Nano-antimicrobial Materials for Biofilm Inactivation in Food Processing and Service Establishments

Thesis Submitted for

Ph. D. V. Sc.

(Hygiene of Meat, Fish and Their Products and Animal By-Products)

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2020

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List of abbreviations

CAR	**Carvacrol**
CDC	**Center for diseases and control**
EHEC	**Enterohaemorraghic *Escherichia coli***
EPEC	**Enteropathogenic *Escherichia coli***
EPS	**Extracellular polymeric substances**
ETEC	**Enterotoxigenic *Escherichia coli***
FCS	**Food contact surfaces**
FTIR	**Fourier transform infrared**
MBP	**Moderate biofilm producer**
MHB	**Muller-Hinton broth**
MIC	**Minimum inhibitory concentration**
NFCS	**Non-food contact surfaces**
NPs	**Nanoparticles**
OD	**Optical density**
ODc	**Cut-off optical density**
SBP	**Strong biofilm producer**
STEC	**Shiga toxin-producing *Escherichia coli***
SD	**Standard deviation**
TSB	**Tryptic soya broth**
TEM	**Transmission Electron Microscope**
WBP	**Weak biofilm producers**
XRD	**X-ray diffraction**

Introduction

Foodborne pathogens are the main factors behind foodborne diseases and food poisoning and thus pose a great threat to food safety. Many outbreaks can be associated with biofilms. It is well documented that biofilms have become an urgent problem in the current food industry as biofilms can renders inhabitants on such films resistant to antimicrobial agents and cleaning **(Zhao et al., 2017).**

Biofilms are aggregation of microorganism attached to and growing on a surface**.** The formation and development of biofilms is affected by many factors, including the specific bacterial strain, material surface properties, and environmental parameters such as the pH and nutrient levels as well as temperature **(Donlan, 2002)**.

Bacterial cells are normally released from biofilms, causing discontinuous secondary contamination of foods during their processing. Sessile cell populations (biofilm-associated cells) are fundamentally different from planktonic cells and display increased tolerance to biocides and disinfectants than their planktonic counterparts **(Bridier et al., 2015).**

The methods used to eliminate existing biofilms can be physical, chemical and biological. The number of usable chemicals in food and eco-food industry is limited and their use can often produce unpleasant by-products. Nowadays, customers prefer products that are minimally processed and have fewer chemicals so it has become necessary to find natural and effective cleaning and preserving products. There is a growing interest in using essential oils and their bioactive compounds as

natural preservatives and sanitizers in the food industry **(Kerekes et al, 2015**).

Essential oils and their principal components especially Carvacrol (CAR) exhibit promising antimicrobial effects against potential foodborne pathogens. Thus, CAR can interfere with the physiology of microorganisms through different mechanisms of action and may often interfere with membrane functions, production of virulence factors, and the formation of bacterial biofilms **(Marchese et al., 2018).**

Recently, the development of nanomaterials and corresponding technologies provides a novel opportunity for the development of antimicrobial agents to control microbial biofilms. These nanoparticles (NPs) have numerous useful properties including stability, lower toxicity, resistance elimination, and high specific surface area giving more active sites for NPs to interact with bacteria. At present, the main types of nanosystems used to deliver bioactive substances include polymeric NPs **(Ling et al., 2020).**

For decades, polymeric NPs have attracted much attention, especially in food industries **(Acosta, 2009),** owing to their functional characteristics. Actually, NPs have been used as an encapsulant or shell to protect bioactive compounds loaded inside (the "core") from direct contact with severe conditions, e.g., light, heat and oxygen **(Jang and Lee, 2008).**

In the recent years, there has been considerable interest in developing biodegradable NPs as effective lipophilic bioactive food components delivery systems. Chitosan is receiving a lot of interest in the

encapsulation of bioactive compounds due to its biocompatibility, low toxicity and biodegradability **(Donsi et al., 2011).**

A series of techniques have been extensively applied to load bioactive compounds into chitosan NPs, including ionotropic gelation method. Ionotropic gelation is a simple and mild method based on the complexation of positively charged polymers when coming in contact with specific polyanions to form inter and intramolecular cross-linkages **(Janes et al., 2001**).

CAR is a major component of the essential oils derived from oregano, thyme, marjoram and summer savory, and is generally recognized as a safe food additive. CAR is a volatile compound which easily evaporates and/or decomposes during food processing, drug and cosmetic formulation, antimicrobial film preparation, etc., owing to direct exposure to heat, pressure, light or oxygen **(Chalier et al., 2007).**

It has been used in several products as a flavoring agent, antimicrobial agent and/or antioxidant**.** Encapsulation of CAR is an alternative way to extend its shelf life and retain its functional properties **(Liolios et al., 2009).**

Objectives and justification

The ability of foodborne pathogens to form biofilm facilitates their persistence in food processing environment, and repeated product contamination. Suitable intervention methods must be developed to eliminate biofilm. Chemical sanitizers are routinely used, but their toxicity and potential for carry over to finished products make them

undesirable. Our objective is to develop an eco-friendly and biodegradable antibacterial nanomaterial to inactivate and inhibit the biofilm formation in the food service establishments.

Sub-objectives that will lead to achievement of the main objective:

- Isolation and identification of pathogenic *E. coli*, *Salmonella* spp. and *L. monocytogenes* from different slaughterhouses.

- Assessment of biofilm formation ability for the isolated strains by microtiter plate method.

- Synthesis and characterization of chitosan NPs and CAR loaded chitosan NPs.

- Investigation of the antimicrobial activity of carvacrol alone, chitosan NPs and CAR loaded chitosan NPs against the isolated bacterial strains and their biofilms.

Review of literature

1. The problem of biofilm in food processing environment

Food processing environments are associated with high nutrient and moisture concentrations that favor the growth of surface-associated microorganisms and their polymers, known as biofilms. Biofilm bacteria are quite resistant to control strategies and biocides, a feature that contributes to rapid biofilm re-growth. The possibility of outbreaks of foodborne illness following biofilm food cross-contamination is a distinct concern, together with the considerable costs associated with biofilm control **(Korber et al., 2009).**

Biofilms are surface-attached microbial communities with distinct properties, which have a great impact on public health and food safety. In the meat industry, biofilms remain a serious concern because many foodborne pathogens can form biofilms in areas at meat plants that are difficult to sanitize properly. Also, biofilm cells are more tolerant to sanitization than their planktonic counterparts. Furthermore, nearly all biofilms in commercial environments consist of multiple species of microorganisms, and the complex interactions within the community significantly influence the architecture, activity and sanitizer tolerance of the biofilm society **(Wang, 2019)**.

Biofilms are complex microbial ecosystems formed by one or more species immersed in an extracellular matrix of different compositions depending on the type of food manufacturing environment and the colonizing species. The presence of more than one bacterial species in a biofilm has important ecological advantages because it can facilitate the biofilm's attachment to a surface. For some species, this can even occur in the absence of specialized fimbriae. Mixed biofilms show higher

resistance to disinfectants such as quaternary ammonium compounds and other biocides **(Meyer, 2015).**

Biofilms are mainly composed of 90% matrix and 10% microorganisms. However, 97% of the matrix is water, which is capable of absorbing nutrients, metabolites and cell lysis products. The remaining 3% of the extra polymeric substances contains proteins, polysaccharides, DNA, RNA, peptidoglycan, lipids and phospholipids **(Flemming and Wingender, 2010).**

Microorganisms have natural affinity to surfaces. When attached, cells are still readily removed by mild rinsing, but when left to proliferate they can also adsorb irreversibly and form layers of bacteria and extra-cellular products called biofilms, are more difficult to remove from a surface **(Jefferson, 2004).**

Bacteria residing in the biofilm are approximately 10 to 1000 times less susceptible to antimicrobial agents than planktonic bacteria, because extracellular polymeric substances of the biofilm act as a barrier to prevent contact with antimicrobial agents **(Penesyan et al., 2015).**

Biofilm formation of foodborne microorganisms is considered a serious problem for public health and food industries. The presence of these undesirable biofilms in food processing or storage environments may lead to food spoilage, as well as foodborne diseases **(Al-Shabib et al., 2017).**

Biofilm formed in food processing environments is of special importance as it has the potential to act as the chronic source of microbial contamination that may lead to food spoilage or transmission of diseases. Bacteria in biofilms exhibit enhanced resistance to cleaning and sanitation **(Joseph et al. 2001).**

Many foodborne pathogens, such as *E. coli* O157:H7, *Salmonella enterica*, and *L. monocytogenes* are able to develop biofilms in many areas of food processing plants, including floors, walls and pipes, etc. **(Sofos and Georgnaras. 2010).**

Food contact surfaces (FCS) and processing equipment made of various materials, including stainless steel, rubber, plastic, and Teflon, etc., may be subject to biofilm formation. In particular, areas such as floor drains and the backside of conveyor belts, as well as other contact and non-contact surfaces in the processing environment, are hot spots that attract biofilm development due to poor accessibility and the resultant difficulty for regular hygiene maintenance **(Wang, 2019).**

Biofilm-forming ability is known to be mostly strain dependent and closely related to bacterial strain properties of cell surface structures. Among those, the importance of extracellular polymeric substances (EPS), such as curli fimbriae, cellulose, capsular polysaccharide, lipopolysaccharide, and outer membrane proteins, for biofilm formation has been well appreciated. The EPS structures also are associated with bacterial capability to compete and establish themselves during mixed biofilm development **(Wang et al., 2013a)**.

1.1. Biofilm formation

The formation of biofilms and their properties are affected by several factors: 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" and the hydrodynamic of the fluid "velocity and turbulence" **(Melo, 2003).**

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

In food processing environments, stainless steel equipment surfaces and utensils are common sites of bacterial adhesion and biofilm formation **(Uchida et al., 2015).**

Four stages are distinguished in biofilm production: Attachment and colonization by primary reversible adhesion between microbial cell surfaces and desired substratum (Stage1), irreversible attachment (Stage 2), biofilm architecture formation and maturation (Stage 3), detachment and dispersal of biofilm cells (Stage 4**) (Abed et al., 2012)**.

Biofilms mature resulting in a complex architecture through the secretion of EPS. This requires quorum sensing (QS), 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 **(Clutterbuck, et al., 2007)**.

The maturation of biofilms occurs in two stages. During the first stage the thickness of the biofilm is >10μm and there is a profound difference in protein expression compared to planktonic cells. In the second stage the thickness reaches up 100μm 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)**.

During the last stage (stage 4) of biofilm production which is the 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)**.

1.2. Biofilms in food industry and the associated health aspects

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)**.

Biofilm may be formed 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 *L. monocytogenes*, *Salmonella, Campylobacter, E. coli* O157:H7*, Pseudomonas* and lactic acid producing bacteria. 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 Georgnaras, 2010).**

Cross-contamination from surfaces plays a vital role in the transmission of bacterial foodborne diseases, as bacterial cells can adhere to surfaces, colonize, and subsequently form biofilms **(Srey et al., 2013).**

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).**

Food-borne diseases associated with bacterial biofilms may arise via intoxications or infections. Toxins, for example, can be secreted by biofilm found within food processing environment. From there, they can contaminate a food matrix, causing individual or multiple (in the case of an outbreak) intoxications. The main locations for biofilm development may include water, and other liquid pipelines, tables, employee gloves, animal carcasses, contact surfaces, storage silos for raw materials and additives, dispensing tubing, packing material, etc. **(Camargo et al., 2017).**

The presence of biofilms is a relevant risk factor in the food industry due to the potential contamination of food products with pathogenic and spoilage microorganisms. Biofilms can be formed on surfaces becoming permanent reservoirs of bacteria. Most important, biofilms may act as reservoirs of pathogenic and spoilage bacteria, in which these microorganisms can persist against the cleaning and disinfection processes. For example, contamination of equipment with biofilms was a contributing factor to 59% of food-borne disease outbreaks investigated in France **(Midelet and Carpentier, 2004).**

The presence of biofilms is common in food industry and represents a concern because bacteria can adhere to almost any type of surface, such

as plastic, metal, glass, soil particles, wood food products **(Gandhi and Chikindas, 2007).**

Many factors could potentially affect the transfer efficiency of biofilm cells to food products. For instance, bacterial surface EPS structures, such as curli, flagella, fibrillae, capsular polysaccharides, lipopolysaccharides, and outer membrane proteins, etc., could assist cell adhesion and affect the degree of attachment strength between bacteria and the contact surface. Other specific properties of the biofilms, such as biofilm cell density, the 3D structure, and the coexistence of other bacterial species in the mixed biofilm community, also would affect bacterial transfer efficiency (**Pérez-Rodríguez et al., 2008)**.

1.3.The role of *E. coli, Salmonella* spp. and *L. monocytogenes* in biofilm formation

A wide variety of foodborne pathogens are able to attach, colonize, and form biofilms, such as the O157 and non- O157 Shiga toxin–producing *E. coli* (STEC), *S. enterica*, and *L. monocytogenes*, etc. In contrast to the single-strain biofilm studies often performed in laboratories, the microbial society in commercial environments is heavily biased toward multispecies communities. Numerous studies have shown the coexistence of STEC O157 and non-O157 serotypes as well as *S. enterica* on veal hides and carcass samples in commercial meat plants **(Bosilevac et al., 2017).**

Escherichia coli, *Salmonella* spp. and *L. monocytogenes* are indicated as the major biofilm-forming pathogens in the food industry (**Sofos and Georgnaras, 2010).**

1.3.1. *Escherichia coli*

Shiga toxin–producing *E. coli* (STEC) are a leading cause of foodborne illnesses worldwide, with beef and beef products as a common food reservoir. STEC strains may be present in beef-processing environments in the form of biofilms **(Ma et al., 2019).**

Shiga toxin–producing *E. coli* are important foodborne pathogens associated with large outbreaks, hemolytic uremic syndrome, kidney failure, or even death **(Peco-Antic, 2016)**.

Contamination of beef with STEC may occur during slaughter, dressing, chilling, or cutting. Consequently, STEC populations may be distributed on surfaces of equipment used to produce meat during slaughter and fabrication, contaminating carcasses and fresh meat products **(Toro et al., 2018).**

Enterohemorrhagic *E. coli* (EHEC) strains are foodborne pathogens responsible for outbreaks of diarrhea and hemolytic uremic syndrome, which can lead to death **(Atnafie et al., 2017).**

Although STEC O157 is the most widely recognized, other serogroups; O26, O45, O103, O111, O121 and O145 have been increasingly implicated in cases of foodborne human diseases **(Bettelheim, 2007).**

A variety of *E. coli* pathotypes can cause enteric infections spreading to the susceptible host via food or water. The regular presence of these strains makes them markers of fecal contamination and indicators of poor hygiene and sanitation conditions **(Newell, et al., 2010).**

Escherichia coli O157:H7 causes diseases range from watery diarrhea to hemorrhagic colitis and life-threatening hemolytic uremic syndrome, with much higher hospitalization and fatality rates compared to other enteric pathogens such as *Salmonella*. Also, it is able to form biofilm on various surfaces, which makes their elimination from food processing facilities impossible **(Kim et al., 2015).**

Escherichia coli strains can attach to a variety of surfaces including stainless steel, Teflon, glass, polystyrene, polypropilene, PVC and biotic surfaces. The hydrophobicity of the surface material plays an important role in biofilm formation by this species **(Van Houdt and Michiels, 2010).**

Escherichia coli survival under stress conditions and its biofilm formation abilities are serotype-dependent. For example, the serotype O157:H7 (a common STEC strain) displayed a high resistance to temperature, high pressure and common food industry disinfectants when compared to other pathogenic and non-pathogenic *E. coli* strains, such as O111 and O26 **(Chagnot et al., 2014).**

Various *E. coli* serogroups, including O157:H7, have the ability to form biofilms which are defined as microorganism aggregates that attach

to a specific surface and are enclosed by extracellular matrix components. The biofilm acts as a barrier to assist cells in the resistance against antimicrobials and sterilizing agents, making organisms difficult to eradicate and control. The presence of a biofilm makes it one of the major sources of cross contamination during processing, distribution, and consumption **(Corzo-Ariyama et al., 2019).**

In reality, it is more likely that one particular type of pathogen would form mixed biofilms with commensal or spoilage bacteria that are more commonly present in the environment, a severe outbreak by multiple foodborne pathogens, such as virulent STEC serotypes O157:H7 and O111:H8, causing serious public health consequences has been reported **(Watahiki et al., 2014)**.

Among EHEC, *E. coli* O26 is one of the most common non-O157 serogroups associated with serious foodborne outbreaks worldwide with a number of food outbreaks linked to consumption of beef products. Also, it has been shown that *E. coli* O26 have the capacity to form biofilms **(Nesse et al., 2014).**

Thirty five isolates of *E. coli* were assessed for biofilm formation and it was observed that only 1 strain detected as a moderate biofilm producer**,** 32 strains detected as weak biofilm producers and 2 negative biofilm producers strains **(Bakhtiari and Javadmakoei, 2017).**

1.3.2. *Salmonella* spp.

Salmonellosis is one of the most common foodborne diseases and millions of human cases are reported worldwide every year, resulting in thousands of deaths. In most countries, foods containing meat are the leading causes of human enteritis outbreaks involving *Salmonella* **(WHO, 2017).**

According to CDC, about 1.2 million salmonellosis cases are reported annually in USA resulting in 378 deaths with an estimated economic losses exceeding $4.4 billion. Among different *Salmonella* serovars, *S. enterica* serovar Enteritidis and Typhimurium are the two most frequently reported serovars implicated in foodborne outbreaks worldwide **(Abdalhaseib et al., 2016).**

persistence of Salmonella in food processing environments, after conventional cleaning and disinfection, may be related to acquired disinfection resistance through biofilm formation. Thus, *Salmonella* spp. found in their planktonic phase are usually susceptible to inactivation by using disinfectants or antibiotics, but in biofilms these are much more resistant **(Corcoran et al., 2014).**

Salmonella spp. including *S.* Typhimurium, are able to form a biofilm on plant and meat products as well as various abiotic surfaces of food processing facilities, such as glasses, plastics, and metals **(Brandl, 2006).**

The persistence of Salmonella in food processing environments and food matrices is associated with the ability of Salmonella to form and reside within single or multispecies biofilms Since the biofilm is resistant

to chemical, physical, and mechanical stresses, biofilm formation is important to the spread of Salmonella **(Soni, et al., 2013).**

Biofilm formation by *Salmonella* on FCS has been recognized as a contributing factor for foodborne outbreaks. Surfaces employed in food processing wear out after repeated use and are more likely to accumulate debris and bacteria (**Rodrigues et al., 2013).**

Salmonella Typhimurium is a major serotype responsible for distressing public health concern worldwide and is able to form biofilm on produced foods and on surfaces of instruments used in food processing facilities, such as stainless steel, plastic, polystyrene, and glass **(Eng et al., 2015).**

Common sites for the presence of *Salmonella* spp. in food-processing plants are filling or packaging equipments, floor drains, walls, cooling pipes, conveyors, collators for assembling product for packaging, racks for transporting products, hand tools or gloves, freezers, etc, which are usually made of plastics **(Pompermayer and Gaylarde, 2000).**

Salmonella enterica can survive in a biofilm on stainless steel for over a year under dry conditions. From there, it is possible for this bacterium to contaminate thousands of food batches **(Morita et al., 2011).**

Salmonella enterica is capable of attaching to meat and other food matrixes easily, eventually leading to cross-contamination between food batches in a manufacturing plant or supermarket, a fact that further underscores the serious health concern this bacterium poses with respect to outbreaks risk, for example associated to refrigerated poultry products in shelves during food processing or sale in a supermarket (**Wang et al., 2013a**).

The main source of contamination by *S. enterica* is biofilm formation in infrastructures used during pre-cooked foods manufacturing (such as pre-cooked chicken), a process that has given rise to outbreaks affecting thousands of people **(Wang et al., 2013b).**

The persistence of *Salmonella* in food processing environments, after conventional cleaning and disinfection, may be related to inadequate sanitation processes, but also to acquired disinfection resistance through biofilm formation **(Speranza et al., 2016).**

Many foodborne outbreaks have been linked to surface colonization by biofilm-forming pathogens, such as *Salmonella*, troubling seriously the international food industry and public health authorities **(EFSA, 2018).**

Most of the *Salmonella* spp. isolates recovered from food and environmental sources (85%) produced biofilm on polystyrene surfaces as assessed by microtiter plate assay. About 67.5% isolates were weak biofilm producers and 17.5% were moderate biofilm producers Whereas, 15% of isolates were categorized as non biofilm producers **(Nair et al., 2015).**

1.3.3. *Listeria monocytogenes*

Listeriosis is an important food-borne disease responsible for high rates of morbidity and mortality. *L. monocytogenes* has been the cause of several food borne outbreaks and its ability to adapt and survive in a wide range of environmental conditions makes eradication difficult **(Stratakos et al., 2020).**

Listeria monocytogenes associated illness is not as common as that of other food borne pathogens like Salmonella or *E. coli*, its mortality rate can be considered the highest. Approximately, 30 % of invasive listeriosis cases lead to mortalities with most requiring hospitalization, and therefore demanding *L. monocytogenes* can be considered as a food borne pathogen of public health importance **(Véghová et al., 2016).**

Persistence of *L. monocytogenes* in food processing environment has been considered the single most important factor in product contamination, which is facilitated by biofilm formation **(Ferreira et al., 2014).**

High mortality and hospitalization rates have seen *L. monocytogenes* as a foodborne pathogen of public health importance for many years and of particular concern for high-risk population groups. Also, the capacity of *L. monocytogenes* strains to colonize food production environments (FPEs) can lead to repeated identification of *L. monocytogenes* in FPE surveillance. Poor equipment design, facility layout, and worn or damaged equipment can result in Listeria hotspots and biofilms where traditional cleaning and disinfecting procedures may be inadequate **(Gray et al., 2018).**

In response to adverse conditions, *L. monocytogenes* has the ability to form biofilms. In addition, food-processing surfaces are one of the primary sources of cross-contamination and re-contamination of this microorganism. These characteristics mean that the control of *Listeria* spp. in food-processing environments requires strategies, such as prevention of *L. monocytogenes* establishment and growth **(Allen et al., 2016).**

Listeria monocytogenes poses a food safety risk due to its ubiquitous nature as it is frequently introduced into the processing environment through raw ingredients. *L. monocytogenes* can adhere to a variety of abiotic surfaces with some strains persisting for numerous years and acting as a source of continuous cross contamination **(Colagiorgi et al., 2017).**

Listeria monocytogenes biofilms are mainly composed of teichoic acids and can grow on polypropylene, steel, rubber or glass surfaces throughout the industry. From there, this pathogen spreads to food batches, where it can replicate at refrigeration temperatures **(Silva et al., 2008).**

Some conditions in food industry can promote the bacterial attachment and subsequent biofilm development, such as flowing water, raw materials, organic load or suitable surfaces. In particular, the surfaces of equipment used for food handling, storage, or processing are recognized as the major source of microbial contamination because bacteria have the ability to attach to different types of materials, such as polystyrene, hydroxyapatite, glass, rubber, and stainless steel **(Bae et al., 2012).**

Biofilm cell density was found to have the greatest influence on bacterial transfer efficiency. Studies investigating *L. monocytogenes* biofilm transfer showed that the strong biofilm-forming strains could transfer a significantly higher number of bacteria to beef food products **(Midelet and Carpentier, 2002).**

The microtiter plate biofilm assay was used to quantify biofilm production by *L. monocytogenes* strains,127 of 138 strains (92.0%) were classified as weak, 9 of 138 strains (6.5%) as moderate and only 2 of 138 strains (1.5%) as strong biofilm formers **(Harveya et al., 2007).**

Biofilm forming ability assessment of *L. monocytogenes* strains isolated from food contact surfaces and food products revealed degrees of biofilm-forming ability based on their OD values: 32% were weak, 47% were moderate and 21% were strong biofilm producers **(Henriques and Fraqueza, 2017).**

2. Biofilm investigation assays

The study of microbial biofilms has received significant attention over the past decades. Biofilm is defined as an assemblage of microbial cells that is associated with a surface and enclosed in an extracellular matrix principally of polysaccharide material **(Donlan, 2002).**

A number of methods have been developed for cultivation and quantification of biofilms such as tube test, microtiter plate test, radiolabeling, microscopy, Congo red agar plate test, etc but no standardized protocol for assessment of biofilm formation by different bacterial species has been established so far **(Deighton et al., 2001).**

Quantification of biofilms started with a method based on the cultivation of biofilm on the wall of a test tube and subsequent detection by stain for biofilm recognition. Later, the wells of microtiter plate were used as culture vessel, and the results were measured spectrophotometrically (**Christensen et al., 1985)**.

The 96-well microtiter plate test has been the most frequently used assay for high throughput quantitative evaluation of biofilm-forming ability by bacteria. Over the years, modifications have been made to improve its accuracy. It is generally performed under static conditions using different media, such as Mueller Hinton Broth or Tryptic Soy Broth and enables quantitative biofilm determination through the application of different dyes such as crystal violet, resazurin, or dimethyl methylene blue **(Pettit et al., 2005)**.

The microtiter plate method remains among the most frequently used assays for investigation of biofilm, and a number of modifications have been developed for the in vitro cultivation and quantification of bacterial biofilms (**Stepanovic et al., 2007**).

Crystal violet is a basic dye that binds to negatively charged molecules present on both the surface of bacteria and the extracellular matrix of biofilm. It is used for quantification of biofilm biomass, as it stains both viable and non-viable cells and is easily measured by absorbance at 550 nm **(Peeters et al., 2008).**

The microtiter plate biofilm formation assay is a method for the study of early biofilm formation on abiotic surfaces. It is a colorimetric technique that uses dyes, such as crystal violet, to stain attached biofilms and to quantify by using an absorbance microtiter plate reader **(De-Jesus and Dedeles, 2019).**

3. Controlling the biofilm problem

Sanitizer tolerance presents a serious public health risk highlights the importance of properly sanitizing food processing equipment and contact surfaces to control and inactivate biofilms. The current sanitization methods mostly rely on the application of chemical disinfectants; however, studies have shown that many common and traditional sanitizers were not able to completely eradicate mature biofilms on food contact surfaces. In particular, those treatments that use individual sanitizer products exhibit limited effectiveness on biofilms, even with prolonged exposure time (**Corcoran et al., 2014**).

Despite the significant problems caused by biofilms of foodborne pathogens, effective control of biofilms is still challenging. Since ideal techniques that are able to successfully prevent or control undesirable biofilms without adverse side effects are not known, new control strategies for microbial contamination and biofilm establishment have been constantly recommended **(Simoes et al., 2010).**

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 **(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 **(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, enzymes; energy uncoupling, cell wall hydrolysis and the application of bacteriophages **(Malaeb et al., 2013).**

Control processes for biofilms may include frequent cleaning and disinfection of food contact surfaces several times per day, while cleaning and disinfection of non-food contact surfaces may occur only once per day. Further, equipment design must minimize the presence of hard to clean crevices, gaskets, surfaces, and dead ends. Cleaning in place procedures of food equipment usually involves a pre-rinse step to thoroughly remove food residues, circulation of a detergent, rinsing to remove detergent, disinfection, and final rinse to remove disinfectant **(Simões et al., 2010).**

Nowadays, there is an increasing effort to combat antimicrobial resistance and develop safe, eco-friendly and efficient anti-biofilm strategies and therapeutic approaches **(Giaouris and Simões, 2018).**

The consumer trend for 'greener' and chemical free approaches, puts pressure on the food industry to develop novel, more efficient, sustainable, and low cost anti-microbial methods to deal effectively with emerging hazards. Nanotechnology is a promising technology to be utilized for food safety and quality and identified as one of the key-enabling technologies impacting the food industry **(Peters et al., 2016).**

Nanotechnology is an emerging area of technology dealing with production, processing, and application of materials with size less than 100 nm **(Bagchi et al., 2013)**.

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).**

Nano-materials are also categorized based on their major constituents, organic and inorganic, into 3 classes: organic polymer (e.g. emulsions and liposomes), inorganic metallic (e.g. metals, metals oxides and magnetic materials) and semiconductor (e.g. quantum dots) **(Luo and Stutzenberger, 2008).**

Nano-materials are synthesized through two main techniques, i.e. the top down and bottom-up methods. 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 mono dispersity **(Ju-Nam and Lead, 2008).**

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).**

Various physical and chemical techniques, such as separation, spectrometric and microscopy techniques, have been employed to characterize the NPs' composition, morphology, coating and size **(Capaldi 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).**

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).**

The small size, in combination with the chemical composition and surface structure gives NPs their unique features and huge potential for applications, 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).**

Application of nanotechnology 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).**

The main purposes of applying nanotechnology 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**).

Chitosan is an inexpensive, nontoxic polycationic natural biopolymer industrially produced by alkaline (40-50% NaOH) deacetylation of chitin from shrimp and crab shell **(Rabea et al., 2003).**

Chitosan is a polysaccharide derived via deacetylation from chitin. Chitin is naturally occurring and abundantly available as it is commonly found in the structural components of many invertebrates and in the cell walls of most fungi and some algae **(Wang et al., 2004).**

Chitosan is considered to be an incredibly versatile polymer due to its chemical, physical and functional characteristics. These advantageous properties include it's; cationic nature, biodegradability, good adsorption capacity, biocompatibility, permeability-enhancing effect, film-forming

capabilities, adhesive characteristics and many more, whilst being considered safe and cost-effective (**Fan et al., 2012).**

Chitosan possesses a wide spectrum of inhibition against bacterial and fungal species, with antimicrobial activity being heavily dependent on molecular weight **(Kong et al., 2008).**

Antimicrobial function of chitosan is known against wide variety of Gram-positive, Gram-negative bacteria, mould and yeast. The polycationic property of chitosan exhibits antimicrobial activity due to its ability to interact with negatively charged cell membranes of these microbes **(Goy et al., 2009)**.

Chitosan NPs synthesized by ionotropic gelation method with tripolyphosphate showed a great antibacterial effect in the minimum inhibitory concentration. In addition, these NPs exhibited biofilm inhibition and were able to eradicate the pre-existing biofilm **(Aguayo et al., 2020).**

Essential oils are natural food antimicrobials, and the use of these substances or their individual constituents as antimicrobial preservatives in foods and sanitizers on FCS has been suggested **(oliveira et al., 2010)**.

Several strategies for controlling bacterial adhesion to surfaces have been proposed, including the use of natural compounds. In this sense, essential oils and their main constituents have shown potential to inhibit bacterial cells in planktonic and sessile state (biofilms). CAR, a hydrophobic terpene component of oregano essential oil, has been

proposed as a potential inhibitor of biofilm formation and other virulence factors of many bacteria **(Tapia-Rodriguez et al., 2017).**

The adhesion of microorganisms to food contact surfaces is an important concern in food industries because the attachment can promote not only cell survival but also biofilm formation, that can lead to cross contamination, reduced product shelf life and foodborne diseases **(Bridier et al., 2015).**

New approaches to sanitize surfaces in the food industry are currently being studied to prevent and treat microbial contamination by biofilm formation inhibition. Among the alternatives, essential oils and their bioactive components are naturally occurring antimicrobial compounds that can be used as natural antimicrobial agents to prevent the limitations of conventional disinfectants, such as low effectiveness and safety issues **(Giaouris et al., 2014).**

Essential oils are composed by complex mixtures of low molecular weight molecules, whose major typical components depend on the plant source. Among these, CAR, a monoterpene phenol (2-meth-yl-5-(1-methylethyl) phenol) present in the volatile oils of *Thymus vulgaris*, *Carum copticum* and *Oreganum* species **(Nabavi et al., 2015).**

CAR is a "generally recognized as safe" food additive, possesses antimicrobial properties and is approved by the U.S. Food and Drug Administration for use in foods and drinks, considering it to be without significant toxic effects in the amounts commonly used **(Marinelli et al., 2018).**

CAR displays abroad spectrum antimicrobial activity toward food spoilage organisms and foodborne pathogens such as *Bacillus cereus, Staphylococcus aureus, S. epidermidis, E. coli, Pseudomonas aeruginosa*, *Salmonella* spp., and *L. monocytogenes* **(Miladi et al., 2016)**.

The antimicrobial activity of CAR has a wide spectrum extended to pathogenic bacteria, fungi and yeast including drug-resistant and biofilm forming microorganisms **(Raei et al., 2017).**

CAR is effective against different microbial biofilms developed on stainless steel and, more importantly, it is able to drastically reduce the possible re-growth of injured cells from the treated biofilms. These findings support the possibility to use natural compounds to formulate new sanitizers able to reduce bacterial biofilm on food contact surfaces. Considering that biofilms produced in food processing environments are presumably multi-species and influenced by several environmental parameters, such as food residues, low temperature or acid condition **(Campana and Baffone, 2018)**.

Recently, the antibiofilm activity of some essential oils and their components has been described against certain pathogenic and spoilage microorganisms. Besides, the combination of conventional and alternative methodologies as well as the combination of novel processes are approaches that arise the interest the food industry needs. Recent examples of this strategy are the combined use of CAR nano emulsion with other compounds (**Dos Santos Rodrigues et al., 2017)**.

CAR exhibited antibiofilm activity against *S.* Typhimurium and *S.* Enteritidis. It was also observed a reduction of established *S.* Typhimurium biofilms about 5 logs with 156 µl/mL (MIC) and 312 µl/mL (2 x MIC) of carvacrol treatment **(Amaral et al., 2015).**

CAR was able to inhibit at sub-lethal concentrations (<0.5 mM), the formation of biofilms by *S.* Typhimurium. In contrast, carvacrol had (up to 8 mM) very little or no activity against existing biofilms, showing that biofilm formation may also confer protection against this compound **(Burt et al., 2014).**

The effect of CAR against *Salmonella* spp. Was evaluated and the MICs of 187.5 and 375 µg mL-1 and an MBC value of 750 µg mL-1 were identified **(Du et al., 2015**).

In the 24-well polystyrene plate assay, 0.1% CAR concentration was enough to inactivate 1-day-old biofilm cells of *L. monocytogenes* completely, whereas concentrations of 0.25 to 0.5% were necessary to completely inactivate 4 day old biofilm cells on stainless steel coupons **(Desai et al., 2012).**

Materials and Methods

Part I

Isolation and identification of pathogenic *E. coli, Salmonella* spp. and *L. monocytogenes* from slaughterhouses

Collection of samples

A total of 180 swabs were collected from slaughterhouses in Assiut Governorate**.** The collected swabs included 90 swabs from non-food contact surfaces (NFCS) represented by walls and floors (45 of each) and 90 swabs from food contact surfaces (FCS) represented by knives, hooks and cutting boards (30 of each).

Swabbing procedure and preparation of samples:

Sampling was done using sterile cotton tipped swabs with wooden shaft. Just before sampling, the swab was moistened with sterile peptone water then rubbed over 10 cm^2 surface area to be sampled. The swabs were transferred directly to the laboratory in an ice box with a minimum of delay where each tube containing the swab was vortexed for 10 seconds to assure mixture of the sample and prepared for the bacteriological examination **(Bodur and Cagri-Mehmetoglu, 2012).**

A- Isolation techniques

1- Isolation and identification of pathogenic *E. coli*:

1.1. Isolation of pathogenic *E. coli*:

One ml from the well mixed sample was inoculated in tryptic soya broth and incubated at 37°C for 24 hours. Loopful from inoculated broth

were cultured onto MacConkey agar plates **(Murray et al., 2003).** Suspected isolates of *E. coli* were identified according to **(MacFaddin, 2000)**.

1.2. Identification of pathogenic *E. coli*

1.2.1. Microscopical examination (Cruickshank et al., 1975):

Films of pure suspected cultures were stained with Gram's stain and examined microscopically. Gram negative, medium size, stained evenly coccobacilli were suspected to be *E. coli*.

1.2.2. Motility test:

Motility medium was inoculated by the stabbing technique to a depth of 5 mm and then incubated at 37°C for 24 hours. A circular growth from the line of stabbing represented a positive test.

1.2.3. Biochemical identification (Kreig and Holt, 1984):

1.2.3.1. Indole test:

To 48 hours culture incubated at 37°C in 1% peptone water, 1 ml of ethyl ether was added. The tubes were vigorously shaken and allowed to stand until ether rises to the surface. To each tube 0.5 ml of the Kovac's reagent was trickled down the side of the tube. The formation of a red ring (surface layer) after 10 minutes was considered a positive reaction.

1.2.3.2. Methyl Red Test:

Five ml buffered glucose broth tube were inoculated with pure culture and incubated at 37°C for 24 hours. To each tube, 5 drops of Methyl Red reagent were added. The development of a red color was considered a positive test.

1.2.3.3. Voges – Praskauer test:

From 48 hours culture incubated at 37°C in 5 ml buffered glucose phosphate broth, 1 ml was taken in a test tube and 0.6 ml of alcoholic solution of alpha–naphthol and 0.2 ml of 4% potassium hydroxide solution were added. The tubes were stand for 24 hours. Pink coloration of the mixture was recorded as a positive test.

1.2.3.4. Citrate utilization test:

Slants and butts of Simmon citrate agar tubes were stabbed from pure cultures and incubated at 37°C for 48 hours. The development of blue coloration indicated utilization of citrate.

1.2.3.5. Urease test:

Christensen medium was inoculated with suspected isolates and incubated at 37°C for 24 hours. Development of pink colour denoted a hydrolysis of urea. Negative tubes were re-examined after further incubation for 24 hours.

1.2.3.6. Hydrogen sulphide production test:

On Triple Sugar Iron (TSI) agar, isolated organisms were stabbed into the bottom of the butt with a needle, and then it was drawn over the slant, for production of a sufficient surface growth. The inoculated tubes were incubated at 37°C for 24 hours. Hydrogen sulphide production was noted by blacking the medium.

1.2.3.7. Gelatin hydrolysis test:

Nutrient gelatin stab cultures were grown at room temperature and observed daily after cooling to about 18°C.

1.2.3.8. Nitrate reduction test:

Culture to be examined was inoculated into 5 ml of peptone broth containing 0.1% potassium nitrate, and incubated at 37°C for 4 days, then 1 ml of solution containing 8 grams sulphanilic acid in 100 ml of 5 N acetic acid was added and mixed, then a solution containing 5 grams of alpha –naphthylamine in 100 ml of 5 N acetic acid was also added drop by drop. A positive test was indicated by development of a red color.

1.2.3.9. Detection of Ornithine decarboxylase (ODC):

Suspected colonies were inoculated into ornithine decarboxylase medium just below the surface. One ml of sterile mineral oil was added to the top of the medium and incubated at 37°C for 24 hours. Turbidity and violet color after incubation indicate a positive ODC.

1.2.3.10. Detection of L- lysine decarboxylase (LDC):

Suspected colonies were inoculated into L-lysine decarboxylase medium just below the surface. One ml of sterile mineral oil was added to the top of the medium and incubated at 37°C for 24 hours. Turbidity and violet color after incubation indicate a positive LDC.

1.2.3.11. Detection of Arginine decarboxylase (ADC):

Suspected colonies were inoculated into arginine decarboxylase medium just below the surface. One ml of sterile mineral oil was added to the top of the medium and incubated at 37°C for 24 hours. Turbidity and violet color after incubation indicate a positive ADC.

1.2.3.12. Detection of β- galactosidase (ONPG):

The suspect colonies were inoculated into a sterile tube containing 2.5% NaCl solution and mixed. A drop of toluene was added and the tube was shaken. The tube was placed in the water bath adjusted at 37°C and left to stand for approximately 5 min. About 0.25 ml of the reagent was added for detection of β- galactosidase (2- ortho-Nitrophenyl-β-D-galactopyranoside) and mixed. The tube was replaced in the water bath set at 37°C and left to stand for 24 hours then examined from time to time. A yellow color indicates positive β- galactosidase.

1.2.3.13. Fermentation of sugars:

To 5 ml tubes of 1% peptone water containing 0.2% bromocresol purple indicator, inverted Durham's tubes and 1% of the following sugars were added (lactose, glucose, sucrose, dulcitol, salicin, arabinose, inositol and xylose). After incubation at 37°C, the reaction of the inoculated tubes was noticed every day for 7 successive days. Appearance of pink color indicates positive result.

1.2.4. Serological identification of pathogenic *E. coli*:

The isolates were serologically identified according to (**Kok et al., 1996)** by using rapid diagnostic *E.coli* antisera sets **(**DENKA SEIKEN Co., Japan) for diagnosis of the Enteropathogenic types according to the manufacture directions.

The diagnostic *E. coli* antisera sets used for identification include the following sets:

Set 1 : O- antisera:

Polyvalent antisera 1: O1, O4, O26, O86a, O111, O119, O127a and O128.

Polyvalent antisera 2: O44, O55, O113, O125, O126, O146 and O166.

Polyvalent antisera 3: O18, O114, O142, O151, O157 and O158.

Polyvalent antisera 4: O2, O6, O7, O27, O78, O148, O159 and O168.

Polyvalent antisera 5: O20, O25, O63, O91, O153, O163 and O167.

Polyvalent antisera 6: O8, O15, O17, O115, O169 and O171.

Polyvalent antisera 7: O28ac, O112ac, O124, O136 and O144.

Polyvalent antisera 8: O29, O121, O143, O152 and O164.

Set 2 : H- sera.

H2, H4, H6, H7, H11, H18 and H21.

2- Isolation and identification of *Salmonella* spp. (ISO-6579: 2002)

2.1. Isolation

Swabs were pre-enriched in Buffered Peptone Water then transferred to sterile flask and incubated at 37°C for 18 hours. The pre-enrichment culture was then sub cultured onto Rappaport-Vasiliadis Soy (RVS) broth (Biolife, CA3501) and incubated at 41.5 °C for 24 hours.

A loopful from incubated RVS tubes was inoculated onto Xylose Lysine Deoxycholate (XLD) Agar plates (Biolife, CB55052) and incubated at 37^{o}C for 24 hours. Representative number of colonies showing typical or atypical (in absence of typical colonies) Salmonella were subcultured on Nutrient Agar slant and incubated at 37^{o}C for 24 hours. The isolates were maintained at 4 ^{o}C for further confirmation and identification. Suspected isolates of Salmonella organisms were identified according to **MacFaddin (2000)**.

2.2- Identification

2.2.1. Microscopical examination (Cruickshank et al., 1975):

As previously mentioned.

2.2.2. Motility test:

As previously mentioned.

2.2.3. Biochemical identification (Kreig and Holt, 1984):

The reactions of suspected salmonella isolates on Triple sugar iron (TSI), Lysine Iron Agar, Urease test, Indole production medium, Methyl Red test, Voges-Proskauer test, Citrate utilization test were carried out as previously mentioned

2.2.4. Serological identification of *Salmonella* spp.:

Serological identification of Salmonellae was carried out according to Kauffman – White scheme **(Kauffman, 1974)** for the determination of Somatic (O) and flagellar (H) antigens using Salmonella antiserum (DENKA SEIKEN Co., Japan) according manufacture directions .

3. Isolation and identification of *Listeria* spp.:

3.1. Isolation (Hitchins, 1990):

One ml from the prepared sample was inoculated in Listeria enrichment broth (LEB, (Oxoid, CM0862) mixed by swirling and incubated at 30°C for 48 hours. A loopful from the LEB culture was streaked onto Oxford agar (Biolife, 401600) plates. Then the plates were incubated at 37°C for 48 hours. Listeria like colonies (about 1 mm diameter black colonies surrounded by black haloes) were picked

and streaked onto Tryptose soya agar plus 0.6% Yeast extract which were incubated at 37°C for 24 hours for further identification.

3.2. Identification

3.2.1. Microscopical examination (Cruickshank et al., 1975):

Films were prepared from pure cultures of the isolated microorganism stained with Gram's stain and examined microscopically. Listeria organisms appeared as Gram positive rods.

3.2.2. Motility test:

Motility medium was inoculated by the stabbing technique to a depth of 5 mm and then incubated at 37°C for 24 hours. A circular growth from the line of stabbing (Umbrella –like) represented a positive test.

3.2.3. Biochemical identification (MacFaddin, 2000):

3.2.3.1. Detection of haemolysis:

A loopful from inoculated brain heart infusion (BHI) broth were streaked on the surface of sheep blood agar plates and incubated at 37°C for 24 hours for detection of haemolysis. *L. monocytogenes* gives β-hemolysis.

3.2.3.2. Catalase activity:

The purified suspected colonies were picked up with a sterile loop and transferred into the surface of glass slide. Accurately, one or two drops of hydrogen peroxide solution (3%) were added. The rapid appearance of gas bubbles was considered a positive reaction. *L. monocytogenes* gives positive result.

3.2.3.3. Oxidase test:

Oxidase test was done by streaking of the pure culture onto filter paper moistened with oxidase reagent. The test is positive if the color turns to mauve, violet or deep purple within 10 seconds. *L. monocytogenes* gives positive result.

3.2.3.4. Bile esculin test:

A loopful from isolated organism was inoculated into test tube containing Bile esculin agar slant which incubated at 37°C for 24 hours. If an organism can hydrolyze esculin, the media will turn dark brown or black. However, the test is interpreted as a positive result only if more than half the medium is dark brown or black after incubation. *L. monocytogenes* gives positive result.

3.2.3.5. Starch hydrolysis:

Starch agar plate was streaked with the tested organism and incubated at 30 ºC for 14 days, then flooded with 5 ml iodine solution. The hydrolysis of starch was indicated by clear zone surrounding the colonies, while reddish brown zone indicated partial hydrolysis of starch.

3.2.3.6. Fermentation of sugars:

To 5 ml tubes of 1% peptone water containing 0.2% bromocresol purple indicator, inverted Durham's tubes and 1% of the following sugars were added (lactose, glucose, sucrose, dulcitol, salicin, arabinose, inositol and xylose). After incubation at 37°C, the reaction of the inoculated tubes was noticed every day for 7 successive days. Appearance of pink color indicates positive result.

3.3. Listeria Latex Agglutination Kit:

Oxoid Listeria Test Kit (Oxoid, Basingstoke, Hampshire, England) is a rapid latex agglutination test for the presumptive identification of *Listeria* spp. in selective and/or enrichment cultures. The Oxoid Listeria Test Kit was used according manufacture directions.

B- Assessment of biofilm formation capacity for the isolated bacterial strains

Bacterial strains

A total of 63 strains, isolated from slaughterhouses, were used in this study represented as follow: *S.* Typhimurium (9), *S.* Enteritidis (10) , *L. monocytogenes* (10), *E. coli* O157:H7 (1), *E. coli* O128:H2 (11), *E. coli* O111:H2 (4), *E. coli* O26:H11 (8), *E. coli* O121:H7 (4) and *E. coli* O91:H21 (6). Each strain was transferred from the stock cultures into Tryptic soya broth and incubated overnight at 37°C. Then subsequently subcultured one more time under the same conditions. The grown cultures were used for inoculation of TSB in the wells of polystyrene microplates for subsequent quantification of biofilm production**.**

Quantification of biofilm formation

Quantification of biofilm production in polystyrene microtitre plates was based on the previously described method **(Stepanovic et al., 2004)** and recommendations of **(Stepanovic et al., 2007).** Approximately, 180 µl of TSB was aseptically transferred to the wells of sterile 96-well flat bottomed polystyrene microplate. A quantity of 20 µl of overnight bacterial culture diluted to 0.5 Macfereland scale was added into each well. The negative control wells contained broth only. The plates were incubated aerobically for 24 hours at 37°C. Each strain was tested in

triplicate. In each trial the content of the plate was then poured off and the wells washed three times with 300 µl of sterile distilled water. The remaining attached bacteria were fixed with 200 µl of methanol per well. After 15 minutes microplates were emptied and air dried. The microplates were stained with 200 µl per well of 0.1 % Crystal violet for 15 min. Excess stain was rinsed off by placing the microplate under running tap water. After the microplates were air dried, the dye bound to the adherent cells was resolubilized with 200 µl of 96% ethanol per well. The optical density (OD) of each well was measured at 570 nm using microplate reader (Epoch, 14041512, USA). Based on the OD produced by bacterial films, strains were classified into the following categories: non biofilm, weak, moderate or strong biofilm producers. The average OD values were calculated for all tested strains and negative controls, since all tests are performed in triplicate and repeated three times. The cut-off value (ODc) was calculated as follow:

ODc = average OD of negative control + (3SD of negative control). Final OD value of a tested strain is expressed as average OD value of the strain reduced by ODc value where (OD = average OD of a strain _ ODc). ODc value was calculated for each microtiter plate separately. If a negative value is obtained, it should be presented as zero, while any positive value indicates biofilm production. For easier interpretation of the results, strains can be divided into the following categories based upon the previously calculated OD values:

$OD \leq ODc$ = non biofilm producer;

$ODc < OD \leq 2ODc$ = weak biofilm producer;

$2ODc < OD \leq 4ODc$ = moderate biofilm producer;

$4ODc < OD$ = strong biofilm producer.

Part II

Study the effect of carvacrol, chitosan nanoparticles and carvacrol loaded chitosan nanoparticles on planktonic cells of the isolated bacterial strains and their biofilms

1- Synthesis and characterization of nanomaterials

1.1. Synthesis of chitosan nanoparticles

Chitosan nanoparticles (NPs) were prepared according to procedures described by **(Zimet et al., 2018).** Briefly, 0.5 g of chitosan was dissolved in acetic acid solution (1%, v/v). Next, the solution was continuous stirred at 500 rpm for 12 hours at room temperature. After that, NaOH solution (1M) was added dropwise until the formation of chitosan NPs. The solution was kept under vigorous stirring for 1hour. The NPs was then separated using centrifuge at 6000 rpm for 20 min. Later, the NPs were washed using double distilled water and kept at –20°C overnight. Finally, the chitosan NPs was freeze dried using Lyophilization (Virtis freeze dryer, Model 6KBTES-55, SP scientific, USA).

1.2. Preparation of carvacrol loaded chitosan nanoparticles

Carvacrol loaded chitosan particles were prepared according to **(Keawchaoon and Yoksan, 2011).** Briefly, 40 mL of chitosan solution (1.2% w/v) was prepared by dissolving chitosan flakes in aqueous acetic acid solution (1% v/v) at an ambient temperature overnight. Tween 80

was then added to the solution and stirred at 60 ◦C for 2 hours to obtain a homogeneous mixture. Carvacrol was gradually dropped into the stirring mixture, and agitation was carried out for 20 min. A weight of 0.48 g of carvacrol were used to obtain a weight ratio of chitosan to carvacrol of 1:1 (W:W). Subsequently, 40 mL of sodium tripolyphosphate (TPP) solution (0.5% w/v) was slowly dropped into an o/w emulsion while stirring; agitation was continuously done for 30 min. The final pH of mixture solution was ~5.0. The particles were collected by centrifugation at 6,000 rpm for 20 min at 25°C and washed with aqueous Tween 80 solution (1% v/v) and distilled water four times to remove free carvacrol. The obtained wet particles were dispersed in distilled water (25 mL). Carvacrol loaded chitosan NPs was freeze dried using Lyophilization.

1.3. Characterization (Keawchaoon and Yoksan, 2011)

1.3.1. Fourier Transformed Infrared Spectroscopy (FTIR)

The chitosan NPs (5 mg) was thoroughly mixed with dry potassium bromide. The infrared spectra between 400 and 4000 cm^{-1} were obtained with a tablet containing KBr and chitosan NPs using a Thermo Nicolet Nexus 470 ESP FTIR spectrometer (Thermo Nicolet, Madison, WI, USA). Thirty-two scans at a resolution of 4 cm^{-1} were evaluated and referenced against air.

1.3.2. X-Ray Diffraction (XRD)

XRD technique was used to obtain the crystalline structure of the particles**.** The sample is irradiated with a beam of monochromatic X-Ray over a variable incident angle range. Interaction with atoms in the sample results in diffracted X-Ray when the Bragg equation is satisfied. Resulting spectra are characteristics of chemical composition and phase**.** Diffraction patterns were collected on a Panalytical X´Pert PRO MPD (Multipurpose Diffractometer). Analyses were performed at Faculty of Science, Assiut university.

1.3.3. Transmission Electron Microscope (TEM)

High Resolution Transmission Electron Microscopy (HRTEM) coupled with Energy-Dispersive Spectroscopy (EDS) (JEOL. JEM 2100F) used to characterize the morphology and sizes of the NPs.

1.3.4. Particle size analysis

Zeta potential and particle diameter were measured at 20 ◦C using a Malvern model 3600 Zetasizer (UK) equipped with a He–Ne laser operating at 4.0 mW and 633 nm with a fixed scattering angle of 90. Samples were prepared by dispersing a fixed amount of the nanomaterial in a definite volume of water or medium at the desired pH value by ultrasonication treatments.

2- Bacterial strains

The bacterial isolates used in this investigation were *L. monocytogenes*, *S.* Typhimurium, *S.* Enteritidis and *E. coli* O26:H11 that isolated in this study and classified as strong biofilm producers.

3- Determination of the minimum inhibitory concentration (MIC) of carvacrol, chitosan nanoparticles and carvacrol loaded chitosan nanoparticles on planktonic cells

The MICs against the bacterial isolates were determined according to procedures described by Clinical and Laboratory Standard Institute **(CLSI, 2012).** Ninety-six-well plates were prepared by dispensing 50 µL of different concentrations of carvacrol, chitosan NPs and carvacrol loaded chitosan NPs dissolved in Mueller-Hinton broth. Then, 50 µL of calculated bacterial suspension (6 log CFU/mL) were added to each well, providing final concentration of 120 – 0.47 mg/mL (w/v) for carvacrol and 20 - 0.16 mg/ml (w/v) for chitosan NPs and carvacrol loaded chitosan NPs. A positive growth control containing MHB and bacterial culture without compounds, and a negative control containing no bacteria were included in each experiment. The microplate for carvacrol was wrapped loosely with parafilm to prevent bacterial dehydration and ensure that carvacrol would not volatilize. Each plate included controls without antibacterial compound. The systems were incubated at 37°C for 24 hours. After the incubation, 30 µL a liquot of resazurin 0.015 % prepared in aqueous solution was added to each well. Color changes were assessed visually after 2 h of incubation at 37°C. Bacterial growth was indicated by a color change in each well from purple to pink (or colorless). The MIC value was confirmed as the lowest concentration capable of inhibiting the growth of the tested strains (No change in color).

4. Testing the anti-biofilm activity of carvacrol loaded chitosan NPs

4.1. Effect on inhibition of biofilm formation

The ability of nanomaterials to inhibit the formation of biofilm was performed by spectrophotometric assay according to **(Plyuta et al., 2013).** 180 µl of different concentrations of carvacrol loaded chitosan NPs (2 MIC, MIC and 0.5 MIC) prepared separately in TSB were distributed separately in 96-well microtiter plates. Wells were inoculated with 20 µl of the overnight-grown culture of the tested bacterial strains and incubated at 37°C during 24 hours. The suspensions were then removed, and the wells were washed with 200 µL of PBS to remove free floating bacteria. Biofilms formed by adherent cells in the plate were fixed by 200 µl methanol for 20 min then stained with 200 µL of 0.1% crystal violet and incubated at room temperature for 15 min. Excess stain was rinsed off by thorough washing with PBS. After air drying of wells, dye of biofilms lining the walls was resolubilized by 200 µl of 96% ethanol and incubated for 15 min. The resulting reaction was read spectrophotometrically at 570 nm. A set of wells inoculated with bacteria without any treatment were used as the controls. Triplicate samples were included for each treatment, and the experiment was repeated three times. The percentage of biofilm inhibition was calculated using the following equation:

% of inhibition = (OD (control)_OD (treatment) / OD (control) × 100

4.2. Inactivation and reduction of pre-formed biofilms

The effect of different concentrations (2MIC and 4 MIC) of carvacrol loaded chitosan NPs on the reduction of preformed biofilms was tested on polystyrene flat-bottomed microtiter plates as described by **(Soni et al.,**

2013). Each 96-well microtiter plate was filled with 180 µl per well of TSB and inoculated with 20 µl per well of overnight-grown bacterial cultures. Plates were incubated at 37°C for 24 hours in a static condition. After incubation, planktonic cells were removed and each well was washed three times. Subsequently, the biofilm cells that attached to the well surface were treated separately by adding 200 µl per well of TSB containing different concentrations of carvacrol loaded chitosan NPs (2 MIC and 4 MIC). Set of wells containing TSB only without any treatment was also used as control. Plates were incubated at 37°C for 24 hours. Then, individual wells were washed three times with sterile water to remove loosely bound cells. Subsequently, each well was filled with 200 µl of 0.1% crystal violet solution and incubated at room temperature for 15 min. The crystal violet solution was removed, and the wells were further washed five times to remove residual crystal violet. The bound crystal violet stain was solubilized with 200 µl per well of 96% ethanol and quantified by its optical density reading at 570 nm.

Statistical analysis

Results are expressed as mean values with the standard deviation. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test (GraphPad Prism, version 8.4.2, LLC). The differences between groups were considered significant when $P < 0.05$.

Chemicals and Reagents

- Tryptic soya broth (Biolife, CP 4712).
- MacConkey agar (Oxoid, CM0007).
- Sorbitol MacCkonkey agar (Oxoid, PO0702).
- Rappaport-Vasiliadis Soy (RVS) broth (Biolife, CA3501).
- Xylose Lysine Deoxycholate (XLD) (Biolife, CB55052).
- Listeria Enrichment broth (Oxoid, CM0862).
- Oxford agar (Biolife, 401600).
- Muller Hinton broth (Oxoid, CM 0405),
- Peptone water (Oxoid, CM0009).
- Carvacrol (≥ 98 %, 499-75-2, Sigma Aldrich, St. Louis, MO).
- Chitosan Low molecular weight, (Sigma-Aldrich, St. Louis, MO).
- Tween 80 (Oxoid, R21276).
- Sodium tripolyphosphate (TPP) (AVI-CHEM Laboratories, 7758-29-4).
- Resazurin sodium salt (LOBA CHEMIE PVT. LTD, 0555200001).
- Crystal violet (AVI-CHEM Laboratories, 548-62-9).
- Phosphate buffer saline (Oxoid, BR0014).
- Ethanol (HPLC grade).
- Methanol (HPLC grade).
- Acetic acid was provided by Fisher (UK).

Results

Table 1: Incidence of *E. coli* serotypes isolated from NFCS swabs of slaughterhouses (No. of each=45)

Samples	*E. coli* serotypes		O157:H7 EHEC		O111:H2 EHEC		O26:H11 EHEC		O121:H7 EHEC		O91:H21 EHEC		O128:H2 ETEC		O146:H21 EPEC		O119:H6 EPEC	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Walls	11	24.4	0	0	0	0	2	4.4	0	0	3	6.6	4	8.8	2	4.4	0	0
Floors	12	26.7	1	2.2	2	4.4	3	6.6	1	2.2	0	0	3	6.6	0	0	2	4.4
Total (90)	23	25.6	1	1.1	2	2.2	5	5.6	1	1.1	3	3.3	7	7.8	2	2.2	2	2.2

Table 2: Incidence of *E. coli* serotypes isolated from FCS swabs of slaughterhouses (No. of each=30)

Samples	*E. coli* serotypes		O157:H7 EHEC		O111:H2 EHEC		O26:H11 EHEC		O121:H7 EHEC		O91:H21 EHEC		O128:H2 ETEC		O146:H21 EPEC		O44:H18 EPEC	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Knives	5	16.7	0	0	0	0	1	3.3	0	0	1	3.3	1	3.3	2	6.6	0	0
Hooks	6	20	0	0	1	3.3	2	6.6	1	3.3	0	0	2	6.6	0	0	0	0
Cutting boards	9	30	0	0	1	3.3	0	0	2	6.6	2	6.6	1	3.3	1	3.3	2	6.6
Total (90)	20	22.2	0	0	2	2.2	3	3.3	3	3.3	3	3.3	4	4.4	3	3.3	2	2.2

Table 3: Incidence of *Salmonella* spp. isolated from NFCS swabs of slaughterhouses (No. of each=45)

Samples	*Salmonella* spp.		*Salmonella* Typhimurium		*Salmonella* Enteritidis		*Salmonella* Essen		*Salmonella* Infantis		*Salmonella* Tamale		*Salmonella* Muenster	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Walls	7	15.6	2	4.4	1	2.2	2	4.4	1	2.2	0	0	1	2.2
Floors	11	24.4	3	6.6	4	8.8	1	2.2	2	4.4	1	2.2	0	0
Total (90)	18	20	5	5.6	5	5.6	3	3.3	3	3.3	1	1.1	1	1.1

Table 4: Incidence of *Salmonella* spp. isolated from FCS swabs of slaughterhouses (No. of each=30)

Samples	*Salmonella* spp.		*Salmonella* Typhimurium		*Salmonella* Enteritidis		*Salmonella* Essen		*Salmonella* Infantis		*Salmonella* Tamale		*Salmonella* Muenster	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Knives	5	16.7	0	0	1	3.3	2	6.6	1	3.3	0	0	1	3.3
Hooks	8	26.7	3	10	2	6.6	1	3.3	1	3.3	1	3.3	0	0
Cutting boards	6	20	1	3.3	2	6.6	1	3.3	0	0	2	6.6	0	0
Total (90)	19	21.1	4	4.4	5	5.6	4	4.4	2	2.2	3	3.3	1	1.1

Table 5: Incidence of *Listeria* spp. isolated from NFCS swabs of slaughterhouses (No. of each=45)

Samples	*Listeria* spp.		*L. monocytogenes*		*L. innocua*		*L. ivanovii*		*L. welshimeri*		*L. grayi*	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Walls	5	11.1	1	2.2	2	4.4	1	2.2	0	0	1	2.2
Floors	8	17.8	3	6.6	3	6.6	0	0	1	2.2	1	2.2
Total (90)	13	14.4	4	4.4	5	5.6	1	1.1	1	1.1	2	2.2

Table 6: Incidence of *Listeria* spp. isolated from FCS swabs of slaughterhouses (No. of each=30)

Samples	*Listeria* spp.		*L. monocytogenes*		*L. innocua*		*L. ivanovii*		*L. welshimeri*		*L. grayi*	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Knives	5	16.7	1	3.3	1	3.3	1	3.3	0	0	2	6.6
Hooks	7	23.3	3	10	2	6.6	0	0	1	3.3	1	3.3
Cutting boards	4	13.3	2	6.7	1	3.3	1	3.3	0	0	0	0
Total (90)	16	17.8	6	6.7	4	4.4	2	2.2	1	1.1	3	3.3

Table 7 : The cut-off optical density value (ODc) for the tested isolates

Bacterial isolates	The cut off value (ODc)		
	ODc	2ODc	4ODc
E. coli	0.247	0.494	0.988
Salmonella spp.	0.240	0.480	0.96
L. monocytogenes	0.244	0.488	0.976

Figure 1: The optical density (OD_{570}) values for EHEC isolates (O111, O26, O91, O121 and O157)

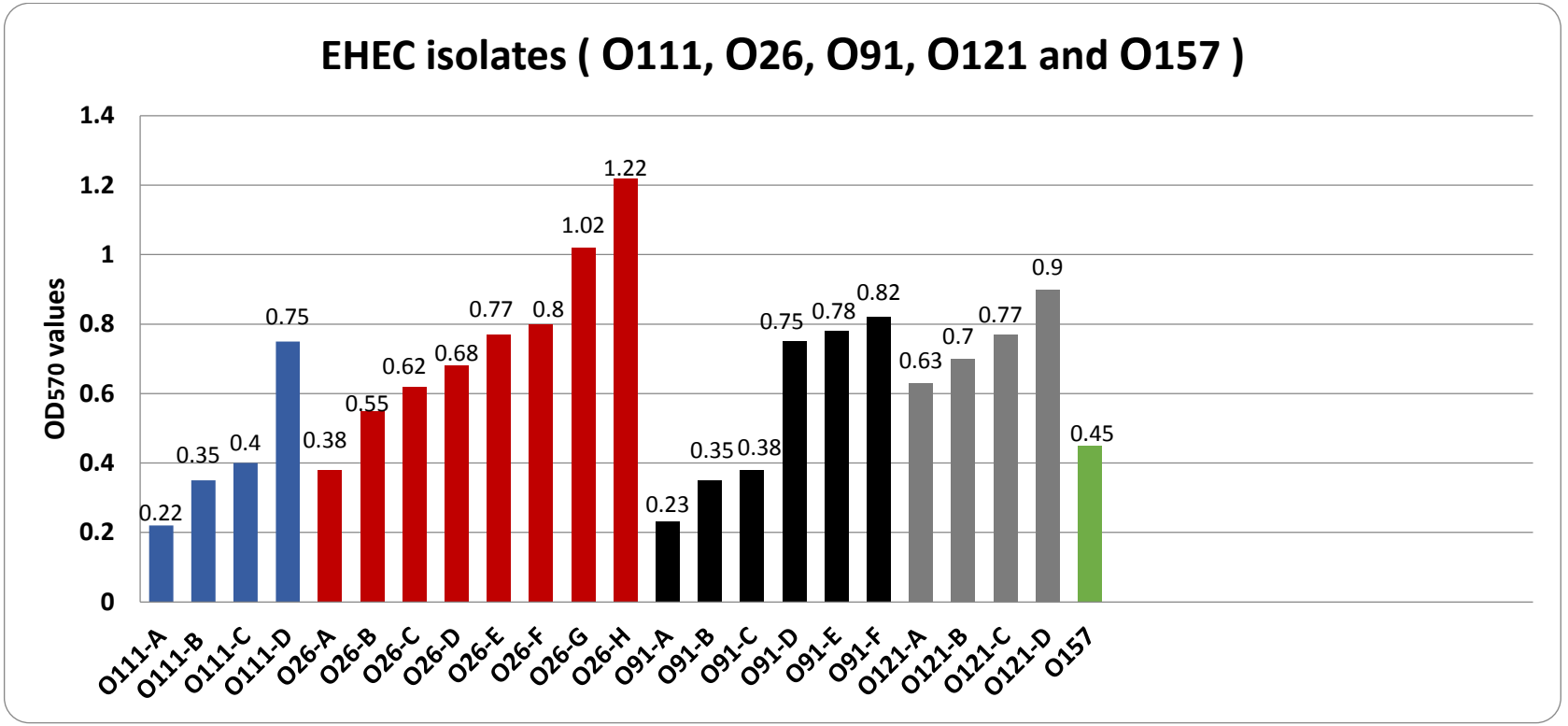


Figure 2: The optical density (OD570) values for ETEC O128 isolates

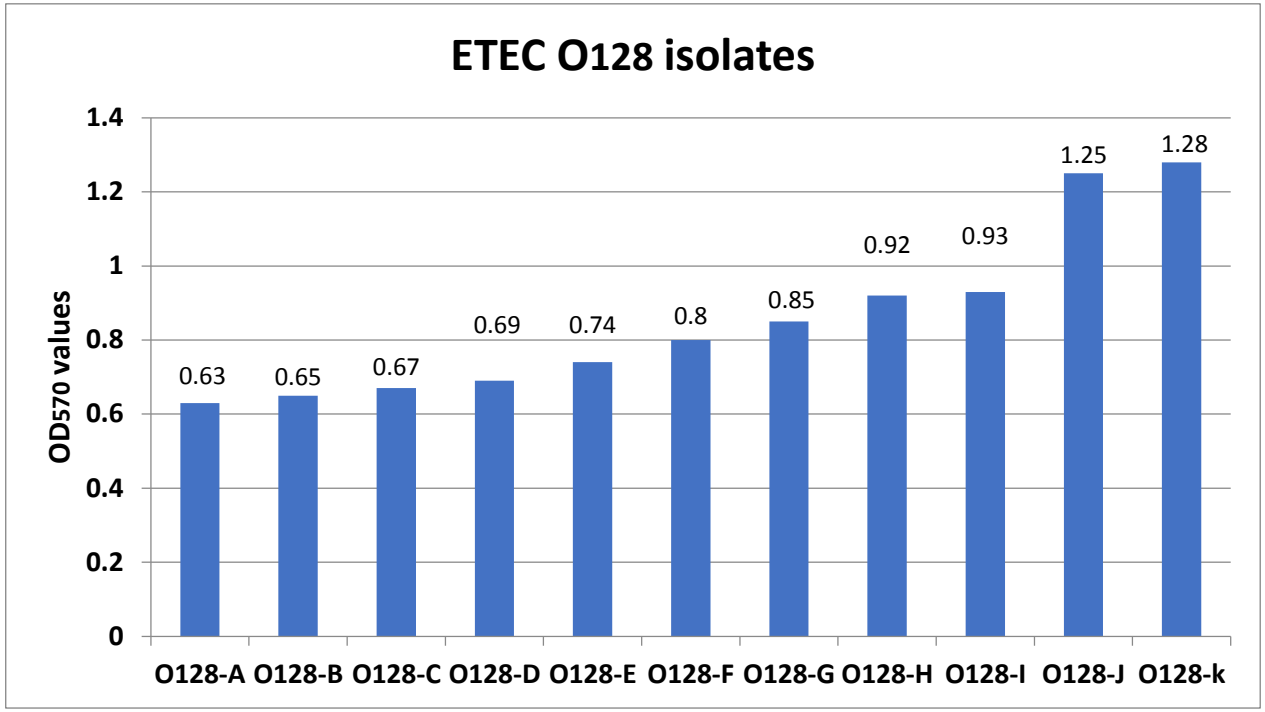


Table 8: Biofilm forming ability of the isolated *E. coli* serotypes

E. coli serogroups	No. of tested strains	Category of biofilm production			
		Undetectable biofilm	Weak producers	Moderate producers	Strong producers
O111	4	1	2	1	0
O26	8	0	1	5	2
O91	6	1	2	3	0
O121	4	0	0	4	0
O128	11	0	0	9	2
O157	1	0	1	0	0
Total	34	2 (5.9%)	6 (17.6%)	22(64.7%)	4 (11.8%)

Figure 3: The optical density (OD570) values for *S.* Typhimurium and *S.* Enteritidis isolates

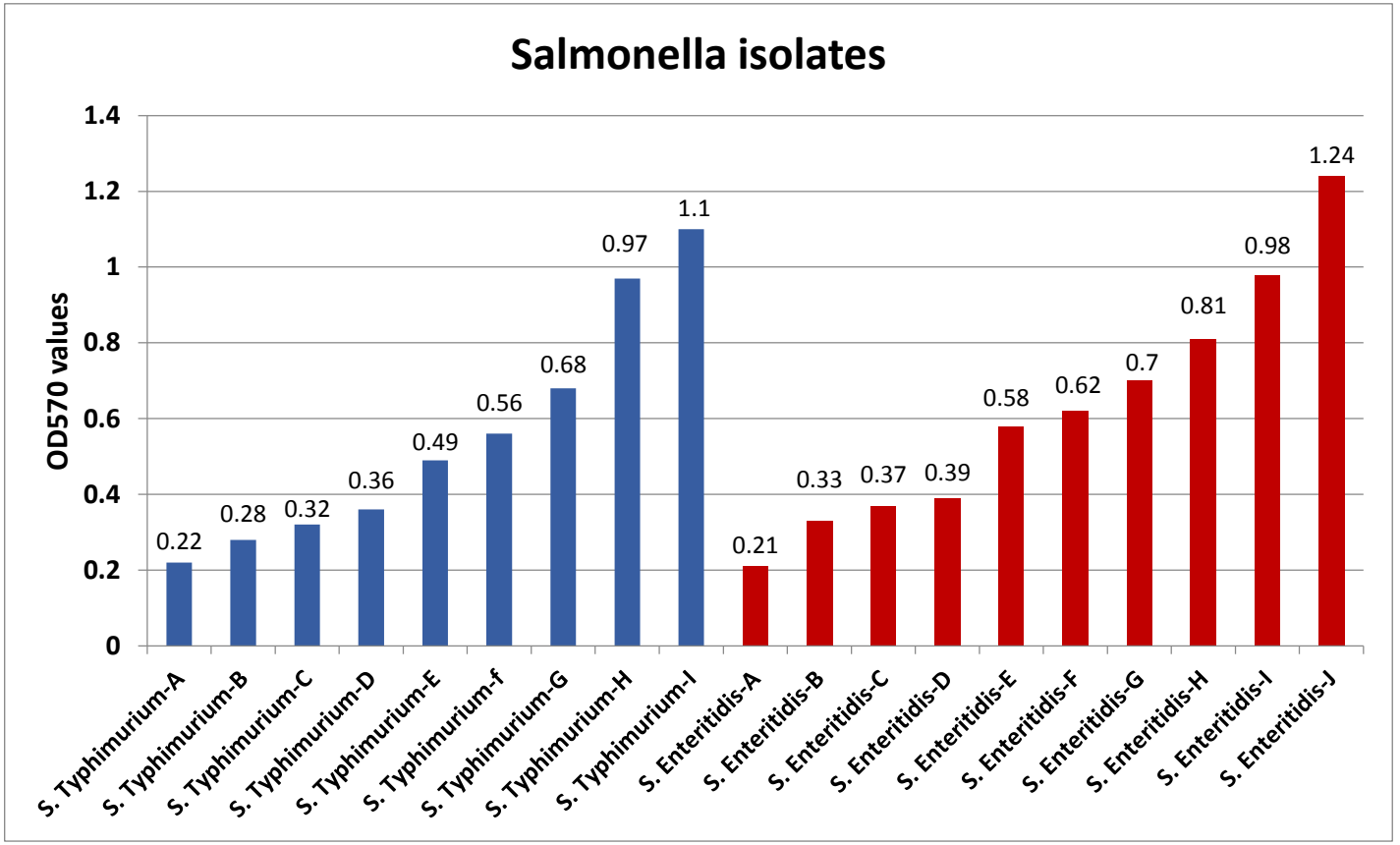


Table 9: Biofilm forming ability of the isolated *Salmonella* spp. isolates

Tested isolates	No.	Category of biofilm production			
		Undetectable biofilm	Weak producers	Moderate producers	Strong producers
Salmonella Typhimurium	9	1	3	3	2
Salmonella Enteritidis	10	1	3	4	2
Total	19	2 (10.5%)	6 (31.6%)	7 (36.8%)	4(21.1%)

Figure 4: The average of OD570 values for *L. monocytogenes* isolates

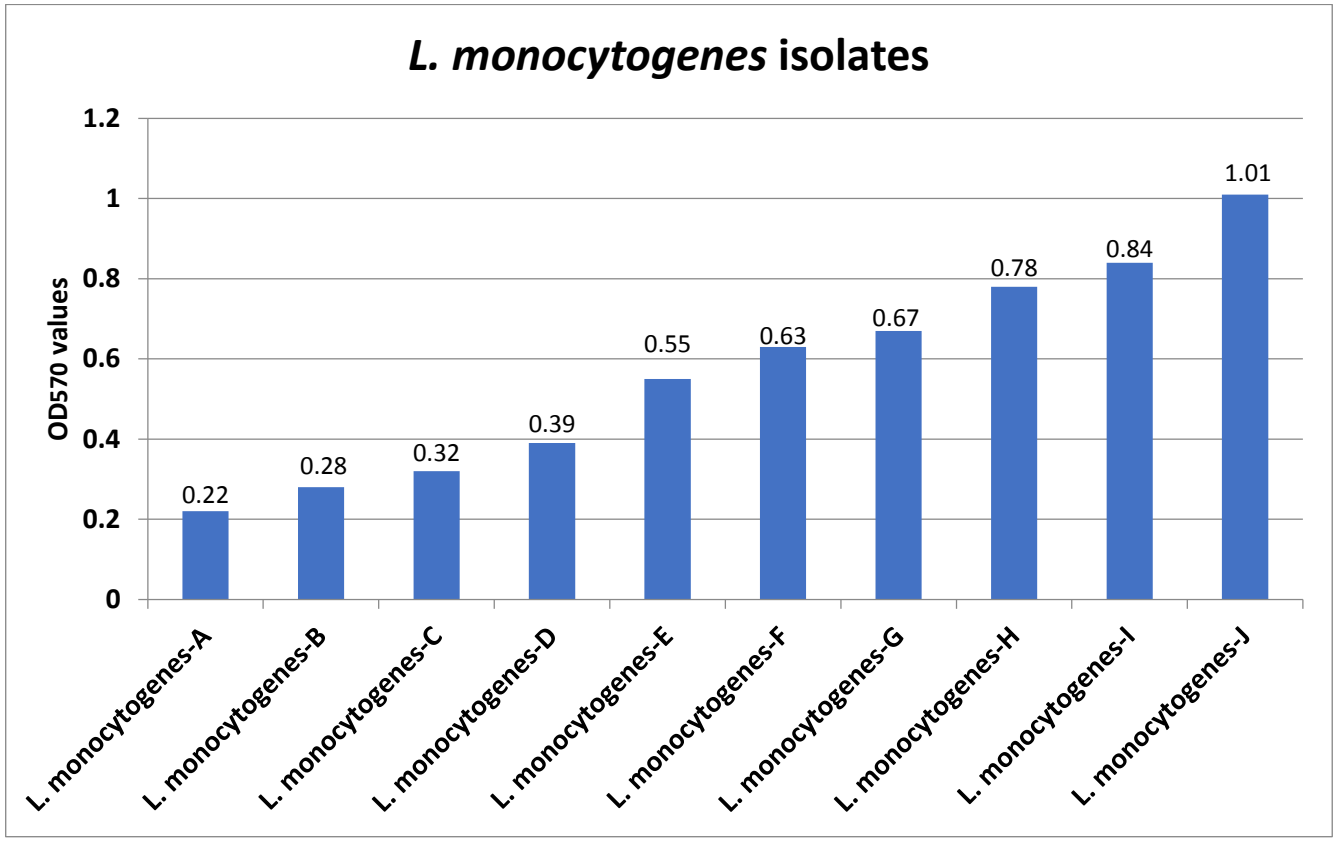


Table 10: Biofilm forming ability of the isolated *L. monocytogenes* isolates

Tested isolates	No.	Category of biofilm production			
		Undetectable biofilm	Weak producers	Moderate producers	Strong producers
Listeria monocytogenes	10	1	3	5	1
Total	10	1 (10%)	3 (30%)	5 (50%)	1(10%)

Figure 5: FTIR spectra for (A) carvacrol, (B) chitosan NPs and (C) carvacrol loaded chitosan NPs

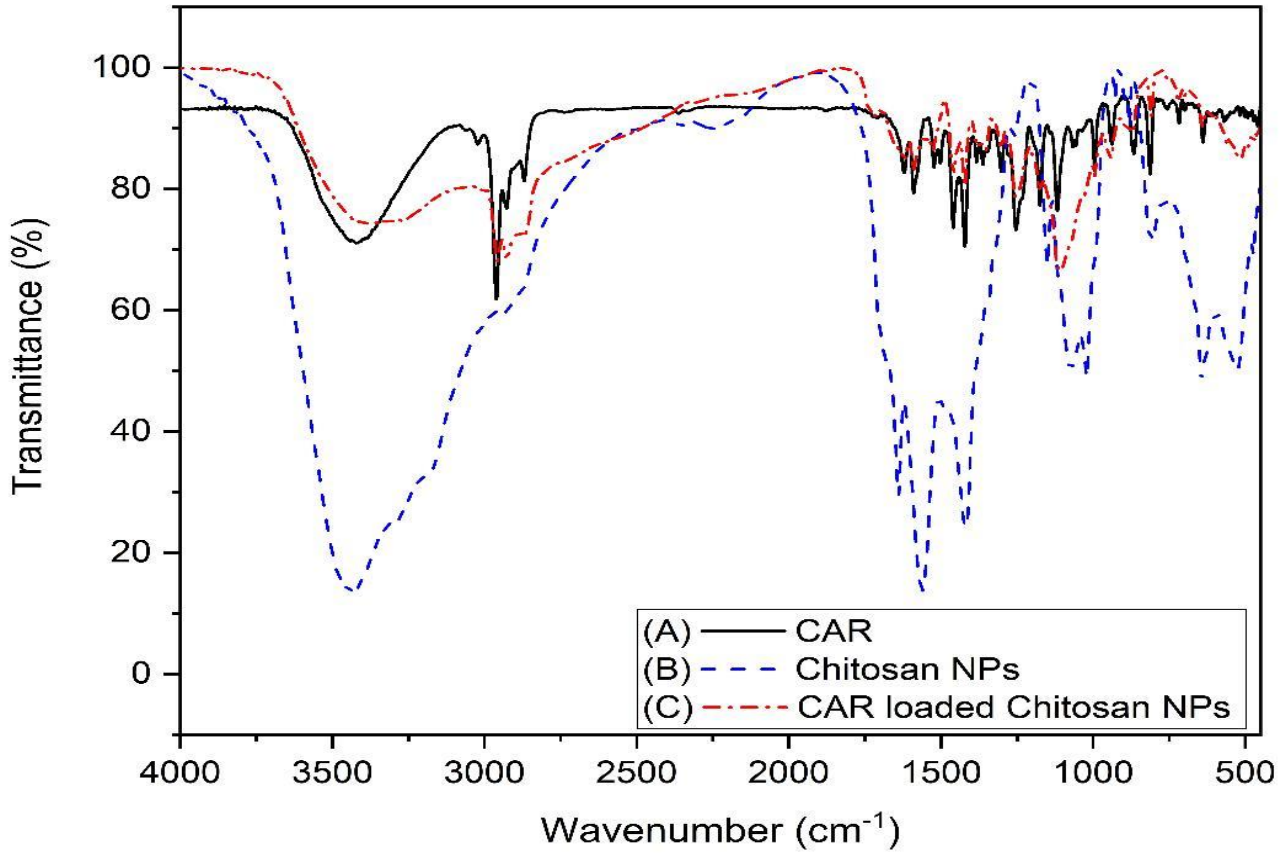


Figure 6: X-ray diffraction (XRD) for chitosan NPs and carvacrol loaded chitosan NPs

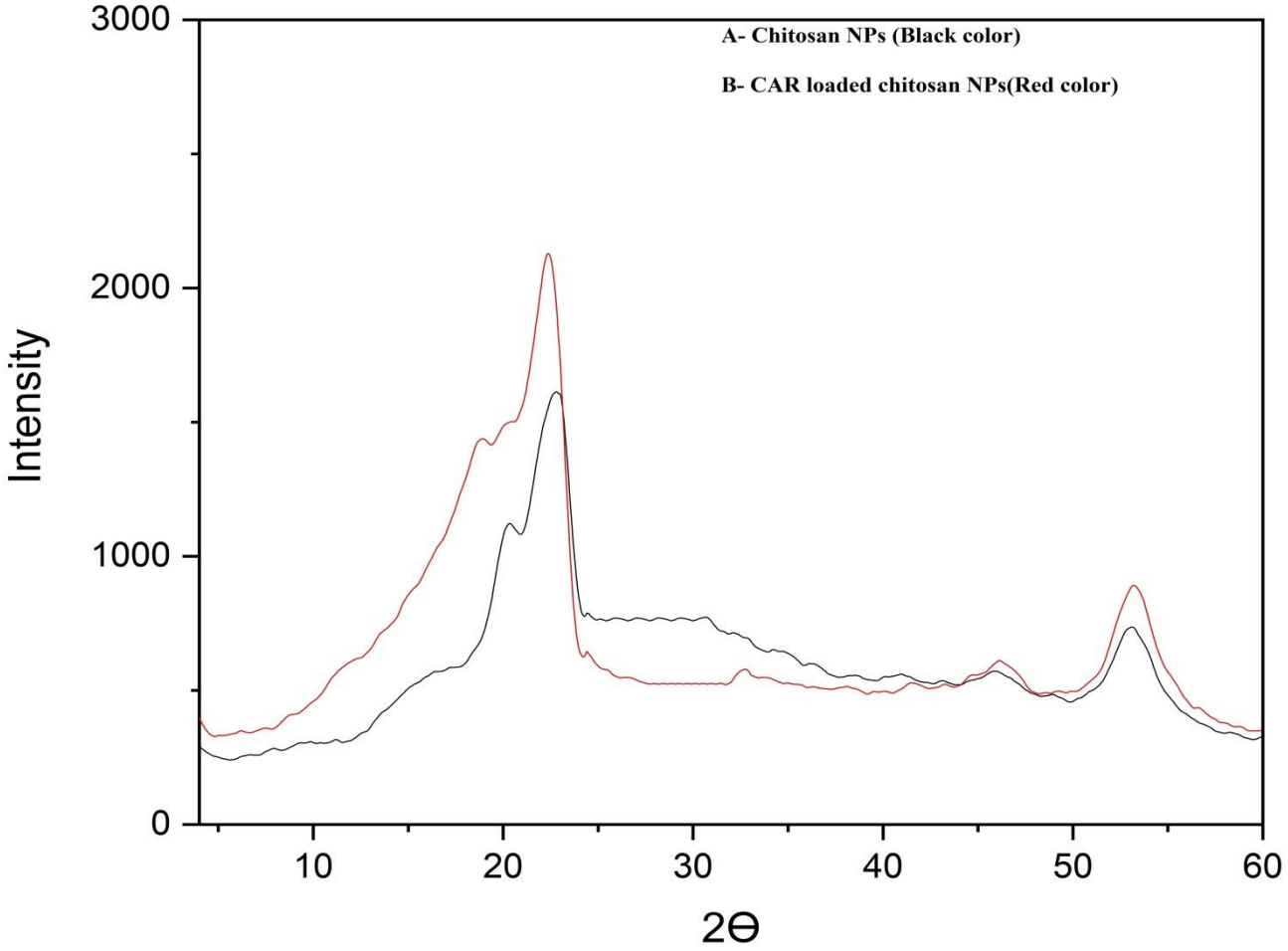


Figure 7: TEM images of (A) chitosan NPs and (B) carvacrol loaded chitosan NPs at the same scale bar

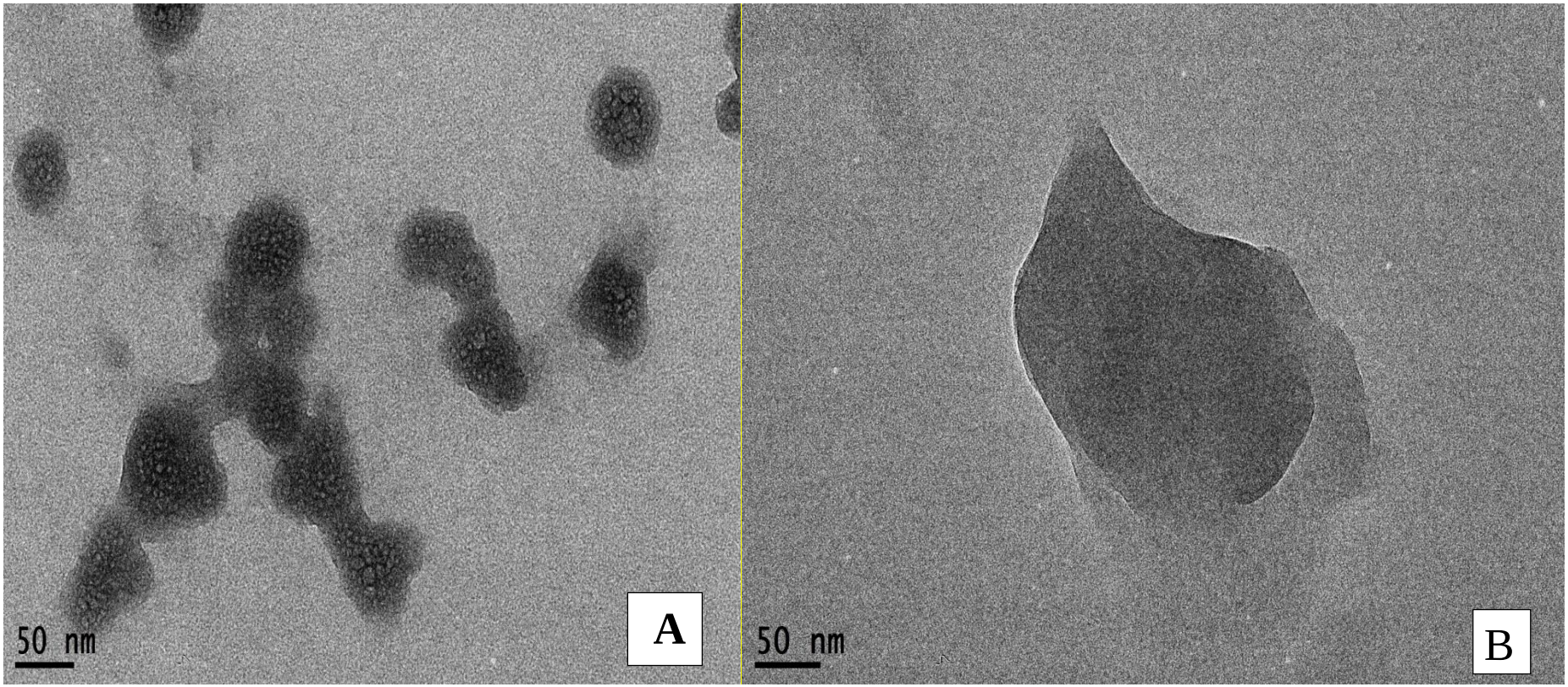


Figure 8: Zeta average size of carvacrol loaded chitosan NPs

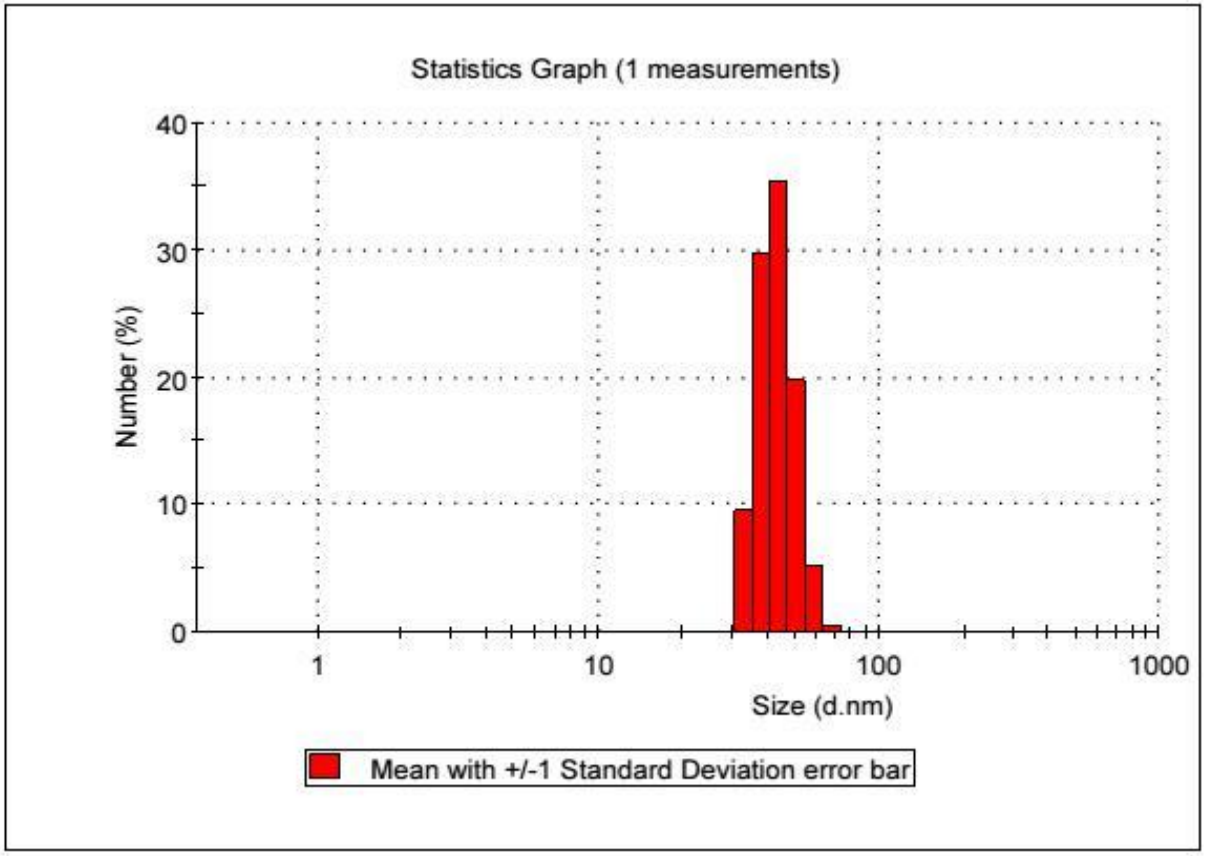


Table 11: Zeta potential value of chitosan NPs and carvacrol loaded chitosan NPs

	Zeta potential (mV)
Chitosan NPs	**+37.44 ± 0.94**
CAR loaded chitosan NPs	**+31.5 ± 0.30**

Table 12: Minimum inhibitory concentrations (MICs) of different antibacterial substances

Bacterial strains	MICs		
	Carvacrol mg/ml	Chitosan NPs mg/ml	Carvacrol loaded chitosan NPs mg/ml
L. monocytogenes	3.75	5	0.62
S. Typhimurium	0.93	2.5	0.31
S. Enteritidis	1.87	2.5	0.62
E. coli O26:H11	0.93	1.25	0.31

Table 13: The effect of carvacrol loaded chitosan NPs on the inhibition of biofilm formation

Strain	% of Biofilm inhibition		
	0.5 MIC	MIC	2 MIC
L. monocytogenes	**35.79 ±9.32^{a}**	**73.37 ±3.62^{b}**	**77.76 ±4.06^{b}**
***S.* Typhimurium**	**44.49 ±10.45^{a}**	**74.26 ±7.39^{b}**	**85.93 ±3.78^{c}**
***S.* Enteritidis**	**36.27 ±7.01^{a}**	**73.47 ±3.49^{b}**	**78.06 ±3.6^{b}**
***E. coli* O26:H11**	**30.89 ±10.67^{a}**	**74.46 ±3.09^{b}**	**87.22 ±10.7^{c}**

Means with different letters (a, b, c) in the same row for each concentration are significantly different ($P < 0.05$)

Figure 9: The effect of carvacrol loaded chitosan NPs on the inhibition of biofilm formation

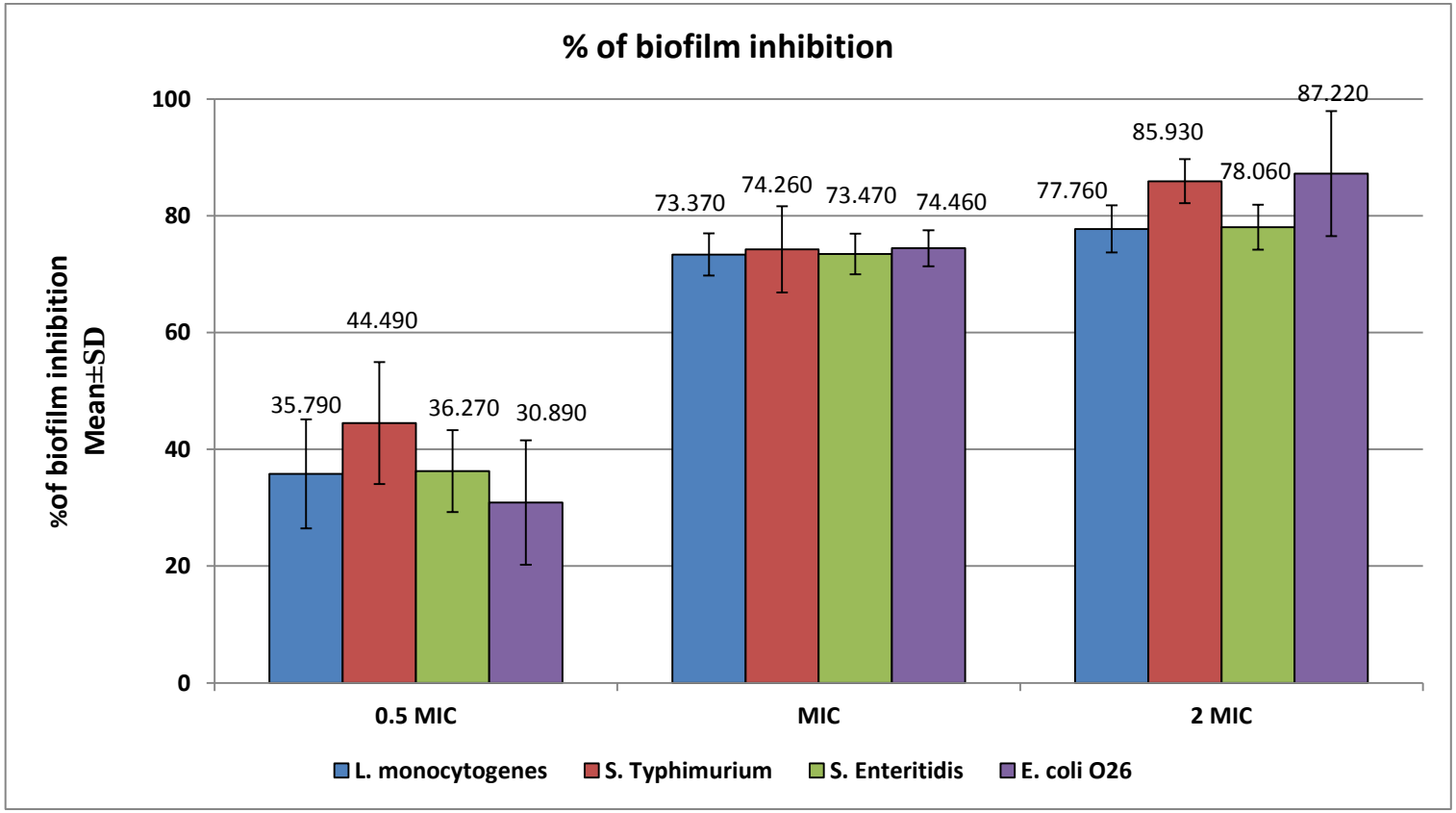
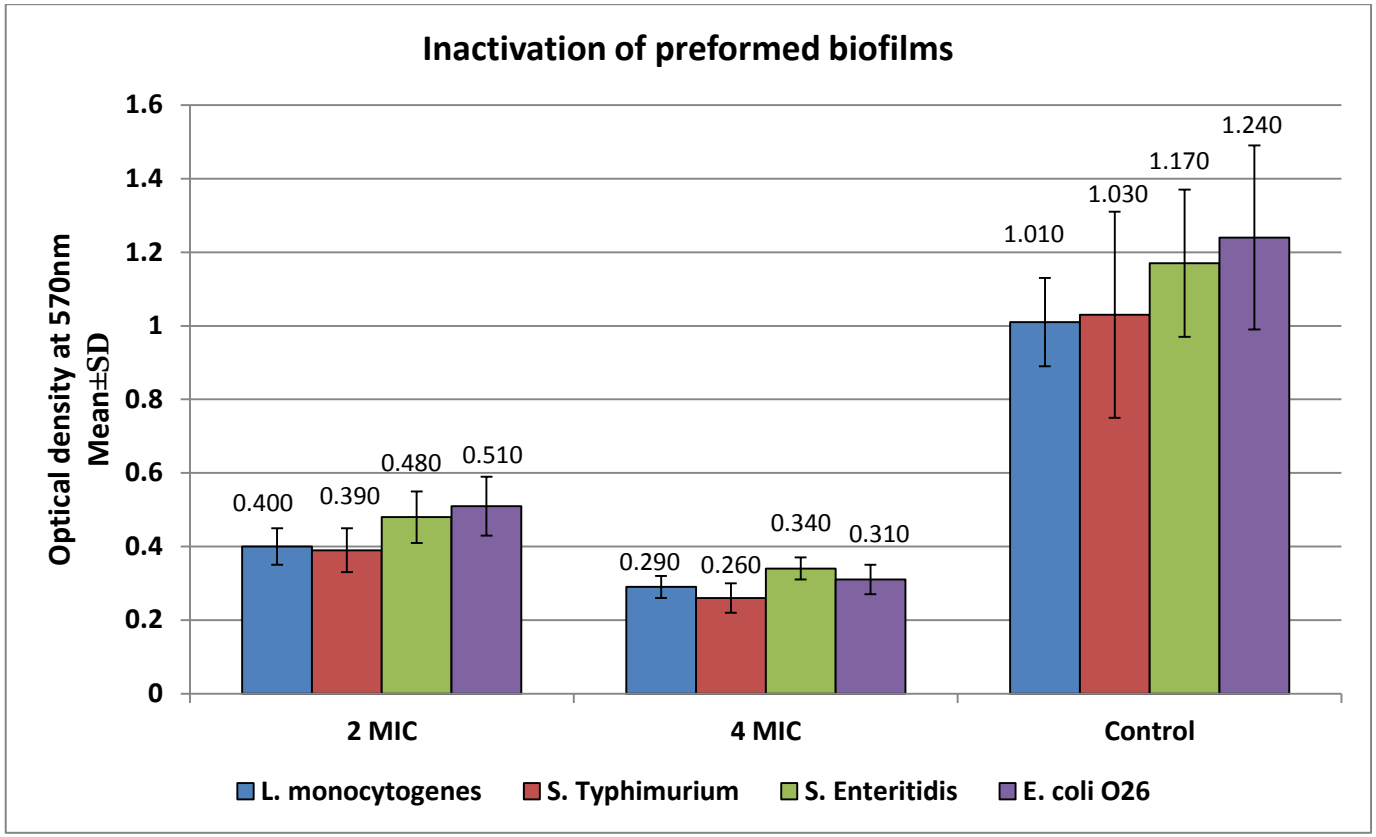


Table 14: Effect of carvacrol loaded chitosan NPs on preformed biofilms

Strain	OD_{570} Mean ± SD		
	2 MIC	4 MIC	control
L. monocytogenes	0.40 ± 0.05^{a}	0.29 ± 0.03^{b}	1.01 ± 0.12^{c}
S. Typhimurium	0.39 ± 0.06^{a}	0.26 ± 0.04^{a}	1.03 ± 0.28^{b}
S. Enteritidis	0.48 ± 0.07^{a}	0.34 ± 0.03^{a}	1.17 ± 0.2^{b}
E. coli O26:H11	0.51 ± 0.08^{a}	0.31 ± 0.04^{b}	1.24 ± 0.25^{c}

Means with different letters (a, b, c) in the same row for each concentration are significantly different ($P<0.05$)

Figure 10: The effect of carvacrol loaded chitosan NPs on preformed biofilms



Discussion

In recent years, the diseases caused by foodborne pathogens have become an important public health problem in many parts of the world, thus pose a great threat to food safety. In addition, many outbreaks have been found to be associated with biofilms. It is well documented that biofilms have become an urgent problem in the current food industry as biofilms can renders inhabitants on such films resistant to antimicrobial agents and cleaning **(Zhao et al., 2017).**

1.The bacteriological analysis of the examined samples:

1.1. Incidence of *E. coli* serotypes in FCS and NFCS.

Various serotypes of shiga toxin–producing *Escherichia coli* (STEC) strains are important foodborne pathogens that pose a serious public health concern, resulting in significant financial loses. These strains have been implicated in numerous outbreaks, with symptoms ranging from bloody diarrhea to other, more severe, diseases such as hemolytic uremic syndrome (HUS), a life-threatening complication that is the major cause of kidney failure for children younger than the age of 5 years **(Peco-Antic´, 2016).**

Outbreaks of STEC are frequently associated with beef and beef products. Contamination of beef with STEC may occur during slaughter, dressing, chilling, or cutting **(Koutsoumanis and Sofos, 2004).**

1.1.1. Incidence of *E. coli* serotypes in NFCS.

The findings outlined in Table 1 indicate that the incidence of *E. coli* isolated from the examined swabs of walls and floors were 11 (24.4%) and 12 (26.7%), respectively. Concerning EHEC strains O157:H7, O111:H2, O26:H11 and O121:H7 incidence were 2.2%, 4.4%, 6.6% and 2.2%, respectively in floor swabs, while the incidence of EHEC O26:H11 and O91:H21 was 4.4% and 6.6% in wall swabs. ETEC O128:H2 incidence was 6.6% in floor swabs and 8.8% in wall swabs. The other serogroups of *E. coli* were classified as EPEC O146:H21 (4.4%) only in walls swabs and O119:H6 (4.4%) in floor swabs only.

Comparatively, **Cetin et al. (2006)** failed to detect *E. coli* from all the wall samples. While in a related study, **Darwish et al. (2018)** revealed that the prevalence rate of *E. coli* in walls and floors was 40 % and 60% respectively. On the other hand, the incidence of *E. coli* O157:H7 (0.9%) in the examined samples of NFCS was revealed by **Orellana (2012).**

The hides and feces of animals presented for slaughter may be the major sources of pathogens in the processing environment (**Barkocy-Gallagher et al., 2001).** Also, holes and crevices are common in floor materials so bacteria in such sites can not be easily removed by cleaning and the attached bacteria become highly resistant to biocidal agents **(Carpentier, 2005).** In addition, the connection between floors and walls didn't have rounded angle which made it not easily cleaned and became highly contaminated with pathogenic microorganisms and facilitate their attachment and persistence **(Lelieveld et al., 2005),** which is the same condition , we found in the slaughterhouses of this study.

1.1.2. Incidence of *E. coli* serotypes in FCS.

The results presented in Table 2 showed that *E. coli* was isolated from 5 (16.7%), 6 (20%) and 9 (30%) in the examined swabs of knives, hooks and cutting boards, respectively. Totally 22.2 % of the examined samples of FCS were contaminated with *E. coli*. EHEC strains O111:H2 and O121:H7 were isolated with incidence 3.3% of each in the examined samples of hooks, while it was 3.3% and 6.6%, respectively for the cutting board samples. The incidence of EHEC O26:H11 and O91:H21 was equal 3.3% for knives samples while it was 6.6% for O26 in hooks and 6.6% for O91 in cutting boards samples. EHEC O157:H7 failed to be detected in any of the examined samples. ETEC O128:H2 incidence was 6.6% in hook samples and 3.3% for both knives and cutting board samples. The other serogroups of *E. coli* were classified as EPEC O146:H21 (6.6%) and (3.3)% for knives and cutting board swabs, respectively. EPEC O44:H18 was recorded in a percentage of 6.6% in cutting board swabs only.

The obtained result in this study was in agreement with **Orellana (2012)** who failed to detect *E. coli* O157:H7 in the examined FCS samples of two meat processing plants. While, high prevalence rate (45%) of *E. coli* was recorded in the examined samples of FCS swabs in cattle slaughterhouse by **Schlegelov et al. (2010)**.

The populations of STEC may be distributed on surfaces of equipment used to produce meat during slaughter and fabrication, contaminating carcasses and fresh meat products **(Stromberg et al., 2018**). Their presence and survival on beef FCS and equipment are often mediated through formation of biofilms **(Wang et al., 2012).** STEC

strains may be present in beef-processing environments in the form of biofilms. The exudate of raw beef, also referred to as beef juice, has been identified as an important source of bacterial contamination on food-processing surfaces (**Ma et al., 2019)**.

Some dead areas in equipments and FCS may contain meat residues which support the growth and multiplication of microbes attached to it and become a source of contamination. For example, if a single cell of *E. coli* is trapped in a dead space filled with 5 ml of slighltly viscous low acid food product at a temperature of 25℃, it could take less than 24 h for the number of microbial cells to increase to 2 $\times 10^8$ per ml (**Lelieveld, 2000).**

1.2. Prevalence of *Salmonella* spp.

1.2.1. Incidence of *Salmonella* spp. in NFCS.

It is evident from the results recorded in Table 3 that for NFCS, isolated Salmonellae from walls samples were serotyped as *S.* Typhimurium (4.4%), *S.* Enteritidis (2.2%), *S.* Essen (4.4%) , *S*. Infantis (2.2%) and *S.* muenster (2.2%). On the other hand, *Salmonella* spp. contaminated floor samples were serotyped as *S.* Typhimurium (6.6%), *S.* Enteritidis (8.8%), *S.* Infantis (4.4%), *S.* Essen (2.2%) and *S.* Tamale (2.2).

In a related study, **Orellana (2012)** revealed that the contamination rate of examined NFCS samples in two meat processing facilities with *S. enterica* was 6.7%.

Floors are an important source of contamination, since they transfer contamination to workers' shoes. The workers, in turn, circulate inside the establishment, thereby disseminating the contamination. Floors can offer a favorable environment for microbial growth, and an important source of propagation and preservation of microorganisms, especially if cleaning is done with water under high pressure **(Barros et al., 2007).**

Moreover, *Salmonella* can be carried asymptomatically in the intestines of healthy animals and are shed into the environment when feces are voided **(Hutchison et al., 2004)**. Consequently, animals sent for slaughter may contaminate the lairage holding and slaughter hall areas by shedding human pathogens in their feces; contamination may also occur through the mechanical transfer of organisms carried on the animals' hides **(Collis et al., 2004).**

1.2.2. Incidence of *Salmonella* spp. in FCS.

It is evident from the results recorded in Table 4 that Salmonellae isolated from knife samples were serotyped as *S.* Enteritidis (3.3%), *S.* Essen (6.6%) , *S.* Infantis (3.3%) and *S.* Muenster (3.3%) from total knives samples. On the other hand, *Salmonella* spp. contaminated hooks samples were serotyped as *S.* Typhimurium (10%), *S.* Enteritidis (6.6%), *S.* Infantis (3.3%), *S.* Essen (3.3%) and *S.* Tamale (3.3%). Regarding to *Salmonella* spp. recovered from cutting board samples were serotyped as *S.* Typhimurium (3.3%), *S.* Enteritidis (6.6%), *S.* Essen (3.3%) and *S.* Tamale (6.6%).

On the contrary, the results of this study were lower than reported by **Upadhyaya et al. (2012)** who revealed that *S.* Typhimurium was

(54.2%) and (55.6%) in cutting boards and knives, respectively. While their findings for *S.* Enteritidis was 13.6% and 18.5% in cutting boards and knives respectively. Also, the results obtained in the present study disagree with **Schlegelov et al. (2010)** who couldn't detect *Salmonella* spp. from the examined FCS swabs in cattle slaughterhouse.

The high contamination of food contact and environmental surface reflects the improper and ineffective cleaning and disinfection. Also, visual observation can be completely false when assessing the cleanliness of a surface **(Upadhyaya et al., 2012)**. Moreover, the detection of *Salmonella* spp. indicates the presence of cross-contamination from multiple sources and poor hygienic measures during meat cutting and handling **(Kirrella et al., 2015)**.

1.3. Prevalence of *Listeria* spp.

1.3.1 Prevalence of *Listeria* spp. in NFCS.

Among 90 examined NFCS swabs (45 from walls and 45 from floors) as demonstrated in Table 5, positive samples of *listeria* spp. in wall samples were identified as *L. monocytogenes* (2.2%), *L. innocua* (4.4%), *L. ivanovii* (2.2%) and *L. grayi* (2.2%). While in floor samples *Listeria* spp. were identified as *L. monocytogenes* (6.6%), *L. innocua* (6.6%), *L. welshimeri* (2.2%) and *L. grayi* (2.2%).

The results obtained in the present study disagree with **Meloni et al. (2014)** who failed to isolate *L. monocytogenes* from NFCS (walls and floors) in food processing environment. On the contrary, high prevalence rate (13%) of *L. monocytogenes* was recorded by **Orellana (2012)** from the all examined NFCS samples.

Paiva et al. (2010) studied *L. monocytogenes* infiltration in concrete blocks and the benefits of using a sealant to prevent harborage in cracks or capillary compartments available in concrete structures. Their findings proved that bacterial cells have the ability to infiltrate concrete blocks, increasing concern of potential harborage sites in NFCS such as ceilings, walls, and floors.

1.3.2 Prevalence of *Listeria* spp. in FCS.

The data outlined in Table 6 illustrated that in 90 examined samples of FCS swabs in the present study (30 from knives, 30 from hooks and 30 from cutting boards), *listeria* spp. in knife samples were identified as *L. monocytogenes* (3.3%), *L. innocua* (3.3%), *L. ivanovii* (3.3%) and *L. grayi* (6.6%). While *Listeria* spp. contaminated hooks samples were identified as *L. monocytogenes* (10%), *L. innocua* (6.6%), *L. welshimeri* (3.3%) and *L. grayi* (3.3%), but in cutting board samples were serotyped as *L. monocytogenes* (6.7%), *L. innocua* (3.3%) and *L. ivanovii* (3.3%).

The prevalence rate (5%) of *L. monocytogenes* from FCS samples obtained by **Schlegelov et al. (2010)** was lower than the present findings**.** On the other hand, the incidence (6%) of *L. monocytogenes* obtained by **Meloni et al. (2014)** in the examined FCS swabs nearly agreed with the findings of the present study. On the contrary, high prevalence rate (15%) of *L. monocytogenes* was obtained by **Henriques and Fraqueza (2017)**.

The ability of foodborne pathogens including *L. monocytogenes* to attach to various FCS and form biofilms making it difficult to adequately clean **(Renier et al., 2011).** Furthermore, several factors influencing the

survival of *L. monocytogenes* strains in food processing environments are recognized: complexity of structure of processing machines, poor hygienic properties, strain-specific properties such as differences in adherence to stainless steel surfaces, and susceptibility to disinfectants **(von Laer et al., 2009).**

2- Biofilm formation capacity for the isolated bacterial strains.

The ability to form biofilm varies greatly not just between species. Even in the same species with different strains and serovars, biofilm formation strength can vary significantly. However, there are some factors that influence biofilm forming capacity, specifically surface properties and nutrient availability, so one type of bacteria can be a strong biofilm producer under a certain environment and become weak in another environment **(Srey et al., 2013).**

2.1. Biofilm forming ability of the isolated *E. coli* serotypes.

The STEC isolates differed in their ability to form biofilms on 96 well microtiter plate. Based on the OD_{570} nm produced by biofilms as demonstrated in Figure 1 for EHEC and Figure 2 for ETEC, strains were classified as non biofilm, weak, moderate, or strong biofilm producers, as previously described. As illustrated in Table 7, The cut off optical density value (ODc) of 0.247 was three standard deviations above the mean OD of negative controls. Isolates were classified as non biofilm producers (NBP) if (OD_{570} of tested isolate < 0.247), weak biofilm producers (WBP) if (0.494 > OD_{570} of tested isolate > 0. 247), moderate biofilm producers (MBP) if (0.988 > OD_{570} of tested isolate > 0.494), or strong biofilm producers (SBP) if (OD_{570} of tested isolate > 0.988).

Out of 33 STEC isolates that were identified as EHEC and ETEC in the present study as demonstrated in Table 8, 31 strains were capable of forming biofilm classified as SBP 4 (11.8%) which serotyped as (2 strains O26 and 2 strains O128), MBP 22 (66.6%) which identified as (1 O111, 5 O26, 3 O91, 4 O121 and 9 O128), and WBP 6 (17.6%) which identified as (2 O111, 1 O26, 2 O91 and 1 O157). On the other hand, 2 strains (5.9%) were not able to form biofilm (O111 and O91).

Similar results were obtained by **Biscola et al. (2011)** who compared biofilm forming ability in 18 *E. coli* O157:H7 isolates and 33 non- O157 strains belonging to serotypes O26, O111, O103 and O145. Their study concluded that, under defined culture conditions, the ability to develop biofilms on abiotic surfaces (96-well plates) varied from strain to strain but was not restricted to any particular serotype

The obtained result was in agreement also with **Wang et al. (2012)** who suggested that STEC biofilm formation on polystyrene surfaces was highly dependent on the isolates but not the serotypes, since no particular serotype among the tested strains showed higher potency compared with the others.

This is consistent with the previous studies conducted by **Dourou et al. (2008)** using a collection of O157:H7 strains. They found that bacterial attachment to beef fabrication surfaces was highly strain-dependent.

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Furthermore, biofilm forming potentials of strains are influenced by an interaction between genetic and environmental factors such as the

nature of the attachment surface and surrounding medium **(Rossi et al., 2016).**

2.2. Biofilm forming ability of the isolated *Salmonella* spp. strains.

Surfaces with Salmonella can serve as a source of food contamination by cross-contamination. Biofilm formation may allow *Salmonella* spp. to survive on surfaces and persist in food processing environments for long periods **(Corcoran et al., 2014).**

Based on the criteria suggested by **Stepanovic et al. (2004)** and the data presented in Table 7, a cut-off value of 0.240 (three standard deviations above the mean OD of negative controls) at OD_{570} was used to categorize the tested isolates as showed in Figure 3, strains were classified as NBP (OD_{570} of test isolate < 0.240), WBP (0.48 > OD_{570} of test isolate > 0. 240), MBP (0.96 > OD_{570} of test isolate > 0.48), or SBP (OD_{570} of test isolate > 0.96).

As summarized in Table 9, among the 19 analyzed *Salmonella* spp. strains (9 *S*. Typhimurium and 10 *S*. Enteritidis), 17 strains were capable of forming biofilm classified as SBP 4 (21.1%) which identified as (2 *S.* Typhimurium and 2 *S*. Enteritidis), MBP 7 (36.8%) which serotyped as (3 *S.* Typhimurium and 4 *S.* Enteritidis) and WBP 6 (31.6%). While 2 (10.5%) were not able to produce biofilm (one strain for each *S.* Typhimurium and *S*. Enteritidis).

Nearly similar results reported by **Borges et al. (2018)** who revealed that most Salmonella strains had the ability to produce biofilms in microtiter plates. Where, only 14.4% of the *S.* Enteritidis strains were not

able to produce biofilm. On the contrary, the results of this study didn't agree with **Nair et al. (2015)** who recorded that about 67.5% of salmonella tested isolates were WBP and 17.5% were MBP Whereas, 15% of isolates were categorized as NBP.

Comparatively, **Ghasemmahdi et al. (2015)** reported that majority of the *Salmonella* isolates were WBP. Meanwhile, moderate biofilm formation by *Salmonella* isolates had also been reported by **Naeem (2014).**

Moreover, the ability of individual strain to form biofilm is generally influenced by incubation time, temperature and also the media used for biofilm production **(Wang et al., 2013b).** Furthermore, the differences in biofilm formation could be attributed to strain variations **(Chelvam et al., 2014).** Also, **Vestby et al. (2009)** suggested that the biofilm forming ability might be an important factor for the persistence of some *Salmonella* serotypes in the food processing industry.

According to **Cabarkapa et al. (2015),** the frequent involvement of *S.* Enteritidis in salmonellosis outbreaks may be a consequence of the strong ability of some strains to produce biofilms. It has been previously demonstrated that *S.* Enteritidis has higher adhesion capabilities on several surfaces that are usually used in the food industry, including different types of glass, stainless steel, polyethylene, polystyrene, polypropylene **(Casarin et al., 2016).**

Biofilm formation abilities of *Salmonella* isolates can be an explanation of widely distribution of *Salmonella* isolates in food. It is also an important public concern because poor sanitation of surfaces that

comes in contact with food causes food borne outbreaks. This situation revealed the importance of microbial food safety and sanitation in industrial processes **(Aksoy, 2019).**

2.3. Biofilm forming ability of *L. monocytogenes* isolates.

Based on the OD_{570} produced by biofilms as demonstrated in Figure 4, isolates were categorized as NBP, WBP, MBP, or SBP, as previously described. The cut off optical density value (ODc) of 0.244 was three standard deviations above the mean OD of negative controls (0.15). Isolates were classified as NBP ($OD_{570} < 0.244$), WBP ($0.488 > OD_{570} > 0.244$), MBP ($0.976 > OD_{570} > 0.488$), or SBP ($OD_{570} > 0.976$).

The data presented in Table 10 revealed that out of 10 tested *L. monocytogenes* isolates for biofilm production, 9 isolates were capable of forming biofilm classified as SBP 1 (10%), MBP 5 (50%), WBP 3 (30%). Meanwhile, one strain was not capable of producing biofilm.

The findings in this study were in consistent with the results obtained by **Henriques and Fraqueza (2017)** who revealed that 32% of the assessed *L. monocytogenes* isolates were WBP, 47% were MBP and 21% were SBP.

In contrast, these results counteract those obtained by **Meloni et al. (2014)**, in which most (65%) of the *L. monocytogenes* strains isolated in meat processing plants presented weak biofilm producing ability.

Verghese et al. (2011) emphasized that different *L. monocytogenes* isolates are well known to persist in meat processing environments and to

contaminate foods for many years due to their ability to grow on surfaces and form biofilms. Furthermore, in food processing plants, food residues accumulate in enclosed areas that are difficult to clean and sanitize and thus represent harborage sites for *L. monocytogenes*. Because they contain food and water for extended periods of time, these harborage sites allow bacteria to become established, multiply and forming biofilms **Chmielewski and Frank (2003).**

Part II

1. Synthesis and characterization of nanomaterials

The application of essential oils in food industry is problematic due to their low solubility in water. Encapsulation techniques in polymeric nanoparticles such as chitosan have been introduced in recent years as efficient means to increase its dispersion in aqueous media **(Hadidi et al., 2020).**

Carvacrol (CAR) loaded chitosan NPs were prepared by two steps process, i.e., droplet formation and droplet solidification. The formation of CAR droplets in chitosan solution was achieved by an oil-in-water emulsion technique. Each droplet was solidified by ionic cross-linking of protonated amino groups along chitosan molecules surrounding the CAR droplet and polyphosphate groups of TPP molecules.

1.1. Fourier Transform Infrared (FTIR) analysis:

Chemical structure of CAR, chitosan NPs and CAR loaded chitosan NPs was characterized by FTIR technique. As demonstrated in Figure 5, carvacrol showed characteristic peaks at 3423, 2960, (1459, 1383 and 1362), and (866 and 812) cm^{-1} which correspond to OH, CH stretching, CH deformation, and aromatic ring, respectively. In addition, the peaks appeared of Chitosan NPs at 3441 (OH), 2922 (CH stretching), 1639 (amide I), 1560 (amide II), 1149 (P = O), 1076 (C-O-C) and 879 cm^{-1} (pyranose ring) indicating electrostatic associations between polyphosphate group of TPP and amine group of chitosan which is agree to the reported by **Hosseini et al. (2013**). It can be seen from the FTIR spectra that the addition of CAR to chitosan NPs led to a significant

increase in the intensity of CH stretching peak at 2871–2958 cm^{-1}, reflecting the existence of CAR in the chitosan matrix. The CH stretching peak was thus used as a probe band for an indirect determination of loaded CAR content.

1.2. X-Ray diffraction (XRD) pattern of CAR loaded chitosan nanoparticles

Figure 6 illustrates the crystallographic structure of chitosan NPs and CAR loaded chitosan NPs. Diffraction spectrum of chitosan NPs depicts a peak at 2Ɵ of 20.32°. The lower intensity of the peak could be attributed to the conversion of chitosan powder into nano-scale form as well as its crosslinking with TPP and the amorphous structure of the chitosan NPs. The results are agreed with the literature documented by **Anand et al. (2018).** As can be seen in the diffractogram of CAR loaded chitosan NPs compared with unloaded chitosan NPs, there is a peak at 18.64° which is confirmed the inclusion of CAR in the complex structure of TPP-chitosan.

1.3. Morphology of CAR loaded chitosan NPs by HRTEM

The morphology of the particles was observed by HRTEM. The individual chitosan NPs exhibited spherical shape with an average diameter of 52 – 88 nm as shown in Figure 7 and CAR loaded chitosan NPs was demonstrated by covering the chitosan NPs with CAR as illustrated in Figure 7.

The obtained result in this study nearly agreed with **Keawchaoon and Yoksan (2011**) who reported that the individual CAR loaded chitosan NPs exhibited a spherical shape with an average diameter of 40–80 nm.

1.4. Size and surface charge of CAR loaded chitosan NPs

Zeta potential is a measure of the magnitude for the electrostatic or charge repulsion/attraction between particles which is a major factor in phenomena like dispersion, flocculation or aggregation and hence a key parameter for evaluating the stability of dispersions, emulsions and suspensions **(Dickinson, 2009).**

Zetasizer was used to determine the particle size of the synthesized nanomaterials. The average particle size of CAR loaded chitosan NPs was about 60 nm, as revealed in Figure 8. In addition, chitosan NPs gave a zeta potential value of +37.44 ± 0.94 mV as showed in Table 11, implying a positively charged surface of the particles. Moreover, the zeta potential value for CAR loaded chitosan NPs was about +31.5 ± 0.3 mV. This reflected that the loading of carvacrol reduced the surface positive charge.

Nearly similar results obtained by **Keawchaoon and Yoksan (2011**) who revealed that a positively charged surface of CAR loaded chitosan NPs with a zeta potential value of 25–29 mV.

2- Minimum inhibitory concentration (MIC) of Carvacrol (CAR), chitosan NPs and CAR loaded chitosan NPs in growth media :

Carvacrol, chitosan NPs and CAR loaded chitosan NPs were tested against the isolated strains of *E. coli* O26:H11, *S.* Typhimurium, *S.* Enteritidis and *L. monocytogenes* that previously categorized as strong biofilm producers.

The improved microdilution method described in this study is enhanced through the addition of resazurin dye as a redox indicator,

which overcomes the problems associated with sparingly soluble test materials. Active bacterial cells reduce the non-fluorescent resazurin (blue) to the fluorescent resorufin (pink) which can be further reduced to hydroresorufin giving a direct quantifiable measure of bacterial metabolic activity and the MIC determined through recording of the colour change observed **(O'Brien et al., 2000)**.

The results presented in Table 12 demonstrated a substantial susceptibility of the tested bacteria toward CAR, chitosan NPs and CAR loaded chitosan NPs. Especially, CAR loaded chitosan NPs displayed a strong inhibitory activity where (MICs values ranged from 0.31 to 0.62 to mg/mL) against the majority of the tested strains compared to carvacrol and Chitosan NPs alone.

Moreover, CAR loaded chitosan NPs exhibited more inhibitory activity than the other materials. Whereas, MIC was 0.31 mg/ml for both *E. coli* O26:H11 and *S.* Typhimurium and 0.62 mg/ml for *S.* Enteritidis and *L. monocytogenes*.

In a related study carried out by **Keawchaoon and Yoksan (2011)** emphasized that CAR loaded chitosan NPs showed antimicrobial activity against *E. coli* with an MIC of 0.257 mg/mL.

Cacciatore et al. (2020) revealed that carvacrol is a natural antimicrobial capable of inhibiting several microorganisms. Also, the authors suggested that the encapsulation of this compound in nanostructures increase its stability, water solubility, provide controlled release and has potential to be used as a surface sanitizer.

Regarding CAR, the MIC was 0.93 mg/ml for *E. coli* O26:H11and *S.* Typhimurium. While, it was 1.87 mg/ml for *S.* Enteritidis and 3.75 mg /ml for *L. monocytogenes*.

Comparatively, **Du et al. (2015)** reported that the MIC of CAR for *S.* Enteritidis was 0.18 mg/ml and 0.37 mg/ml for *S.* Typhimurium and *E. coli*. On the other hand**, Cacciatore et al. (2015)** recorded that MIC of CAR against *E. coli* was 0.60 mg/ml. While, **Ait-Ouazzou et al. (2011)** revealed that MIC of CAR against *L. monocytogenes* was 0.20 mg/ml.

Carvacrol is a phenolic compound with a hydroxyl group on an aromatic ring. The hydroxyl group plays a crucial role in the antibacterial activity of this phytochemical; indeed, carvacrol interacts with the lipid bilayer of the bacterial cytoplasmic membrane due to its hydrophobic nature causing the expansion and destabilization of the membrane structure by increasing its fluidity and permeability for protons and ions. The loss of the ion gradient leads to bacterial cell death **(Ciandrini et al., 2014).**

Furthermore, MIC of chitosan NPs was 1.25 mg/ml against *E. coli* O26, 2.5 mg/ml against *S.* Typhimurium and *S.* Enteritids. While, it was 5 mg/ml against *L. monocytogenes*.

Qi et al. (2004) reported that the MIC of chitosan NPs was 0.125 and 0.25 mg/ml against *E. coli* and *S.* Typhimurium, respectively. On the other hand, the results obtained in this study disagree with **Keawchaoon and Yoksan (2011)** who suggested that chitosan NPs with a concentration below 8.25 mg/mL could not inhibit the growth of *E. coli.*

The antimicrobial activity of chitosan has been demonstrated against many bacteria, fungi and yeasts possessing a high killing rate against Gram-positive and Gram-negative bacteria, but lower toxicity toward mammalian cells **(Kong et al., 2010).**

Severino et al. (2015) attributed the mechanism of chitosan antimicrobial action to the polycationic nature of chitosan as the interaction between positively-charged chitosan molecules and negatively charged microbial cell membranes leads to leakage of intracellular constituents.

3.The anti-biofilm activity of carvacrol loaded chitosan NPs:

3.1. Effect on inhibition of biofilm formation

Besides the great problem of antimicrobial resistance, several of the chemical disinfectants used in food industry may constitute a significant risks to humans, surfaces, and to the environment, since they can cause toxic and / or corrosive effects. Considering the previous, nowadays, there is an increasing effort to combat antimicrobial resistance and develop safe, eco-friendly and efficient anti-biofilm strategies and therapeutic approaches **(Giaouris and Simões, 2018).**

In this study, the in vitro effect of CAR loaded chitosan NPs on biofilms of *L. monocytogenes*, *S.* Typhimurium, *S.* Enteritidis and *E. coli* O26:H11 was evaluated. Despite the different inhibitory effect among the strains, a general attenuated level of biofilm formation in the presence of different concentrations of CAR loaded chitosan NPs was observed as showed in Table 13.

Significant differences ($P < 0.05$) in biofilm inhibition values were observed between different concentrations (0.5 MIC, MIC and 2 MIC). The inhibition was proportional to the concentration. This effect was more evident for *E. coli* O26 and *S.* Typhimurium. On the other hand, there was no significant difference between doses of 2 MIC and MIC for *S.* Enteritidis and *L. monocytogenes*, ($P > 0.05$).

In the presence of CAR loaded chitosan NPs (0.5 MIC), the mean biofilm inhibition values were equal to 30.89, 44.49, 36.27 and 35.79 % for *E. coli* O26:H11, *S.* Typhimurium, *S.* Enteritidis and *L. monocytogenes*, respectively. In the presence of a dose equal to MIC, the mean biofilm inhibition values were equal to 74.46, 74.26, 73.47 and 73.37 % for *E. coli* O26:H1, *S.* Typhimurium, *S.* Enteritidis and *L. monocytogenes*, respectively. Meanwhile, in the presence of 2 MIC, the mean biofilm inhibition values were equal to 87.22, 85.93, 78.06 and 77.76 % for *E. coli* O26:H11, *S.* Typhimurium, *S.* Enteritidis and *L. monocytogenes*, respectively.

The obtained results of this study are in agreement with **Upadhyay et al. (2013)** who suggested that CAR could potentially be used to control *L. monocytogenes* biofilms in food processing environments.

The inhibition of biofilm formation possibly occurs because CAR interacts with microbial proteins, reducing their adhesion to the surfaces **(Nostro et al., 2007).** Besides that, CAR at sublethal concentration can interfere on quorum sensing mechanism, affecting the expression of virulence factors and bacterial motility, thus hindering the biofilm formation **(Andersson and Hughes, 2014)**.

3.2. Effect on preformed biofilms

To analyze the effects of CAR loaded chitosan NPs on (24 hours old) biofilms of the tested strains, various concentrations were separately added to the biofilms (2 MIC and 4 MIC), and the microtitre plates were then incubated at 37 ^{o}C for an additional 24 hours.

Compared to the control, a statistically significant reduction ($P < 0.05$) was noted in biofilms that were treated with CAR loaded chitosan NPs at both 2 MIC and 4 MIC level as demonstrated in Table 14 and Figure 10.

Regarding to *L. monocytogenes*, 2 MIC and 4 MIC treatments reduced the biofilm mass to 0.40 and 0.29, respectively as compared to 1.01 OD_{570} for the control. While for *S.* Typhimurium, the biofilm mass reduced to 0.39 and 0.26 OD_{570} as compared to 1.03 OD_{570} for the control.

On the other hand, 2MIC and 4 MIC treatments of *S.* Enteritidis reduced the OD_{570} measurments to 0.48 and 0.34 OD_{570} comparatively with 1.17 OD_{570} for the control. Meanwhile, for *E. coli* O26 reduced the biofilm mass to 0.51 and 0.31 OD_{570} as compared to 1.24 OD_{570} for the control.

However, 4 MIC of CAR loaded chitosan NPs reduced significantly ($P < 0.05$) the amount of biofilm mass for the strains of *E. coli* O26 and *L. monocytogenes* comparatively with 2 MIC and control treatments. While there is no significant difference between 2 MIC and 4 MIC in the treatment of *S.* Typhimurium and *S.* Enteritidis biofilms.

The antibiofilm effect of carvacrol was evaluated against *S.* Typhimurium and *S.* Enteritidis which exhibited antibiofilm activity against both serovars at sub-inhibitory concentrations. It was also observed a reduction of established *S.* Typhimurium biofilms about 5 logs with MIC and 2 MIC of carvacrol treatment **Amaral et al. (2015)**.

Burt et al. (2014) also reported that CAR was able to inhibit, at sub-lethal concentrations (< 0.5 mM), the formation of biofilms by *S.* Typhimurium, but at concentrations up to 8 mM had a very little or no activity against existing biofilms.

On the other hand, the inhibitory effect of CAR on the *E. coli* biofilms was stated by **Orhan-Yanıkan et al. (2019)** which displayed a significant effect ($p < 0.05$) and obtained a total biomass reduction of 63% for *E. coli* isolates.

CAR shows excellent antimicrobial and anti-biofilm activities, and is a very interesting bioactive compound against a wide range of Gram-positive and Gram-negative bacteria, and being active against both planktonic and sessile pathogens. Moreover, CAR lends itself to being combined with nanomaterials, thus providing an opportunity for preventing biofilm-associated infections by new bio-inspired, anti-microbial materials **(Marchese et al., 2018).**

Furthermore, the antibiofilm activity of chitosan NPs can be enhanced by conjugation with a wide range of bioactive compounds such as CAR. Moreover, the mechanism of its antimicrobial and antibiofilm properties is due to the electrostatic interaction between the positively charged

amino group of chitosan and negatively charged constituents of biofilm matrix such as exopolysaccharides, DNA, surface proteins and lipids. This interaction leads to the change of membrane permeability and dispersal of biofilm matrix **(Khan et al., 2020)**.

Conclusion and Recommendations

The obtained results of this study revealed that the food contact surfaces (FCS) and non-food contact surfaces (NFCS) in slaughterhouse environment may constitute a public health hazard, as it may be associated with food poisoing microorganisms such as pathogenic *E. coli*, *Salmonella* spp. and *L. monocytogenes*. Also, these microorganisms are capable of forming biofilms which considered a major source of contamination, transmission and infection.

Both CAR, chitosan NPs and CAR loaded chitosan NPs exhibited inhibitory activity against *E. coli*, *Salmonella* spp. and *L. monocytogenes*.

CAR loaded chitosan NPs was the potent antimicrobial against planktonic cells of *E. coli*, *S.* Typhimurium, *S.* Enteritidis and *L. monocytogenes* and was able to prevent or at least interfere with biofilm formation at MIC level on polystyrene surfaces. It also has a highly significant effect on the reduction of preformed biofilm at double minimum inhibitory concentration (2MIC) and 4 MIC level.

Based on our data, the following recommendations should be followed:

1- Carvacrol loaded chitosan NPs seems to be promising, cost effective, innovative eco-friendly sanitizers and definitely carry the potential to be developed into effective agents preventing and destroying existing biofilms in food industry.

2- The visited slaughterhouses need the following corrective actions:

- A set of standard sanitary operating procedures with effective cleaning and sanitizing of surfaces to reduce potential hazards.

- Slaughterhouses should have a sufficient number of rooms, appropriate to the operations being carried out such as evisceration and further dressing.

- Floor of slaughaterhouses should be made of suitable non-absorbent, non slip and non-toxic materials include sealed concrete, epoxy resin that are easy to clean, disinfect and maintain. Also, avoid materials that require high levels of maintenance or are not durable.
- Junctions between floors and walls should be smooth, sealed and rounded to facilitate cleaning.
- Smooth wall surfaces extend to a suitable height above the working area and should be light coloured to reflect light and so that dirt can be seen easily.

3- Further extensive and validated in vivo studies are advised to better test the safety and the efficacy of these nanomaterials to reproduce real clinical conditions, toxicity and their pharmacokinetic evaluation.

Summary

The present study was carried out to develop an eco-friendly and biodegradable nanomaterials , evaluate its efficacy against potential food borne pathogens and investigate their antibiofilm activity. Therefore, a total of 180 swabs from different slaughterhouses in Assiut Governorate represented as 90 swabs from food contact surfaces (FCS) and 90 from non-food contact surfaces (NFCS) were examined bacteriologically.

The bacteriological examination of the FCS samples revealed that 20 samples (22.2%) were positive for *E. coli*; represented as 2 (2.2%) for both O111:H2 and O44:H18, 3 (3.3%) for both O26:H11, O121:H7, O91:H21 and O146:H21and 4 (4.4%) for O128:H2. Also, 19 samples were positive for *Salmonella* spp. with an incidence (21.1%) identified as, *S.* Typhimurium (4.4%), *S.* Enteritidis (5.6%), *S.* Essen (4.4%), *S.* Infantis (2.2%), *S.* Tamale (3.3%) and *S.* Muenster (1.1%). Additionaly, 16 isolates of *Listeria* spp. with a percentage (17.8%) were isolated and represented as; 6 (6.7%) *L. monocytogenes*, 4 (4.4%) *L. innocua*, 2 (2.2%) *L. ivanovii* ,1 (1.1%) *L. welshimeri* and 3 (3.3%) *L. grayi*.

On the other hand, the investigation of the examined NFCS samples revealed that 23 samples were positive for *E. coli* with a percentage 25.6%; represented as 2 (2.2%) for both O111:H2, O146:H21and O119:H6, 1 (1.1%) for both O157:H7 and O121:H7, 3 (4.4%) for O91:H21, 5 (5.6%) for O26:H11 and 7 (7.8%) for O128:H2. Also, 18 samples were positive for *Salmonella* spp. with an incidence (20%) identified as, *S.* Typhimurium (5.6%), *S.* Enteritidis (5.6%), *S.* Essen (3.3%), *S.* Infantis(3.3%), *S.* Tamale (1.1%) and *S.* Muenster (1.1%). Additionaly, 13 isolates of *Listeria* spp. with a percentage (14.4%) were isolated and represented as ; 4 (4.4%) *L. monocytogenes*, 5 (5.6%) *L.*

innocua , 1 (1.1%) *L. ivanovii* , 1 (1.1%) *L. welshimeri* and 2 (2.2%) *L. grayi*.

Moreover, Assesment of biofilm formation capacity for 63 isolates was performed by microtiter plate method. Quantification of biofilm based on average optical density (OD) values determined at 570 nm revealed that isolates of *E. coli* were classified as strong biofilm producers (SBP) 4 (11.8%), moderate biofilm producers (MBP) 22 (64.7%), weak biofilm produces (WBP) 6 (17.6%) and 2 (5.9%) couldn't produce biofilm. While *Salmonella* spp. isolates were categorized as SBP 4 (21.1%), MBP 7 (36.8%), WBP 6 (31.6%) and 2 (10.5%) couldn't produce biofilm. Also, the isolates of *L. monocytogenes* were classified as SBP 1(10%), MBP 5 (50%), WBP 3 (30%) and 1(10%) couldn't produce biofilm.

Chitosan nanoparticles (NPs) and carvacrol (CAR) loaded chitosan NPs were synthesized and the formation of NPs was proved by FTIR, XRD, TEM and zeta potential measurements.

The antibacterial activity of CAR, chitosan NPs and CAR loaded chitosan NPs was evaluated against the isolated strains of *E. coli* O26:H11, *S.* Typhimurium, *S.* Enteritidis and *L. monocytogenes* that previously categorized as strong biofilm producers in growth media.

The investigation revealed that the minimum inhibitory concentration (MIC) of CAR was 0.93 mg/ml for both *E. coli* O26:H11 and *S.* Typhimurium, 1.87 mg/ml for *S.* Enteritidis and 3.75 mg/ml for *L. monocytogenes*. Moreover, the MIC of chitosan NPs was 1.25 mg/ml for *E. coli* O26:H11, 2.5 mg/ml for both *S.* Typhimurium and *S.* Enteritidis and 5 mg/ml for *L. monocytogenes*. Concerning CAR loaded chitosan NPs the MIC was 0.31 mg/ml for both E. coli O26:H11 and *S.*

Typhimurium and 0.62 mg/ml for both *S.* Enteritidis and *L. monocytogenes.*

The antibiofilm activity of CAR loaded chitosan NPs was investigated against *E. coli* O26:H11, *S.* Typhimurium , *S.* Enteritidis and *L.* monocytogenes. The findings revealed that CAR loaded chitosan NPs significantly inhibit their biofilm formation. In the presence of (0.5 MIC), the mean biofilm inhibition values were equal to 30.89, 44.4, 36.27 and 35.79 % and in the presence of MIC, the mean biofilm inhibition values were equal to 74.46, 74.26, 73.46 and 73.37 % while in the presence of 2 MIC, the mean biofilm inhibition values were equal to 87.22, 85.93, 78.06 and 77.76 % for *E. coli* O26:H11, *S.* Typhimurium, *S.* Enteritidis and *L. monocytogenes*, respectively.

Furthermore, compared to the control, a statistically significant reduction ($P < 0.05$) was noted in the established mature biofilm at the level of 2MIC and 4MIC of CAR loaded chitosan NPs for the all tested strains.

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الملخص العربي

- البيوفيلم هو تكدس وتجمع معقد للكائنات الحية المجهرية يتسم بإفراز نسيج خارج الخلية محصن ولاصق على أنواع مختلفة من الأسطح. تتميزالبكتريا المحصنة بالبيوفيلم بقدرتها على مقاومة فعالية المضادات الحيوية ما يصل إلى ألف ضعف مقاومة خلايا البكتريا الحرة (الغير محصنة بالبيوفيلم). و قد ثبت أن العديد من البكتريا المنتقلة بواسطة الغذاء تتميز بقدرتها على إفراز البيوفيلم وتعتبر مصدرا رئيسيا للتلوث ونقل العدوى.
- يمكن للعديد من مسببات الأمراض المنقولة عن طريق الغذاء مثل الإيشيركية القولونية و السالمونيلا والليستيريا مونوسيتوجينيس تكوين البيوفيلم علي الأسطح المختلفة سواء كانت ملاصقة للغذاء مثل المعدات و السكاكين و ألواح التقطيع أو غير ملاصقة للغذاء مثل الأرضيات و الجدران.
- تتأثر عملية تكون و تطور البيوفيلم بالعديد من العوامل، بما في ذلك السلالة البكتيرية و خواص الأسطح و بعض العوامل البيئية مثل درجة الحرارة ووجود المغذيات.
- يوجد الكثير من الطرق المستخدمة للقضاء علي البيوفيلم منها الفيزيائية و الكيميائية و البيولوجية. وحيث أن إستخدام المطهرات و المنظفات الكيميائية في البيئة المحيطة بالغذاء محدود لما لها من أثار صحية سلبية علي الإنسان لذا من الضرورى البحث عن بدائل طبيعية فعالة بدلا منها.
- حديثاً، هناك اهتمام كبير و متزايد لإستخدام الزيوت الطبيعية الأساسية و مشتقاتها الحيوية كمطهرات ذات تأثير قاتل للبكتيريا كأحد الحلول الأمنة و الفعالة بدلا من إستخدام المواد الكيميائية.
- و قد أظهر الكارفاكرول كأحد أهم مشتقات الزيوت الأساسية تأثيرات واعدة كمضاد للميكروبات التي تنتقل عن طريق الغذاء.

- فى الآونة الأخيرة اكتسبت المواد المحضرة باستخدام تقنية النانو إهتماما كبيرا، و خاصة في مجال إستخدامها كمواد مضادة للميكروبات.و تعتبر جزيئات الكيتوزان النانونية المستخلصة من القشريات واحدة من تلك المواد والتي تعمل على تثبيط مدى واسع من الميكروبات المفسدة والممرضة المكونة للفيلم الحيوي كما يمكن أن تكون حلا آمنا وصحيا للقضاء علي البيوفيلم
- وقد اجتذبت الجسيمات النانونية البوليمرية مثل جزيئات الكيتوزان النانونية إهتماما كبيرا حيث يتم إستخدامها كحامل للمركبات الحيوية مثل الكارفاكرول لكى تحتفظ بخصائصها الوظيفية ويمنع تكسيرها وتبخرها ويزيد من فاعليتها.
- لذلك فإن الهدف الأساسي من هذه الدراسة هو تخليق مركب جديد من المواد المضادة لميكربات التسمم الغذائى المكونة للبيوفيلم من خلال مواد طبيعية آمنه على صحه المستهلك عن طريق زيادة فاعلية جزيئات الكيتوزان النانونية من خلال تحميلها بالكارفاكرول.

<u>وقد إشتملت هذه الدراسة على جزئين:</u>

- **الجزء الأول: دراسة إستبيانية لمعرفة مدى تلوث الأسطح الملاصقة والغير ملاصقة للغذاء داخل مجازر اللحوم في محافظة أسيوط بميكروبات الإيشيريكية القولونية ، السالمونيلا و الليستيريا مونوسيتوجينيس وتقييم مدى قدرة العترات البكتيرية المعزولة على تكوين البيوفيلم.**
- **جمع العينات:** تم جمع عدد ١٨٠ مسحة من الأسطح المختلفة داخل المجازر وأشتملت على عدد ٩٠ مسحة من الأرضيات و الجدران (٤٥ من كل منها) و عدد ٩٠ مسحة من الأسطح الملاصقة للغذاء (٣٠ خطاطيف، ٣٠ سكاكين و ٣٠ من ألواح التقطيع) وتم نقلها تحت إشتراطات صحية إلي المعمل للفحص البكتريولوجي.

الفحص البكتريولوجى للعينات:

١- عزل بكتيريا الإيشيريكية القولونية

- أوضحت النتائج أن ٢٣ عينة من إجمالى ٩٠ عينة من الأسطح الغير الملاصقة للغذاء تحتوى على عترات مختلفة من ميكروب الإيشيريشيا كولاى بنسبة ٢٥.٥% ، كما تواجد الميكروب فى ٢٠ عينة من إجمالى ٩٠ عينة من الأسطح الملاصقة للغذاء داخل المجازر بنسبة ٢٢.٢%.

٢- عزل ميكروب السالمونيلا

- أوضحت النتائج أن ١٨ عينة من إجمالى ٩٠ عينة من الأسطح الغير الملاصقة للغذاء تحتوى على ميكروب السالمونيلا بنسبة ٢٠% و تم تصنيف سالمونيلا تيفيموريم بنسبة (٥.٥%) و سالمونيلا انتيريتيدس بنسبة (٥.٥%)

.

- كما وجد أن ١٩ عينة من إجمالى ٩٠ عينة من الأسطح الملاصقة للغذاء تحتوى على ميكروب السالمونيلا بنسبة (٢١%) و تم تصنيف سالمونيلا تيفيموريم بنسبة (٤.٤%) و سالمونيلا انتيريتيدس بنسبة (٥.٥%)

٣- عزل ميكروب الليستريا

- أظهر التحليل الميكروبيولوجى للعينات أنه قد تم عزل عترات من الليستريا مونوسيتوجينيس من ٤ عينات من إجمالى عدد عينات الأسطح االغير ملاصقة للغذاء بنسبة (٤.٤%) ومن ٦ عينات من الأسطح الملاصقة للغذاء بنسبة (٦.٦%) .
- تم تقييم قدرة عدد ٦٣ عترة من العترات المعزولة على تكوين البيوفيلم باستخدام طريقة أطباق الميكروتيتر و اشتملت هذه العترات علي عدد سالمونيلا تيفيموريم (٩)، سالمونيلا انتيريتيدس (١٠)، ليستيريا مونوسيتوجينيس(١٠) و الإيشيريكية القولونية (٣٤).

- وقد أظهرت النتائج أنه تم تصنيف عترات الإيشريشيكية القولونية لى عترات قوية الإنتاج للبيوفيلم بنسبة ١١.٧% و عترات متوسطة القوة بنسبة ٦٤.٧% و عترات ضعيفة الإنتاج بنسبة ١٧.٦% بينما العترات التي لم تستطيع تكوين البيوفيلم كانت بنسبة ٥.٨%.
- بينما تصنيف عترات السالمونيلا إلى عترات قوية الإنتاج للبيوفيلم بنسبة ٢١% و عترات متوسطة القوة بنسبة ٣٦.٨% و عترات ضعيفة الإنتاج بنسبة ٣١.٥% بينما العترات التي لم تستطيع تكوين البيوفيلم كانت بنسبة ١٠.٥%.
- ايضا تم تصنيف عترات الليستيريا مونوسيتوجينيس إلى عترات قوية الانتاج للبيوفيلم بنسبة ١٠% و عترات متوسطة القوة بنسبة ٥٠% و عترات ضعيفة الانتاج بنسبة ٣٠% بينما العترات التي لم تستطيع تكوين البيوفيلم كانت بنسبة ١٠%.

الجزء الثاني: تم تخليق جزيئات الكيتوزان النانونية و جزيئات الكيتوزان النانونية المحملة بالكارفاكرول و دراسة تأثير كل منهما بالاضافة الي الكارفاكرول ضد عترات الإيشيريكية القولونية والسالمونيلا والليستيريا مونوسيتوجينيس قوية الإنتاج للبيوفيلم وتأثير جزيئات الكيتوزان النانونية المحملة بالكارفاكرول علي البيوفيلم.

- وقد اظهرت النتائج بالنسبة للكارفاكرول أن أقل تركيز مثبط لنمو بكتيريا الإيشيريكية القولونية و سالمونيلا تيفيميوريم ٠.٩٣ مجم/مل بينما كان ١.٨٧ مجم/مل للسالمونيلا انتيريتيدس و٣.٧٥مجم/مل لليستريا مونوسيتوجينيس.
- بينما فى حال جزيئات الكيتوزان النانونية كان أقل تركيز مثبط لنمو بكتيريا الإيشيريكية القولونية هو ١.٢٥ مجم/مل و ٢.٥ مجم/مل لكل من سالمونيلا تيفيموريم و سالمونيلا انتيريتيدس و ٥ مجم/مل لليستيريا مونوسيتوجينيس.

- بالنسبة لجزيئات الكيتوزان النانونية المحملة بالكارفاكرول كانت الأكثر تأثيرا على الميكروبات محل الدراسة حيث كان اقل تركيز مثبط لنمو بكتيريا الإيشيريكية القولونية و سالمونيلا تيفيموريم ٠.٣١ مجم/مل بينما كان ٠.٦٢ مجم/مل لكل من سالمونيلا انتيريتيدس و ليستيريا مونوسيتوجينيس.
- علاوة على ذلك تم دراسة تأثيرنشاط جزيئات الكيتوزان النانونية المحملة بالكارفاكرول ضد تكوين البيوفيلم وقد وجد أنه تم منع تكوين البيوفيلم بشكل كبير و ملحوظ فى وجود أقل تركيز مثبط لنمو الميكروبات بنسبة ٧٤.٤٦% و ٧٤.٢٦% و ٧٣.٤٦% و ٧٣.٣٧% و فى وجود ضعف أقل تركيز مثبط لنمو البكتيريا بنسبة ٨٧.٢٢% و ٨٥.٩٣% و ٧٨.٠٦ % و ٧٧.٧٦ % لكل من الإيشيريشيا كولاى O26:H11 و سالمونيلا تيفيميوريم و سالمونيلا انتيرتيدس و ليستيريا مونوسيتوجينيس على الترتيب .
- و ايضا تم دراسة تأثير جزيئات الكيتوزان النانونية على البيوفيلم الذي تكون مسبقا ووجد تأثير ملحوظ وكبير في القضاء علي البيوفيلم في وجود ضعف أقل تركيز مثبط لنمو البكتيريا و تركيز ٤ أضعاف أقل تركيز مثبط لنمو كل ميكروب ، و ذلك من خلال تحليل النتائج إحصائياً .
- **هذا وقد تمت مناقشة تأثير الميكروبات المعزولة علي صحة المستهلك و تقييم مدى قدرتها على تكوين البيوفيلم . وقد تم وضع المقترحات الواجب إتباعها لمنع تلوث الأغذية وخاصة اللحوم داخل المجازر بهذه الميكروبات. كما أوصت الدراسة بإستخدام مركب جزيئات الكيتوزان النانونية المحملة بالكارفاكرول فى عملية التطهير للأسطح المختلفة داخل المجازر حيث انها اثبتت كفاءة و فعالية في القضاء على ميكروبات التسمم الغذائي ومنع تكوين البيوفيلم والتخلص منه بالإضافة لكونها مواد امنة و صديقة للبيئة .**