spread of the intestinal microfloras worldwide
- Changing eating habits, such as the consumption of raw or lightly cooked food
- Increasing in the demand for high protein foods, primarily meat and fish products

- Higher proportions of immunologically compromised individuals such as elderly, children and immunosuppressive groups
- Innovating and adopting new farming practices to produce cheaper food and organic food as a response to consumer demands and welfare. This includes the use of unnatural animal feed and cramped farming conditions promoting microbial spread among animals
- Increasing human involvement on native wildlife habitats
- Climate change, for example bringing novel vectors into temperate regions or temperature-associated changes in contamination levels

1.4. Food contamination

The vehicle of transmitting microorganism entering the human hosts through the digestive system can be water and food. Contaminated water can directly be consumed (these are waterborne infections) or may contaminate the food during irrigation or processing (Medema, 2013). Most of the microorganism with the highest chance to colonize or infect a new human host derives directly or indirectly (i.e. via irrigation water) either from animals or from another human being (Medema, 2013). These organisms mostly colonize the intestinal tract of these reservoirs, although there are exceptions from this rule (i.e. *S. aureus* which may contaminate food as a skin colonizer).

An important and unique mode of acquiring food-borne diseases is when the contaminated raw material is being eaten directly or without sufficient processing. Consumption of raw milk (e.g. brucellosis), un-pasteurized cheese (e.g. listeriosis),

raw fish (e.g. various *Vibrio* infections), meet or eggs (salmonellosis) are examples of this category (Unicomb, 2009).

Contamination of the food can take place at any of the three main stages of the food chain, i.e. production in the field, processing, and preparation (Graves, 2011; Leon and Albrecht, 2007). The raw material can be directly contaminated with pathogens from the animals carrying them. Some human pathogens can be present in the cattle gut, such as *E. coli* O157, *Salmonella* spp., in that of swine (e.g. various *Yersinia* sp.) or in the intestinal tract of poultry (e.g. *Campylobacter*). This contamination often takes place between carcasses during transportation, or while slaughtering (Reid et al., 2002; Roberts et al., 2005a). Fruits and vegetables can be contaminated with pathogenic microorganisms from the use of raw sewage, manure fertilizer, water (Roberts et al., 2005b). Insects, birds and rodents are also considered as passive vectors carrying pathogens (Reij and Den Aantrekker, 2004).

Man is also an important source of food contamination. It has been reported that personal hygiene of the food handlers contributed to about 97% of the foodborne illness in food premises and home (Aa et al., 2014). The inadequate hand-hygiene after visiting the toilet is probably the most critical determining factors in spreading enteric pathogens (Lues and Van Tonder, 2007). It should be remembered, however, that not only classical enteric pathogens, but pyogenic bacteria can also be introduced to food by hands with skin and soft tissue infections. This is the typical way how *S. aureus* contaminates food while being processed. Bacteria such as *S.aureus*, *E.coli* and *Salmonella* spp., can survive on hands and on surfaces for hours or days (Kusumaningrum, et al., 2003).

Once introduced to the food-mass, bacteria can survive for a considerable time. Uneven, rough, damaged surfaces of food-processing equipments are important to facilitate colonization and may interfere with cleaning (Reij and Den Aantrekker, 2004).

Several species of bacteria have unique feature to adhere and colonize surfaces (Myszka and Czaczyk, 2009). Adherence to abiotic surfaces is, at least initially, mediated by physical interactions, like electric charge and/or hydrophobicity. However, several food-transmitted microorganisms are capable of forming biofilms. Cells grown in biofilms are considerably protected from all external noxas and hence pose a challenging problem once we try to interfere with food contamination.

1.5. Bacterial biofilms

Biofilms are complex microbial communities composed of interacting cells embedded in a extracellular polymeric substance (EPS) called matrix (Yang et al., 2012). In biofilms these *sessile cells* are attached to a substratum, which could be an abiotic or a biotic surface, or could be an interface in between. Cells embedded in this matrix differ from their *planktonic*, free living counterparts concerning their growth rates and their gene expression (Donlan and Costerton, 2002; Lazar, 2011).

Biofilm are mainly composed of 90% matrix and 10% microorganism (Flemming and Wingender, 2010). However, 97% of the matrix is water, which is capable of absorbing nutrients, metabolites and cell-lysis products. The remaining

3% of the EPS contains proteins, polysaccharides, DNA, RNA, peptidoglycan, lipids and phospholipids (Sutherland, 2001b). Polysaccharides and proteins have been shown to be the key components of the matrix. Also, DNA plays a role in the establishment of the structure. It was also demonstrated that some Gram-negative bacteria, e.g. *S. typhimurium* and *E. coli*, produce cellulose as EPS component. (Branda et al., 2005). The EPS can be neutral or poly-anionic in Gram-negative bacteria, while their nature is cationic in Gram- positive ones. Usually in the anionic type uronic acid and ketal-linked pyruvates increase the binding force through enhancement of the calcium and magnesium association (Vu et al., 2009).

Bacteria embedded in EPS form different structures ranging from patchy monolayers, heterogeneous mosaic models to mushroom or tulip-like models (Wimpenny et al., 2000). The architecture of the biofilm affects the dissemination of nutrients and chemicals within the matrix and thus results in a heterogeneous growth rate and physiological activities of the assembled cells (Folkesson et al., 2008).

1.5.1. Biofilm formation

The formation of biofilms and their properties are affected by several factors: (Melo, 2003)

- The microbial species and strains characteristic
- The composition and roughness of the substratum
- The composition of the fluid environment (e.g. pH, temperature and ionic strength)
- The hydrodynamic of the fluid (velocity and turbulence) (Melo, 2003).

Biofilm can be formed on a variety of surfaces. These can be living tissues, indwelling medical devices, industry equipment, portable water system piping and natural aquatic systems (Rodney, 2002). The stages involved in the biofilm formation are shown on Figure 1.

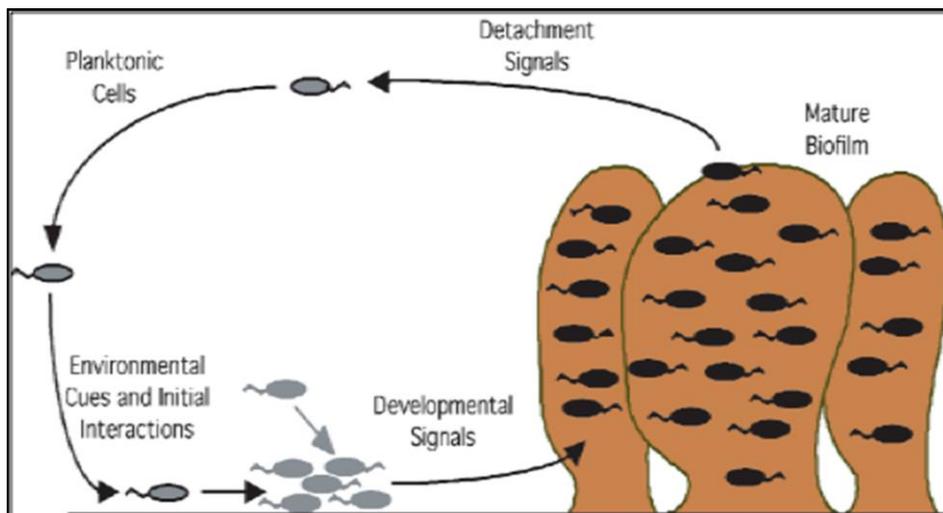


Figure 1: Stages of biofilm formation (Abed et al., 2012)

Usually 4 steps are distinguished in biofilm production:

Stage 1: Attachment /colonization by primary reversible adhesion between microbial cell surfaces and desired substratum;

Stage 2 : Irreversible attachment;

Stage 3: Biofilm architecture formation and maturation;

Stage 4: Detachment and dispersal of biofilm cells

During the initial stage 1 planktonic cells move towards the surface via either flagella or physical forces and establish a connection called reversible attachment and involve cell-pole mediated interactions (Clutterbuck et al., 2007). Upon contact bacteria roll across the surface before settling and initiating their adhesion (Costerton, 1999). The quality of surfaces is crucial. Preconditioning by adhesion of macromolecules (e.g. dust, dirt, leftover material on un-cleaned surfaces or bodily macromolecules in living tissues) facilitate this step. Regarding food environment, it is usually rich in nutrients, which often act as a conditioning film. The conditioning may change the physiochemical properties such electrostatic charges, surface free energy, and hydrophobicity of the surface. The physiochemical properties of the bacterial cell also play a role in this interaction. Initially these are weak interactions, like Van der Waals attraction forces, electrostatic forces and hydrophobic interactions. At this stage bacteria show Brownian motion and can be easily eliminated by the fluids' shear forces (Méndez-Vilas, 2011).

In the irreversible stage (Stage 2) flagella, fimbria, pili and EPS fibrils help cells to anchor to the surface through forces involving dipole-dipole interactions, hydrogen ionic and covalent bonding and hydrophobic interactions. All of these forces increase bacterial adhesion strength and the removal of these cells needs much greater forces, like scrapping. Attached cells start to up-regulate all the necessary genes that express enzymes required for EPS synthesis like a pivotal sigma factor (Kumar and Anand, 1998).

At stage 3 biofilms mature resulting in a complex architecture through the secretion of EPS. This requires quorum sensing (QS) (Clutterbuck, et al., 2007), i.e.

cell to cell communication. During QS cells produce and release QS molecules that are detected by neighboring cells thus gathering information about the density and structure of EPS, i.e. sensing that they are within a biofilm structure. There are several kinds of QS molecules including N-acyl-homoserine lactone and 4-quinolone which produced by Gram-negative bacteria, while the Gram-positive ones produce *AgrD* peptide (Chen and Wen, 2011). In this way cells regulate the expression of specific genes in response to their population density (Kolari, 2003).

The maturation of biofilms occurs in two stages. During the first stage the thickness of the biofilm is $>10\mu\text{m}$ and there is a profound difference in protein expression compared to planktonic cells. In the second stage the thickness reaches up to $100\mu\text{m}$ and there is a significant difference in the protein expression compared to planktonic cells and the first maturation stage. More than 100 proteins were synthesized and 50% of all proteins up-regulated (Sauer et al., 2002).

The last stage (stage 4) is detachment and dispersal of cells from the biofilm. These planktonic cells are considered the source of both infection and contamination in either clinical or public settings. Detachment usually caused by response to decreased nutrient levels via quorum sensing or by shearing off biofilm aggregates due to physical effect (Rodney, 2002).

Based on the strength and frequency of detachment dispersal has three main stages (Rodney, 2002; Stoodley et al., 2001):

- *Erosion* is the continual detachment of single cells and small portions of biofilm mostly present in thinner biofilms and in high shear environments
- *Sloughing* is the rapid and massive loss of biofilm. This process results from the depletion of nutrient and oxygen
- *Abrasion* is collision of particles from the bulk fluid with biofilm

Beside their structural role, EPS also works as a barrier to protect the embedded cells from the effect of antimicrobial agents and harsh environments (Eastman et al., 2011).

Stewart and Costerton suggested the main mechanisms how biofilms protect sessile cells residing in the matrix. First the rate of permeation is considerably reduced through the matrix limiting the amount of compounds reaching the cells (Stewart and William Costerton, 2001). EPS matrix may bind antimicrobial agents, e.g. positively charged aminoglycoside antibiotics bind to negatively charged EPS. The decreased concentration of drugs in the vicinity of the cells may allow that hydrolyzing enzymes with even limited activity (e.g. some narrower spectrum β -lactamases) might be sufficient to protect (Lewis, 2001).

Secondly, the alteration of the chemical microenvironment within the biofilm may create a niche where the drug has a limited activity. Furthermore, the accumulation of acidic waste products results in a change in pH, which may lead some bacteria to enter a non-growing state in which they are more protected from elimination. The alterations of the osmotic stress within biofilm change the relative proportion of porins in such a way that limits the uptake of the drug.

Finally, a subpopulation of micro-organisms in the biofilm may enter a unique and highly protected phenotypic state of cell differentiation which are similar to spore formation (Stewart and William Costerton, 2001).

1.5.2. Biofilms in medicine

According to the National Institute of Health of the USA 80% of bacterial infections were related to biofilms. These infections could include biomaterial-related infections, chronic wounds, cystic fibrosis-related lung infections, endocarditis and otitis media (Fey, 2010). In addition to that, several biofilm-producing organism can inhabit indwelling medical devices. Hence, they are highly resistant to antibiotic treatment and leading to device deterioration, blockages, loss of function and consequently require the replacement of the device. These problems are particularly common in the most vulnerable population, i.e. immune-compromised patients (Lindsay and von Holy, 2006).

Biofilms isolated from the medical device could contain yeasts, Gram-positive and Gram-negative bacteria. The most common medically relevant bacteria are *Enterococcus faecalis*, *S. aureus*, *S. epidermidis*, and *Streptococcus viridans*, *E. coli*, *K. pneumoniae*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* considered as examples of the Gram-negative biofilm producers (Donlan, 2001). Devices most commonly colonized by biofilms are urinary and central venous catheters, prosthetic heart valves, contact lenses, intrauterine devices and dental unit water lines (Donlan and Costerton, 2002).

1.5.3. Biofilms in food industry

On one hand, bacterial biofilms adversely affect any system transporting water via increasing corrosion of piping material (Hallam et al., 2001). It has been reported that 95% of water microorganisms are present inside biofilms while only 5% are floating (Gomes et al., 2014). Despite the low nutrients level in water bacterial cells do colonize water pipes or other wet systems. Poulsen reported that the number of planktonic cells found are 500 to 50,000 times lower than the number of cells in biofilm in the water system (Poulsen, 1999).

From the microbiological perspective, biofilms are predominating in water systems because attached cells are more resistant to chlorine and to other biocides than planktonic counterparts (Berry et al., 2006). Sulfate reducing bacteria in biofilm are responsible for deterioration of the pipelines metal where they are settled the anaerobic niches causing bio-corrosion and bio-fouling. Yearly, the pipeline damage caused by the sulfate reducing bacteria cost the industrial sector from 4-6\$ billion in US (Jayaraman et al., 1999). Moreover, the growth of the biofilm in water system leads to decrease in water quality, increase in energy utilization and decrease in operations efficiency and productivity (Kumar and Anand, 1998).

But food industry is affected by another way, as well, i.e. by bacteria residing in biofilms and thus contaminating food. Actually, most of the microbial contamination of food products are biofilm-related (Cappitelli et al., 2014). Food industry heavily relies, at almost every stage of the processes, on water or on some other kinds of liquid and the environment the food is prepared is almost always wet, moisten. Most of the biofilm problems in food industry are associated with

contamination of the water source. Biofilm may form in any sites in the food environmental area such as walls, floors, pipes and drains. As well as on all food contact surfaces like stainless steel, aluminum, nylon, teflon, rubber, plastic, buna-N, and glass. Bacteria forming biofilms include pathogens and spoilage type organisms such as *Listeria monocytogenes*, *Salmonella*, *Campylobacter*, *Pseudomonas* and lactic acid producing bacteria, *E. coli* O157:H7; they may be present in mixed cultures or as a mono-species biofilm. Some pathogens, such as *L. monocytogenes*, may persist in food plants for several months, even up to several years and can survive in aerosol and pose a re-contamination threat (Sofos and Geornaras, 2010).

Food systems have a variety of environmental conditions that are suitable for biofilm formations like moisture, nutrients, and density of bacteria present in the raw material (Kregiel, 2014). In particular, biofilm caused damage to the ultrafiltration membrane which used in the dairy industry, the growth of the bacteria resulting in membrane blockage, product contamination, and reduction of membrane life (Tang et al., 2010). Furthermore, both *Streptococcus thermophiles* and *Bacillus cereus* were found attached to the heat exchanger in milk processing equipment (Poulsen, 1999).

Another example of the bacterial biofilm in the food facilities is *Pseudomonas*. This bacterium produces very thick ESP and can live together with other species biofilms. Also, *Salmonella* biofilms have been detected in poultry processing equipment in slaughter and evisceration area (Chmielewski and Frank, 2003).

As a conclusion any methods, by which in food processing environments microbial contamination present as biofilms, could be reduced or eliminated is of huge importance.

1.6. Food-transmitted microorganisms studied in the current thesis

There are several dozens of bacterial species that are commonly transmitted by food. The most common foodborne pathogens are *Salmonella* spp., *Staphylococcus* spp., *Shigella*, *Escherichia coli*, *Listeria monocytogenes*, *Campylobacter* spp. and *Clostridium* spp (Salazar et al., 2015). It is important to keep in mind, however, that not only pathogenic species, and certainly not only enteric pathogens enter the host via contaminated food. In the current thesis two food-borne pathogens causing enteric intoxications/infections (*S. enterica* and *S. aureus*), a microorganism which seldom causes enteric infections but very important in food spoilage (*P. aeruginosa*) and one a pathotype of *E. coli* which although colonizes the gut (and hence enters the human body orally, i.e. often by food), but causes infection extra-intestinally were used as model organisms.

1.6.1. *Staphylococcus aureus*

S.aureus inhabit human skin and mucosal membranes of around 15–40% of healthy people (Sospedra et al., 2012). Beyond being one of the most important infectious agents among hospital settings, *S. aureus* is also a common cause of community-acquired, mostly pyogenic infections. Furthermore, it is estimated that approximately quarter of a million cases of foodborne illnesses are caused by *S.aureus* in the US annually (Rodríguez-Lázaro et al. 2014). People, especially food

handlers, are considered an important vehicle for the transmission of *S.aureus* and contributed directly in food contamination during preparation and serving (Sospedra, et al., 2012). Furthermore, it is increasingly recognized that animals, especially cattle suffering from mastitis are important sources mostly contaminating raw milk (Huong et al., 2010).

The most important factors in food-associated *S.aureus* disease are the staphylococcal enterotoxins (SEs) produced by about 50% of these strains (Fetsch et al., 2014). Unlike most exotoxins, the SEs are relatively heat-stable withstanding sub-optimal heat-treatment during food preparation. In the gut SE-induced inflammation is considered responsible for nausea, vomiting and, less frequently, for diarrhea (Jett et al., 1990). In extra-intestinal infections, SEs are also associated with dermatitis, nasal polyposis and have super-antigenic features contributing in an excessive inflammatory response particularly in systemic infections (Gustafson et al., 2014). SEs genes are located on transposons, i.e. mobile genetic elements, which enhance their horizontal transfer among *S.aureus* strains (Song et al., 2015).

S. aureus is a strong biofilm former. The strains either produce exopolysaccharide or a protein biofilm material (Fitzpatrick et al., 2005; Vergara-Irigaray et al., 2009). Among clinical isolates from device-related infections methicillin sensitive isolates formed polysaccharide type biofilms in medium supplemented with NaCl, whereas once grown in glucose, polysaccharide type biofilms were not detected. Glucose-induced biofilm in MRSA was made of protein-type matrix (O'Neill et al., 2007).

S. aureus is a highly clonal species and clonality has been shown to affect biofilm production (Croes et al., 2009).

1.6.2. *Salmonella*

In the last few decades, the prevalence of salmonellosis has increased worldwide. It is reported that annually the species is responsible for 1.4 million human infections, 95% of which were foodborne ones (Wang et al., 2015) resulting in about 16,000 hospitalizations with nearly 600 deaths in the US (Lee et al., 2015). *Salmonella* is a Gram-negative, non-spore forming bacillus and a member of the family *Enterobacteriaceae* (Hur et al., 2012). *Salmonella* species are associated with both animal and human infections and lead to high morbidity and mortality rates (Sánchez-Vargas et al., 2011). In principle, human-pathogenic *salmonella* can be grouped as typhoidal and non-typhoidal *salmonella*, both having important roles in food-related infections. The members of the former group, e.g. *S. typhi*, are highly adapted to man. The source of infection is always the sick or asymptomatic human carrier, often by contaminating food or water. The clinical presentation is enteric fever (typhoid), i.e. a systemic infection with high mortality. Non-typhoidal *Salmonella* spp., on the other hand, are a zoonotic microorganisms that colonize various livestock species (Fashae et al., 2010; Newell, et al., 2010). These infections are associated with the consumption food of animal origin, such as beef, egg and dairy products, and fruits and vegetables that have been contaminated with animal manure (Voetsch et al., 2004). The clinical presentation is gastroenteritis of varying severity, while in immunocompromised, it may turn systemic (i.e. bacteria is distributed throughout the body rather than concentrated in one area) (Andrews-

Polymenis et al., 2009) and even may cause a variety focal infections, such as in the meninges, and bone or joint spaces (Van et al., 2012).

1.6.3. *Pseudomonas*

The genus *Pseudomonas* is a highly heterogeneous group that is abundantly present in natural habitats like soil, fresh water and marine environments. Furthermore, some species were isolated from clinical instruments, aseptic solutions, cosmetics and medical products. (Franzetti and Scarpellini, 2007). The most important member of the family, *P. aeruginosa*, is considered a typical opportunistic pathogen. It is the third most common pathogen responsible for extra-intestinal nosocomial, urinary, blood-stream, airway and soft tissue infections (Matyar et al., 2010).

Pseudomonas spp. are rarely associated with foodborne illnesses (Pagedar and Singh, 2014), although seldom gastroenteritis may result from the consumption of contaminated food (Myszka and Czaczyk, 2009). However, *Pseudomonas* is one of the most important food-spoiling organisms that deteriorates food, changes food textural (Myszka and Czaczyk, 2009) and produce volatile compounds which considered the main source of the off-flavor compounds in food (Franzetti and Scarpellini, 2007). *Pseudomonas* spp. present in the food rich in proteins such as meat, poultry, milk and fish and in several ready-to-eat products (Gram et al., 2002)

P. aeruginosa is an excellent biofilm former and is often resistant to multiple antibiotics (Liu et al., 2012). Most of the strains involved in the infections possess

different surface virulence factors which facilitate their colonization and adherence (Mesaros et al., 2007). There are no major differences in virulence between clinical and environmental isolates (Naves et al., 2008a).

1.6.4. Extraintestinal pathogenic *Escherichia coli*

A variety of *E. coli* pathotypes can cause enteric infections spreading to the susceptible host via food or water (Newell, et al., 2010). Another groups are permanent or temporary members of the gut microbiota. The regular presence of these strains makes them markers of fecal contamination and indicators of poor hygiene and sanitation conditions. Some of them, i.e. the extra-intestinal pathogenic *E. coli* (ExPEC), may cause serious infections outside of the gut once displaced from the intestine (Köhler and Dobrindt, 2011; Vejborg and Klemm, 2009). Nevertheless, their natural habitat is also the gut and these strains also typically enter the macroorganism through food (Capita et al., 2014).

ExPEC strains cause considerable morbidity, mortality and increased health care costs (Johnson et al., 2010). The most common infection is urinary tract infections. Globally, around 130 to 175 million uncomplicated urinary tract infection UTI cases occurred each year and *E.coli* strains were responsible of 80% of those cases (Caroline et al., 2010). Annually the treatment of UTIs in the US healthcare costs \$3.1 billion (MacVane et al., 2014).

Nowadays, ExPEC strains are increasingly resistant to a variety of antibiotics. Perhaps the most serious threat is the resistance of extended spectrum

beta-lactam antibiotics (3rd and 4th generation cephalosporins and in some cases carbapenems) due to the production of extended spectrum beta-lactamases (ESBLs) and carbapenemases. The ESBL genes are commonly located on plasmids which promote its spread between different strains (Cantón et al., 2012). The prevalence of *E.coli* harboring ESBLs genes is increasing in animals (Aidara-Kane et al., 2013), primarily in poultry where the 3rd generation of cephalosporin (ceftiofur) used in chicks and broiler eggs (James et al., 2007). Actually, bacteria contaminating poultry products are increasingly considered as the reservoir for ESBL genes for human pathogens. For instance, there was a notable increase in the prevalence of ESBL-producing *E. coli* in poultry meat retailed in Spain from 62.5% to 93.3% between 2007 to 2010 respectively (Aidara-Kane et al., 2013)

ESBL-producing *E. coli*, particularly those expressing the so called CTX-M-15 type enzymes are widely spread globally. This phenomenon is closely linked to the emergence of a clone carrying the O25b cell wall antigen and belonging to sequence type 131 (ST131) (Platell et al., 2011). Various reasons may associate with the increased prevalence of this clone e.g. its capability to exchange genetic material (Rogers et al., 2011). Importantly, ingestion of contaminated food or water (Peirano and Pitout, 2010) have been noted to contribute to the spread of ST131.

Taken together, although ExPEC in general, and ST131 in particular, are not enteric pathogens, they have a huge impact on human health and food appears to be important in transmitting these bacteria. Consequently, their biological features, e.g. their capacity to form biofilm carries considerable importance from the perspectives of this research work.

1.7. Possible interference with food-born transmission of microorganisms

Food safety includes adherence to certain guidelines at all possible stages of the food chain. Some of these rules, particularly those related to the last stage, i.e. food preparation, are ancient, culturally embedded, mostly common sense procedures (Fleckenstein et al., 2010), while others, applied from agricultural production to industrial scale food preparation and processing are strictly regulated by government agencies. Some are general rules, most related to hygiene, while others target the prevention of contamination with specific pathogens (Examples of such list of guidelines for the USA can be seen at: <http://www.fda.gov/Food/GuidanceRegulation>). As during food processing the role of biofilms are increasingly recognized in contamination and in microbial transmission, the prevention of its development and its elimination are possible ways to improve food safety.

1.7.1. Controlling the biofilm problem

In principle there are two ways to control biofilms. The most important strategy is to prevent their formation by adopting one of several approaches. This can either be achieved by eliminating bacteria before they could form biofilms or by using surfaces resistant to biofilm formation. This latter approach means that the physiochemical properties of surfaces are modified or coated with either antimicrobial agents or other substances, such as benzyldimethyldodecylammonium chloride and silver (Srey et al., 2013).

The methods used to eliminate existing biofilms can be physical, chemical and biological. Physical control includes super-high magnetic fields, ultrasound treatment, high pulsed electrical fields and low electrical fields combined with biocides (Kumar and Anand, 1998). Chemical methods usually represent different types of biocides and sanitizers and they must be effective enough to eliminate EPS in order to facilitate their penetration to the viable cells. The combination of physical and chemical methods could increase their efficacy against biofilms. Halogens, peroxygens, acids, and quaternary ammonium compounds are the major compounds used in the food industry. However, their effectiveness is limited by the presence of soil, water hardness, temperature of applications and the ability to the physically contact to microorganisms (Chmielewski and Frank, 2003; Myszka and Czaczyk, 2009).

Biological approaches have advantages over the other two methods. They have higher effectiveness, lower toxicity, more sustainability and less bacterial resistance. The most important examples of this method are quorum quenching (QQ), enzymes; energy uncoupling, cell wall hydrolysis and the application of bacteriophages (Malaeb et al., 2013).

1.8. Nanotechnology

In his famous lecture: “There is Plenty of Room at the Bottom”, in 1959, Richard Feynman had introduced the first concept of nanotechnology (NT). He stated that the boundary of knowledge and technology could be found not only in physics but also in other nano-sized fields. Afterward, Norio Taniguchi was the first who

used and proposed the term "*nanotechnology*" in 1974 and it was referred to the precise and accurate tolerances required for machining and finishing materials (Ashby et al., 2009a). Later on, significant discoveries had been developed in different fields and more investment has been spent particularly in the field of fullerenes and carbon tubes (Miyazaki and Islam, 2007). Today, the term "*nanotechnology*" indicates a technology of design, fabrication, and applications of nanostructures and nanomaterials (Cao and Wang, 2011) .

The word "*nano*" is derived from Greek and means "*dwarf*" referring to tiny things with the size one billionth of a meter (10^{-9} m) (Narayanan and Sakthivel, 2010). The national Nanotechnology Initiative has proposed the definition of nanotechnology as "the understanding and control of matter at dimensions of roughly 1-100 nm", where the materials below the sub-microscopic level were produced by manipulating their atoms and molecules (Adams and Barbante, 2013).

1.8.1. Nanoparticles

Nanoparticles are recognized as the essential backbone of nanotechnology where assembling of precursor particles and related structures is fundamental of developing nanostructure materials (Roco, 1999). Previously, particles characterized by their small size less than 100 nm were termed as ultra-fine particles or submicron, but since 2000 the word nanoparticle has become the term accepted (Kruis and Joshi, 2005). The denotations proposed by different organizations are summarized in Table1 (Horikoshi and Serpone, 2013).

Table 1: Definitions of nanoparticles used by different organizations

Organization	Nanoparticles definition
ISO	A particle spanning 1–100 nm (diameter)
ASTM	An ultrafine particle whose length in 2 or 3 places is 1–100 nm
NIOSH	A particle with diameter between 1 and 100 nm, or a fiber spanning the range 1–100 nm.
SCCP	At least one side is in the nanoscale range.
BSI	All the fields or diameters are in the nanoscale range.
BAuA	All the fields or diameters are in the nanoscale range.

ISO- International Organization for Standardization, ASTM- American Society for Testing and Materials, NIOSH- National Institute for Occupational Safety and Health, SCCP- Committee on Consumer Products, BSI- British Standards Institution, BAuA- Bundesanstalt für Arbeitsschutz und Arbeitsmedizin.

Generally, NPs can be defined as materials with two or three dimensions between 1 to 100 nm (Fahrner, 2005). Precisely, NPs are amorphous semi-crystalline 0-dimension nanostructures with dimensions larger than 10 nm, and relatively larger ($\geq 15\%$) size dispersion, whereas, the nanostructures materials smaller in size (1-10 nm) and narrow size distribution called nanoclusters (Fahlman, 2011).

1.8.2 Classification of nanoparticles

NPs originate from two main routes. Incidental NPs are byproducts of various processes, while engineered NPs are intentionally prepared for specific purposes (Iavicoli et al., 2013). Nano-materials (NMs) can be categorized based on origin, dimensions and structural content. NMs have been classified into 4 categories based on the number of their dimensions which are not restricted to the nanoscale range (Ashby et al., 2009b). These are 0-dimension (nanoparticles), 1-dimension

(nanorods, nanowires or nanotubes), 2-dimension (thin nanofilm, nanolayers) and 3-dimension (dispersions of NPs and bundles of nanowires (Ashby et al., 2009b; Vollath, 2013)).

NMs are also categorized based on their major constituents, organic and inorganic, into 3 classes, [1] organic polymer (e.g. emulsions, liposomes and dendrimers), [2] inorganic metallic (e.g. metals, metals oxides and magnetic materials), [3] semiconductor (e.g. quantum dots) (Luo and Stutzenberger, 2008).

1.8.3. Synthesis of nanoparticles

Nano-materials are synthesized through two main techniques, i.e. the top-down and bottom-up methods (Figure 2). The experimental condition of NPs production in both laboratory and industrial areas should be controlled in order to produce identical NPs in terms of size, morphology, chemical composition, crystal structure and monodispersity (Ju-Nam and Lead, 2008).

The top-down approach is more applicable for the commercial purpose in which the bulk materials were reduced to their nano-size by different ways, such as milling, nanolithography or precision engineering (Azeredo, 2009). The bottom-up approach is commonly used for chemical and biological synthesis of NPs where atoms or molecules are combined to molecular structures (Narayanan and Sakthivel, 2010). In this method the NPs assembled are generally produced from bulk materials (solid phase) to generate the nanofraction of the materials by using various

distribution tools (Ju-Nam and Lead, 2008) such as milling and lithography (Cao and Wang, 2011).

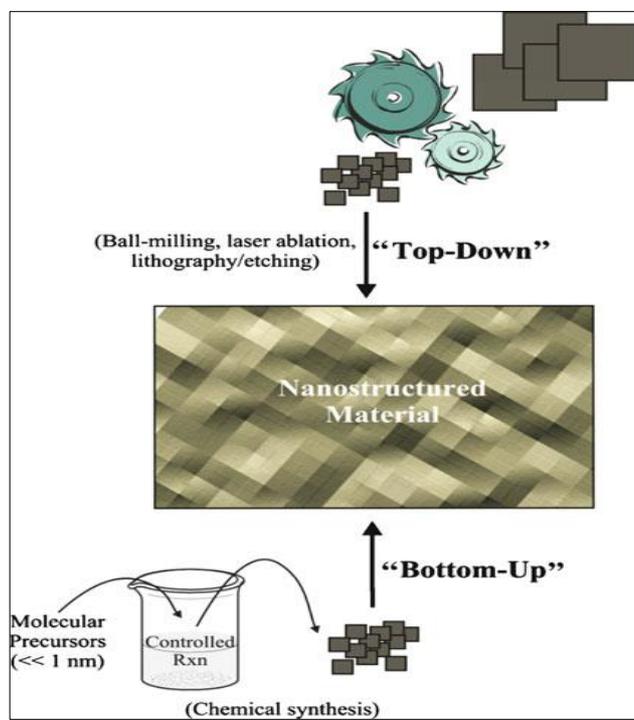


Figure 2: The synthesis of nanoparticles (Fahlman, 2011).

Grinding system (e.g. dry and wet grinding), mechanochemical methods (e.g. mills and ultrasonic wave) and mechanical alloying methods are examples of this approach (Horikoshi and Serpone, 2013).

NPs can be build up from the bottom atom by atom, molecule by molecule or cluster by cluster (Cao and Wang, 2011). The techniques based on this method are summarized in Table 2.

Table 2: Nanoparticle preparation techniques (Vemal et al., 2011)

Method	Description of method	Advantages	Disadvantages
Molecular beam	Beams are directed towards specific metal targets using a variety of methods; laser vaporization, pulsed arc, ion and magnetron sputtering. This creates clusters of metallic nanoparticles including nanoparticles	<ul style="list-style-type: none"> Any type of nanoparticle or nanoalloy can be created from metallic/alloy targets. Nanoparticle synthesis is quick and not lengthy 	<ul style="list-style-type: none"> Process is expensive, and requires equipment setup in most cases
Chemical reduction	Use of precursor salts, reducing agents and stabilizer to synthesize nanoparticles. In most cases a catalyst and some heating is used.	<ul style="list-style-type: none"> Can readily produce bulk quantities of nanoparticles and nanoparticles . Process can be easily scaled up to meet mass manufacturing needs. Process enables synthesis of particles close to 1 nm and this can easily be controlled. Process is relatively cheaper compared to other synthesis methods since the technology is quite standard. 	<ul style="list-style-type: none"> Mass use of chemicals and some may be harmful to the environment. Processing is time consuming and depends on many parameters.
Thermal decomposition of metals	Thermal decomposition of metal or metal complexes (for nanoparticles) is produced using high temperature mediums or solvents.	<ul style="list-style-type: none"> Nanoparticles can be created at relatively low temperatures. Process can create nanoparticles in a wide range of sizes. 	<ul style="list-style-type: none"> Requires use of chemicals and solvents, which may be harmful to the environment.
Ion implantation	This method is used to create NPs by implanting two or more metal ions into a specific matrix. This generates metallic/bimetallic clusters.	<ul style="list-style-type: none"> Metallic ions can be implanted into exact positions in a matrix. Various combinations of ions can be used to yield nanoalloy clusters. 	<ul style="list-style-type: none"> Requires equipment setup which is relatively expensive compared to chemical reduction.

Table 2. - cont.

Method	Description of method	Advantages	Disadvantages
Electro-chemical synthesis	Using an electrolysis cell and two electrodes of metallic elements, bimetallic NPs/nanoparticles can be created in solution. Core-shell structures have also been created via this method.	<ul style="list-style-type: none"> • Various nanoparticles combinations can be synthesized. • Cell setup is rather easy and does not need extensive equipments. 	<ul style="list-style-type: none"> • Use of chemicals as electrolytes which may yield harmful/toxic gases as by products from the process.
Radiolysis	Radiolysis of an aqueous solution of metal ions to produce nanoparticles. This method has also been used to create nanoparticles /bimetallic particles.	<ul style="list-style-type: none"> • Irradiation of molecules is able to create nanoparticles with a wide range of sizes, as well as very narrow sizes. 	<ul style="list-style-type: none"> • Requires expensive equipment setup. • Radiation is harmful to the health of living organisms, including humans. • The use of this method requires extensive clearance from concerned authorities.
Sonochemical synthesis	Irradiation of metal salt solutions using ultrasound to create nanoparticles and nanoparticles.	<ul style="list-style-type: none"> • Sonic wave irradiation is able to create narrow particle sizes. 	
Biosynthesis	Biological means are used to synthesize nanoparticles and nanoparticles using microorganisms or plants. Synthesis of nanoparticles using these mediums can make the nanoparticles more bio-compatible.	<ul style="list-style-type: none"> • This method is cheap and uses sources from nature. • The method does not produce waste detrimental to human beings. • Does not require extensive equipment setup. 	<ul style="list-style-type: none"> • This method is slow and takes time.

The manufacturing of the NPs involves three main steps: co-precipitation or nucleation, growth and agglomeration. NPs tend to agglomerate, under the action of Van der Waals forces, as a way to decrease their high surface area (Ribeiro and Leite, 2009). This approach is divided into gaseous phase methods and liquid phase methods. The liquid phase methods are the major technique to engineer NPs and they can be sub-divided into liquid/liquid methods and sedimentation methods. Gel-sol process is a prime example of sedimentation method and greatly used in metal oxides NPs synthesis. Chemical reduction of the metal ions is a typical example of liquid/liquid method (Horikoshi and Serpone, 2013).

1.8.3.1. The chemical reduction method

The reduction method is applicable to produce either monometallic or bimetallic NPs from their transition metal salts in a dry powder form by using different types of stabilizers and reducing agents with narrow size distribution (Bönnemann and Richards, 2001). Also, it is easy to control the primary structure (e.g. size, shape, composition) of the NPS (Toshima et al., 2008).

The principle of the chemical reduction is that the transition metals are reduced to produce a zerovalent metal colloids in aqueous (hydrosols) or organic media (organosols) (Boönnemann and Nagabhushana, 2008). Several steps are involved. First the process starts with the reduction of the metal precursor by a reducing agent, followed by elementary nucleation. After the nuclei are formed, they grow slowly via deposition onto the solid surface till the complete formation

of NPs (Yu et al., 2008). Various types of stabilizers could be used at any stage of synthesis to prevent NPs agglomeration (Hubenthal, 2011).

Bimetallic NPs has a potential synergetic effect (Toshima et al., 2008), diversity structures, and composition (Liu et al., 2014). Generally, bimetallic NPs categorized into four divisions based on their structure:

- Core shell segregated structure, a shell of one type of atom surrounds a core of the other atom.
- Hetero-structure is formed by independent nucleation process and growth of two kinds of metal. Atoms of the two different metals share a mixed interface or have only a small number of bonds.
- Intermetallic or alloyed structure, a homogeneous mixture of two metals exists in the form of a solid solution.
- Multi-shell structure, layered or onion-like alternating shells that are usually metastable or stable exist (Liu et al., 2014).

1.8.3.2. The biological, “green chemistry” method

The main advantages of the green method are the utilization of nontoxic materials, the use of environment friendly solvents and of renewable, biodegradable materials, and the minimized energy requirement (Aiad et al., 2014; Stevanović et al., 2012). Green chemistry is a bottom-up method where the microbial enzymes or the plant phytochemicals are responsible for metals reduction (Nath and Banerjee, 2013).

Many types of bacteria have been known to produce metal structures either intra or extracellular (Nath and Banerjee, 2013) as a resistance mechanism or to conserve energy for growth (Hennebel et al., 2009). For instance, *Pseudomonas stutzeri* is resistant to silver, and this property is attributed to the intracellular accumulation of silver crystals of approximately 200 nm in diameter with a well-defined composition and shape (Hennebel et al., 2009). Various microbes are well known to reduce Ag metal to NPs, e.g. *K. pneumoniae*, *E. coli*, and *Enterobacter cloacae* has been investigated in this regard (Sharma et al., 2009).

In the recent decade, various biological agents have been investigated to produce different types of metallic nanoparticles like copper, zinc, titanium, gold, and silver NPs (Durán and Marcato, 2012; Durán et al., 2011; Manjumeena Rajarathinam et al., 2013). Polymers like chitosan, starch, and polypeptide also have been studied for their application as reducing and stabilizing agent in NPs preparation (Zhao et al., 2014).

1.9. Properties of nanoparticles

The unique properties of NPs are attributed to their small size, and to the high surface to volume ratio resulting in a high percentage of atoms on the particle's surface. Consequently, reactivity is increased and, depending on the application, it can provide increased surface catalysis, improved loading of the surface or greater release of ions into solution (Perni et al., 2014). Their ultrafine size is similar to that of biological macromolecules like proteins and structures like viruses (Fadeel and Garcia-Bennett, 2010). These unique characteristics

include shape, surface properties, purity, stability, molecular weight, composition, identity and solubility (Lin et al., 2014).

The full understanding of the physiochemical characteristic of NPs is important to realize their toxicity to biological system (Oberdörster et al., 2005). Some NPs have tendency to form aggregates or agglomerates under ambient condition. Various forces play a role in NP-NP interactions such as weak van der Waals forces and stronger polar and electrostatic or covalent interactions. This kind of interaction forces between either NP-NP or NP-aqueous solution are the basis for chemical and physical processes (Niazi and Gu, 2009).

Size is one of the most critical factors among NPs properties as it could play an important role in physiological interactions. For example, it regulates NPs movement, penetration and localization of specific targets (Amol S Amritkar et al., 2011). Surface composition is also an important factor relevant to the dissolution, aggregation and accumulation of NPs. Surface charge controls the dispersion stability or aggregation of nanoparticles (Lin et al., 2014).

1.10. Characterization of nanoparticles

Various physical and chemical techniques, such as separation, spectrometric and microscopy techniques, have been employed to characterize the NPs' composition, morphology, coating and size (Peralta-Videa et al., 2011) (Capaldi Arruda et al., 2015). Separation methods mostly developed for the size determination (Shen and Yu, 2009). Cloud-point extraction, chromatographic

methods, electrophoresis and density-gradient centrifugation are the most frequently used methods (Liu et al., 2012). Among these HPLC is considered the most powerful and efficient technique because it capable to separate small sizes (<10 nm) NPs (Capaldi Arruda et al., 2015).

Electron microscopy, transmission and scanning electron microscopy are used to visualize nanoparticles and determine their size, polydispersity, and shape (Maskos and Stauber, 2011). On the other hand, the spectrometric methods, X-ray diffraction, dispersive spectroscopy X-ray and inductively Coupled Plasma Mass Spectrometry (ICP-MS) are used to quantify the elementary information of NPs (Capaldi Arruda et al., 2015).

Zeta potential has also been used to characterize the surface charge of NPs. Those particles with high Zeta potential value, (above ± 30), usually exhibit stability in suspension because the surface charge prevents aggregation of those NPs (Lin et al., 2014).

1.11. Application of Nanoparticles

Their small size, in combination with the chemical composition and surface structure gives NPs their unique features and huge potential for applications (Bouwmeester et al., 2009; de Faria et al., 2014). This is the driving force behind developing new products with new properties to meet the increased demand in the industrial areas (de Faria, et al., 2014). The enormous potential of the technology is shown by the fact that three to four new nanotechnology

consumer products introduced into the markets weekly (Gajewicz et al., 2012). It is speculated that nanotechnology markets will reach 1 trillion dollars by 2015. Nanotechnology has been involved in four main areas nanomedicine, nanofabrication, nanometrology and nanomaterials or nanoparticles (Aitken et al., 2006). Nanotechnology has caused a dramatic change in many disciplines from science, industry to agriculture (Yao et al., 2013).

Application of NT has considerably increased recently and it is estimated that in 2010 only \$1.64 billion were spent on the advancement of nanotechnologies in the US (Cushen et al., 2012). Due to their capacity to adsorb and carry drugs, probes and protein, they have been widely used in the different medical fields (Nath and Banerjee, 2013) such as pharmaceuticals, medical imaging and diagnosis, cancer treatment, implantable materials, and tissue regeneration (Etheridge et al.). Furthermore, nanoscale materials are used for cosmetics, house paints, clothing, and computers (Vaseashta, 2009).

1.11.1 Application of nanotechnology in food sector

In nature, several food ingredients have nanoscale properties, e.g. the native beta-lactoglobulin food protein has about 3.6nm length. Nanotechnology could be utilized all through the food sector "from farm to fork" (Sekhon, 2010) as summarized in Figure 3.

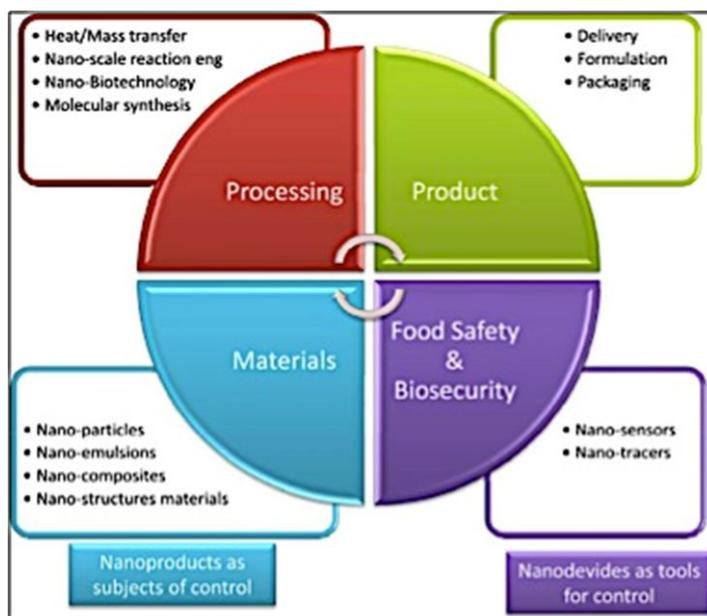


Figure 3: Application of nanotechnology in the food sector

The main purposes of applying NT in the food area are to improve food quality and safety. NPs have been used to alter food texture, encapsulate food components, develop new tastes and sensation, control flavor release and bioavailability of nutritional components (Chaudhry et al., 2008). Along with that, the new innovation in NTs help to create new food packaging materials with enhanced mechanical, barrier, antioxidant and antimicrobial properties (Jiménez and Ruseckaite, 2012).

In order to enhance the functionality of the packaging material, they were manufactured from nanocomposites that are comprised of a single, or mixture of polymers with at least one organic or inorganic nano-filler such as SiO₂, clay, TiO₂, silicates, and noncellulose. These new formula are capable to enhance mechanical properties, e.g. stiffness, toughness, tensile, shear strength, and barriers properties for the diffusion of the permeant molecules (Mihindukulasuriya and Lim, 2014).

Recently, plenty of polymers have been innovated as nanocomposite film such as Durethan. This nanomaterial film is enriched with an enormous number of silicate nanoparticles that reduce entry of oxygen and other gases and the exit of moisture, thus preventing food from spoiling (Sekhon 2010). Clay nanoparticle is another example of nanocomposites emerged on the market as food packaging materials. The nanoclay mineral used in these nanocomposites is montmorillonite which has a natural nano-layer structure that limits the permeation of gases, and provides substantial improvements in gas barrier properties of nanocomposites. This material has a potential use in a variety of food-packaging applications, such as processed meats, cheese, as well as in extrusion-coating applications for fruit juices and dairy products, or co-extrusion processes for the manufacture of carbonated drinks bottles (Chaudhry et al. 2008).

Currently, antimicrobial nanocomposite films were incorporated into food packaging materials to work as growth inhibitor, killing agents or antibiotic carriers. Polymers based their structure on silver NPs mostly are used as antimicrobial coating films (Azeredo, 2009). Zapata and coworkers demonstrated that polyethylene nanocomposites coated with Ag NPs has 99.99 % of efficacy against bacteria compared with the uncoated one (Zapata et al., 2011).

It has been claimed that Ag NPs exhibit other functions beside their antimicrobial effect, such as extending shelf life of fruits and vegetables through absorption and decomposition of ethylene, and retarding senescence (Azeredo, 2009). Furthermore, Cu-nanofiber was shown to enhance the package tensile

strength and worked as oxygen barriers and antimicrobial agent when it incorporated with high density polyethylene (Bikiaris and Triantafyllidis, 2013).

NP-based biosensors have been shown to improve sensitivity, specificity and carry the promise to develop new innovations to detect pathogens. Three nanotechnologies have been proposed for this purpose i.e. nanoarrays, nanofluids and nanotransduction (Driskell and Tripp, 2009). Yang and coworkers reported that a test based on NPs capable to detect of *E. coli* O157 inoculated in ground beef with as low detection limit as 1.6×10^2 cfu/ml. Along with that, this technology was sensitive enough to detect *L. monocytogenes* in mono or two species biofilm models (Yang et al., 2008). Moreover, nanosensors were developed for traceability and monitoring condition of food during transportation and storage (K.A. Abbas et al., 2009) where these sensors can interact with either food components or external environment and generate a response in correlation with the food status such as oxygen indicators (Mihindukulasuriya and Lim, 2014), food freshness sensor (Maynor et al., 2007), and time-temperature indicators (Zeng et al., 2010).

NPs have a great impact on the water and wastewater treatments where they have been engineered as a filter membrane with excellent properties. Membranes were designed with nano-adsorbents materials that are capable to remove organic and inorganic contaminates in water (Qu et al., 2013). Along with what mentioned above, also NPs used as carriers of synthesized pesticides (de Oliveira et al., 2014), nanoceuticals (Chellaram et al., 2014), animal feed nanosupplements and mycotoxin binders (Handford et al., 2014).

1.11.2. Antimicrobial and anti-biofilm effect of metals and nanoparticles

Although various metals are essential micronutrients for bacterial growth, some of them are toxic at certain level such as iron, zinc, cobalt, copper and nickel (Matyar et al., 2014). The mechanism of toxicity of metals is strongly dependent on its oxidation state and on the role of the structure or function of chemical species in the cell. Most of the metals ions can bind to thiol groups, affecting the activity of essential enzymes or the role of structural proteins. Similarly, metals cations may displace essential cations within the enzymes, modifying their functionality or causing an imbalance of the redox state of the cell leading to oxidative stress (Borsetti et al., 2009). Some toxic metals have potency to inhibit and kill sessile cells and increase detachment of bacteria and EPS from biofilms. The effectiveness of the metal depends on metal speciation and concentration and on microbial species. It has been documented that at certain metal concentrations, EPS production may be enhanced and this may lead to retardation of metal transport in EPS matrix (Yang et al., 2013).

In recent years, the use of metals in the form of nanoparticles as antimicrobial agents has gaining noticeable attention (Allaker et al., 2011). Different types of nanomaterials like copper, zinc, titanium, magnesium, gold, alginate and silver were introduced but silver NPs have proved to be most effective NPs (Rai et al., 2009). Also, metal oxide showed a remarkable antibacterial activity due to high surface areas and unusual crystalline morphologies with a high number of edges and corners, and other potentially reactive sites (Sathyanarayanan et al., 2013).

The antimicrobial mechanisms of metal NPs is attributed to their high surface to volume ratio rather than to the effect of metal-ion release. Such characteristics allow nanoparticles to interact closely with cell membrane, damage their structure, and inactivate bacterial enzyme (Sathyanarayanan, et al., 2013). The potency of NPs is determined by their physical properties (i.e. size and shape) and their chemical composition and stability (Ivask et al., 2012). Size has a strong reverse relationship with NPs antimicrobial activity, i.e. smaller size Ag NPs appeared to be more toxic than larger particles (Choi et al., 2009). Shape also affects activity of nanoparticles. It has been shown that silver nanoparticles with truncated triangular shape displayed the greatest bactericidal effect compared with spherical and rod shaped nanoparticles (Allaker, 2012).

Information on the susceptibility of biofilms to nanoparticles is limited and their antimicrobial effects are unclear. A study conducted by Raftery and coworkers revealed that Ag NPs were found to inhibit biofilm formation of *P. aeruginosa* and *S. epidermidis* by 95 % and caused sloughing of *P. putida* biofilms (Raftery et al., 2013). Kalishwaralal and coworker reported that Ag NPs could directly transport into the biofilm through the water channel, diffuse through biofilm matrix and block the synthesis of EPS by bacteria (Kalishwaralal et al., 2010). Another study demonstrated that ZnO NPs produce reactive oxygen species (ROS) that interfere with *E. coli* and *S. aureus* biofilm formation. Also, superparamagnetic iron oxide showed the highest antibacterial activity against biofilms, they have a considerable capability to penetrate into biofilms by using external magnetic fields (Hajipour et al., 2012)

The diffusing capacity of NPs into biofilm is controlled by their mobility, aggregation, and toxicity. The diffusion of NPs may be restricted by (1) local accumulation of NPs by cells, non-diffusing macromolecules or the EPS matrix; (2) the porous structure of the biofilm, and (3) the adsorption of the solute to freely diffusing species, abiotic surface or gas bubbles (Martinez-Gutierrez et al., 2013).

Although, application of NPs is sharply increased, the knowledge on their potential toxicity is still limited (Bouwmeester et al., 2009). Despite, the unique chemical and physical properties of NPs, their potential risk must be taken in to consideration. Based on their nature NPs have different potential toxicity mechanism (Niazi and Gu, 2009). There are three possible routes where NPs can access human body: dermal exposure, inhalation and ingestion (Chau et al., 2007). Due to the reactive surface of the small particles size, harmful free oxygen radicals are generated and initiate several side effects. First, NPs have high ability to attack cell membrane, DNA and proteins. Second, their capability to penetrate the body and cells enhance their access to reach sites where they are not normally present. Third, as their bio-distribution is unknown, it could promote their accumulation in the body over time which may lead to a potential hazard (Liu et al., 2014). The adverse health effects of the engineered nanomaterial on various organs could be present after long term exposure. These effects may include oxidative stress on immune and activation of pro-inflammatory cytokines in lungs, liver, heart and brain (Bouwmeester, et al., 2009).

Taken together, nanoparticles have several features, which make them attractive candidates to be used in interfering with food-contaminating microorganisms. During my work I intended to explore some of these possibilities.

Chapter 2. Aims and objectives

In order to establish more effective preventive strategies, the long term aim of the study was to contribute to a better understanding of how bacteria colonize niches related to food-production, in particular how they establish biofilms on inanimate surfaces and also to clarify how metal NPs applied in forms of nanoparticles could eliminate some of the most common food-borne bacteria, prevent the formation and reduce existing biofilms. It is believed that by achieving our objectives an information highly relevant for combatting food-transmitted infections could be provided.

Specific objectives

1. to investigate the biofilm producing capacity of *E. coli* ST131 strains, i.e. a multidrug resistant extra-intestinal pathogen often transmitted by food, under various growth conditions.
2. to reveal the clonal nature of biofilm formation in *E. coli* ST131.
3. to relate biofilm formation to particular genes
4. to evaluate the antibacterial effect of the nanoparticles against a selected groups of food-borne pathogens
5. to investigate the capacity of the nanoparticles to kill bacteria in existing biofilms.
6. to reveal whether sensitivities in biofilms to nanoparticles have any clonal relation in *S. aureus*

7. to prove the sanitary effect of nanoparticles against food-borne pathogens when applied on stain-less steal surfaces

Chapter 3. Materials and methods

3.1. Bacterial strains

For routine susceptibility testing of planktonic and sessile cells in biofilms, as well as for assessing the sanitary effect of nanoparticles (NPs) clinical isolates methicillin resistant *Staphylococcus aureus* (MRSA) S800, *Escherichia coli* TPC244, SZ29659 and 90405, *Pseudomonas aeruginosa* AG1 and *Salmonella* BC789 were used from our own strain collection (Medical Microbiology and Immunology Department, College of Medicine and Health Sciences, UAEU). The clonality of nanoparticle efficacy in *S. aureus* was tested using 10 methicillin resistant *S. aureus* (MRSA) strains recovered in Tawam and Al Ain hospitals (Al Ain, UAE) from various clinical materials, representing various clonal lineages (see later). The biofilm forming capacity of *E. coli* ST131 clone was tested on a pool of 115 non-repeat isolates recovered from blood and urine samples of patients in Tawam hospital (Al Ain, UAE) (95 isolates) and in three Hungarians university hospitals (Szeged, Debrecen and Budapest, Hungary) (20 isolates). *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were used as controls for antibiotic susceptibility testing.

All isolates were stored in duplicates in Tryptic Soy Broth (TSB, Mast, UK) containing 10% glycerol (Univar, US) in two separate -80 °C freezers being on independent electric circuits. Prior each experiment strains were propagated on Tryptic Soy Agar (TSA, Mast, UK) and incubated for 24 h at 37 °C.

3.2. Susceptibility testing

Qualitative antibiotic susceptibility testing was conducted by the Kirby-Bauer (disc diffusion) method. Overnight cultures grown at 37 °C on Mueller Hinton agar (MHA, Mast, UK) were used to suspend bacteria into 0.9% NaCl. This suspension was applied by a sterile swab (Citoswab, China) onto MHA plates and the antibiotic discs were placed on by an applicator, seven discs per an 90mm dish. Plates were incubated for 18 h at 37 °C. This mode of inoculation yielded a semi-confluent growth. For Gram-negative bacteria (*P. aeruginosa* and *E. coli*) the following antimicrobials were tested using discs (MAST: ceftazidime, imipenem, meropenem, gentamicin, amikacin, doxycyclin, ciprofloxacin and trimethoprim-sulphamethoxazole). For *S. aureus* the following discs were used: cefoxitin, mupirocin, clindamycin, erythromycin, gentamicin, amikacin, kanamycin, streptomycin, neomycin, rifampicin, fusidic acid, teicoplanin, linezolid, trimethoprim-sulphamethoxazole, ciprofloxacin and tetracycline. The diameters of the zone of inhibition were measured with a caliper.

To determine the Minimal Inhibitory Concentration (MIC) of nanoparticles three to four isolated colonies were picked, suspended into 5 ml Mueller Hinton broth (MHB, Oxoid, UK) and incubated at 37 °C for 4 h under shaking of 300 rpm. After incubation, the optical density (OD) of the culture was measured at 600nm (DU-70 spectrophotometer, Beckman, US) and converted to cfu/ml value using a standard curve. Subsequently, the suspension was diluted in MHB in order to obtain approximately 5×10^5 cfu/ml.

Antibiotic stocks were prepared to yield doubling multiplicities of 1 mg/L concentration and diluted serially in 96 wells microtiter plates (Nunc, US) containing 50 μ l MHB. NP stock solutions at 1 mg/ml concentration were freshly prepared in MHB and sonicated for 20 min (Ultrasonic Homogenizer 4710 series, Cole-Parmer, US). Serial dilutions of NPs were prepared in a sterile 96 wells plate containing 50 μ l MHB.

Aliquots (50 μ l) of the bacterial suspension were dispensed into each well except the last one, which was used as a sterility control. Plates were sealed with Parafilm to avoid evaporation and were incubated at 37 °C for 24 h under shaking of 250 rpm using Orbi shaker MP (Benchmark, US) to prevent sedimentation of the nanoparticles. On the following day, the OD of the wells was measured at 600nm using plate reader (Tecan, Austria). The MIC was defined as the lowest concentration of the test compounds that inhibited visible growth (i.e. OD not exceeding that of the sterility control well). All tests were performed in triplicates.

For antibiotics, susceptibility was determined according to the CLSI guidelines (CLSI, 2014). For nanoparticles, as they do not have established "clinical breakpoints" only the mg/L value was registered without any further interpretation.

3.3. Molecular methods

3.3.1. DNA extraction

Three to four isolated colonies of *E. coli* from fresh TSB culture were suspended in 200 µl sterile distilled water, incubated at 99 °C for 10 min and centrifuged at a maximum speed (14,800rpm) for 10 minutes (Sigma Microfuge, US). Afterward, the supernatants were collected, transferred into new Eppendorf tubes, kept at 4 °C and used as samples in PCR reactions.

S. aureus DNA was extracted according to the method described (Louie et al., 2002). Three to four colonies were inoculated into 95 µl Triton X lysis buffer and 5µl lysostaphin (400 mg/L). Samples were incubated at 37 °C for 10 min and followed by another incubation at 99 °C for 10 min. Later on, the tubes were centrifuged at high speed (14,800 rpm) for 10 min. Finally, the supernatants were transferred into new Eppendorf tubes and kept at 4 °C until used.

3.3.2. Genotyping *Escherichia coli* by Polymerase Chain Reaction

PCR amplifications were carried on Applied Biosystems 2700 and 2720 thermocyclers. Typically, the reaction was initiated at 94 °C for 5 min followed by 20-35 cycles at different annealing, denaturation and extension temperatures, depending on the primers used. After the last PCR cycle the product was kept for a final extension at 72 °C for 7-10 min. All genes targeted, the used primers, and the reaction conditions were summarized in Table 3.

Table 3: PCR reactions of *E. coli* adhesion genes

Gene	Primer	Sequences (5'-3')	Product size	Initial Denaturation	Cycle	Final extension	Ref
Detection of fimbria genes							
<i>papC</i>	PapC f PapC r	GTGGCAGTATGAGTAATGACCGTTA ATATCCTTTCTGCAGGGATGCAATA	220 bp				
<i>papAH</i>	PapA f PapA r	ATGGCAGTGGTGTCTTTTGGTG CGTCCCACCATACGTGCTCTTC	720 bp				
<i>papEF</i>	PapEF f PapEF r	GCAACAGCAACGCTGGTTGCATCAT AGAGAGAGCCACTCTTATACGGACA	336 bp				
<i>fimH</i>	FimH f FimH r	TGCAGAACGGATAAGCCGTGG GCAGTCACCTGCCCTCCGGTA	508 bp				
<i>bmaE</i>	bmaE-f bmaE-r	ATGGCGCTAACTTGCCATGCTG AGGGGGACATATAGCCCCCTTC	507 bp				
<i>sfa/focDE</i>	sfa1 sfa2	CTCCGGAGAACTGGGTGCATCTTAC CGGAGGAGTAATTACAAACCTGGCA	410 bp	5' at 94°C	25X (30" at 94°C, 30" at °C 63 and 60" at 72°C)	10' at 72°C	(Johnson and Stell, 2000)
<i>gafD</i>	gafD-f gafD-r	TGTTGGACCGTCTCAGGGCTC CTCCCGGAACTCGCTGTTACT	952 bp				
<i>focG</i>	FocG f FocG r	CAGCACAGGCAGTGGATACGA GAATGTCGCCTGCCCATGCT	360 bp				
<i>afa/draBC</i>	Afa f Afa r	GGCAGAGGGCCGGCAACAGGC CCCCTAACGCGCCAGCATCTC	559 bp				
<i>sfaS</i>	SfaS f SfaS r	GTGGATACGACGATTACTGTG CCGCCAGCATTCCCTGTATTC	240 bp				
<i>nfaE</i>	nfaE-f nfaE-r	GCTTACTGATTCTGGGATGGA GCTTACTGATTCTGGGATGGA	559 bp				

Cont. Table 3.

Gene	Primer	Sequences (5'- 3')	product size	Initial Denaturation	Cycle	Final extension	Ref
Detection of fimbria genes							
<i>iha</i>	iha-I iha-II	CAG TTCAGTTTCGCATTCACC GTA TGG.CTC.TGATGCGATGG	1305 bp	5' at 94°C	35X (30" at 94°C, 30" at 53°C and 90" at 72°C)	10'at 72°C	(Toma et al., 2004)
Detection of Autotransporter UPA&C genes							
<i>UpaH</i>	upaH f 524 upaH r 525	AGT GAA GGG GCA AAA ACC TT TGA AAC CAC CAC CAT TCT GA	328 bp	5' at 94°C	30X (30" at 94°C, 30" at 50°C and 60" at 72°C)	7'at 72°C	(Allsopp et al., 2012)
<i>UpaB</i>	CO426.s-5 CO426.s-3	GGA AAG GCA AAG TTT CAG GG GGT GGT ATG TTT CTG TTT AC	462 bp				
Detection of curli fimbriae							
<i>Crl</i>	M571F M570R	TTTCGATTGTCTGGCTGTATG CTTCAGATTCAGCGTCGTC	250bp	5' at 94°C	30X (30" at 94°C, 30" at 50°C and 60" at 72°C)	7'at 72°C	(Maurer et al., 1998)
<i>CsgA</i>	M464F M465R	ACTCTGACTTGACTATTACC AGATGCAGTCTGGTCAAC	456bp				
Detection of cellulose synthase gene							
<i>BcsA</i>	BCSA74 F BCSA86 R	GCAACAGATTCAATTTCTGCCCTTC GCACCCGCGCTGGCAGCGTATTCG	860bp	5' at 94°C	35X (30" at 94°C, 30" at 58°C and 1'30" at 72°C)	7'at 72°C	(Zogaj et al., 2003)

Cont. Table 3.

Gene	Primer	Sequences (5'-3')	product size	Initial Denaturation	Cycle	Final extension	Ref
Detection of Autotransporter Ag43 genes							
Ag43	agn43 F agn43 R	CTGGAAACCGGTCTGCCCTT CCTGAACGCCCAGGGTGATA	433bp				
Ag43-K12	agn43K12 F agn43K12 R	CCGGCGGGCAATGGGTACA CAGCTCTCACAATCTGGCGAC	386 bp				
Ag43-EDL	agn43EDL933 F agn43EDL933 R	CGTATCGCTGTGCCCGATAAC CCGTATACGAGTTGTCAGAATCA	707 bp				
Ag Rs218	agn43RS218 F agn43RS218 R	CGGATTTACCACCGTTAACC CATCCACCAGTGTTTTCCAGG	240 bp	1' at 95°C	30X (1' at 95°C, 1' at 58°C and 1' 20''at 72°C)	10'at 72°C	(Restieri et al., 2007)
Ag43a-cft073	agn43aCFT073 F agn43aCFT073 R	AGGCAGGAGGAACTGCCAGT TAAATGAGGGTGTCCCGTGCC	340 bp				
Ag43b-cft073	agn43bCFT073 F agn43bCFT073 R	CAGCCGATCTGCGGCACT ACTCTGGTGTCTGGCTGTT	440 bp				

Amplified DNA was analyzed using 1-2% agarose gels (Promega, US) in the presence of ethidium bromide (Sigma, US), and then the gels were examined and photographed at 302nm using the Biometra Gel Documentation System (Biometra, Gottingen, Germany). To assess the molecular mass of the amplicons Gene Ruler 100 bp DNA ladder standards (Fermentas, US) were run on each gel.

3.3.3. Multilocus sequence MLST typing

In order to study the relatedness of the MRSA strains, their genomic DNA were typed by MLST method based on the protocol described by Enright coworkers (Enright et al., 2000). The seven housekeeping genes (*arc*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, *yqiL*) were amplified using the primers shown in Table 4. The PCR product was purified according to the manufacturer's instruction with PCR and gel purification kit (Promega, US). The MLST was established according to the *S. aureus* MLST webpage (<http://saureus.mlst.net/>).

3.3.4. DNA sequencing

Sequencing of the column-purified PCR amplicons was carried out on both strands using a 3130X genetic analyzer (Applied Biosystems). The sequences were aligned using the MEGA5 program (Tamura et al., 2011).

Table 4: Primers and amplification parameters for Multilocus Sequence Typing of *S. aureus*

Gene	Primer	Sequences (5'- 3')	Product size	Initial Denaturation	Cycle	Final extension	Ref
arcC	arcC-Up	5'TTG ATT CAC CAG CGC GTA TTG TC 3'	456bp	5' at 94°C	30X (1' at 94°C, 1' at °C 55 and 1' at 72°C)	7'at 72°C	(Enright, et al., 2000)
	arcC-Dn	5' AGG TAT CTG CTT CAA TCA GCG 3'					
aroE	aroE-Up	5'ATC GGA AATCCT ATT TCA CAT TC 3'	456bp				
	aroE-Dn	5'GGT GTT GTA TTA ATA ACG ATA TC 3'					
glpF	glpF-Up	5'CTA GGA ACT GCA ATC TTA ATC C 3'	465bp				
	glpF-Dn	5'TGG TAA AAT CGC ATG TCC AAT TC 3'					
gmk	gmk-Up	5'ATC GTT TTA TCG GGA CCA TC 3'	429bp				
	gmk-Dn	5'TCA TTA ACT ACA ACG TAA TCG TA 3'					
pta	pta-Up	5'GTT AAA ATC GTA TTA CCT GAA GG 3'	474bp				
	pta-Dn	5'GAC CCT TTT GTT GAA AAG CTT AA 3'					
tpi	tpi-Up	5'TCG TTC ATT CTG AAC GTC GTG AA 3'	402bp				
	tpi-Dn	5'TTT GCA CCT TCT AAC AAT TGT AC 3'					
yqiL	yqiL-Up	5'CAG CAT ACA GGA CAC CTA TTG GC 3'	516bp				
	yqiL- Dn	5'CGT TGA GGA ATC GAT ACT GGA AC 3'					

3.3.5. Macrorestriction analysis with pulsed field gel electrophoresis

In this technique a restriction endonuclease with rare cutting sites (XbaI) was applied to digest the entire bacterial chromosome embedded into agarose gel plugs to protect DNA from mechanical fragmentation. The large DNA fragments generated by enzymatic digestion were electrophoretically separated in an electrical field with changing vector of the current (Gautom, 1997). The pattern of the fragments provided the basis of comparison.

Bacterial strains grown on TSA plates were suspended in 2 ml of cell suspension buffer up to a density of 3 McFarland units. Suspensions were kept on ice. Simultaneously, 1% plug agarose (Sigma, US) was melted in 1% SDS in TE buffer and kept at 54 °C. 500 µl bacterial suspensions, 25 µl of proteinase K (Invitrogen, US) (20 mg/ml) and 525 µl of 1% plug agarose were combined, mixed carefully, quickly transferred into 1 ml syringes and kept for 15-30 minutes at room temperature to allow the solidification of the agarose. Aliquots of 5 ml cell lysis buffer and 25 µl proteinase K 20 mg/mL were distributed into 50 ml tubes and 1mm thick slices of agarose plugs were directly cut into them. They were incubated for 2 hours at 50 °C in a shaker water bath (200 rpm) (Lab line, US). Subsequently, the plugs were washed twice with 10 ml of preheated sterile MilliQ water for 20 minutes in a 50 °C shaker water bath. Plugs were washed four times for 20 minutes with 10 ml of preheated TE buffer. Finally, plugs were stored in 5 ml of fresh TE buffer at 4 °C.

Genomic DNA within the plugs were digested overnight at 37 °C in a 100 µl restriction mixture made of 10µl of NE buffer 4 (Biolabs, New England), 1 µl of BSA (Biolabs, New England), 30 U (1.5 µl) of *XbaI* enzyme (Biolabs, New England) and 87.5 µl of sterile distilled water. Following digestion, the restriction mixtures were removed and the plugs were incubated in 250 µl of 0.5X TBE buffer for 30 minutes at room temperature. Subsequently, plugs were inserted into wells of 1.4% of agarose gel (Pulse Field Running Agarose A2929, Sigma,US) prepared in 0.5X TBE buffer. The two wells at the two sides of each gel contained a lambda-ladder PFGE marker (Biolabs, New England) for standardization. Gels were run in CHEF Mapper (Biorad, US) electrophoresis chamber in 0.5X TBE buffer pre-chilled to 14 °C. The running program consisted of 26 hours run at 6 V/cm with 120° angle and an initial switch time of 2.2 seconds and a final switch time of 54.2 seconds with linear ramp.

The gels were stained with ethidium bromide for 20 minutes, followed by de-staining in MilliQ water. Bands were detected and photographed under UV light in a Biometra gel documentation system. Gel pictures were stored as .tif files for further analysis. The Gel Compare II software (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyze the banding patterns. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree graphically showing the level of relatedness between the isolates was created based on the Dice similarity coefficient (SD) (Dice, with a 1.5% position tolerance). Strains showing patterns with $SD \geq 80\%$ were arbitrarily considered to represent a pulsotype.

3.4. Preparation of nanoparticles and their NPs

Nanoparticles were prepared by the co-precipitation. Ag, Cu, Ag-Cu and Ag-Cu-B nanoparticles were prepared with volume ratios shown in Table 5.

Table 5: Nanoparticles prepared with different volume ratios

Composition	Volume ratio
Ag-Cu-B	70:20:10
	60:30:10
	50:40:10
	40:50:10
	45:45:10
	40:40:20
	35:35:30
	30:30:40
	30:60:10
	20:10:10
Ag	100
Cu	100
Ag-Cu	90:10
	10:90
	80:20
	20:80
	70:30
	30:70
	60:40
40:60	
50:50	

3.4.1. The chemical co-precipitation method

By this method nanoparticles were produced by the reduction of metal salts dissolved in an organic solvent or water (Ferrando et al., 2008). In this study, silver nitrate (AgNO_3 , 99.99%), copper sulfate (CuSO_4) (both from Interchem, US) and boric acid (H_3BO_3) (Panreac, Spain) salts were used as metal precursors. Based on each volume ratios 25 ml 0.1 M solutions of each salts were prepared in deionized water and sonicated for once 10 min with power output at 40% (maximum 100W) and pulsar 70-80% (Ultrasonic Homogenizer 4710 series, Cole Parmer, USA). Then, drop-wise, 1 ml of 2 M NaOH (AJAX chemical, Australia) was added to the mixture until the formation of black precipitate was visible. The mixture was sonicated for 20 more minutes while the precipitate turned to greyish black. The precipitate formed was rinsed three times in deionized water by centrifugation at 4000 rpm for 10 minutes. Washed NPs were dried at 60 °C for 1 h and were stored under ambient room temperature.

3.4.2. The green approach: The microwave method

In this technique (Mirzaei and Davoodnia, 2012), three nanoparticles were synthesized: Ag-Cu-B, Ag-Mg-B and Ag-Na-B, all in volume ratio of 70:20:10, respectively. Each NPs was prepared by mixing 0.1 M from the salts of each metal and 1 ml of 2 M NaOH in 50 ml borosilicate tubes. The mixtures were reacted under microwave oven (CEM, Discover, US), at 300 W and pressure of 1 Atm for 5 min. The precipitates were subjected to four cycles of centrifugation and washing using deionized water. Finally, the NPs formed were dried at 60 °C and stored under ambient room temperature.

3.4.3. Characterization of nanoparticles

The NPs engineered were characterized by using transmission electron microscope (TEM, Tecnai G2, Netherlands) to measure their morphology and Zeta Sizer (Malvern, England) to measure their charge and size distribution.

3.5. Experiments with biofilm models

The biofilm model was used for two purposes: on one hand to assess the extent of biofilm production under various growth conditions in an important group of *E. coli* strains transmitted by food and, on the other hand, to investigate efficacy of nanoparticles to kill microbial pathogens in biofilms of different composition. For each experiments growth conditions reported to be optimal for biofilm production for the respective pathogens were explored (see below). For both type of experiments biofilms were grown in 96 wells tissue culture plates (Nunc).

3.5.1. Biofilm formation of *E. coli* ST131

Biofilm formation of *E. coli* ST131 was studied with bacteria grown in rich media and also in media more restricted in nutrients, in this both cases at 37 °C and at room temperature. While growing in nutrient restricted environment, the effect of anaerobiosis was also studied.

3.5.1.1. The nutrient rich method

For the nutrient rich environment the methods of Donelli et al. was used with some modification (Donelli et al., 2004). *E. coli* strains were grown overnight in 2 ml Brain Heart Infusion (BHI, Oxiod, UK) at 37 °C. Next morning, 10 µl of these cultures were transferred into 2 ml of TSB or Luria Broth (LB, Invitrogen, US) with or without 1% glucose. Immediately, 100 µl aliquots of these suspensions were distributed into 96-wells plate, 3 wells per strain, and were incubated at both 37 °C or at room temperature for 48 h. Un-inoculated wells containing medium only served as negative controls. Following incubation, the content of the wells were aspirated and the wells were carefully washed three times with 200 µl PBS to remove non-adherent cells. Adherent bacteria (i.e. biofilms) were fixed with 100 µl 2% formalin in PBS for 1 min., then were washed once with PBS. Subsequently, plates were stained with 200 µl of 0.13% crystal violet for 20 min and rinsed three times with PBS to remove excess dye. After air-drying, the dye retained by the biomass was extracted (i.e. the biofilms lysed) with 200 µl lysis buffer. The OD of the wells was measured at 570nm by using plate reader (Biotek, US). The results of three separate, independent experiments were evaluated.

The results were evaluated using an arbitrary scheme (Novais et al., 2012) defining the cut-off value as the mean + three standard deviations of OD of the sterility control (OD_c). The strength of biofilm production was scored as seen on Table 6.

Table 6: Scoring system for the extent of biofilm production

No biofilm	$OD \leq OD_c$
Weak	$OD_c < OD \leq 2X OD_c$
Moderate	$2 OD_c < OD \leq 4X OD_c$
Strong	$OD > 4X OD_c$

3.5.1.2. The nutrient restricted method

The biofilm production of *E. coli* strains was also studied under nutrient-restricted conditions (O'Toole and Kolter, 1998). Bacteria were grown in LB for overnight at 37 °C. A 10 µl of this culture was transferred into 2 ml of 1X M63 broth supplemented with 20% casamino acid (Bacto, France) and 0.2% glucose. Hundred µl aliquots of the culture were distributed into wells of 96-wells tissue culture plates (Nunc). The plates were incubated aerobically and anaerobically at 37 °C and at room temperature for 18 h. Anaerobiosis was secured in a Gaspack Anarobic System (BBL) using AnaeroGen gas generating bags (Oxoid, UK). After the incubation, 25 µl of 1% crystal violet were added and the plates (i.e. to the original culture, this time without previous washing and fixation) kept at room temperature for 15 min. Then the wells were emptied, rinsed three times with 200 µl sterile distilled water and air-dried. The lysis of biofilms, spectrophotometric measurements and evaluation were carried as described above.

3.5.2. Susceptibility of biofilms to nanoparticles

The bactericidal activity of nanoparticles on biofilms was studied by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Tang et al. 2011). The test quantitatively assesses the viability of cells based on their capacity to reduce the water-soluble yellow salt MTT to insoluble formazan. The formazan is subsequently solubilized, and its concentration is measured by optical density estimation at 575nm. The formazan concentration is directly proportional to the number of metabolically active cells in the biofilm (Tang et al. 2011).

For this purpose *S. aureus* and *P. aeruginosa* biofilms were induced by using the semi-quantitative method described by O'Neill and coworkers (O'Neill et al., 2008). Briefly, bacteria were inoculated into 2 ml BHI broth and incubated overnight at 37 °C. Then, 10 µl of culture was inoculated into 2 ml BHI supplemented with either 1% glucose or 4% NaCl. When *E. coli* was used, 10 µl of freshly grown culture in BHI was inoculated into 2 ml MHB. 100 µl aliquots of these bacterial suspensions were distributed into 11 wells of 96-wells microtiter plates while one well in each row left to contain sterile broth, only. Plates were incubated at 37 °C for 48 h. After this incubation period all the supernatants were aspirated to remove the non-adherent cells and biofilms were rinsed gently once with 200 µl sterile distilled water.

Ten wells were filled with 100 µl of serial dilutions of NPs prepared in the respective media representing concentrations between 500 mg/L to 0.97 mg/L and

incubated overnight at 37 °C. The untreated wells were served as control. After exposure to NPs, the content of the wells was gently aspirated and replaced with 150 µl PBS containing 0.3% MTT (Sigma) salt and incubated at 37 °C for 2 h.

MTT was re-solubilized in 150 µl of dimethyl-sulfoxide (DMSO, Sigma) solution and 25 µl of 0.1 M glycine buffer (pH 10.2) and kept for 15 minutes at room temperature. The amount of the formazan produced was measured at 570 nm by using plate reader (Biotek, US). Experiments were performed in triplicates.

Data were presented as "percentage eradication" in the biofilm growth in the presence of NPs compared to untreated biofilm masses. The % of eradication was calculated as the following equation (Pitts et al., 2003): % of biofilm eradication = $[(OD_{\text{untreated biofilm}} - OD_{\text{treated biofilm}}) / OD_{\text{untreated biofilm}}] \times 100$

3.6. Sanitary effect of nanoparticles on stainless steel surfaces

The antimicrobial efficacy of Ag-Cu-B NPs prepared by the co-precipitation method applied by saturated sanitary wipes was tested on stainless steel surfaces that commonly come into contact with food. *S. aureus* (S800) and *Salmonella* group D (BC789) strains were utilized to contaminate the surfaces. Two to three isolated colonies grown overnight on TSA plate were picked, suspended into 2 ml PBS and the optical density was set to 0.5 McFarland standards and used as inoculum. The sanitary effect of the nanoparticles was evaluated on 1 mm thick, 10 x 10 cm stainless-steel squares (304 mirror type)

supplied by Emirates Italian Restaurant Equipment & Supplies (Al Ain, UAE). The coupons were conditioned by soaking in 70% ethanol for 24 h, rinsed with 20% bleach and distilled water. The coupons were then autoclaved and oven-dried. A 0.5 ml aliquot of each inoculum was distributed and spread using sterile plastic spreaders over the stainless steel surfaces and air-dried for 1-2 h under ambient room temperature.

A 7.5 x 7.5 cm squares sterile gauze swabs (Topper 8, Johnson& Johnson) were used as fabric wipes. The fabrics were soaked for 24 h in 100 mg/L nanoparticles dispersed in autoclaved tap water. Three sets of stainless steel coupons were contaminated. One set was used to estimate the initial level of contamination; the second set was treated with wipes soaked in NPs, while the third set was treated with wipes containing sterile tap water. Each test wipes was squeezed to remove excess solution before application and then surfaces were wiped forward and backward with the folded wipes once. Subsequently, the surfaces were sampled using cotton swabs (Citoswab, China) pre-wetted with PBS, 0, 60 and 120 min after exposure. The surface area was swabbed vertically and horizontally from top to down and then from left to right twice. After sampling, each swab was cut close to the tip and dipped into 2 ml PBS. The swabs were vortexed for 60s, the suspension serially diluted and the appropriate dilutions plated on duplicate TSA plates. The plates were incubated overnight at 37 °C. The log reduction was defined as the log difference between untreated surface and treated one. To be considered effective sanitizers with the surface test, NPs must achieved at least 3 log reduction in bacterial populations (Riazi and Matthews, 2011).

3.6.1 Time killing assay

In order to determine the minimum exposure time for the effective bactericidal activity of ACBC, bacterial populations was estimated over a period of one hour. Surfaces were contaminated, wiped and sampled according to the procedures described above. After the wiping step, the number of viable cells were determined at 0, 15 ,30 and 60 min., respectively. At each time, three coupons were swabbed.

3.8. Statistical analysis

All experiments were run in triplicates. For nanoparticles efficacy, statistical analysis was performed using Megastat for Microsoft excel (version 10.2, release 2.1, Canada). Comparison were carried out at 95% confidence by using one-way analysis of variance (ANOVA) with post hoc Tukey test.

For the *E.coli* biofilm production, a generalized linear mixed effect model with a binomial distribution and a logit link was used. This allows taking into account that strains within the same level of PFGE are correlated. The function “glmer” from the package “lme4” of the R software version 3.1.2 was used. To select the best combinations of predictors for each dependent variable the stepwise forward and backward procedures were used. Before doing so, a univariate logistic regression was carried out. The odds ratio was used as the main measure for the

association between dependent and independent variables. The stepwise procedure uses a rejection alpha of 10%.

To compare the different growth conditions on biofilm production the Spearman rank correlation and Wilcoxon tests were used.

Chapter 4. Results

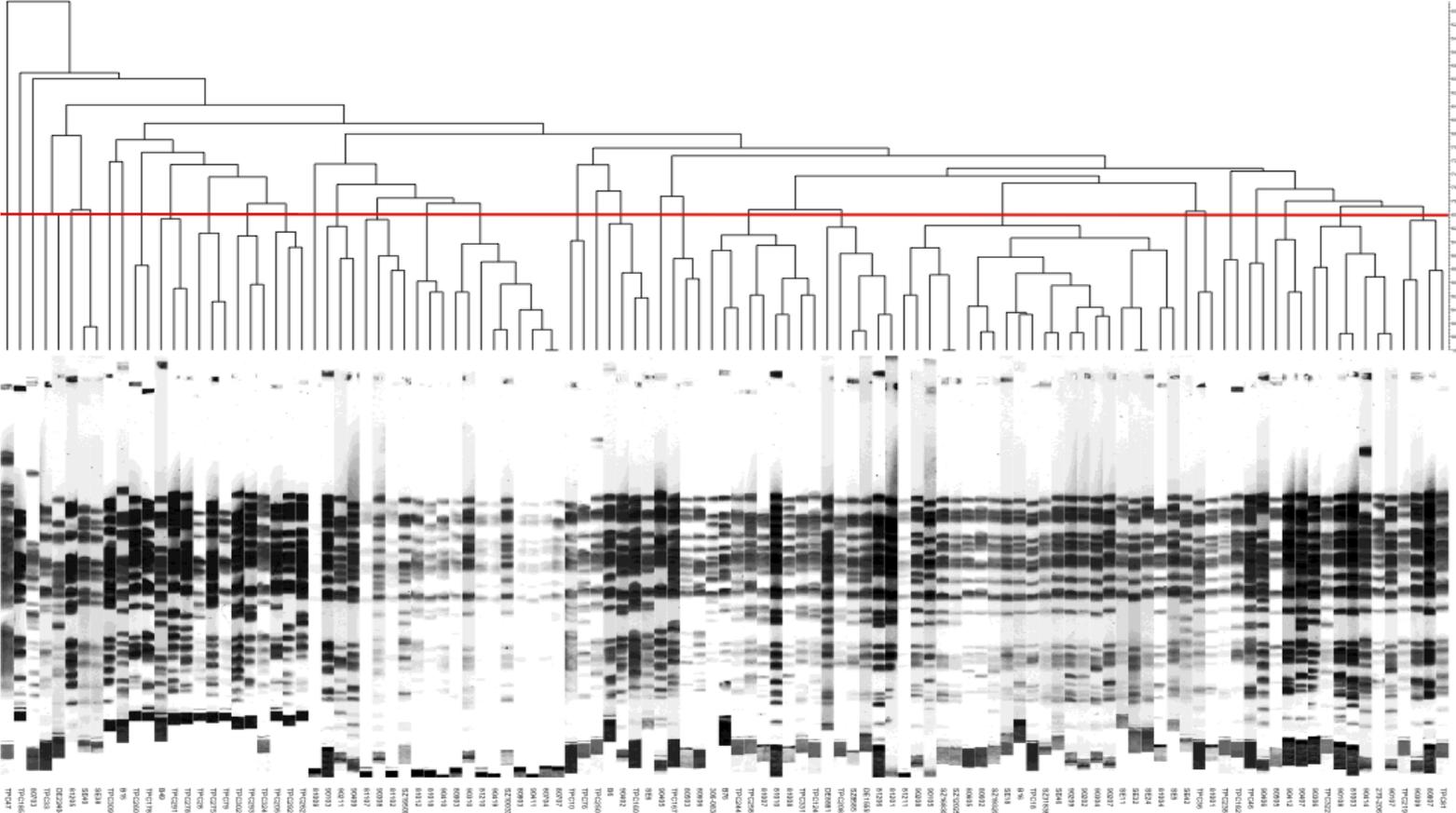
4.1. Biofilm formation of *E. coli* ST131 strains

Recently, multidrug resistant *E. coli* ST131 strains have been increasingly recognized as causative agents of various extra-intestinal infections. As most of these infections derive from the gut colonized by these bacteria (Dhanji et al., 2011; Li et al., 2010) a food-related transmission is very likely (Egea et al., 2012; Kawamura et al., 2013; Lazarus et al., 2015; Platell, et al., 2011; Vincent et al., 2010; Zurfluh et al., 2015). Therefore the details of biofilm formation of this important group of organisms were studied.

4.1.1. Clonality of the *E. coli* ST131 strains studied

A 115 independent *E. coli* ST131 strains were investigated. 52.2% of the isolates were recovered from urine while the rest was blood isolates. First, the distribution of clones within this sequence type was established based on the *Xba*I digested macro-restriction pattern of the isolates. Two isolates were untypable. The PFGE picture of the strains is shown on Figure 4. Using an 80% similarity cut-off value the clustering of the strains are shown in Table 7.

Figure 4: Macrorestriction patterns of *E. coli* ST131 strains



The red line shows the 80% similarity threshold

Table 7: Macrorestriction clusters of *E. coli* ST131 strains

Size of the cluster	N of clusters
1	12
2	6
3	8
4	3
6	1
7	1
9	2
22	1

These data showed that the collection of strains represents a variety of clusters suitable for studying the biofilm forming capacity of the isolates.

4.1.2. Virulence factor gene distribution of the *E. coli* ST131 strains studied

In order to be able to correlate the biofilm forming capacity of the strains to known adhesin genes the strains were subjected to PCR genotyping targeting 23 such genes and that of the *bla*_{CTX-M} beta-lactamase gene characteristically present in the majority of ST131 isolates. The percent distribution of these genes is shown in Table 8.

Table 8: Distribution of bla_{CTX-M} and virulence genes among *E. coli* ST131 strains

Genes	(%)
<i>bla</i> _{CTX-M}	78.3
<i>Crl</i>	100.0
<i>CsgA</i>	100.0
<i>BcsA</i>	87.8
<i>Ag43</i>	99.1
<i>Ag43 EDL933</i>	0.0
<i>Ag43-K12</i>	87.0
<i>Ag43-CFT073 a</i>	53.0
<i>Ag43-CFT073 b</i>	0.0
<i>Ag43-RS218</i>	40.9
<i>PapC</i>	18.3
<i>PapAH</i>	18.3
<i>PapEF</i>	13.0
<i>fimH</i>	98.3
<i>FimH30</i>	91.3
<i>BmaE</i>	0.0
<i>Sfa/foc DE</i>	0.9
<i>FocG</i>	0.0
<i>SfaS</i>	0.0
<i>GafD</i>	0.0
<i>NfaE</i>	0.9
<i>iha</i>	57.4
<i>Afa/dra</i>	50.4
<i>UpaB</i>	99.1

As expected, the beta-lactamase gene *bla*_{CTX-M}, causing 3rd generation cephalosporin resistance in the strains, was present in the majority of the isolates. The presence of the different adhesin genes exhibited broad variation.

During the subsequent experiments the effect of various culture conditions on the biofilm forming capacity of the strains, as well as its relation to the clusters identified and to the presence of the various genes were studied.

4.1.3. Biofilm forming by *E. coli* ST131 grown under various culture conditions.

Altogether 12 culture conditions were studied. Biofilms were developed in TSB and in LB with and without glucose at 37 °C and at room temperature and, in separate experiments, in different experimental settings (for the details see Materials and Methods) under limited nutrient conditions using M63 minimal medium supplemented with 20% casamino acid and 0.2% glucose at 37 °C and at room temperature, under aerobic and anaerobic conditions. The strength of biofilm formation was scored on a scale 0-3.

Table 9 shows the average score and the distribution of biofilm strength among the strains grown under different conditions.

Table 9: Distribution of biofilm strength in *E. coli* ST131 strains grown under different conditions

Conditions	Score ($\bar{X} \pm SD$)	Frequency (%) of biofilm strength			
		None (Score 0)	Weak (Score 1)	Moderate (Score 2)	Strong (Score 3)
TSB 37	0.84±1.09	52.2	27.0	5.2	15.7
TSB RT	0.1±0.33	90.4	8.7	0.9	0.0
TSB Glu 37	0.1±0.3	90.4	9.6	0.0	0.0
TSB Glu RT	0.06±0.24	93.9	6.1	0.0	0.0
LB 37	0.62±0.91	60.9	23.5	8.7	7.0
LB RT	0.24±0.57	81.7	13.0	4.3	0.9
LB Glu 37	0.16±0.36	84.3	15.7	0.0	0.0
LB Glu RT	0.19±0.62	88.7	6.1	3.5	1.7
CAA AE 37	1.65±0.88	12.2	25.2	47.8	14.8
CAA AE RT	2.45±0.57	0.0	3.5	60.0	36.5
CAA AN 37	2.33±0.54	0.0	3.5	47.8	48.7
CAA AN RT	2.72±0.51	0.0	2.6	22.6	74.8

TSB - Tryptic Soy Broth, LB - Luria Broth, CAA - M63 minimal medium supplemented with 20% casamino acid and 0.2% glucose, 37 - 37 °C, RT - Room temperature, AE - aerobic, AN - anaerobic

In the first set of experiments (using TSB and LB, i.e. rich media), generally speaking, all conditions induced no or weak biofilm production in the majority of the strains. It was shown that in rich media it is primarily the growth temperature that affects biofilm production, i.e. it was significantly stronger at 37 °C than at room temperature in TSB (Wilcoxon test, $P < 0.0001$), as well as in LB ($P = 0.0003$), while TSB somewhat outperformed LB ($P = 0.0244$). The addition of glucose significantly reduced biofilm production in both media at 37 °C ($P < 0.0001$, in both cases).

This was further corroborated by the fact that the biofilm forming capacity of the strains, irrespective of the media used, at the same temperatures (i.e. either 37 °C or room temperature) correlated well (Spearman rank correlation, $P = 0.01$, correlation coefficients varying between 0.377 to 0.628) while all other comparisons failed to exhibit the same level of association.

When low nutrient culture conditions were used (i.e. casamino acid and glucose supplemented M63 minimal medium), biofilm production shifted towards stronger scores and the majority of the isolates did produce moderate or strong biofilms. Importantly, under these conditions the lower growth temperature induced stronger biofilms ($P = < 0.0001$) and at both temperatures anaerobic growth conditions favored the development of biofilms over growing at ambient air ($P < 0.0001$ at both temperatures).

The clonal association of biofilm production was assessed by investigating whether members of any of the PFGE clusters containing more than 3 members

exhibited a biofilm strength score beyond the $X \pm SD$ of the one characteristic to all strains grown under the same condition. Only one such clone (PFGE cluster 1) was identified when growing at 37 °C in TSB. Its score was 2.25, compared to the average of all strains, i.e. 0.84 ± 1.09 . No other indication of clonality in biofilm production by these criteria was identified.

4.1.4. Correlation between biofilm production and specific genes in *E. coli* ST131

Multiple logistic regression analysis, a stepwise procedure to select the set of genes which presence are predictors of biofilm formation (using the grouping described in the Material and Methods section) identified a few associations. However, the associated genes exhibited a considerable variation depending on the growth conditions used as it is summarized in Table 10., which shows all significant associations revealed.

Importantly, the strains positive for the CFT073a allelic variant of the *ag43* gene, encoding Antigen 43, formed significantly more likely biofilm when tested in different conditions (i.e. in TSB at 37 °C, and in nutrient limited anaerobic or aerobic conditions at 37 °C or at room temperature in anaerobic condition). Other allelic variants of the *ag43* gene were negatively associated with biofilm formation, but this was observed in certain condition, only: namely, strains positive for *ag43*-K12 allele were significantly less likely to produce biofilm when tested in LB broth supplemented with glucose at 37 °C, and strains positive for *ag43*-RS218 allele were significantly less likely to produce biofilm

when tested in casamino acid and glucose supplemented M63 minimal media incubated either anaerobically at room temperature, or in ambient air at 37 °C.

Table 10: Significant associations revealed between genes and biofilm formation of *E. coli* ST131

Condition	Genes	Genes Present N (%)	With Biofilm Present			
			OR	SE	P	95% CI
TSB 37	<i>bla</i> _{CTX-M}	90 (78.3)	4.43	2.36	0.005	1.56 – 12.60
	<i>Ag43-CFT073 a</i>	61 (53.0)	2.67	1.07	0.015	1.21 – 5.89
LB 37	<i>PapC PCR</i>	21 (18.3)	7.17	4.01	0.000	2.39 – 21.44
LB glucose 37	<i>Ag43-K12</i>	100 (87.0)	0.13	0.21	0.001	0.037 – 0.45
CAA 37 AE	<i>Ag43-CFT073 a</i>	61 (53.0)	3.30	1.62	0.015	1.26 – 8.63
	<i>Ag43-RS218</i>	47 (40.9)	0.32	0.16	0.021	0.12 – 0.84
CAA 37 AN	<i>Ag43-CFT073 a</i>	61 (53.0)	4.85	2.10	0.000	2.07 – 11.37
CAA RT AE	<i>afa/dra</i>	50 (50.4)	0.42	0.17	0.032	0.19 – 0.93
	<i>PapA PCR</i>	21 (18.3)	5.06	3.06	0.007	1.55 – 16.57
CAA RT AN	<i>Ag43-CFT073 a</i>	61 (53.0)	4.31	2.61	0.016	1.32 – 14.13
	<i>Ag43-RS218</i>	47 (40.9)	0.24	0.15	0.021	0.069 – 0.80

OR: Odds Ratio; SE: Standard Error; P: Probability Value; CI: Confidence Interval, TSB - Tryptic Soy Broth, LB - Luria Broth, CAA - M63 minimal medium supplemented with 20% casamino acid and 0.2% glucose, 37 - 37 °C, RT - Room temperature, AE - aerobic, AN - anaerobic

Other adhesin genes exhibiting significant association with biofilm production were the *papA* and *papC* genes of the P fimbria, the latter being associated with biofilm production in LB broth incubated at 37 °C and the former showing association with strong biofilm production in casamino acid and glucose supplemented M63 minimal media incubated in ambient air at room temperature. In this latter condition another significant association was seen: the afimbrial adhesin *afa/dra* positive strains were less likely to form biofilm.

The most interesting association was established with the presence of *bla*_{CTX-M} extended spectrum beta-lactamase and biofilm production in TSB incubated at 37 °C. Strains carrying this antibiotic resistant gene were 4.43 times more likely to produce biofilm in this condition than the ones not possessing the gene.

4.2. The effect of nanoparticles against biofilms produced by food-transmitted bacteria

4.2.1. Characterization of nanoparticles

Various nanoparticles were synthesized by co-precipitation and microwave methods and characterized by using TEM and Zeta Sizer to measure their morphology, size distribution and charge. The characteristics of all NPs are given in Table 11. All particles had negative charge and varied in size from 27 to 84 nm. Ag-Cu-B NPs prepared by microwave possessed the smallest size.

Table 11: Physio-chemical characteristics of the nanoparticles

NPs type	Size distribution (nm)	Charge	Shape
Ag-Na-B	53	negative	spherical
Ag-Mg-B	79	negative	spherical
Ag-Cu-B	27	negative	spherical
Ag-Cu-B (co)	84	negative	spherical and platelet

* (co) - co-precipitation

Transmission electron microscopic analysis revealed that the distribution of all NPs varied in size. Moreover, it was obvious that NPs, synthesized using the microwave method were spherical in shape, whereas Ag-Cu-B prepared by co-precipitation technique have two types of morphology: spherical and platelet (Figure 5).

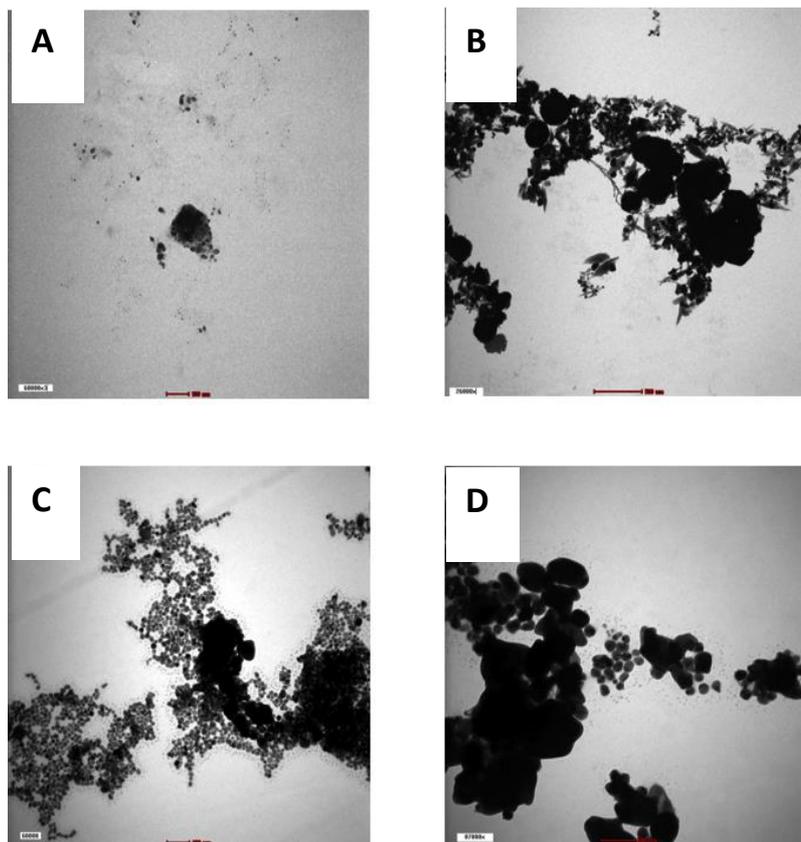


Figure 5: TEM micrographs of the NPs. Magnification 105,000X

A: Ag-Cu-B, B: Ag-Cu-B, C: Ag-Na-B, D: Ag-Mg-B.
A, C, D - microwave methods, B - co-precipitation method

4.2.2. Antimicrobial effect of the nanoparticles on planktonic cells

In order to select the most effective nanoparticles, initially, the antimicrobial efficacy of different NPs prepared by co-precipitation method against planktonic cells of *S. aureus* S800 was evaluated. The results are summarized in Table 12.

Table 12: MIC of various nanoparticles against planktonic cells of *S. aureus*

NPs composition	volume ratio	MIC (mg/L)*
Ag	100	416.7
Cu	100	500.0
Ag-Cu	90:10	166.7
	10:90	250.0
	80:20	166.7
	20:80	208.3
	70:30	166.7
	30:70	166.7
	60:40	125.0
	40:60	125.0
	50:50	125.0
	70:20:10	125.0
Ag-Cu-B	60:30:10	125.0
	50:40:10	104.2
	40:50:10	104.2
	45:45:10	125.0
	40:40:20	125.0
	35:35:30	125.0
	30:30:40	125.0
	30:60:10	125.0
	21:10:10	125.0

* average of 3 experiments

The results clearly showed that mono-component Ag and Cu NPs exhibited considerably higher MIC values than either the Ag-Cu or Ag-Cu-B nanoparticles, of which some approached values as low as 100 mg/L.

In an attempt to prepare highly effective NPs with unique physiochemical and biological characteristic and alloy with a specific volume ratio with a MIC

close to 100 mg/L was selected for further studies. The highly dispersed nanoparticles (i.e. Ag-Cu-B alloy with volume ratio "70:20:10") was chosen and prepared also by the microwave technique together with a new batch of the same alloy using the co-precipitation technique. Furthermore, the Cu metal has been substituted with other elements (i.e. Na and Mg, maintaining the same ratio) as a further attempt to improve antimicrobial activity of Ag-NPs.

The antimicrobial effect of these NPs was tested against planktonic cells of a variety of bacteria. Multi-antibiotic resistant strains of *S. aureus* S800, *P.aeruginosa* AG1, and 3 strains of *E.coli* ST131 (TPC244, SZ29659 and 90405) were investigated. As shown in Table 13, it is evident that all NPs possessed varied antimicrobial effect against tested bacteria. Nanoparticles were the least active against *S. aureus* and the most effective one, in general, was the Ag-Cu-B NPs prepared by co-precipitation (Table 13).

Table 13: Activity of the NPs against a variety of test bacteria

NPs type	MIC mg/L*				
	<i>S.aureus</i>	<i>P.aeruginosa</i>	<i>E.coli</i> ST131		
			90405	SZ29659	TPC244
Ag-Cu-B	23.6	13.7	11.7	9.8	19.5
Ag-Cu-B (Co)	19.7	11.8	9.8	6.8	9.8
Ag-Na-B	23.4	19.5	21.5	19.0	17.6
Ag-Mg-B	23.4	17.6	13.7	15.1	13.7

(Co) - co-precipitation

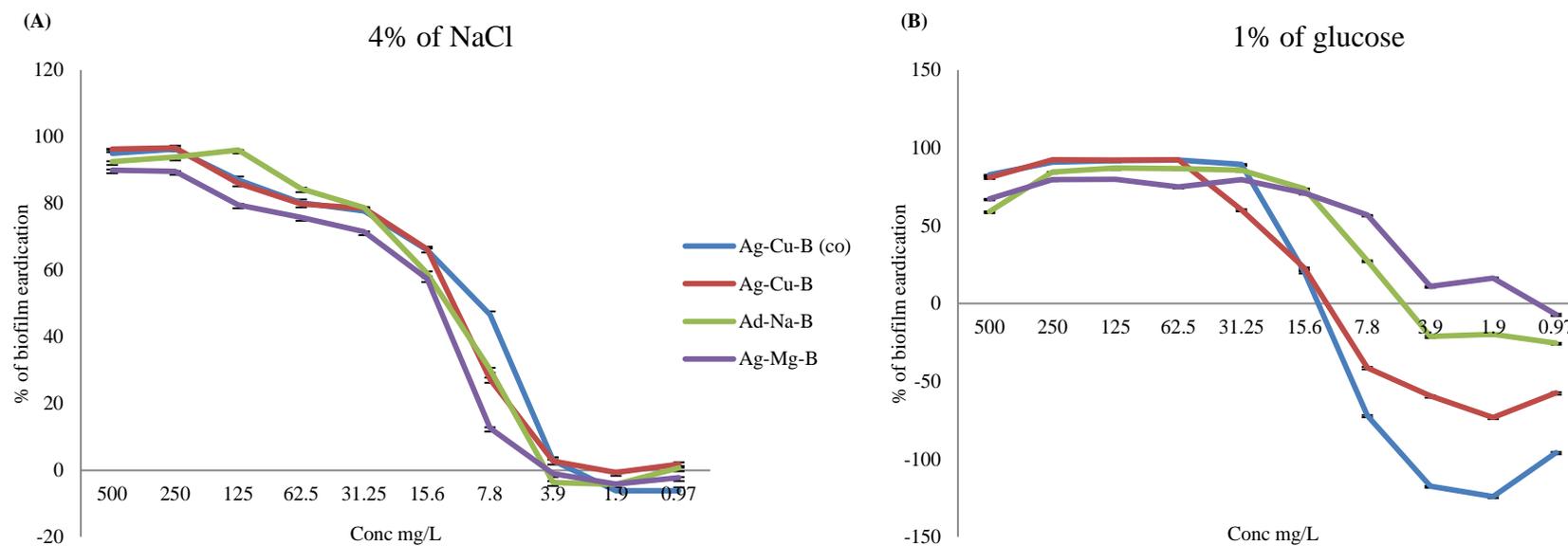
* average of 3 experiments

4.2.3. Efficacy of nanoparticles against biofilms

The same nanoparticles were also tested against the same target bacteria but this time grown in biofilms. For *S. aureus* and *P. aeruginosa* biofilms were established in BHI with 1% glucose or with 4% NaCl, while *E. coli* was grown in MHB. The biofilms were exposed to graded doses of the nanoparticles. The results are shown in Figures 9-11 and in Table 14 and 15.

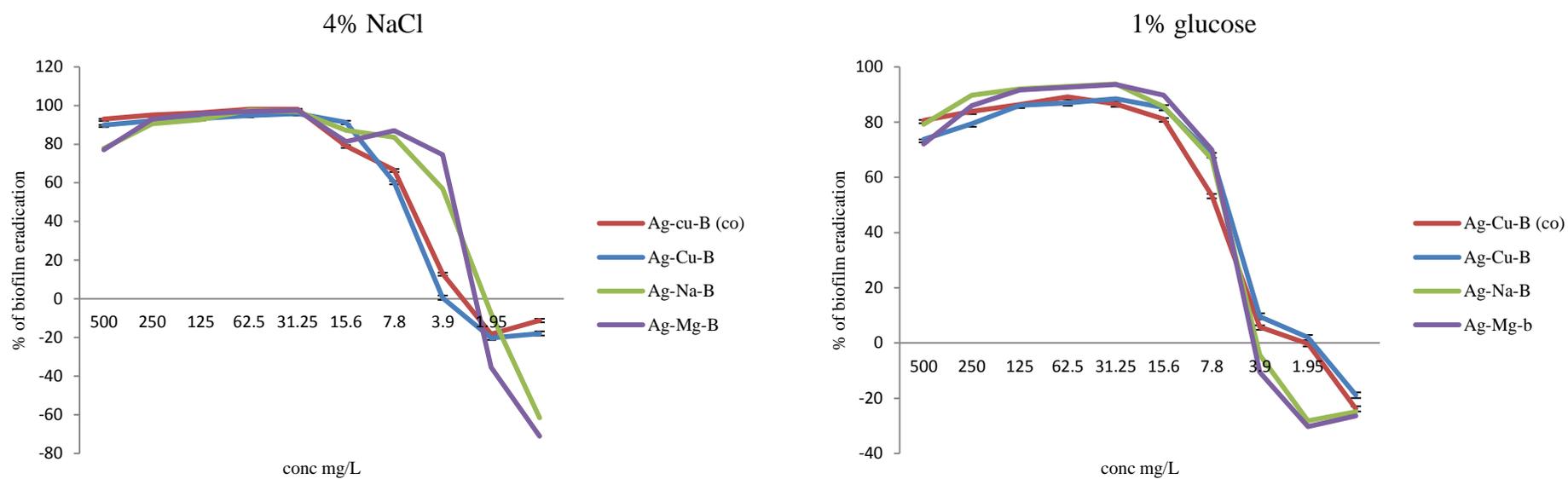
The biofilm reduction by NPs, when used in high concentration exceeded at least 50%, and exhibited some effect even at a concentration as low as 15.6 mg/L irrespective of the nanoparticles, the species or growth conditions. However, there were some quantitative differences. As expected, based on susceptibilities of their planktonic cells, Gram-negative bacteria, particularly two strains of *E. coli* (SZ29659 and TPC244) were more susceptible than *S. aureus*. Within the latter species biofilms developed in 4% NaCl supplemented media.

Figure 6: Biofilm reducing effect of nanoparticles against *S. aureus* S800



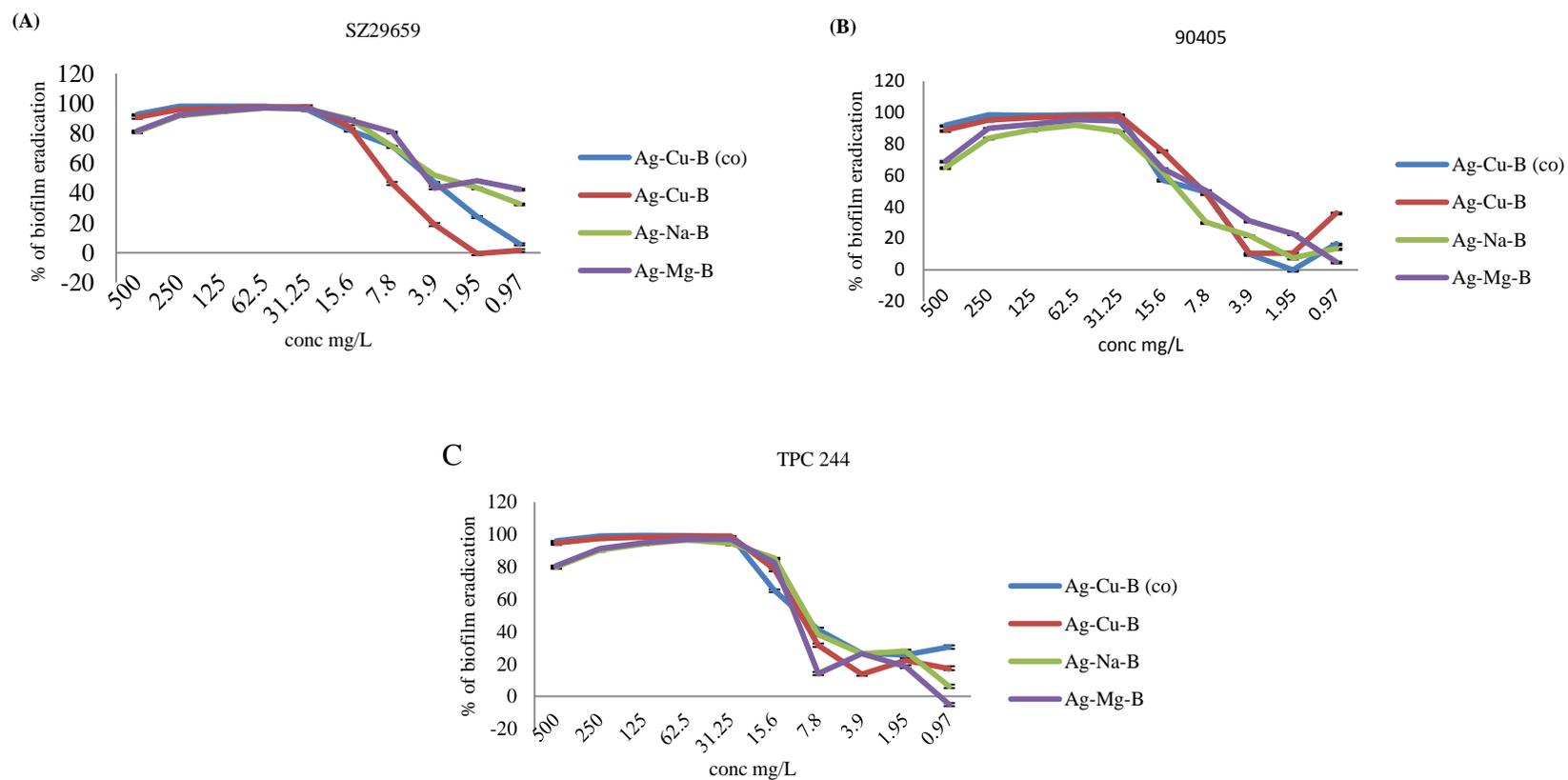
(Co) by coprecipitation, all other NPs were prepared by the microwave technique

Figure 7: Biofilm reducing effect of nanoparticles against *P. aeruginosa* AG1



(Co) by coprecipitation, all other NPs were prepared by the microwave technique

Figure 8: Biofilm reducing effect of nanoparticles against various *E. coli* ST131 strains



(Co) by coprecipitation, all other NPs were prepared by the microwave technique

Table 14: Minimum concentration of nanoparticles causing 10% biofilm reduction

Strains	Type of biofilm	Medium	Concentration of nanoparticles (mg/L)			
			Ag-Cu-B (Co)	Ag-Cu-B	Ag-Na-B	Ag-Mg-B
<i>S. aureus</i> S800	PS/PROT	BHI +NaCl	7.8	7.8	7.8	7.8
		BHI+glucose	15.6	15.6	7.8	7.8
<i>P. aeruginosa</i> AG1		BHI+NaCl	7.8	3.9	3.9	3.9
		BHI+glucose	7.8	7.8	7.8	7.8
<i>E. coli</i> SZ29659		MH	1.9	3.9	0.97	0.97
<i>E. coli</i> TBC 244		MH	0.97	0.97	3.9	7.8
<i>E. coli</i> 90405		MH	7.8	7.8	3.9	1.9

PS- polysaccharide, PROT - protein, BHI - Brain Heart Infusion, MH - Mueller-Hinton broth

Table 15: Minimum concentration of nanoparticles causing 80% biofilm reduction

Strains	Type of biofilm	Medium	Concentration of nanoparticles (mg/L)			
			Ag-Cu-B (Co)	Ag-Cu-B	Ag-Na-B	Ag-Mg-B
<i>S. aureus</i> S800	PS/PROT	BHI +NaCl	62.5	125	62.5	250
		BHI+glucose	31.25	62.5	31.25	>500
<i>P. aeruginosa</i> AG1		BHI+NaCl	15.6	31.25	7.8	7.8
		BHI+glucose	15.6	15.6	15.6	15.6
<i>E. coli</i> SZ29659		MH	15.6	15.6	15.6	7.8
<i>E. coli</i> TBC 244		MH	31.25	31.25	15.6	15.6
<i>E. coli</i> 90405		MH	31.25	31.25	31.25	31.25

PS- polysaccharide, PROT - protein, BHI - Brain Heart Infusion, MH - Mueller-Hinton broth

i.e. being mostly polysaccharide in nature, were more resistant. Also, Ag-Mg-B NPs were quite ineffective in any type of *Staphylococcus aureus* biofilm, and especially the one produced in glucose supplemented media (i.e. even the highest concentration of it, otherwise active against other species, could not reduce the biofilm by 80%). Otherwise, the difference in concentrations causing min 80% or min 10% biofilm reduction protein and polysaccharide biofilms never exceeded the two-fold differences. Regarding *P. aeruginosa* grown under similar conditions the trends were the opposite, biofilm reduction effect of NPs was generally greater in biofilm produced in 4% NaCl supplemented media.

For the other Gram-negative organism, i.e. *E. coli*, grown in MH all NPs caused at least a 80% reduction at 31.25 mg/L and for some strains even 7.8 mg/L was enough of exhibit the same effect. From these data no clear preference emerged for any of the particular NPs over the others.

4.2.4. Efficacy of nanoparticles against *S. aureus* of different clones

Randomly 9 MRSA strains capable of producing different kinds of biofilms when grown in different conditions were selected. First strains were confirmed that they are clonally independent, indeed. The results of the multi-locus sequence typing is shown in Table 16. They showed that the strains represent independent lineages, indeed.

Next the nanoparticles were tested against the planktonic cells of the strains. As shown in Table 17, although there were some variations in the MIC of the NPs against the various strains, still the Ag-Cu-B (Co) nanoparticles were the most efficacious for all strains not exceeding the difference by one dilution.

Table 16: Multi-locus Sequence types of the *S. aureus* strains studied

Strains	MLST type	Type of biofilm	
		Polysaccharide	Protein
12/07	ST149	+	-
75/08	ST5	+	-
172/08	SLV of ST772	+	-
205/08	ST6	+	-
274/08	ST8	-	+
T27/9	ST239	-	+
48/08	ST80	+	+
177/08	ST80	+	+
201/08	SLV of ST1	+	+
S800	ST779	+	+

SLV: Single Locus variant, ST: sequence type

Table 17: Susceptibility of the *S. aureus* strains to different types of nanoparticles

Strain	Nanoparticles (MIC mg/L)			
	Ag-Cu-B (Co)	Ag-Cu-B	Ag-Na-B	Ag-Mg-B
12/07	46.9	46.9	93.8	46.9
48/08	46.9	46.9	93.8	46.9
75/08	31.3	46.9	46.9	31.3
172/08	46.9	46.9	93.8	46.9
177/08	31.3	46.9	62.5	46.9
201/08	46.9	46.9	93.8	46.9
205/08	46.9	46.9	93.8	46.9
274/08	46.9	46.9	93.8	46.9
T27/9	39.1	46.9	78.1	46.9
S800	23.4	46.9	46.9	23.4

Subsequently, the efficacy of the four nanoparticles was tested on the biofilms formed by these strains. The results are presented in Figures 9-17 and Tables 18-19.

Comparing strains, it was obvious from the results the susceptibility of their biofilms may exhibit considerable variation. Strain T27/9, when grown in glucose medium promoting a protein biofilm produced a highly resistant matt for which extreme concentration of NPs were needed to achieve 80% reduction, and Ag-Na-B and Ag-Mg-B were not able to do that even the maximal concentration.

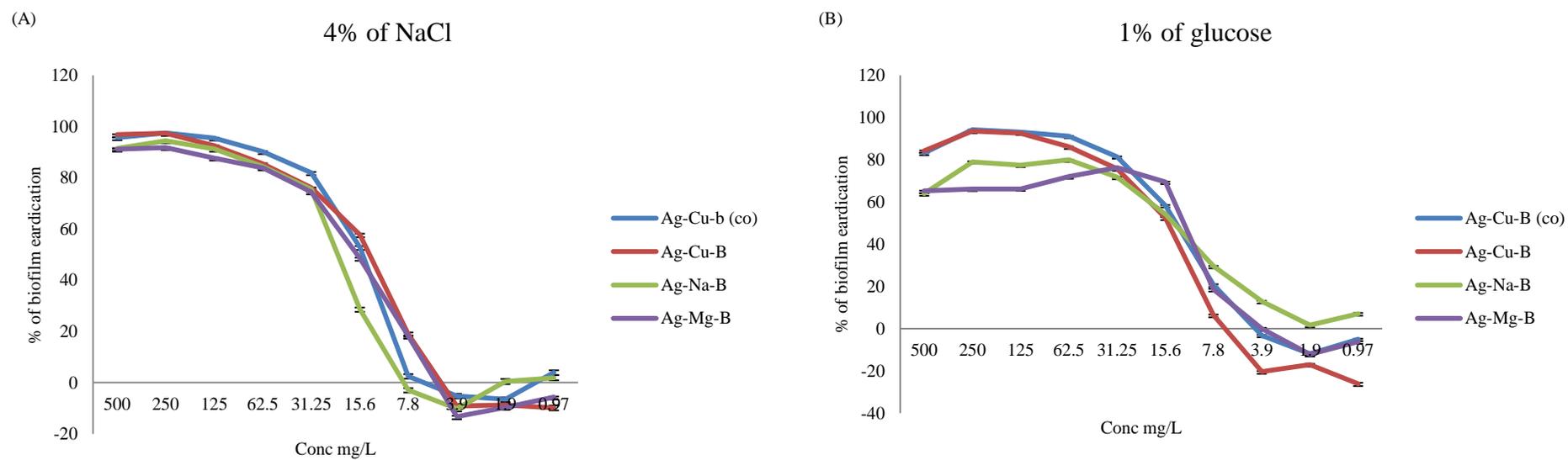
Interestingly, this was not obvious when the minimum concentrations needed to achieve some (10%) reduction were considered. The high resistance of protein type biofilms was the most obvious when tested with Ag-Mg-B as this

alloy showed the least efficacy in achieving 80% reduction with some other strains, as well, e.g. S800, while other NPs achieved that at much lower concentrations. Nevertheless, No clear differences between the susceptibility of protein or polysaccharide type biofilms could be identified, as extreme resistance was also seen in some polysaccharide type mats, too (e.g. 201/08).

A surprise finding was the highly resistant biofilm produced by 12/07 in glucose enriched medium, as this strain was not supposed to form protein type biofilms.

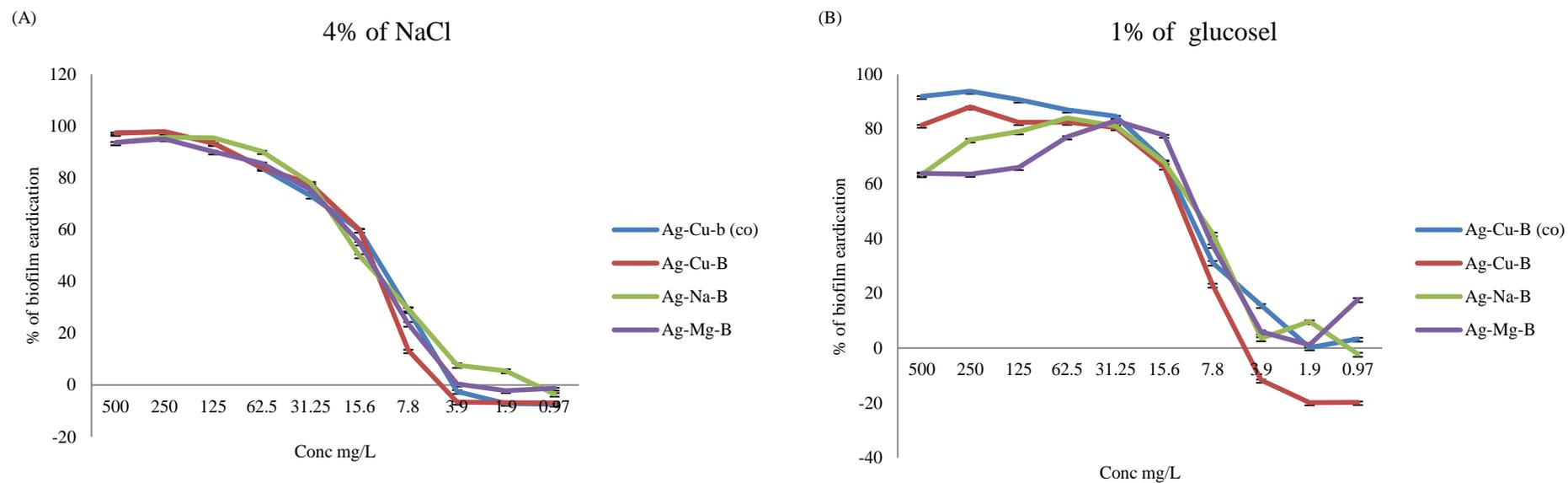
While there were no significant differences between nanoparticles when the concentrations to achieve minimal, 10% reduction was considered, in inducing 80% reduction Ag-Cu-B (Co), particularly if prepared by co-precipitation often were more active than their Na or Mg containing counterparts.

Figure 9: Efficacy of nanoparticles against *S. aureus* 12/07



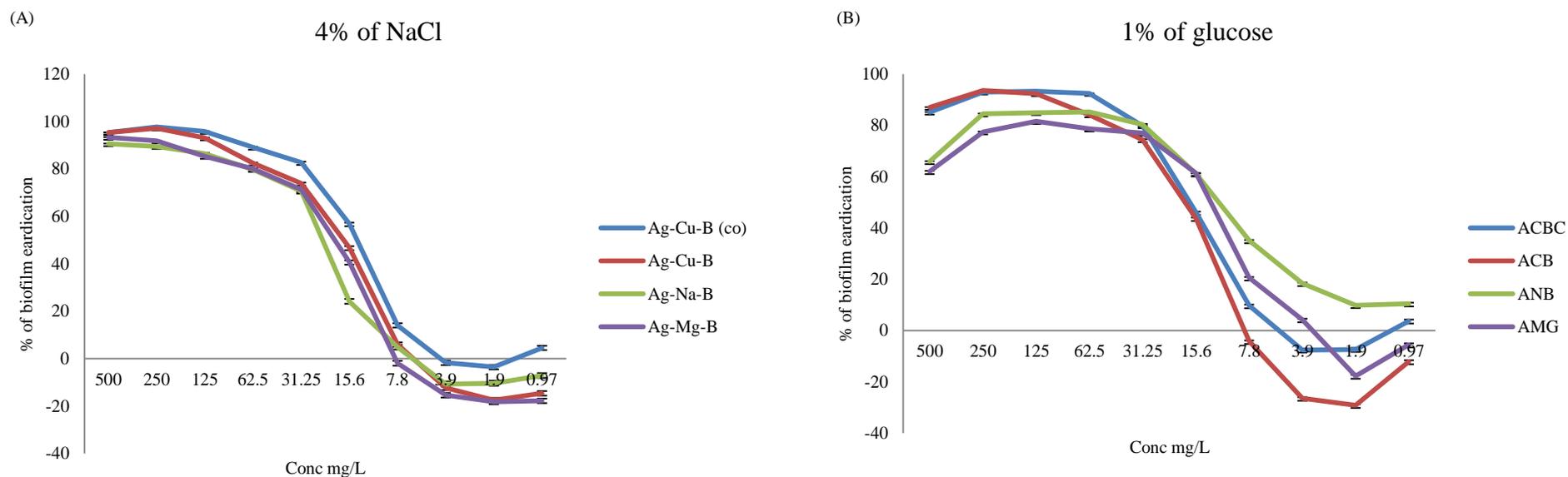
(Co) by coprecipitation, all other NPs were prepared by the microwave technique

Figure 10: Efficacy of nanoparticles against *S. aureus* 48/08



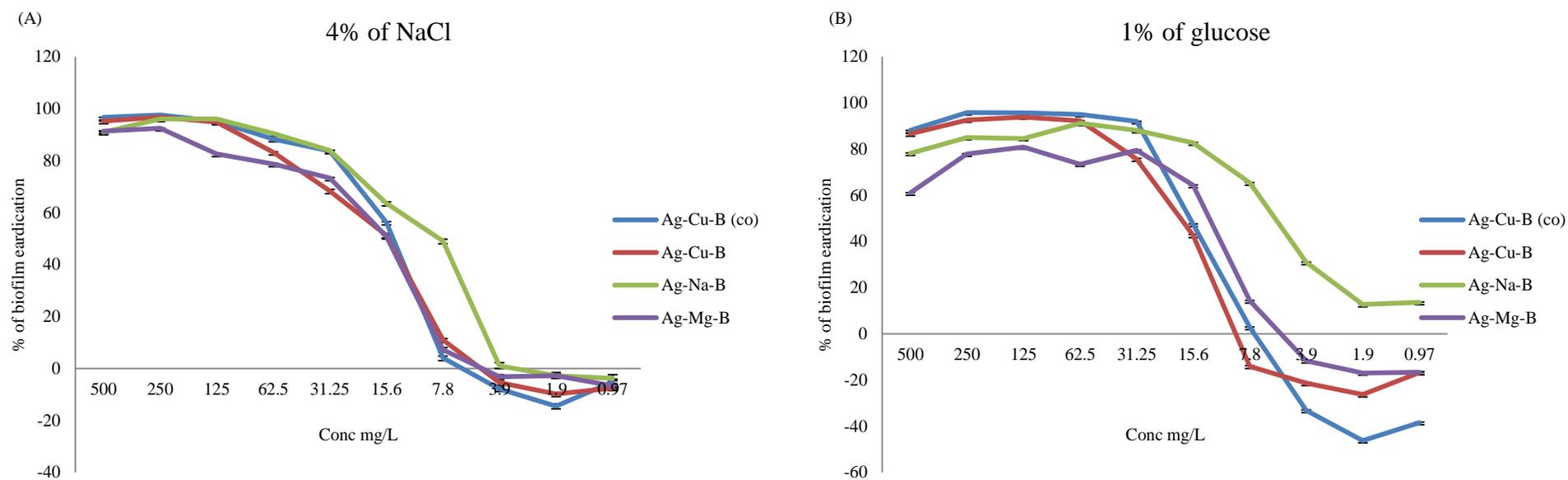
(Co) by coprecipitation, all other NPs were prepared by the microwave technique

Figure 11: Efficacy of nanoparticles against *S. aureus* 75/08



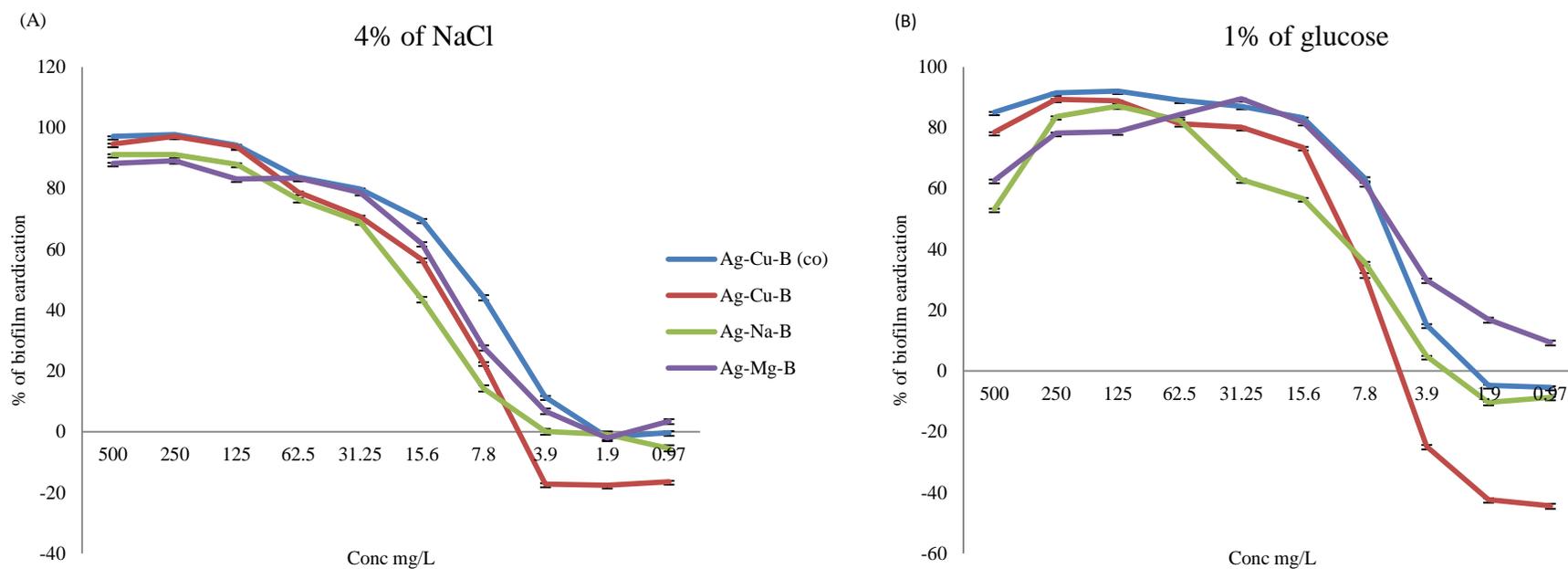
(Co) by coprecipitation, all other NPs were prepared by the microwave technique

Figure 12: Efficacy of nanoparticles against *S. aureus* 172/08



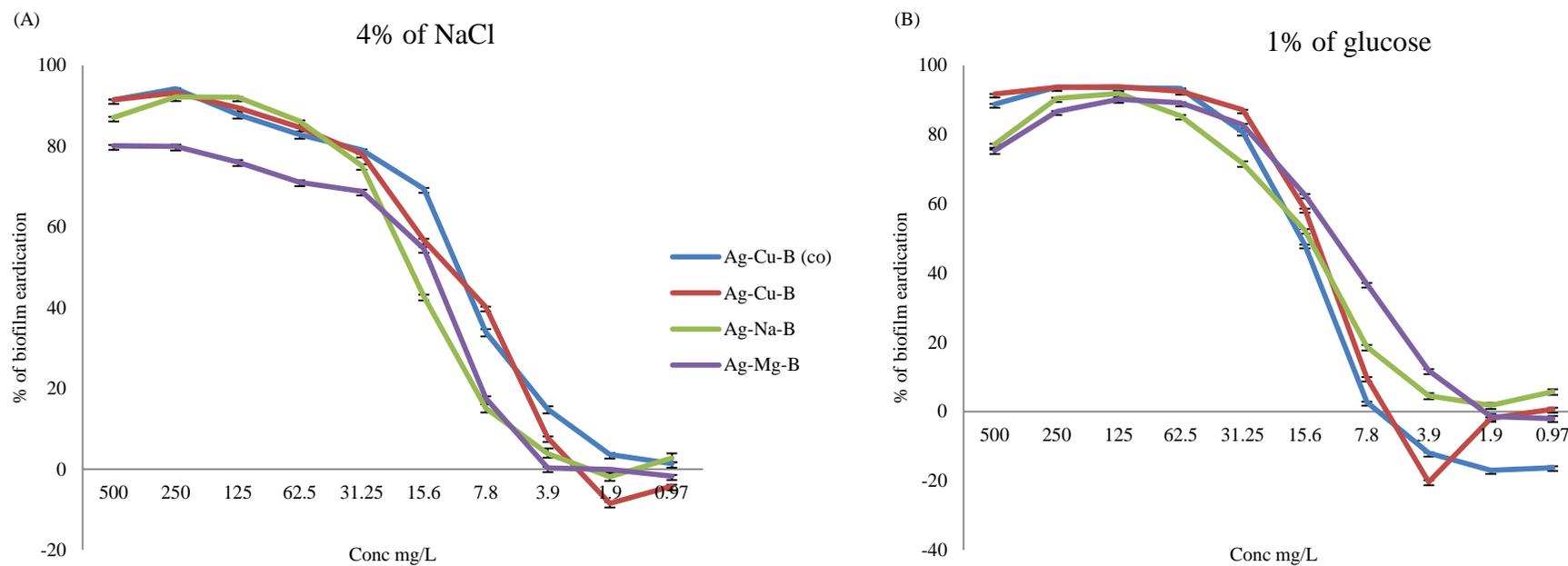
(Co) by coprecipitation, all other NPs were prepared by the microwave technique

Figure 13: Efficacy of nanoparticles against *S. aureus* 177/08



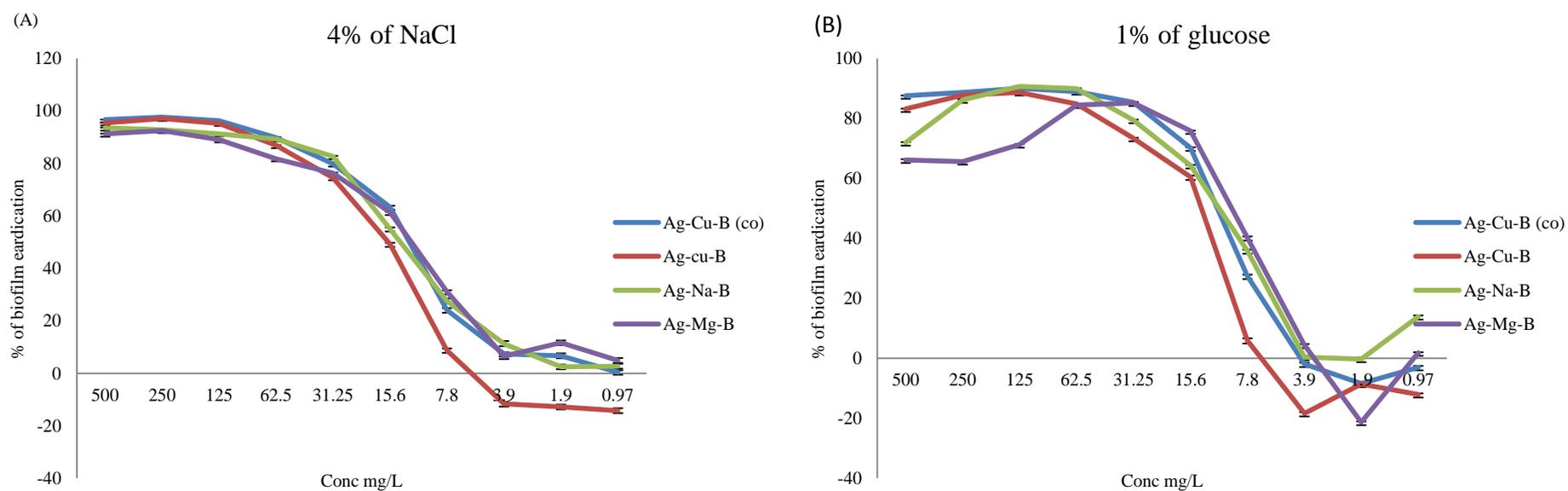
(Co) by coprecipitation, all other NPs were prepared by the microwave technique

Figure 14: Efficacy of nanoparticles against *S. aureus* 201/08



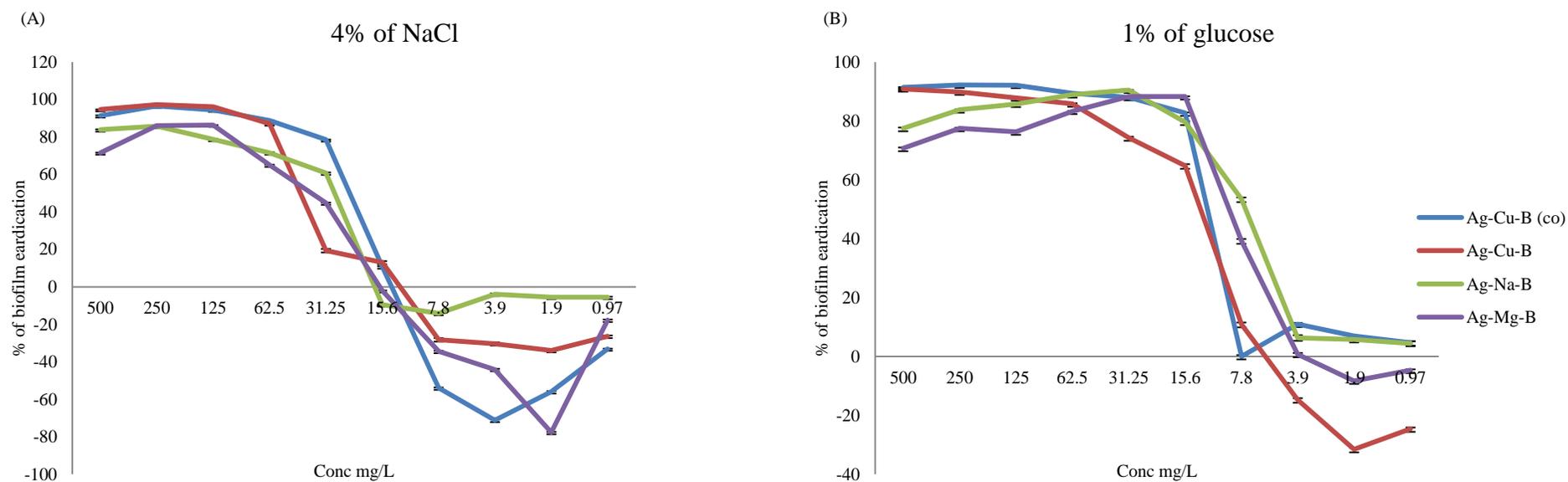
(Co) by coprecipitation, all other NPs were prepared by the microwave technique

Figure 15: Efficacy of nanoparticles against *S. aureus* 205/08



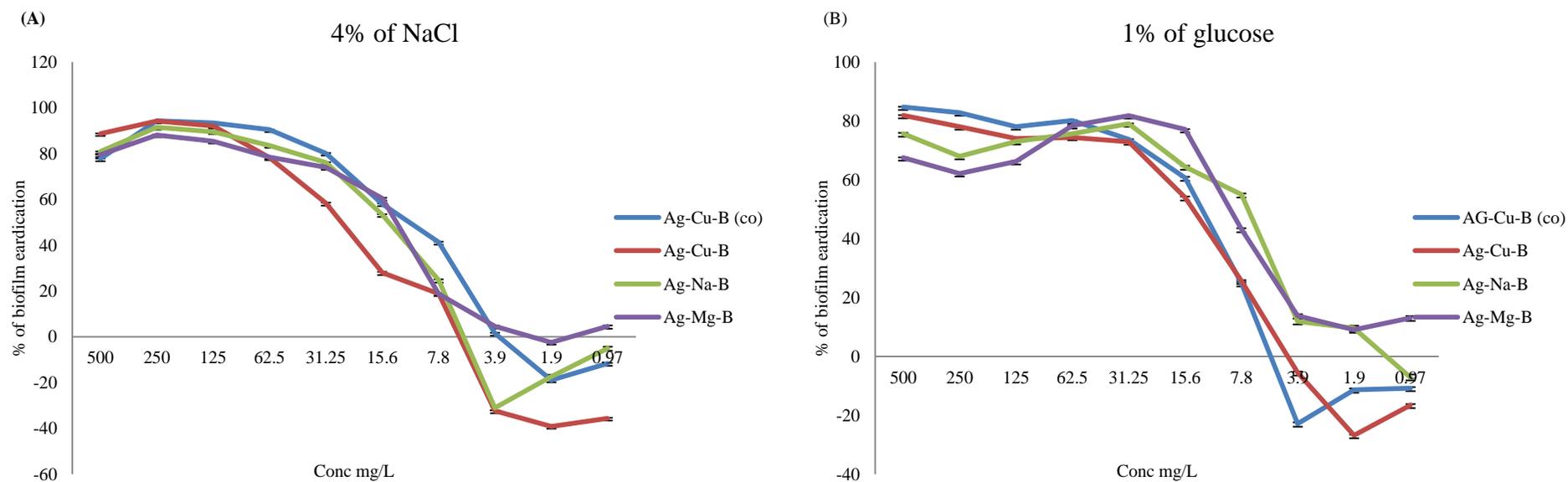
(Co) by coprecipitation, all other NPs were prepared by the microwave technique

Figure 16: Efficacy of nanoparticles against *S. aureus* 274/08



(Co) by coprecipitation, all other NPs were prepared by the microwave technique

Figure 17: Efficacy of nanoparticles against *S. aureus* T27/9



(Co) by coprecipitation, all other NPs were prepared by the microwave technique

Table 18: Minimum concentration of nanoparticles causing 10% biofilm reduction

Strains	Type of biofilm	Medium	Concentration of nanoparticles (mg/L)			
			Ag-Cu-B (Co)	Ag-Cu-B	Ag-Na-B	Ag-Mg-B
12/07	PS	BHI +NaCl	15.6	7.8	15.6	7.8
		BHI+glucose	7.8	15.6	3.9	7.8
75/08	PS	BHI +NaCl	7.8	15.6	15.6	15.6
		BHI+glucose	15.6	15.6	3.9	7.8
172/08	PS	BHI +NaCl	15.6	7.8	7.8	15.6
		BHI+glucose	15.6	15.6	1.9	7.8
205/08	PS	BHI +NaCl	7.8	15.6	3.9	7.8
		BHI+glucose	7.8	15.6	7.8	7.8
274/08	PROT	BHI +NaCl	31.25	15.6	31.25	31.25
		BHI+glucose	15.6	15.6	7.8	7.8
T27/9	PROT	BHI +NaCl	7.8	7.8	7.8	7.8
		BHI+glucose	7.8	7.8	3.9	3.9
48/08	PS/PROT	BHI +NaCl	7.8	7.8	7.8	7.8
		BHI+glucose	3.9	7.8	7.8	7.8

Table.18. *Cont*

Strains	Type of biofilm	Medium	Concentration of nanoparticles (mg/L)			
			Ag-Cu-B (Co)	Ag-Cu-B	Ag-Na-B	Ag-Mg-B
177/08	PS/PROT	BHI +NaCl	3.9	7.8	7.8	7.8
		BHI+glucose	3.9	7.8	7.8	1.9
201/08	PS/PROT	BHI +NaCl	3.9	7.8	7.8	7.8
		BHI+glucose	15.6	15.6	7.8	3.9
S800	PS/PROT	BHI +NaCl	7.8	7.8	7.8	7.8
		BHI+glucose	15.6	15.6	7.8	7.8

PS- polysaccharide, PROT - protein, BHI - Brain Heart Infusion

Table 19: Minimum concentration of nanoparticles causing 80% biofilm reduction

Strains	Type of biofilm	Medium	Concentration of nanoparticles (mg/L)			
			Ag-Cu-B (Co)	Ag-Cu-B	Ag-Na-B	Ag-Mg-B
12/07	PS	BHI +NaCl	31.25	62.5	62.5	62.5
		BHI+glucose	31.25	62.5	>500	>500
75/08	PS	BHI +NaCl	31.25	62.5	125	62.5
		BHI+glucose	62.5	62.5	31.25	125
172/08	PS	BHI +NaCl	31.25	62.5	31.25	125
		BHI+glucose	31.25	62.5	15.6	125
205/08	PS	BHI +NaCl	62.5	62.5	31.25	62.5
		BHI+glucose	31.25	62.5	62.5	31.25
274/08	PROT	BHI +NaCl	62.5	62.5	250	125
		BHI+glucose	15.6	62.5	31.25	15.6
T27/9	PROT	BHI +NaCl	31.25	125	62.5	125
		BHI+glucose	250	500	>500	>500
48/08	PS/PROT	BHI +NaCl	62.5	62.5	62.5	62.5
		BHI+glucose	31.25	31.25	62.5	31.25

Table.19. *Cont*

Strains	Type of biofilm	Medium	Concentration of nanoparticles (mg/L)			
			Ag-Cu-B (Co)	Ag-Cu-B	Ag-Na-B	Ag-Mg-B
177/08	PS/PROT	BHI +NaCl	62.5	125	125	62.5
		BHI+glucose	15.6	31.25	62.5	15.6
201/08	PS/PROT	BHI +NaCl	62.5	62.5	62.5	500
		BHI+glucose	31.25	31.25	62.5	31.25
S800	PS/PROT	BHI +NaCl	62.5	125	62.5	250
		BHI+glucose	31.25	62.5	31.25	>500

PS- polysaccharide, PROT - protein, BHI - Brain Heart Infusion

4.3. The sanitary effect of nanoparticles

Based on the previous experiments Ag-Cu-B nanoparticles prepared by co-precipitation were selected to assess their sanitary effect on stainless-steel surfaces. *S. aureus* (S800) and a group D Salmonella strain (BC789) were applied in these experiments. The MIC value for the latter strains was 15.6 mg/L.

Wipes were soaked in 100 mg/L concentration of the Ag-Cu-B (Co) NPs dispersed in sterile tap water. The results of wiping contaminated surfaces is shown in Table 20.

For the *S. aureus* strain the log reduction was more than what seen with water-soaked wipes, and after 2 hours no microorganisms were detected. With Salmonella a nearly complete elimination was achieved even by 1 hour with some residual colonies seen after 2 hours, still log reduction was more than what seen with water.

When the speed of killing was studied (Table 21) it was shown that it take much longer against a Gram positive cell to achieve good sanitary effect, while for the Gram negative salmonella it was much faster. Nevertheless, against both organisms a log 3 reduction was safely achieved 1 hour after treatment.

Table 20: The effect of Ag-Cu-B (Co) nanoparticles as a sanitary agent

Strain	Untreated	Log reduction					
		Time after treated with					
		0 time		1 hour		2 hours	
		Water	Nano	Water	Nano	Water	Nano
<i>S. aureus</i> S800	5.9	3.4	3.1	4	4.6	3.5	ND
<i>Salmonella</i> BC789	4.9	1.4	2.5	2.2	ND	2.5	3.2

Table 21: The time-kill effect of Ag-Cu-B (Co) nanoparticles as a sanitary agent

Strain	Untreated	Log reduction							
		Time after treated with							
		0 time		15 min		30 min		60 min	
		Water	Nano	Water	Nano	Water	Nano	Water	Nano
<i>S. aureus</i> S800	5.9	2.3	2.7	3.3	3	3.4	3.4	2.8	3.7
<i>Salmonella</i> BC789	4.3	1.6	ND	1.9	ND	1.9	ND	2.3	3

ND- not detected

Chapter 5. Discussion

The past few years have seen an increase in the incidence of food-borne infections and the emergence of new food-borne pathogens (Turner et al., 2003). Food globalization, the new trends in food production and distribution, changes in consumer habits and population susceptibility are pointed as the main contributing factors. One of the main concerns in food safety is the transmission of pathogenic Gram-negative *Enterobacteriaceae*, (e.g. *E. coli*, *Salmonella* spp, *Shigella* spp) and *Pseudomonas* because of their high incidence in food-borne illness and the emergence of new virulent serotypes and transmission routes (Gálvez et al., 2010). Consequently, in order to successfully deal with the problem of food-transmitted infections more efforts are needed to better understand the mechanisms how these microorganisms contaminate and survive in food and what interventions can be used to prevent or eliminate such contaminations.

The species *E. coli* represents a diverse collection of types of organisms that occur as pathogens and as commensals in the mammalian intestinal tract. An extraintestinal pathogen *E. coli*, ExPEC, is the most common Gram-negative bacterium associated with urinary tract and bloodstream infections (Riley, 2014). ExPEC infections have been described previously as sporadic infections, however, recently ExPEC have been associated with outbreaks which suggest that ExPEC can be spread in the community by a common source or vehicle (Bergeron et al., 2012). Investigations of these outbreaks has proposed that environmental sources, possibly

contaminated meat and other foods, may play a role in the local spread of related *E. coli* strains (Vincent et al., 2010).

Bergeron and co-worker characterized the genetic similarities between *E. coli* isolates recovered from the cecal contents of slaughtered food animals (beef cattle, chickens, and pigs) and ExPEC in humans causing community-acquired urinary tract infections (UTIs), they found genetic similarities between *E. coli* from animals, particularly chickens, and ExPEC caused UTIs in human (Bergeron et al., 2012). This finding is similar to that reported by Caroline and co-workers who also characterized the genetic similarities between *E. coli* isolates recovered from retail meat (poultry, pork and beef) and ExPEC isolated from human. They identified two clonal groups (i.e. ST95 & ST131) containing isolates from retail chicken meat and human infections and this evidence suggested that chicken was likely to be the primary reservoir of ExPEC in humans (Caroline et al., 2010). The outcomes of these two studies provide a strong support for the role of food reservoirs, principally chicken, or foodborne transmission in the prevalence of ExPEC causing UTIs.

Recently one of these multidrug-resistant *E. coli* clones, i.e. ST131 has emerged globally. One of the diverse factors explaining its rapid dissemination was transmission through human animal contact and consumption of contaminated food. A high degree of similarity has been shown among certain ST131 isolates from humans, companion animals, and poultry based on resistance characteristics and genomic background (Platell et al., 2011).

One common survival strategy employed by the pathogenic bacteria is to form a biofilm, an amorphous and dynamic structure (Chen and Wen, 2011) that is 10 to 1000 times more resistant to the effect of antimicrobial agents than their planktonic counterparts (Jefferson, 2004). This makes their eradication and control extremely difficult in many environment such as medical and food industrial areas (Mah et al., 2003). So far, very little has been known on the biofilm forming capacity of *E. coli* ST131. Hence, one of the aims of the current study was to measure the biofilm forming capacity of food-transmitted *E. coli* of ST131 clone.

5.1. Biofilm formation of *E. coli* ST131 strains

Biofilm-forming ability of 115 *E. coli* ST131 was examined under 12 different environmental conditions (i.e. nutrient rich and restricted methods). The relationship among the isolates, as assessed by PFGE, suggested a considerably heterogeneity within the ST131 sequence type (Table 7) making the pool of isolates optimal to study the targeted phenotype and its relation to growth conditions.

Several genes have been implicated to contribute to the formation of biofilms by *E. coli* that are flagella, various classes of fimbriae, curli, antigen 43 (*Ag43*), and the extracellular matrix compounds (Beloin et al., 2006). The isolates were screened for the presence of different adherence genes. The level of variations corroborated the findings of PFGE, i.e. a considerable heterogeneity of the strains (Table 8). Five different genes were frequently present in all isolates (*Crl*, *CsgA*, *Ag43*, *FimH* and *UpaB*). Notably, the majority of the members of *E. coli* ST131 clonal group was

found multi-drug resistant and possessed the *bla*_{CTX-M} beta-lactamase gene. Our results are in accordance with previous studies reporting that ST131 clone associated with *bla*_{CTX-M} extended spectrum β -lactamase (ESBL) production, with CTX-M enzyme (Cagnacci et al., 2008; Hernández et al., 2005; Mugnaioli et al., 2006; Oteo et al., 2006). ESBL dissemination in *E. coli* is usually due to plasmid transmission between unrelated strains (Hernández et al., 2005) and this can potentially increase the spread of antibiotic resistance within the bacterial population through horizontal gene transfer. Epidemiological and environmental studies are needed to identify transmission routes, and reservoirs for these multidrug resistant bacteria on the local level.

The ability of the isolates to form biofilms on abiotic surfaces was examined under restricted and under rich nutrient conditions. Our results revealed that, albeit very much in a medium-dependent manner, the *E. coli* strains of the clone ST131 are capable of producing biofilm. This finding is in agreement with Clermont and co-workers who measured adherence capabilities in four representatives O25:H4-ST131 CTX-M producing *E. coli* isolates collected from Europe and Africa and their findings demonstrated that two isolates produced biofilm (Clermont et al., 2008). In contrast, Novais and co-workers studied 31 ST131 strains obtained between 1991 and 2010 isolated from healthy volunteers, animals and environmental samples from different geographical areas. All tested strains were found weak biofilm producers (Novais et al., 2012). The difference in findings between the studies could be attributed to the applied growth condition in each study. Naves and co-workers evaluated the impact of methodological approaches in determining biofilm formation by four clinical

isolates of *Escherichia coli* in static assays. They concluded that *E. coli* forms biofilms on static assays in a method dependent fashion, depending on strain, and it is strongly modulated by culture conditions (Naves et al., 2008b).

Actually, our study, with a much higher strain number also proved the importance and role of the medium and growth temperature (Table 9). If grown in rich media, the determining factor was temperature, i.e. 37 °C. In contrast, once nutrients were limited, the degree of biofilm formation was generally higher. It was higher at room temperature, and particularly high under anaerobic conditions. This findings, in contrast to some earlier observations (Pratt and Kolter, 1998) and are in line with other previously published reports showing that biofilm formation increases under low nutrient media (Reisner et al., 2006; Skyberg et al., 2007; Yang et al., 2004).

An important observation of this study was that the only clonal clustering of the biofilm forming capacity was observed in nutrient rich environment (TSB) at 37 °C. This finding was interpreted as that the capacities to establish stable mats on solid surfaces is an attribute of the entire clone, albeit the expression of the actual capacity exhibits considerable inter-strain variations. As a conclusion, *E. coli* ST131 should be considered as an organism able to contaminate various niches of the food-production chain where both nutrient rich, as well as nutrient limited environments, with variable existing temperatures can be anticipated.

When using multiple logistic regression analysis to evaluate the relation between biofilm formation and the presence or absence of different genes a strong correlation between *bla*_{CTX-M} beta-lactamase gene was found and the ability of the isolates to produce biofilm at 37 °C in TSB. This is a particularly interesting observation as earlier it was found that non-betalactamase producer isolates were the major biofilm producers, while strains possess the betalactamase produced either a minor biofilm or no biofilm at all (Gallant et al., 2005). The authors speculated that among their strains plasmid vectors encoding the common betalactamase marker TEM-1 caused defects in twitching motility (mediated by type IV pili), adherence and biofilm formation (Gallant et al., 2005).

However, one should keep in mind that the study was not about this particular *E. coli* clone, i.e. ST131 and here the situation could be entirely different. It is hard to envision that it is the *bla*_{CTX-M} gene, itself which directly affect biofilm formation. It more likely to be the effect of a linkage between the *bla*_{CTX-M} gene and a yet to be identified gene present. The *bla*_{CTX-M} gene is commonly localized on mobile plasmids (Zhao and Hu, 2013), which would explain our finding. However, the identification of such gene(s) were beyond the scope of the current project.

Further significant correlations were identified between biofilm and the *ag43*-CFT073a and *ag43*-RS218 allelic variants of the *ag43* gene in different conditions (Table 10). *Ag43* is a surface Autotransporter proteins transport all necessary information to the bacterial cell membrane and secretion through cell surface within the protein itself (Kjærsgaard et al., 2000; Sherlock et al., 2006). It was shown

promoting cell to cell adhesion and cells autoaggregation via intercellular Ag43-Ag43 handshaking mechanism (Sherlock et al., 2006) rather than Ag43 being directly involved in the initial adhesion to abiotic surface. This leads to the conclusion that autoaggregation appears to contribute to the development of the three dimensional biofilm structure (Beloin et al., 2008) and micro-colony formation (Van Houdt and Michiels, 2005).

Both P and type 1 fimbria are produced by uropathogenic strains of *E. coli* and they have several functions in mediating colonization, invasion and biofilm formation (Miller et al., 2006; Otto et al., 2001). Type 1 fimbria are encoded in the *Fim* operon while P fimbria localize within the *Pap* operon (Lillington et al., 2014). Earlier, type 1 fimbria adhesin gene *FimH* was shown to play a significant role in mediating attachment to biotic and abiotic surfaces by some *E. coli* strains (Hung et al., 2013), but based on our results it seems not to be the case in the ST131 clone, at least not under the conditions used to culture our isolates. Our findings revealed that biofilm production in *E. coli* ST131 strains were more related to the presence of P fimbria genes rather than type 1 fimbria.

Although, the prevalence of *papA* and *papC* genes of the P fimbria was relatively rare among the strains (18.3%), a strong relationship was observed between these genes and biofilm formation in LB at 37 °C and CAA at room temperature suggesting that once present, it does play a role in the biofilm formation of *E. coli* ST131, as well.

Taken together, our findings showed the *E. coli* ST131 strains do form biofilm, and this capacity, in a culture-condition dependent manner, with varying extent, is present across the clone. This suggests the biofilm formation is to be expected in food-processing related niches and should be taken into consideration when measures to decrease this contamination are designed. Biofilms within the clone appear to be of multiple nature, some linked to the *bla*CTX-M gene, according to our hypothesis indirectly, by gene co-localization on mobile genetic elements.

5.2. The effect of nanoparticles against biofilms produced by food-transmitted bacteria

Over the past few decades inorganic nanoparticles have been developed and, due to their nanoscale size, recognized to have remarkable physical, chemical, and biological properties. Metallic nanoparticles (NPs), particularly silver and copper NPs are promising new antibacterial agents due to their high surface area to volume ratio (Pelgrift and Friedman, 2013). The antimicrobial properties of silver against different microorganisms are well established (Ghosh et al., 2010; Kim et al., 2007; Mirzajani et al., 2011; Sondi and Salopek-Sondi, 2004). In comparison with silver relatively few studies addressed the antimicrobial properties of Cu-NPs (Allaker, 2012).

Although, the bactericidal effect of Ag-NPs and Cu-NPs, as a single element was explored, few studies investigated the effect bimetallic Ag-Cu nanoparticles on different microorganisms. Boron in the form of nanoparticles was also studied for its

antimicrobial effect in combination with other elements (Wang et al., 2014; Xue et al., 2013).

5.2.1. Susceptibility of planktonic bacterial cells to nanoparticles

In order to select the most effective NPs, a preliminary study on bactericidal effect of various nanoparticles with different volume ratios prepared by coprecipitation method was conducted on *S. aureus* (Table 12). The findings revealed that *S. aureus* exhibited almost similar sensitivity to Ag-NPs and Cu-NPs. This finding is in agreement with what reported by Rupareli and coworkers (Ruparelia et al., 2008). Although the single Ag-NPs and Cu-NPs were effective, the combination of Ag and Cu acts synergistically against *S. aureus*. The minimum inhibitory effect of Ag-Cu nanoparticles with different volume ratios varied between 166 and 250 mg/L. Furthermore, the synergistic antibacterial activity of Ag, Cu and B complexes with all volume ratios exhibited similar inhibitory effect (MIC 125 mg/L). This, however, does not seem to be a general rule. Martinez-Gutierrez and coworkers investigated the antimicrobial effect of 15 types of nanoparticles using silver, TiO₂, or a combination of both compounds against a panel of opportunistic and pathogenic bacteria and fungal strains. They concluded that no significant activities were observed when TiO₂-NPs were combined with silver compared to the Ag-NPs (Martinez-Gutierrez et al., 2010). Based on our findings the Ag-Cu-B NPs with volume ratio 70:20:10 were selected and subjected it to further investigation. Simultaneously, the same formula was also prepared by the microwave technique and the Cu component was also substituted with Na or Mg. These nanoNPs were used to test their antimicrobial efficacy against a

range of drug resistant bacteria i.e. *S.aureus*, *P. aurgnosia* and three *E. coli* strains of ST131 clone (Table 13).

Although the Gram positive *S. aureus* was found slightly more resistant than the Gram negative isolates, this finding in agreement with the previous findings of Martínez-Gutierrez and coworkers (Martinez-Gutierrez et al., 2012). Investigating the antibacterial effect of 24nm Ag-NPs on five pathogens commonly isolated from medical devices (i.e. *E. coli*, *E. faecalis*, *P. aeruginosa*, *S. aureus*, and *S. maltophilia*) they found no significant differences in the antibacterial activities against Gram-positive and Gram-negative organisms (Martinez-Gutierrez, et al., 2012). On the other hand, Fayaz and coworkers reported MIC values of 30–35 mg/L for Gram-negative bacteria compared to the MIC values of 65–80 mg/L for Gram-positive isolates (Fayaz et al., 2010). Conversely, Premanathan found that the MIC of ZnO-NPs against the Gram-negative bacteria *E. coli* and *P. aeruginosa* (MIC 500 mg/L) was more than that against the Gram-positive bacterium *S. aureus* (MIC 125 mg/L), (Premanathan et al., 2011). The susceptibility to NPs could be a function of the composition of the NP itself. Actually the differences in susceptibilities can easily be attributed to the cell-wall structure of the Gram-positive and Gram-negative bacteria. Importantly, however, the compounds which have been studying appear to be effective against both groups of organisms.

On the nanoparticles' side, several factors could influence their efficacy. Previously, it was found that Ag-NPs having a size of 20–25nm were more effective than those which have 80-90nm size (Martinez-Gutierrez et al., 2010). In our study,

although Ag-Cu-B prepared by co-precipitation possessed the biggest size among the other NPs, it demonstrated the strongest antibacterial effect on all bacteria compared. This apparent controversy could be attributed to several factors. The superior potency of this NP could be due to its spherical/platelet shape (Figure 5).

Pal and co-workers reported that the antimicrobial activity of Ag-NPs is shape dependent. A truncated triangular Ag-NPs, with a lattice plane as the basal plane, were found to be more effective on the growth of *E. coli* than spherical particles. The inhibition of bacterial growth by truncated triangular shape was found at 1 mg/L, whereas, in the case of spherical shape bacterial inhibition was 12.5 mg/L (Pal et al., 2007). The shape-dependent antibacterial property of CuO-NPs was also evaluated by Ananth and coworkers against four bacterial strains, namely *Streptococcus iniae* and *Streptococcus parauberis* (Gram positive) and *Escherichia coli* and *Vibrio anguillarum* (Gram negative). Their results indicated that the plate-like CuO displayed more powerful antibacterial activity than grain or needle shaped CuO NPs (Ananth et al., 2015).

However, several studies actually showed that some nanoparticles did not exhibit considerable bactericidal effects. Platinum nanoparticles having size 3 ± 1 nm did not show antimicrobial activity for *S. aureus* and *E. coli* in some studies (Cho et al., 2005). Furthermore, silica, silica/iron oxide, and gold failed to inhibit growth of *E. coli* (Williams et al., 2006). Several members of the environment microbiota are tolerant to NPs that are present in the environment. Wu and coworkers reported that Cu-doped TiO₂-NPs actually increased the survival rates of *Shewanella oneidensis*

MR-1 by over 10,000-fold (Wu et al., 2011). In another study, *Shewanella oneidensis* was able to tolerate higher concentration of Cu-doped TiO₂-NPs. One explanation could be that a large amount of extracellular polymeric substances (EPS), in particular extracellular protein, was produced by the strain used and it was also speculated that the bacteria cell membrane was able to adsorb NPs and enzymatically reduced ionic copper in the culture medium (Wu et al., 2010). In fact, several bacteria are able to tolerate NO-NPs using various mechanisms. For example *P. aeruginosa*, *E. coli*, and *S. typhimurium* induce the expression of genes that are responsible for repairing of DNA and altering the metal homeostasis in the presence of NO-NPs. In this condition, *K. pneumoniae* produces the enzyme flavohemoglobin, which neutralizes nitrosative stress (Hajipour et al., 2012)

Taken together, it was felt that NPs used, particularly the Ag-Cu-B alloy prepared with co-precipitation exhibit promising features and can be taken a step further to try its efficacy in biofilm models.

5.2.2. Susceptibility of bacterial biofilms to nanoparticles

Compared to killing planktonic cells, a mature biofilm is difficult to eradicate. Biofilms are resistant to antibiotics, antimicrobial agents and toxic chemicals such as heavy metals (Harrison et al., 2005). In this part of study, the anti-biofilm efficacy of the same synthesized nanoparticles (i.e. Ag-Cu-B (Co), Ag-Cu-B, Ag-Na-B and Ag-Mg-B) was investigated on biofilm models of *S.aureus* (S800), *P. aeruginosa* (AG1) and three strains of *E. coli* ST131.

As expected, nanoparticles were more effective on Gram-negative bacteria than *S. aureus*, particularly with biofilm formed by the latter in the presence of glucose (i.e. protein type biofilms). Our results were in good agreement with those reported by Hetrick and co-workers. They found that the anti-biofilm efficacy of NO-releasing Si-NPs at the highest dose (8 mg/L) was greatest against the *P. aeruginosa* and *E. coli*, with ≥ 5 logs of killing for both, whereas the highest dose of those NPs killed ~ 2 logs of *S. aureus* and *S. epidermidis* biofilms (Hetrick et al., 2009).

One may speculate that the difference in the toxicity of the nanoparticles between Gram-negative and Gram-positive biofilms may be due to differences in the extracellular polymeric substance (EPS) properties of each type of biofilm. The EPS synthesized by bacterial cells greatly vary in their composition, chemical and physical properties (Sutherland, 2001a). Both *P. aeruginosa* and *S. aureus* are known to synthesize exopolysaccharides. Jena and co-workers examined the ability of starch-stabilized Ag-NPs (CS-AgNPs) in disrupting the biofilm formation of *P. aeruginosa* and *S. aureus*. The results indicated that the inhibition of biofilm formation was more potent in *P. aeruginosa*. This distinction could be because of the presence of abundant EPS, which leads to strong interaction with CS-Ag-NPs, produced by *P. aeruginosa* (Jena et al., 2012). This is consistent with previous research reported that Chitosan (CS-NPs) and Zinc Oxide (ZnO-NPs) possessed significant anti-biofilm activities and were capable of disrupting the multilayered biofilm structure of the *Enterococcus faecalis* (Gram-positive) (Shrestha et al., 2010). On the other hand, Sheng and Liu observed that genera of *Kelbsiella* produced

a large amount of EPS and were relatively tolerant to Ag-NPs activity (Sheng and Liu, 2011).

Toxicity of these nanoparticles could be, at least partly, attributed to their negative charge which enhance their penetration and diffusion into the EPS. Many bacterial species are characterized by a negatively-charged biofilm matrix due to the presence of either uronic acids or ketal-linked pyruvate (Sutherland, 2001a). It was found that the penetration of the positively charged drugs, such as aminoglycosides and polypeptides, was low through the *P. aeruginosa* biofilm matrix whereas negatively charged drugs showed good penetration. Direct binding of positively charged agents to negatively-charged EPS has been reported to account for their poor penetration while the penetration of the negatively charge were not inhibited (Kumon et al., 1994).

On the other hand, previous studies reported that NPs prepared by biological method exhibited relatively similar effect on both Gram-negative and Gram-positive bacteria . It was shown that 100nM of Ag-NPs resulted in 95–98 % reduction in *P. aeruginosa* and *S. epidermidis* biofilms (Kalishwaralal et al., 2010) while Ag-NPs and Au-NPs showed different effect on Gram-positive and Gram-negative bacteria. Ag-NPs have a good biofilm disruption, with the highest of 88% in *A. baumannii*, 67% in *E. coli*, 78% in *S. aureus*, whereas, Au-NPs showed lower biofilm disruption of around 40% in Gram-negative bacteria and with a maximum of 95% in *S. aureus* biofilm (Salunke et al., 2014). The difference between the anti-biofilm activities of different NPs in different studies could be explained by variability in several factors

between the studies, such as strains, types of NPs , NPs preparation technique and the used methodologies.

An interesting observation was that some of the NPs at low concentration actually increased biofilm formation, while at higher concentration they showed good anti-biofilm efficacy. This was present in both *S. aureus* and in *P. aeruginosa*, being more pronounced when strains were grown in glucose (Figures 6 and 7). Others reported similar phenomenon earlier, as well. It was shown that *P. aeruginosa* biofilm increased in the presence of 0.2 mg/mL of FeO-NPs (Carl Haney et al., 2012). Also, 0.01 mg/mL Au-NPs and FeO-NPs showed increased in *S. aureus* and *P. aeruginosa* biofilm growth (Sathyanarayanan et al., 2013). Furthermore, non UV-irradiated TiO₂-NPs increased biofilm formation by *Listeria monocytogenes* (Ammendolia et al., 2014). Currently, the basis of this phenomenon is not clear, which however, does not appear to affect the potential utility of NPs.

5.3. Efficacy of nanoparticles against *S. aureus* of different clones

Using a limited number of strains, only to know whether the results could be applicable to other isolates, as well. Testing further 9 MRSA strains in their planktonic, as well as biofilm forms representing independent lineages (Table 16) revealed some variations between the strains. For planktonic cells the Ag-Cu-B (Co) nanoparticles were the most efficacious for all of them (Table 17). Regarding the biofilm system, it was observed that the susceptibility of pre-existing MRSA biofilms exhibit considerable variation (Table 18-19). Although, no clear differences between

the susceptibility of protein or polysaccharide type biofilms was observed, protein biofilm produced a highly resistant matt for which extreme concentration of NPs were needed to achieve 80% reduction. No clear differences between the susceptibility of protein or polysaccharide type biofilms could be identified, as extreme resistance was also seen in some polysaccharide type matts. Noticeably, Ag-Na-B and Ag-Mg-B NPs were not able to inhibit biofilm of certain strains under the presence of glucose (i.e. 12/07, T27/9 and S800) even the maximal concentration. It could be speculated that in these cases the NPs may have been hindered by EPS. Actually, EPS acting as a physical barrier might result in a gradient of nanoparticles affecting the biofilm bacterial cells (Shrestha et al., 2010). Also, it is possible that NPs are trapped by EPS and can't reach the biofilm cells. In addition, the EPS might also serve as a chemical barrier by adsorbing the harmful reactive oxygen species (ROS) from reaching the cell surface, thereby decreasing the effect of radical oxidative stress formed by NPs (Shrestha et al., 2010).

However, most of NPs could reduce above 80% of the biofilms, although 100% viability loss did not occur even at the highest concentrations tested, suggesting some biofilm tolerance to the nanoparticles effect. This finding is in line with those reported by Wirth and co-workers who observed that *Pseudomonas fluorescens* biofilms showing some tolerance to highest concentration of AgNPs (Wirth et al., 2012). Tolerance could be associated with the physical barrier, which derived from the presence of EPS. EPS could act as a barrier to antimicrobial transport into biofilms as it plays a role in the extraordinary antimicrobial tolerance of biofilms (Wirth et al., 2012). Furthermore, this tolerance may be attributed to the presence of survivor cells

(presisters). Presisters are largely responsible for high levels of biofilm tolerance to antimicrobials (Keren et al., 2004).

As a conclusion, nanoparticles could considerably reduce established biofilms. This is definitely true for Gram negative bacteria, while certain Gram positive strains exhibit high level of resistance. It should be noted, however, that our model used well-established, mature biofilms and further studies are needed to clarify how the timely application of these compounds affect biofilms of resistant strains being in their earlier stages of development. The results certainly warrant further studies with these compounds to develop them into agents to be used on surfaces which cannot be replaced or subjected to harsher cleaning methods.

5.4. The sanitary effect of nanoparticles

Contamination of food contact surfaces is a major safety concern for food-service facilities. It was reported that approximately 80% of the foodborne outbreaks were traced back to food-service facilities with major contributing factors including inadequate personal hygiene, temperature abuse and cross contamination (Masuku et al., 2012).

Keeping the food contact surfaces clean, i.e. either preventing their exposure to microorganisms, or removing the organism before they more or less permanently colonize them is a critical issue. This is particularly true as it is known that the effectiveness of sanitizers on the planktonic cells are greater than their sessile

counterparts. Furthermore, with the emergence and prevalence of the multidrug resistant bacteria, the demand for improving and enhancing disinfectants efficiencies should be increased (Zarei et al., 2014).

Cleaning process refers to the mechanical removal of dirt soil and microorganisms from surfaces while sanitizing refers to the reduction of microbial contamination on inanimate surfaces to acceptable level (Koo et al., 2013). Choosing both cleaning materials and disinfectants is required to ensure food surface hygiene. Lee and co-workers showed that the application of wipes integrated with sanitizer was capable of reducing the viability of bacteria, yeast and fungal spores from different surfaces (Lee et al., 2007).

Masuku and co-workers found that the combination of a sanitizer (silver dihydrogen citrate) and physical removal of microbes with wiping cloths is essential (Masuku et al., 2012). DeVere and Purchase compared the effectiveness of two wipes and two sprays disinfectant on a range of food contact surfaces contaminated with *E. coli* and *S. aureus*. The findings revealed that wipes were found to be less effective than sprays and this could be due to the amount of disinfectant received by the surface during the treatment (DeVere and Purchase, 2007). In contrast, various studies reported that by the impregnation of fabric material with disinfectant lead to eliminate variety of microorganisms on different food surfaces. Tebbutt reported that surfaces cleaned with clothes soaked in a detergent and hypochlorite solution were more likely to be successfully cleaned than those wiped with un-soaked cloths (Tebbutt, 1991). Also, clothes impregnated with quaternary ammonium disinfectant

significantly reduced number of *Enterobacteriaceae* and *Pseudomonads* (Scott and Bloomfield, 1993).

However, data on the application of NPs in this context is very limited. The bactericidal effect of Ag-NPs were tested and compared with two commonly used disinfectants, sodium hypochlorite (NaClO) and phenol (C₆H₅OH) on *E. coli* using suspension test. It was noticed that the two chemical disinfectants exhibited rapid bactericidal activity within about 10 min. In contrast, Ag-NPs exhibit slow but long-term bactericidal effect within 6 h (Chamakura et al., 2011).

The effectiveness of NPs integrated into fabric material was assessed to reduce microbial loads to acceptable level. Noticeably, it was found, that Ag-Cu-B NPs wipes could reduce the viability of both *S. aureus* and *salmonella* more than 10 times more what seen with water-soaked wipes. Furthermore, NP-soaked wipes could totally eliminate *S. aureus* cells after 2 hour. In fact, a 3 log reduction was safely achieved after 1 hour after treatment (Table 20 and 21). It could be speculated that the sanitary effect of NPs on the contaminated surfaces can be explained by this NPs' antimicrobial activity (have shown in our earlier experiments) beyond the additional effect of mechanical action, detected by water-soaked wipes. Clearly, however, it was the NPs which had a significantly greater sanitary effect.

Our results indicate, that after submitting these compounds to appropriate toxicology testing, Ag-Cu-B NPs carry the promise to be developed into effective

antimicrobials in food industry, both in eliminating existing biofilms, as well as to be applied as sanitary agents.

Chapter 6. Conclusions and recommendations

Based on our data the following conclusions and recommendations could be drawn:

E. coli ST131 strains do form biofilms, albeit the capacity of the strains exhibit considerable variation without showing, with very limited exceptions, any considerable clonal clustering. Biofilm formation within this sequence type is highly variable according to growth conditions. In nutrient rich environment 37 °C temperature is an important factor promoting biofilm formation, while under nutrient limitation anaerobiosis seems to be a considerable stimulant.

Therefore the study recommends that food-industry should be aware of the potential threat by this group of bacteria as strains can easily be encountered armored with capacities to form biofilms under a broad variety of conditions created by different procedures of food industry.

Biofilm forming capacity exhibited only a limited association with any of the known adhesions genes suggesting new, yet to be identified adherence mechanisms. However, the strong correlation at 37 °C with the *bla*_{CTX-M} beta lactamase gene indicates a possible co-location of the unknown biofilm gene(s) with the *bla*.

The study suggests further studies to identify these genetic factors facilitating the rapid detection of strains with biofilm forming capacity.

Ag-Cu-B nanoparticles exhibit strong anti-microbial activity against planktonic cells of bacteria, particularly in 70:20:10 ratio when prepared by the co-precipitation method. It also exhibits considerable capacity to kill bacteria within existing biofilms of different nature and produced by different bacteria. Consequently, these nano-NPs definitely carry the potential to be developed into effective agents preventing and destroying existing biofilms in food industry.

The study recommends further studies with these nanoparticles , particularly as far as its standardization of production and toxicity (release and contamination of food by metal ions) are concerned, i.e. investigations that went beyond the scope of the current project.

The study concludes that these nanoparticles are fast and effective compounds to be used as sanitary wipes, i.e. a task much needed in food industry and food-related activities.

Beyond the above mentioned toxicology studies, study also recommends investigations in cooperation with the industry to develop the most appropriate vehicle for this nanoparticles to be applied as wipes.

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Appendix

Composition of buffers, solutions and media used

BHI broth: 3.7 grams of Brain Heart Infusion medium (Oxoid, Basingstoke) was added to 100 ml distilled water then autoclaved.

BHI medium supplemented with 4% NaCl was prepared by adding 3.7 grams of Brain Heart Infusion medium (Oxoid, Basingstoke) and 4 grams of NaCl (95.5%, sigma) to 100 ml distilled water then autoclaved.

BHI medium with 1 % glucose was prepared by adding 2 ml of 20% sterile glucose (95.5%, sigma) solution (sterilized with 0.22 μ m syringe filter) to 38 ml of autoclaved BHI broth.

Casamino acid solution (20%) was prepared by adding 20 g of casamino powder (Bacto, France) into 100 ml distilled water, then autoclaved.

Cell lysis buffer 25 ml of 1M Tris buffer (pH 8.0) and 50 ml of 0.5 M Na-EDTA (pH 8.0) were added to 50 ml of 10% Sarkosyl, then the solution was filled up to 500 ml sterile distilled water.

Cell suspension buffer was prepared by adding 10 ml of 1 M Tris buffer (pH 8.0) to 20 ml of 0.5 M Na-EDTA (pH 8.0), then the solution was filled up to 1 liter sterile distilled water.

Fixing solution was prepared by adding 10 ml of formalin to 490 ml of autoclaved 1X PBS.

Glycine buffer (0.1 M) was prepared by adding 0.75 g of glycine (sigma, US) into 100 ml distilled water. Glycine solution's pH was adjusted to 10.2 with 10N NaOH, then autoclaved.

LB medium with 1 % glucose was prepared by adding 2 ml of 20% sterile glucose to 38 ml of autoclaved LB.

LB medium: 0.2 grams of Luria broth medium (Invitrogen, US) was added to 100 ml distilled water then autoclaved.

Lysing solution was prepared by adding 5 ml of 10% SDS to 250 ml of 99% ethanol, then filled up to 500 ml with autoclaved 1X PBS.

Lysostaphin was prepared by adding 10 mg of lysostaphin powder into 1ml sterile distilled water, then aliquoted in 500 μ l tubes and stored at -20 °C.

M63 (5X) medium broth : 20 g ammonium sulfate ($(\text{NH}_4)_2\text{PO}_4$), 136 g potassium Phosphate (KH_2PO_4) and 5 mg ferrous sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) were dissolved into 1.8 liter distilled water. M63 Solution's pH was adjusted to 7 with 10 N KOH. The solution was filled up to 2 liter with distilled water, then autoclaved.

M63 broth supplemented with 20% casaminoacid and 0.2% glucose: was prepared by adding 20 ml 5X M63, 5 ml casaminoacid, 1 ml 20% sterile glucose and 100 μ l 1M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ into 74 ml sterile distilled water.

Magnesium sulfate solution 1M: was prepared by adding 24.64 g of magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) into 100 ml distilled water. Then solution was sterilized with 0.22 μ m syringe filter.

MHA: 3.6 grams of Mueller Hinton agar medium (MHA, Mast) was added to 100 ml distilled water and then autoclaved.

MHB : 2.1 grams of Mueller Hinton broth medium (Oxoid, UK) was added to 100 ml distilled water and then autoclaved.

MTT solution (0.3%) was prepared by adding 60 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (sigma, >99.5%,USA) into 20 ml sterile 1X PBS.

PBS (10X): 80 g NaCl, 2 g KCl, 14.4 g Na₂HPO₄ and 2.4 g KH₂PO₄ were dissolved in 800 ml ultrapure water. PBS Buffer Solution's pH was adjusted to 7.4 with 1 N HCl. The solution was filled up to 1 liter with ultrapure water and sterilized by autoclave.

Phosphate buffered saline (PBS): 1X PBS was prepared by adding 10 ml of 10X PBS solutions to 90 ml of distilled water then autoclaved.

Proteinase K solution (20 mg/ml) was prepared by adding 0.120 g of proteinase K powder to 6 ml of sterile distilled water, then aliquoted in 500 µl tubes and stored at -20 °C. Ten milliliter of 1 mg/ml solution was prepared from the stock by adding 9.5 ml sterile distilled water to 500 µl proteinase K 20 mg/ml immediately before use.

SDS (20%) was prepared by dissolving 20 g of Sodium dodecyl sulfate (SDS) in 100 ml sterile distilled water.

Staining solution was prepared by adding 0.659 g of crystal violet to 2.5 ml of 99% ethanol and 10 ml of formalin plus 487.5 ml of autoclaved 1X PBS.

TE buffer was prepared by adding 10 ml of 1M Tris buffer (pH 8.0) to 2ml of 0.5M Na-EDTA (pH 8.0), then the solution was filled up to 1 liter sterile distilled water.

Triton X lysis buffer: 1 ml of 100 mM NaCl, 100 μ l of 1 mM TRIS-HCl (pH8.0), 0.5 ml of 1mM Na-EDTA (pH9.0) and 100 μ l of 1% Triton X were added to 8.8 ml sterile distilled water.

TSA: 3.7 grams of tryptic soy agar medium (Mast, UK) was added to 100 ml distilled water and then autoclaved.

TSB medium supplemented with 1% glucose was prepared by adding 2 ml of 20% sterile glucose to 38 ml of autoclaved TSB.

TSB: 3.7 grams of tryptic soy broth medium (Mast, UK) was added to 100 ml distilled water and then autoclaved.

NPs stock solution (1mg/ml): 1 mg of NPs powder was added to 1 ml MHB and sonicated for 20 minutes.