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Study of the biofilm forming capacity of E. coli strains of animal origin.

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Dedication

To my adorable mother Saliha

To the woman who has always been by my side to support and encourage me to face various obstacles, your kindness guides me, and your presence by my side has always been my source of strength.

My dear father Djeloule

To the man who suffered without letting me suffer,
who never said no to my demands, and who spared no effort to encourage.
me and make me happy.

To my sister Khadidja

I wish you success in your life and to be filled with happiness. Thank you for always being by my side and continuously encouraging me.

To my brothers Mohamed and Sofiane

Whom I adore immensely, so they can be proud of their little sister.

To my friends Fatiha, Djihed, Hadjer, Meriem, and Houda

We formed a great team with beautiful moments. I thank you for everything you brought to me over the past year.

To all my colleagues and to my entire microbiology class

Dedication

To my dear Mom Nora

the most extraordinary mom in the world,who made me who I am today.
Thank you for all your sacrifices and your eternal love, for encouraging me and always believing in me.

I love you, Mama

To my beloved father Nour Eddine

I miss you so much. Making you proud is one of my goals. I will never forget your precious advice and the love you gave me.

To my brothers Mohamed and Abd Elrahmane

who have always been there for me, my success is your success too.

I love you.

To my sisters Khadidja and Rahma

My best, my strength.Thank you for always being there for me and for your support.

. I love you so much, my dears

To my sister Asmaa and my beloved nieces Alaa and Abd allah

Thank you for the love and happiness you bring into my life.

I love you, my dears..

To my partner Wassila

It is such a pleasure to work together. Thank you for your kindness, trust, and especially for your understanding throughout this work. You are the best

To my friends Djihed, Hadjer, Meriem, and Houda

We formed a great team with beautiful moments. I thank you for everything you brought to me over the past year.

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Our thanks and appreciation also go to all the people, near and far, who contributed with us, even if with just a word of encouragement.

-Abstract:

A biofilm is a structured community of microorganisms that adhere to surfaces and are embedded within a self-produced matrix of extracellular polymeric substances (EPS), biofilms can form on various biotic and abiotic surfaces. It plays a significant role in various fields including medicine, industry, food safety, and environmental science.

This study focuses on the formation of biofilms by enterobacteria, which are responsible for colibacillosis in Algeria, the primary objective of this study is to assess the biofilm forming capacity using tissue culture plate method (TCP) in 96 well plates, and optical density (OD) value of the formed biofilm that measured on an ELISA Microplate reader at 600 nm on a total of 23 bacterial isolates from the liver of broiler chicken.

The disinfectants utilized in this study include Chlorhexidine, and quaternary ammonium,the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of these disinfectants are determined. Additionally, the antibiotic resistance profile of the selected isolates is established by VITEK 2 system.

Furthermore, the f*liC,tsh,csgA* and *PapG II* genes are detected using PCR in 23 strains of *E. coli*.

The study results show that all strains of enterobacteria can form biofilms, although to different degrees. Chlorhexidine emerges as the most effective disinfectant then quaternary ammonium. Additionally, every strain exhibits resistance to at least one of the antibiotics tested.

The identification of *fliC,tsh,csgA* and *PapG II* genes confirms the presence of those genes in certain selected isolates which provide important insights into their ability to form biofilm ,these findings emphasize the importance of implementing robust preventive and control measures to combat the formation of biofilms and antibiotic resistance in enterobacteria responsible for colibacillosis.

Keywords: Biofilm, Enterobacteria, *Escherichia coli*, colibacillosis,Disinfectants, antibiotic resistance.

-Résumé :

Un biofilm est une communauté structurée de microorganismes qui adhèrent aux surfaces et sont intégrés dans une matrice autoproduite de substances polymériques extracellulaires (EPS). Les biofilms peuvent se former sur diverses surfaces biotiques et abiotiques. Ils jouent un rôle significatif dans divers domaines, notamment la médecine, l'industrie, la sécurité alimentaire et les sciences environnementales.

Cette étude se concentre sur la formation de biofilms par les entérobactéries, responsables de la colibacillose en Algérie. L'objectif principal de cette étude est d'évaluer la capacité de formation de biofilm en utilisant la méthode de la plaque de culture tissulaire (TCP) dans des plaques à 96 puits, et la valeur de densité optique (DO) du biofilm formé mesurée sur un lecteur de microplaques ELISA à 600 nm sur un total de 23 isolats bactériens provenant du foie de poulets de chair.

Les désinfectants utilisés dans cette étude comprennent la chlorhexidine et les ammoniums quaternaires, et la concentration inhibitrice minimale (CIM) et la concentration bactéricide minimale (CBM) de ces désinfectants sont déterminées. De plus, le profil de résistance aux antibiotiques des isolats sélectionnés est établi par le système VITEK 2.De plus, les gènes *fliC, tsh, csgA* et *PapG II* sont détectés par PCR dans 23 souches d'E. coli.

Les résultats de l'étude montrent que toutes les souches d'entérobactéries peuvent former des biofilms, bien que à des degrés différents. La chlorhexidine émerge comme le désinfectant le plus efficace que l’ammoniums quaternaires. De plus, chaque souche présente une résistance à au moins l'un des antibiotiques testés.

L'identification des gènes *fliC, tsh, csgA* et *PapG II* confirme la présence de ces gènes dans certains isolats sélectionnés, fournissant des informations importantes sur leur capacité à former des biofilms. Ces résultats soulignent l'importance de mettre en œuvre des mesures préventives et de contrôle robustes pour lutter contre la formation de biofilms et la résistance aux antibiotiques chez les entérobactéries responsables de la colibacilloses.

Motsclés: Biofilm,Entérobactérie,*Escherichia coli*, colibacillose, Désinfectants, résistance aux antibiotiques.

-ملخص:

الأغشية الحيوية عبارة عن مجتمع منظم من الكائنات الحية الدقيقة التي تلتصق بالأسطح وتكون مدمجة داخل مصفوفة منتجة ذاتيًا من المواد البوليمرية خارج الخلية (EPS)، ويمكن أن تتشكل الأغشية الحيوية على أسطح حيوية وغير حيوية مختلفة، تلعب دورًا مهمًا في مختلف المجالات بما في ذلك الطب والصناعة وسلامة الأغذية وعلوم البيئة.

تركز هذه الدراسة على تكوين الأغشية الحيوية بواسطة البكتيريا المعوية المسؤولة عن داء العصيات القولونية في الجزائر،و الهدف الأساسي من هذه الدراسة هو تقيم قدرة تكوين الاغشية الحيوية باستخدام طريقة لوحة زراعة الانسجة (TCP) في96 بئر،والكثافة البصرية .(OD)قيمة الاغشية الحيوية المتكونة التي تم قياسها على قارئ صحيفة ELISAعند600 نانومتر على اجمالي 23عزلة بكتيرية من كبد الدجاج اللاحم .

المطهرات المستخدمة في هذه الدراسة تشمل الكلورهيكسيدين والأمونيوم الرباعي، وتم تحديد الحد الأدنى للتركيز المثبط (MIC) والحد الأدنى لتركيز مبيد الجراثيم (MBC) لهذه المطهرات. بالإضافة إلى ذلك، تم إنشاء ملف مقاومة المضادات الحيوية للعزلات المختارة بواسطة نظام VITEK2 .

علاوة على ذلك، تم الكشف عن جينات *fliC* و*tsh* و*csgA* و*PapG II* باستخدام تفاعل البوليميراز المتسلسل في 23 سلالة من الإشريكية القولونية .

أظهرت نتائج الدراسة أن جميع سلالات البكتيريا المعوية يمكن أن تشكل أغشية حيوية، و بدرجات مختلفة. يظهر الكلورهيكسيدين باعتباره المطهر الأكثر فعالية من الأمونيوم الرباعي. بالإضافة إلى ذلك، تظهر كل سلالة مقاومة لواحد على الأقل من المضادات الحيوية التي تم اختبارها.

إن تحديد جينات *fliC* و*tsh* و*csgA* و*PapG II* يؤكد وجود تلك الجينات في بعض العزلات المختارة والتي توفر رؤى مهمة حول قدرتها على تكوين الأغشية الحيوية، وتؤكد هذه النتائج على أهمية تنفيذ تدابير وقائية ومراقبة قوية لمكافحة تكوين الأغشية الحيوية ومقاومة المضادات الحيوية في البكتيريا المعوية المسؤولة عن داء العصيات القولونية.

الكلمات المفتاحية: الأغشية الحيوية، البكتيريا المعوية، الإشريكية القولونية، العصيات القولونية، المطهرات، مقاومة المضادات الحيوية.

List of Abbreviations

DNA : Deoxyribonucleic Acid.

ATB : Antibiotic

EB : Ethidium Bromide.

MDR : Multidrug-Resistant

QACs : Quaternary Ammonium Compounds.

MBC : Minimum Bactericidal Concentration.

MIC : Minimum Inhibitory Concentration.

CV : Crystal Violet.

OD : Optical Density.

E. COLI : Escherichia coli.

DE : Distilled Water.

ELISA : Enzyme-Linked Immunosorbent Assay.

EPS : Exopolysaccharides

MH : Mueller Hinton.

PBS : Phosphate Buffered Saline.

PCR : Polymerase Chain Reaction .

PH : Potential Hydrogen .

QQ : Quorum Quenching .

QS : Quorum Sensing.

QSI : Quorum Sensing Inhibitors

R : Resistant.

S : Sensitive.

I : Intermediate

TBE : Tris-Borate-EDTA .

TCP : Tissue Culture Plate .

TSB : Tryptic Soy Broth .

UV : Ultraviolet.

SBP : Strong biofilm production.

MBP : Moderate biofilm production.

WBP : Week biofilm production.

NBP : Non biofilm formation.

AMP : Ampicillin .

AMC : Amoxicillin

CHX : Chlorhexidine.

CIP : Ciprofloxacin

AN : Amikacin.

IMP : Imipenem.

ETP : Ertapenem.

CAZ : Ceftazidime.

CTX : Cefotaxime.

FOS : Fosfomycin.

CZ : Cefazolin.

CXT : Cefoxitin.

PIP : Piperacillin.

NTR : Nitrofurantion.

SXT : Strimethoprim-sulfamethoxazole.

CHL : Chloramphenicol.

GN : Gentamicin.

UIT : Urinary tract infection

ID : Identification.

FLI : Freidrich Loeffler institut.

IBIZ : Institut of bactereil infection and zoonose.

BP : Base pair.

MIC 90 : Minimum Inhibatory Concentration that kill 90%

UPEC : Uropathogenic Escherichia coli.

APEC : Avian Pathogenic Escherichia coli

MALDI/TOF-MS : Matrix-Assisted Laser Desorption/Ionization.

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Introduction.

Introduction: -

Microorganisms have an extraordinary ability to live in almost any environment and protect themselves from external agents through sophisticated survival mechanisms. Bacteria can be found either in planktonic form or, under specific conditions, as sessile aggregates on biotic and abiotic surfaces, forming complex structures known as biofilms.

Biofilms are complex, structured communities of microorganisms that adhere to surfaces and are embedded within a self-produced matrix of extracellular polymeric substances (EPS) (Costerton et al., 1999)[1]. These microbial consortia can form on a wide array of biotic and abiotic surfaces, playing crucial roles in various fields such as medicine, industry, food safety, and environmental science (Hall-Stoodley et al., 2004)[2]. In the context of animal health, biofilm formation by pathogenic bacteria poses significant challenges, particularly in poultry farming, where enterobacteria like *Escherichia coli* are known to cause severe infections such as colibacillosis (Dho-Moulin and Fairbrother, 1999)[3].

Colibacillosis, primarily affecting broiler chickens, is a major concern in Algeria's poultry industry due to its impact on animal health and productivity (Mellata, 2013)[4]. The ability of *E. coli* to form biofilms enhances its survival in hostile environments, contributes to its virulence, and complicates treatment by providing a protective barrier against antibiotics and disinfectants (Donlan, 2002)[5]. This poses a major problem in terms of animal and public health.

Faced with this problematic, this study seeks to address the following research questions: What is the extent of biofilm formation among different *E. coli* strains isolated from broiler chickens in Algeria? How effective are common disinfectants, such as chlorhexidine and quaternary ammonium compounds, against these biofilms? What is the antibiotic resistance profile of these biofilm-*forming E. coli* strains? Additionally, which biofilm-associated genes (e.g., *fliC, tsh, csgA,* and *PapG II*) are present in these strains, and how do they contribute to biofilm formation?

To achieve this objective, this thesis will focus on biofilm formation capacity and evaluating various disinfectants and to determine their effectiveness against Enterobacteria biofilms ,the antibiotic resistance profile and study the presence of the genes responsible for biofilm formation.

Introduction.

The aim of our study is to:

• Identify the species of Enterobacteria(23 poultry samples) responsible of colibacillosis using MALDI-TOF.

• Determine the biofilm production potential using the tissue culture plate method (TCP).

• Determine the level of tolerance to two disinfectants (Quaternary Ammonium, Chlorhexidine) in terms of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

• Determine resistance to different classes of antibiotics.

• Detect the *fliC, tsh, csgA and PapG II* genes in selected strains.

-Understanding the biofilm forming capacity of *E. coli* strains from animal origins is essential for developing effective preventive and control strategies.

CHAPTER I

Overview of biofilm

I.1.Biofilm:

I.1.1.Definition of biofilm:

In 1978, William J. Costerton introduced the term "biofilm" to describe the collective presence of bacteria adhering to surfaces (Hajiagha and Kafil 2023) [1] . They described a biofilm as a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (Van Houdt and Michiels 2005) [2]. This biofilm mode of growth can be seen as a specific lifestyle compared to the planktonic lifestyle and formation of a biofilm.

I.1.2. Biofilm formation in Enterobacteria, *E.coli* as Example:

Several species of enterobacteria have the ability to form biofilms (Harkins et al., 2015) [3]. However, the significant genetic diversity within the same species means that the genes and proteins used during biofilm formation can vary from one strain to another (Giaouris, Heir et al., 2015)[4].

I.2. Escherichia coli :

Escherichia coli (E. coli) is a Gram-negative, rod-shaped, facultative anaerobic bacterium. Most *E. coli* strains harmlessly colonize the gastrointestinal tract of humans and animals as a normal flora(Lim, Yoon, and Hovde 2010) [5]. It has the ability to secrete toxins, polysaccharides, and form a biofilm . It can also form a biofilm in vitro (Naves et al., 2010) [6]. The capsules of *E. coli* consist of high molecular weight molecules attached to the surface of the cell. These capsules indirectly contribute to the formation of the biofilm by protecting the bacterial surface adhesion. Various environmental conditions influence E. coli's capacity to form a biofilm (Larson EL, et al., 2003) [7].

I.3. The stages of biofilm formation :

Formation of the biofilm matrix inducs a unique environment for bacteria that allows the dynamic biofilm mode of life. Biofilms, and the resulting lifestyle, are built in specific, defined steps, producing a bacterial community that is heterogeneous in space and time (Flemming et al., 2010) [8].

I .3.1. Adhesion Phase:

Zobell demonstrated that adhesion consists of two alternative stages: reversible adhesion involves a physicochemical process and constitutes a non-specific stage, while

irreversible adhesion involves a biological phenomenon, which is considered a specific stage(Zobell .,1943)[9].

I.3.1.1. Reversible Adhesion:

In a liquid environment or when exposed to moisture, planktonic bacteria approach a solid surface through Brownian motion, sedimentation, or active mobility (presence of flagella). They attach reversibly through non-specific, electrostatic, and electrodynamic interactions. This stage is influenced by environmental conditions including pH, osmolarity of the medium, temperature, and concentration and nutrient concentration, as well as the fluid's hydrodynamics (Beloin et al., 2008)[10]. Bacterial adhesion is also influenced by the nature of the surface, particularly its roughness and hydrophobicity. Bacteria adhere more easily to rough, hydrophobic, and non-polar surfaces.

I.3.1.2. Irreversible Adhesion:

During this adhesion phase, there is a specific fixation of microorganisms on a support. This stable adhesion is made possible by the secretion of extracellular polymers that form binding bridges between the cell and the surface or between two cells (Beloin, 2008)[10].

In Irreversible Adhesion, by contrast, bacteria adhering to the surface do not move,either by swimming or Brownian motion. In general, bacteria that have become immobilized on the surface are described as irreversibly adhered to the surface, while cells that can still swim along the surface are described as reversibly adhered_(Vigeant,2002)[11].

A certain number of reversibly adsorbed cells remain immobilized and become irreversibly adsorbed. As bacteria multiply, their adhesion becomes stronger, and they differentiate by altering their gene expression pattern to promote their survival (Garrett et al., 2008)[12];(Philips et al., 2011)[13]. fimbriae and curli in *E. coli* interact with specific receptors present on the surface (Beloin ,2008)[14].

I.3.2. Micro-Colony Aggregation :

The microorganisms, adhering to the carrier surface, grow and multiply slowly to form Colonies,they aggregate with each other and form micro-colonies, which are protected by the EPS matrix (Jacobsen, 2008)[15],Then, the continuous proliferation of microorganisms makes their biological growth space crowded with a lack of nutrients and accumulation of toxic substances. At

this stage, the quorum-sensing (QS) signals, a cell-to-cell communication mechanism in

bacteria, could induce the *E. coli* into the biofilm lifestyle by regulating the synthesis of biofilm matrix compounds (Yang,2010) [16]. During this process, EPS is significantly increased, forming
a gel phase on the surface of the carrier to protect the microorganisms(Simões,2010)[17.

I.3.3. Biofilm Maturation :

The complex architecture of a biofilm forms with the development of aqueous channels and pores between microcolonies, allowing the transport of oxygen and nutrients necessary for the growth of microorganisms, as well as the removal of waste (Filloux ,2003)[18]. The production and secretion of enzymes or toxins cause the degradation of the surrounding host tissues, thereby releasing nutrients (Jacobsen , 2008)[19]. A biofilm is composed of approximately 85% extracellular matrix and 15% microorganisms (Behlau, 2008)[20]. The extracellular matrix is primarily composed of water (97%) and also includes exopolysaccharide polymers, proteins, nucleic acids, phospholipids, nutrients, and metabolites (Beloin, 2008)[10]. This matrix has both protective and structural roles.

Bacteria acquire a mode of growth, physiology, and metabolism different from planktonic bacteria. These phenotypic changes result from an evolution of their gene expression profiles. For instance, in *E. coli*, there is a 38% difference in gene expression between bacteria attached to biofilms and planktonic bacteria (Tolker-Nielsen, 2000)[21]. All these transformations are coordinated by a communication system among bacteria of the same species within the biofilm, called quorum sensing (Behlau, 2008)[20]. This system is based on the production of diffusible molecules by grown bacteria, such as acyl-homoserine lactones in Gram-negative bacteria, which provide an indication of cell density in a given environment (Filloux, 2003)[18].

Mature biofilms possess a three-dimensional structure with several different microenvironments that evolve according to osmolarity, pH, and cell density. Due to this heterogeneity, the metabolic state of a bacterium varies depending on its location, resulting in a variety of bacterial phenotypes within a biofilm.

Close contacts between microorganisms promote the horizontal transfer of genetic material. This phenomenon can notably facilitate the spread of antibiotic resistance genes (Trautner, 2004)[22].

I.3.4. Bacterial Detachment :

Bacteria detach from the biofilm and disperse into the surrounding environment after reverting to the planktonic state. Traditionally, bacterial detachment is considered a passive phenomenon, largely dependent on the flow forces of the medium in which the biofilm is located. However, bacterial detachment can also be an active strategy initiated by the bacteria themselves, allowing them to colonize new surfaces and survive when space and nutrients become limited. Bacteria can detach individually or in small or large clusters depending on the mechanisms involved (Kaplan, 2010)[23]. Like other stages, bacterial detachment is a complex process that involves environmental signals and communication between the bacteria.

Thus, an established biofilm serves as a reservoir of viable bacteria capable of colonizing other surfaces. Bacteria acquire a mode of growth, physiology, and metabolism different from planktonic bacteria. These phenotypic changes result from the evolution of their gene expression profiles. For instance, in *E. coli*, there is a 38% difference in gene expression between bacteria attached to biofilms and planktonic bacteria (Tolker-Nielsen, 2000)[21]. All these transformations are coordinated by a communication system among bacteria of the same species within the biofilm, called quorum sensing (Behlau, 2008)[20]. This system is based on the production of diffusible molecules by bacteria, such as acyl-homoserine lactones in Gram-negative bacteria, which provide an indication of cell density in a given environment (Filloux, 2003)[18].

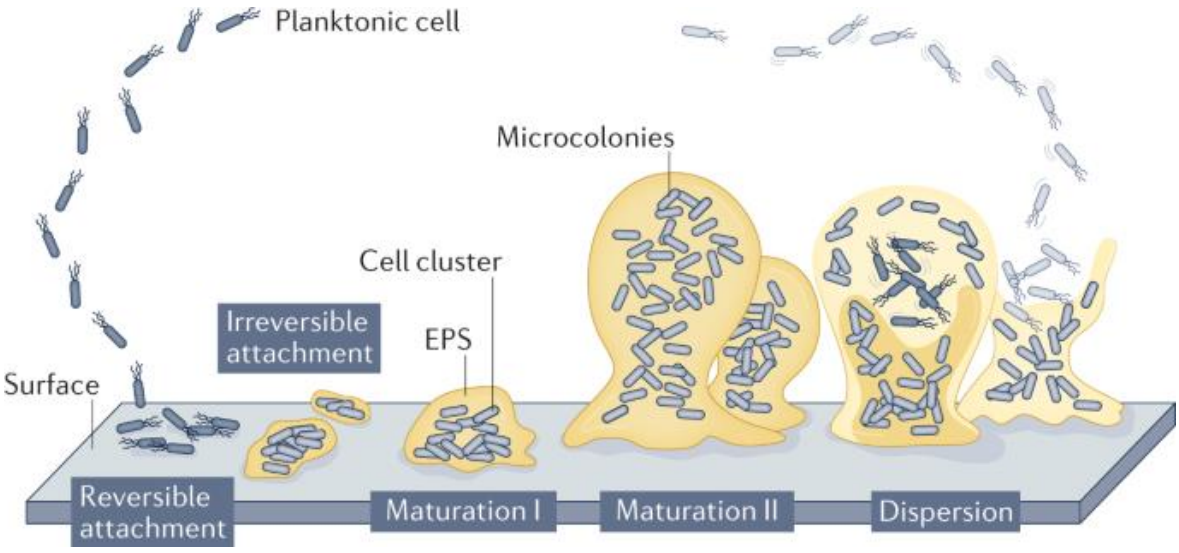


Figure I .1: Stages of biofilm formation.

I .4 . The factors influencing the formation of biofilm:

I .4 .1. The surface:

Cellular attachment is significant on rough surfaces, and the physico-chemical properties exert an influence on it. Microorganisms adhere more easily to hydrophobic and non-polarized surfaces (such as glass, metals, etc.), and the prior presence of protein films (blood, tears, urine, etc.) influences bacterial attachment and promotes biofilm formation (Bezoui., 2016)[24].

- **Surface Roughness:** The rougher the surface, the greater the colonization of that surface by micro-colonies. Rough surfaces are preferentially colonized due to weaker repulsive forces and increased attachment surfaces facilitated by surface roughness. However, certain strains of wild bacteria also thrive on smooth surfaces (Donlan & Costerton, 2002)[25].
- **Physical and Chemical Properties of the Surface***:* The physicochemical properties of the surface affect the rate and extent of adhesion. Microorganisms adhere more readily to hydrophobic and non-polar surfaces such as Teflon or other plastics than to hydrophilic materials such as glass or metal.
- **Presence of Protein Films on the Surface:** The presence of protein polymers on the surface of a material alters its physicochemical properties and directly influences bacterial attachment to that surface. Indeed, the prior presence of a protein film such as blood, tears, urine, saliva, interstitial fluid, and respiratory secretions on a biomaterial affects bacterial adhesion to its surface and promotes biofilm formation (Nobbs, 2009)[26].

I .4. 2. Characteristics of the environment:

The formation and dispersion of biofilm require precise enzymatic equipment and specific structural entities, the activation of which depends on key environmental factors (Donlan., 2002) [27].

- **Temperature:** Temperature is important not only because it affects bacterial metabolism and enzymatic activity, but also because it affects certain physicochemical parameters (pH, ionic activity, thermal agitation, and gas solubility) as well as bacterial surface properties. Growth temperature can have a dramatic effect

on cell electrophoretic mobility and flagella production, thereby affecting adhesion (Bellifa Samia, 2014)[28].

The formation of biofilms by *E. coli* was significantly reduced at lower temperatures (4-20°C) compared to its optimal growth temperature of 37°C, indicating the importance of metabolic activity for biofilm development.(Jahid,2014)[29].

- **pH:** The pH of the surrounding environment alters the surface charge of microbes and solid support, as the ionization balance (protonation/deprotonation) of exposed functional groups shifts according to their pKa (Bellifa Samia, 2014)[28]. Repulsive electrostatic interactions detrimental to adhesion are thus reduced or increased (Boutaleb, 2007)[30]. The formation of biofilms by *E. coli* is significantly influenced by pH levels. Studies have shown that E. coli biofilm formation is optimal at neutral to slightly acidic conditions. For instance, biofilm formation was strongly supported at pH 7.4, biofilm formation was minimal. Conversely, at pH 5.5.(Mathlouthi,2018)[31].
- **Available carbon sources:** They influence biofilm formation and maturation (Martinez, 2007)[32] glucose and glycerol, affect the gene expression and production of extracellular matrix components in *E. coli*. The results indicate that glucose significantly enhances the production of biofilm matrix components.(Jackson,2002) [33].
- ***Nutrient concentrations*:** In a static environment, nutrient concentration must be high for biofilm formation to occur; this is not the case for a hydrodynamic environment (Spormann, 2008)[34].The composition of the nutrient medium significantly impacts E. coli biofilm development. For instance, *E. coli* biofilms were shown an increase in the number of adherent cells with E. coli strains in nutrient-rich medium such as LB, without salt LB or tryptic soy broth (TSB).(Lajhar,2018)[35].

I .4.3. Cell wall hydrophobicity Properties :

E. coli cells with increased surface hydrophobicity exhibited enhanced initial attachment to surfaces and formed denser and more stable biofilms compared to wild-type cells. This demonstrated that the hydrophobicity *of E. coli* cell surfaces directly influenced their ability to adhere to surfaces and form biofilms (Rosenberg,2019)[36].

I.5. Determinants of Biofilm Formation in E. coli:

Among the many virulence factors described in *E. coli*, several are known to be involved to varying degrees in biofilm formation. These include flagella and curli and EPS.

I.5.1. Exopolysaccharides :

The EPS represent the "house of the biofilm cells." The EPS determine the immediate conditions of life of biofilm cells living in this microenvironment by affecting porosity, density, water content, charge, sorption properties, hydrophobicity, and mechanical stability (Flemming et al.,2007)[37]. Exopolysaccharides (EPS) are important in the attachment of bacteria to substrata and thus development of biofilms, Exopolysaccharides are excreted from multiple bacterial species, which make biofilms a good source for screening exopolysaccharides producing bacteria, During the process of colonization on particular surfaces, bacteria produces extracellular polymeric substance which construct the biofilm matrix(Kumar, Anandapandian, and Parthiban 2011)[38].

I.5.2. Flagella:

Flagella are extracellular filamentous appendages with a helical structure, inserted into the inner membrane. They allow the bacterium to move in liquid or semi-liquid environments and play a role in chemotaxis. A study demonstrated the decisive role of flagella in the initiation of biofilm formation. They promote the initial attachment of bacteria to a surface by overcoming repulsive electrostatic forces and enable their spread along this surface (Pratt & Kolter, 1998)[39]. However, some non-motile strains are also capable of producing biofilm. This is the case with enteroaggregative *E. coli* (Sheikh et al., 2001)[40] or strains overexpressing surface adhesins such as curli (Prigent-Combaret et al., 2000)[41].

I .5.3. Curli:

Curli are very fine protein fibers that aggregate on the surface of bacteria to form structures 6 to 12 nm in diameter and 0.5 to 1 µm long (Beloin, 2008)[14]. They can bind to extracellular matrix proteins such as fibronectin, laminin, and plasminogen, allowing bacteria to adhere to different types of human cells. They also facilitate initial attachment to inert surfaces (Beloin ,2008)[14]. Curli enable bacteria to aggregate and form clusters, a property used in the initial stage of biofilm development. They are also believed to induce the host inflammatory response (Barnhart, 2006)[42].

The genes involved in curli synthesis are located on the *csgBAC and csgDEFG* operons, transcribed in opposite directions. The *CsgA* protein, called curlin.

The synthesis of curli is thus under the influence of complex regulation, depending notably on temperature, osmolarity, pH, and oxygenation of the environment (Beloin ,2008)[10].

Curli were initially described in *E. coli* but are also present in other species of Enterobacteriaceae (*Shigella sp., Citrobacter sp., and Enterobacter sp*.) (Beloin,2008)[10].

Among *E. coli* strains, curli are expressed in most enterohemorrhagic and enterotoxigenic strains but are absent in enteroinvasive and enteropathogenic strains (Van Houdt, 2005)[43].

I.6. Regulatory Mechanism of Biofilm Formation :

I.6.1. Quorum Sensing (QS) :

The formation of a biofilm is controlled by regulatory mechanisms such as Quorum Sensing (QS). These are control mechanisms within cells, manifested by cell-to-cell signals, via chemical mediators, and dependent on the quantity of cells present: these are referred to as mechanisms of cell density perception. These mechanisms are based on the principle of critical mass. Once signal molecule concentrations reach a threshold value, transcriptional regulators are activated and control specific genes (Costerton, 1999)[44]. QS regulates biofilm physiology by modulating biofilm population size and taking into account environmental conditions. It initiates phenomena of dispersion and swarming of planktonic bacteria from the biofilm (Irie, 2008)[45].

I.6.2. Quorum Quenching (QQ):

Disruption of quorum sensing (QS) communication mechanisms can lead to significant phenotypic changes in biofilm microorganisms, such as increased sensitivity to antibiotics or antiseptics, as well as abnormalities in the biofilm development cycle, particularly during formation and dispersion stages (Irie, 2008)[45]. Various strategies have been developed to inhibit these QS-regulated behaviors by blocking communication between bacteria. This inhibition method of QS is known as "quorum quenching" (QQ). Since the production and detection of signaling molecules are key components of QS, QQ can interfere with this system in different ways, whether at the intracellular or extracellular level. The use of inhibitory molecules, called QS inhibitors (QSI), allows intervention at the bacterial cell level to inhibit the production or perception of signaling molecules. Other strategies directly target communication molecules secreted into the environment before they reach their target. QQ has the advantage of not affecting bacterial growth, unlike antibiotics, significantly

reducing selection pressure and potentially limiting resistance emergence (Mion, 2019)[46]. Therefore, QQ represents an ideal approach for the prevention and treatment of bacterial infections and could serve as an alternative to antibiotics (Mion, 2019)[46].

I.7. The relationship between biofilm and virulence :

The link between a strain's ability to form biofilm and its virulence is often depicted as direct. However, recent studies have highlighted that environmental signals regulate, in opposite ways, the abilities of bacteria to form biofilms or to be virulent. This regulatory mechanism offers bacteria greater adaptability to environmental stresses. During the planktonic phase of infection, bacteria release virulence factors and alter host tissues. Once in a suitable environment, bacteria may implant and persist on a surface through the expression of factors involved in biofilm formation. These results suggest that biofilm plays a role in bacteria persistence in a given environment rather than directly in the infectious mechanism. Biofilms thus constitute reservoirs of infections(Roux and Ghigo 2006)[47].

The results of the present and previous study demonstrated a significant association between virulence gene expression involved in bacterial attachment and biofilm formation, indicating that biofilm-forming bacteria are more pathogenic than the planktonic form in urinary tract infections, and that biofilm formation cause increasing the rate of bacterial virulence as well as the severity of disease, making biofilm-based UTI very difficult to cure.

Different results in various studies may be due to regional differences in hygiene status and increasing resistance to antibiotics. The limitations of the present study are that 1. The number of evaluated genes involved in virulence and biofilm formation was lower than in other studies and 2. the targeted community in this study was selected from one specific geographical area. These may explain the difference in results compared to other studies.

CHAPTER II

Effect of disinfectant on biofilm

II.1. Definition of disinfectant:

Disinfectants can effectively control infectious diseases by inhibiting or destroying the pathogenic growth, including bacteria and viruses in the transmission medium (Tong et al. 2021)[1].

II.2. Types of disinfectant:

◊ **BACTERICIDE:** Anything that destroys bacteria(Patterson 1932)[2].

◊ **ANTISEPTIC:** A substance that opposes sepsis, putrefaction or decay; one that prevents or arrests the growth or action of microorganisms, either by inhibiting their activity or by destroying them; used especially of agents applied to living tissue(Patterson 1932)[3]

◊ ***GERMICIDE*:** Anything that destroys germs (microorganisms), applied especially to agents that kill disease germs.(Patterson 1932)[4].

II.3. Characteristics of the ideal disinfectant:

- kill claims for the most prevalent healthcare pathogens.
- fast kill times and acceptable wet-contact time to ensure proper disinfection of noncritical surfaces and patient care equipment.
- safety.
- ease of use.
- other factors: such as the training and support offered by the manufacturer, costs, and standardization.
- have a broad spectrum of effectiveness.
- be used at low concentration.(Rutala and Weber 2014)[5].

II.4. The different classes of disinfectants:

Among the most commonly used products, we can mention:

- **Alcohols***:* Such as ethanol and isopropanol, are among the most used. They are active against Gram-positive and Gram-negative bacteria and act quickly (about 30 seconds). However, they are ineffective against spores, not very effective on viruses,

and evaporate quickly. Additionally, they are inactivated by organic matter and tend to cause organic debris to stick (Massicotte, 2009)[6].

- Aldehydes: Such as formaldehyde and glutaraldehyde, are also among the main disinfectants in this category. They are bactericidal at high concentrations on Gram-negative bacteria. They are also attributed fungicidal, virucidal, mycobactericidal, and sporicidal properties (Massicotte, 2009)[6]. Moreover, they have a broad spectrum of action and remain active over a wide pH range (Quittet, 1999)[7].

- Biguanides*:* Are widely used disinfectants and antiseptics. They have a chemical structure containing two guanidine groups connected by a carbon chain. Biguanides have extensive antimicrobial activity against bacteria, fungi, and viruses. Among biguanides, chlorhexidine is particularly valued for its exceptional properties such as effectiveness, persistence, and prolonged residual activity. It has many applications, including oral hygiene, wound disinfection, and skin preparation before surgery (Massicotte, 2009)[6].

- Halogen Compounds: Such as chlorine and its derivatives, as well as iodine and its derivatives, are widely used due to their effectiveness, even at low concentrations of active chlorine. For example, mixing iodine with a surfactant results in strong bactericidal action at low concentrations (Bimbenet, 2002)[8]. Additionally, they have powerful deodorizing activity and a broad spectrum of activity on microorganisms (Trueman, 1971)[9].

- *Phenolic Compounds*: Exhibit bactericidal and fungicidal action. At low concentrations (0.2%), they have a bacteriostatic effect, while at concentrations of 1% or higher, they become bactericidal. Phenolic derivatives dissolved in water demonstrate excellent activity against mycobacteria and viruses. On the market, new formulas combining several phenols capable of destroying hydrophilic viruses are available. These compounds are mainly used for cleaning surfaces contaminated with organic matter (Massicotte, 2009)[6].

- **Oxidants:** Are oxygen-based products that typically contain atoms working in pairs. Examples include peroxide, peracetic acid, peroxyphthalate, perglutarate, and peroxymonosulfate. Peracetic acid: Peracetic acid is a highly effective disinfectant obtained by mixing acetic acid and hydrogen peroxide. It has a broad spectrum of action, even at low concentrations, by altering the cell membrane permeability of microorganisms (Bimbenet, 2002)[8].
- **Quaternary ammonium compounds (QACs):** are cationic surfactants introduced in the late 1930s along with the first antibiotics, sulfonamides. They are classified as 'high production volume' chemicals (Tezel and Pavlostathis 2015)[10]. QACs used preoperatively as a skin antiseptic and can be applied to mucus membranes. QACs are also used in deodorants and disinfectants of hard surfaces (Kudinha 2022)[11].

  1) Wide germicidal range, noncorrosive and low toxicity.

  2) Reduced efficiency and residual activity in the presence of organic matter.

  3) Not sporicidal, effective against vegetative bacteria, fungi and viruses, some activity against Cryptosporidium (10 percent Ammonium).

  4) Limited effectiveness in soaps, detergents and hard water salts.

  5) Good disinfectant for use on cleaned surfaces and low cost.
- **Chlorhexidine***:* is probably the most widely used biocide in antiseptic products, in particular in handwashing and oral products but also as a disinfectant and preservative. This is due in particular to its broad-spectrum efficacy, substantivity for the skin, and low irritation. Of note, irritability has been described and in many cases may be product specific Despite the advantages of chlorhexidine, its activity is pH dependent and is greatly reduced in the presence of organic matter (McDonnell and Russell 1999)[12].

  1) Wide germicidal activity, but ineffective against some important species.

  2) Some activity in the presence of organic matter.

  3) Some residual activity but must be in contact for at least five minutes.

  4) Fair effectiveness as sporicidal agents.

  5) Effective at low concentrations for disinfecting objects.

  6) Low cost but requires frequent applications.

  7) Nontoxic.

II.5. Limitation of diffusion biofilm formation:

Bacterial biofilms are much more resistant to disinfection than free-floating bacteria. The mechanism of biofilm resistance is attributed to the extracellular matrix, which forms a complex and compact structure in which biocides struggle to penetrate antibacterial agents, as well as the production of glycocalyx, a sticky substance secreted by bacteria that covers the bacterial surface to provide a buffering effect (Tong et al., 2021) [13].

The biofilm matrix significantly impacts the efficacy of disinfectants due to its complex structure and protective mechanisms. Biofilms, which are communities of microorganisms embedded in an extracellular polymeric substance (EPS), present a major challenge in both medical and industrial settings because they can be up to 1,000 times more resistant to disinfectants than planktonic (free-floating) bacteria.

One of the key reasons for this resistance is the physical barrier provided by the EPS, which impedes the penetration of disinfectants. Additionally, biofilms have a high cell density that facilitates quorum sensing, further enhancing their resistance by regulating the expression of resistance genes and metabolic activity based on cell density and biofilm maturity[(Maillard ,2023)[14].

Studies have shown that different disinfectants vary in their effectiveness against biofilms

Biofilm age and species composition also play crucial roles in resistance. Older biofilms tend to be more resistant due to more developed EPS and greater cell differentiation. Furthermore, multispecies biofilms often exhibit enhanced resistance compared to single-species biofilms due to cooperative interactions among different microbial species (Simões ,2010)[15].

CHAPTER III

Materials and Methods

III .1. Materials and Methods :

III.1.1. Type and setting of the study :

The present study was carried out as part of the preparation of a Master's thesis in Applied Microbiology at Yahia Fares University in Medea. The lab work was conducted in the Microbiology and molecular biology laboratory, Department of Biology, Faculty of Natural and Life Sciences, Dr. Yahia Fares University, Medea, during a period from February to May 2024.

The aim was to develop a new reproducible method for testing the efficacy of disinfectants on bacterial biofilms and to evaluate the effectiveness of different disinfectants against biofilms. And to explore the genetic carriage of different genes related to the formation of biofilms.

III.1.2. The Objectives:

- Study the biofilm formation capacity of *e.coli strains* isolated from avian.
- Evaluate the effectiveness of different disinfectants on the biofilm.
- Analyze the sensitivity of isolated strains to commonly used antibiotics in avian.
- Molecular investigation of some biofilm-related genes by PCR technique and analyze their genetic profile, particularly, with those demonstrating a strong biofilm formation capacity.

These research steps help improve our understanding of biofilm formation in enterobacteria isolated from avian. Additionally, they aid in assessing the effectiveness of disinfectants and the susceptibility of these strains to antibiotics.

III.2.Materials:

III.2.1. non biological material:

The Annex contains a list of all equipment utilized throughout the different procedures.

III.2.2. biological material:

In our study, we used strains that were previously isolated, identified, and analyzed.

III.3. Origin and identification of strains:

The strains studied are of animal origin and were obtained from cases of colibacillosis in broiler chickens in Algeria. The samples were taken from the liver, and primary isolations

were done previously under a collaboration project with the institut of freidrich loeffler institut (FLI), institut of bacteial infection and zoonoses (IBIZ), Jena, Germany (unpublished Data). The final identification of these strains was performed previously in FLI/IBIZ, Germany using the MALDI/TOF-MS technique (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry). Table 1 presented the result of indentification and the origin of each strain used in the present study.

Table III .1 : Number and origins of the strains used.

Sampels	MAL_TOF	Region
A1	Escherichia coli	Medea
A2	Escherichia coli	Medea
A3	Escherichia coli	Medea
A4	Escherichia coli	Medea
A5	Escherichia coli	Medea
A6	Escherichia coli	Medea
A7	Escherichia coli	Medea
A10	Escherichia coli	Medea
A11	Escherichia coli	Medea
A12	Escherichia coli	Djelfa
A14	Escherichia coli	Djelfa
A15	Escherichia coli	Djelfa
A16	Escherichia coli	Djelfa
A17	Escherichia coli	Djelfa
A18	Escherichia coli	Djelfa
A20	Escherichia coli	Djelfa
A21	Escherichia coli	Djelfa
A22	Escherichia coli	Djelfa
A23	Escherichia coli	Chlef
A24	Escherichia coli	Chlef
A25	Escherichia coli	Djelfa

III.4. Methodology:

III.4.1. Revival and purity verification of strains:

Initially, the strains that had been previously preserved in glycerol (-20°C) were revived. After vortex homogenization, the strains were revived in TSB broth (tryptone soy broth). Incubation was carried out at 37°C for 24 hours. Purity verification of the strains was performed on MacConkey agar, a selective culture medium commonly used to isolate and identify Gram-negative enteric bacteria (such as *Escherichia coli, Salmonella, and Shigella*).

The purification was achieved by streaking onto agar plates, followed by an incubation at 37°C for 24 hours, until colonies with uniform size, shape, and color appeared, indicating the purity of the strains.

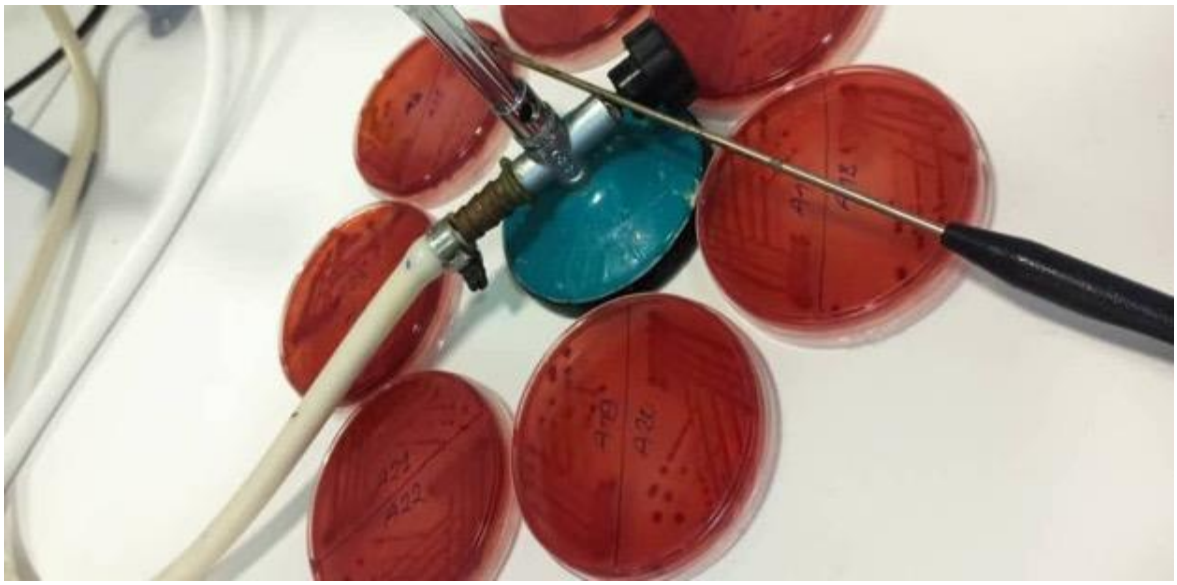


Figure III.2: Inoculation on MacConkey medium after enrichment in TSB.

III.5. In vitro Biofilm Formation Assessment Technique:

III.5.1.detection of biofilm formation:

In this study, we evaluated the biofilm production capacity of 23 isolates in vitro using the Tissue Culture Plate (TCP) assay as described by Christensen's *et al*., 1995 is the most widely used method and is considered as the standard method for detection of biofilm formation. The isolates from fresh agar plates were inoculated in 5 ml of Trypticase soy broth and were kept for incubation at 37°C for 24 h(Harika et al. 2020)[1].

⇒ **protocol:**

as described by (Christensen,1985)[2], Few isolated and characteristic colonies taken from the initial MacConkey purification culture, were individually cultured for 18 to 24 hours in 5 ml of TSB at 37°C. Each culture was adjusted to reach a density of 0.5 McFarland (Grant-bio®) units (diluted in TSB). A volume of 200 µl of the bacterial suspension was deposited into the sterile polystyrene wells of a U-shaped 96-well microplate (PorLab Scientific Co.,Ltd®). Each bacterial culture was used 3 times to ensure result accuracy. The 3 wells in the first column were filled with the same volume of 200 µl of non-inoculated TSB, serving as a negative control. The covered microplate was then incubated at 37°C for 24 hours, followed by an additional 48 hours without agitation.

After the incubation period, the contents of the microplate wells were emptied. Three consecutive washes were performed with 200 µl of phosphate-buffered saline solution (PBS,

pH=7.4) to remove free planktonic bacteria. Then, the adherent cells were fixed in an oven for 60 minutes at 60°C. Bacterial cells adhering to the inner wall of the wells, forming a biofilm, were stained with 0.1% (w/v) crystal violet(CV). After 10 to 15 minutes, the crystal violet solution was removed, and the wells were rinsed 3 times with sterile distilled water to remove any residual unbound dye. Subsequently, the microplates were left to air dry at room temperature.

Finally, the wells were refilled with 150 µl of 95% ethanol solution for at least 30 minutes at room temperature to solubilize the dye contained within the cells trapped in the formed biofilm Optical density was measured at 600 nm using a microplate reader (ELISA) (model MP96 from Safas ® Monaco) connected to a computer.



Fill the tubes with TSB.



Replating.



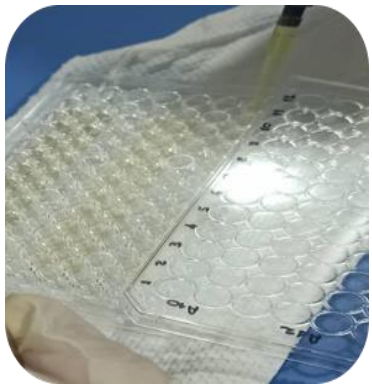
Inoculation into TSB.



Incubation.



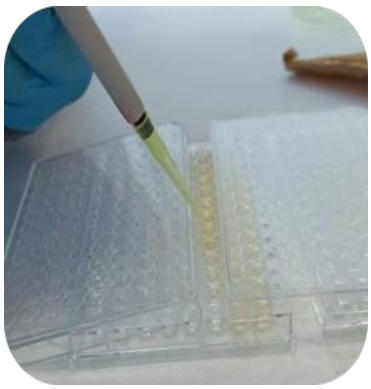
Dilution in TSB medium.



filling the microplate



Incubation of microplate.



emptying the microplate.



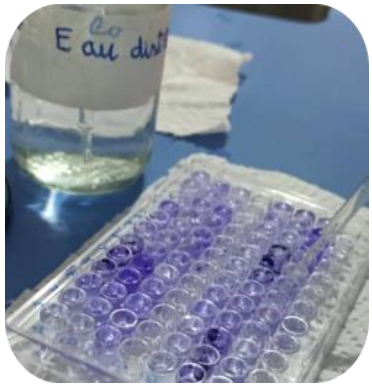
Rinsing with PBS.



Fixation.



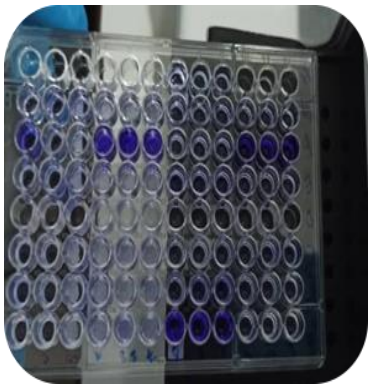
staining with crystal violet.



Wash 3 times with DW.



Resolubilization with ethanol.



Place microplate into MP96 reader.



Reading by MP96 reader.

Figure III.3: the steps of TCP method.

III.5.1.1. Lecture of the microplate:

Visual reading of the biofilm in the microplate after staining allows for an approximate estimation of its density in each well (figure 04). However, it is recommended to use more precise quantification methods, such as absorbance measurement or biomass measurement, to more reliably assess biofilm formation.

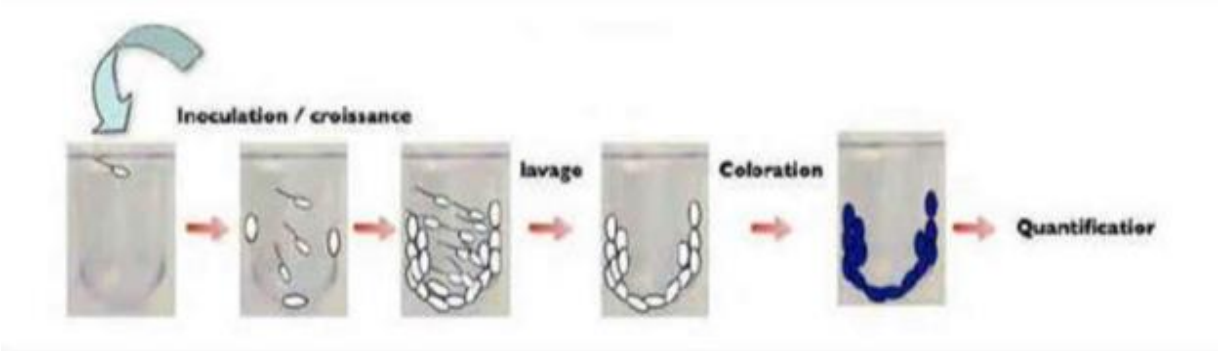


Figure III .4: Principle of biofilm quantification techniques in microplates (Laurent, 2011).

To facilitate result interpretation, strains can be classified into the following categories established by Stepanovic *et al.* (2000): strains not producing biofilm (category 7), those producing biofilm weakly (category 8), those producing biofilm moderately (category 9), and those producing biofilm strongly (category 10). (Stepanovic *et al.* (2000)[3].

Table III.2: classification of biofilm formation according to(Stepanovic et al,2007)

Average of DO values	Biofilm formation	Interpretation
DO read≤DOnc (7)	–	*Absence*
DOnc<DO read≤2X DOnc (8)	±	*Weak*
2X DOnc<DOread≤4X DOnc (9)	+	*Moderate*
DO read>4X DOnc (10)	+ +	*Strong*

III.5.2. Evaluation of the effectiveness of some disinfectants on biofilm

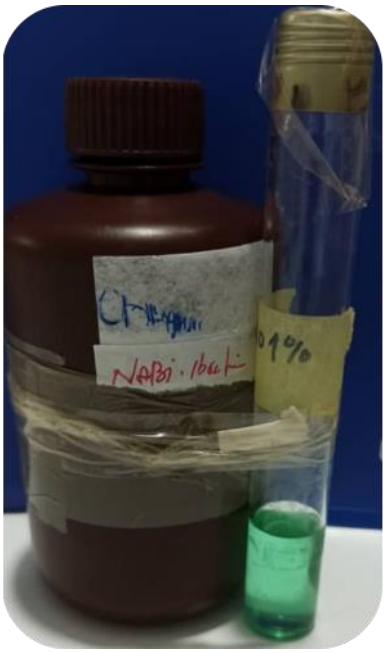
Formation in vitro:

During this stage of the experimental study, the effectiveness of three disinfectants was assessed on strains previously characterized for their ability to form biofilms

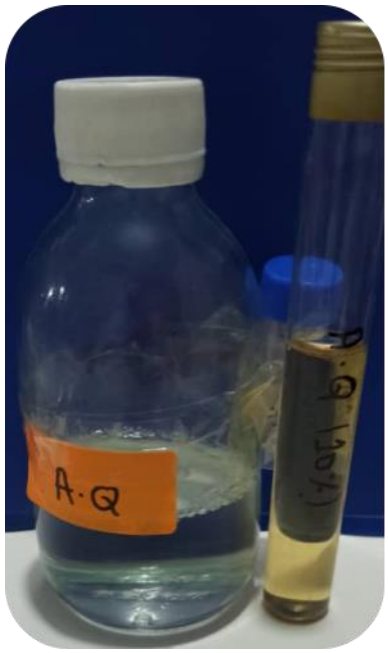
(MIC/MBC). Among these, three strains were selected to investigate the efficacy of different disinfectants on biofilm formation. The choice of these strains was based on their strong biofilm-forming potential.

Table III.3: Strains used to test the effectiveness of disinfectants on biofilm formation.

Strains		DO Average
Avian strains	*A10*	*2.96*
	A12	*3.41*
	A24	*3.55*



Chlorhexidine.



Quaternary ammonium.

Figure III .5: the used disinfectants.

III.5.2.1. Determination of the Minimum Inhibitory Concentration (MIC):

Minimum Inhibitory Concentration MIC is the lowest concentration of an antibacterial agent expressed in (mg/L)(µg/mL) which, under strictly controlled in vitro conditions, completely prevents visible growth of the test strain of an organism(Kowalska-Krochmal and Dudek-Wicher 2021)[4].

⇒ **protocol:**

To begin, bacterial colonies are collected and inoculated into 5 ml of TSB broth, then incubated at a temperature of 37°C for a period of 18 to 24 hours. Next, all wells of the plate are filled with 100 µl of TSB broth, except for wells in column n°01 and n°12. Subsequently,

a dilution of the disinfectant is prepared at the highest concentration, and column n°1 is filled with 200 µl of this prepared concentration. Then, 100 µl from well n°01 is transferred to the next well (well n°02) and thoroughly mixed. This process is repeated successively for the following wells up to well n°11. The additional 100 µl from well n°10 is discarded. Every two rows correspond to a selected bacterial strain, and wells n°1 to n°11 are used for decreasing dilutions of the tested disinfectant. Well n°12 is reserved respectively as a positive control. The operation is then repeated for the other rows. An inoculum is prepared for each isolate by diluting bacterial suspensions in TSB broth to obtain a bacterial concentration of 0.5 McFarland.

Then, 100 µl of inoculum is added to each well from n°01 to n°11. Finally, column n°12 is filled with 200 µl of inoculum (positive control). The microplate is covered and incubated at 37°C for 24 hours. Finally, the growth rate in the wells containing the antimicrobial agent can be observed with the naked eye.

III.5.2.2 . Determination of the Minimum Bactericidal Concentration (MBC):

The minimum bactericidal concentration was shown to be considerably influenced by technical and definitional factors, particularly by the survival of some organisms on the walls of test tubes and by the growth phase of the inoculum. Attention to technical detail greatly improved reproducibility, and log-phase cultures of all strains showed greater than 99.9% killing in 24 h, at or close to the minimum inhibitory concentration (Taylor et al. 1983)[5].

⇒ **Protocol:**

• Prepare Petri dishes containing Muller-Hinton(MH) culture medium with a 4 mm thickness of agar, ensuring that the dishes are properly dried.

• Precisely place a drop of 10 µl of bacterial suspension in separate wells, including one well corresponding to the Minimum Inhibitory Concentration (MIC) and other wells with concentrations higher than the MIC of the tested disinfectant, where no bacterial growth should be detected.

• Incubate the dishes at a temperature of 37 °C for a duration of 18 to 24 hours.

• Proceed with the evaluation of bacterial growth presence. The absence of bacterial growth in the well with the lowest concentration indicates the Minimum Bactericidal Concentration (MBC) of the tested disinfectant. The MBC may be equivalent to the MIC or higher than it.

Note: Don't forget to label the dishes according to strain identifiers.

III.6. Determination of Antibiotic Sensitivity:

In the present study, a commercial Automated bacterial susceptibility testing systems have been used. The VITEK 2 system (bioMérieux), which uses a new fluorescence-based technology.

◊ There are six types of cards available for the identification of different microorganisms, the selection of the card based on the gram stain reaction and microscopic observation of the culture

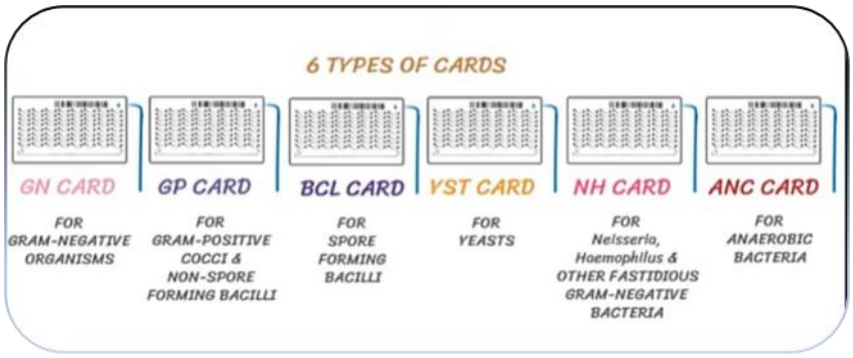


Figure III.6: Types of VITEK2 cards.

◊ The instrument utilizes reagent cards that have 64 wells each well contains individual test substrates, these substrates measure various metabolic activities like: acidification , alkalinization, enzyme hydrolysis and growth in the presence of inhibitory substances.

◊ Each card is integrated with a transfer tube for the transfer of microbial culture to the card, each card has a barcode that contains information (lot number, expiry date, identification number).

A total of 23 isolates *E. coli* strains Escherichia coli were subjected to antimicrobial susceptibility testing according to the protocol described by [Basma M. Hamed , Mona I. Elenbaawy]using AST-N635 TEST KIT(bioMérieux, Incorporation., Durham, NC) using VITEK 2 system version 9.02 software version according tomanufacturer's instructions. Minimal Inhibitory Concentration (MIC) interpretation guideline was done according to (CLSI, 2020), The cards overflowed with an inoculum ready by transferring 145 µL of culture suspension from the 0.5 to 0.63McFarland culture suspension. The VITEK-2 system

automatically processes the antimicrobial susceptibility cards until MICs are obtained (Joyanes et al., 2001)[6].

⇒ **Protocol:**

III.6.1.Preparation of suspension:

- take a polystyrene test tube and aseptically transfer 3ml sterile saline.



- Prepare a homogeneous suspension using isolated colonies of test microorganism with the help of a sterile cotton swab or inoculation loop.



- adjust the density of the suspension using the density check meter based on the card chosen for microorganism, ensure that the density check is calibrated using standards.

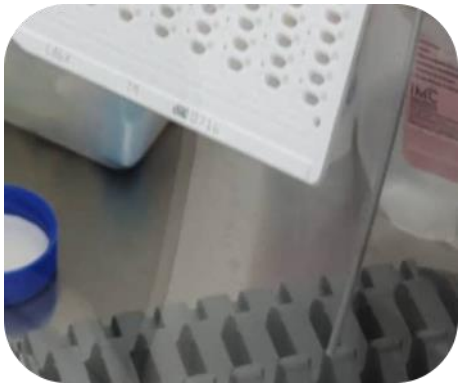


III.6.2. Transfer of culture:

- **place the polystyrene tube into a special cassette.**



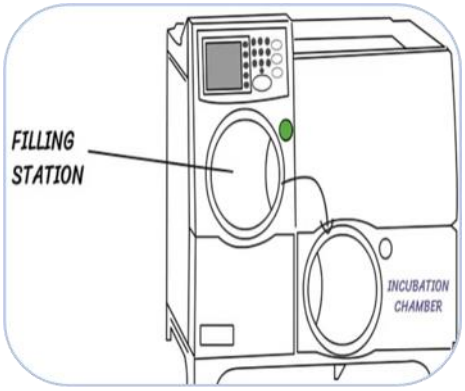
- **the identification card is placed in the adjacent slat such that the transfer to tube goes into the corresponding suspension tube.**



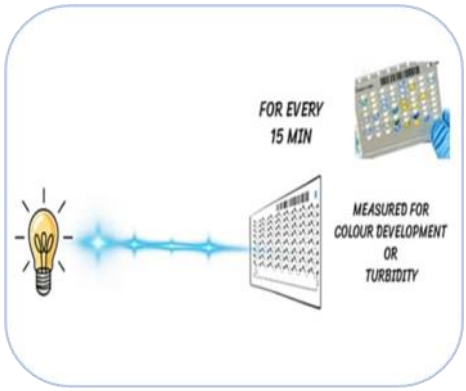
- **place the cassette into the filling station of VITEK2 compact.**



- **once the transfer of suspension into the identification card is completed, the system will indicate to transfer the cassette by blinking a light.**
- **Then manually transfer the cassette from filling station to the incubation chamber.**



- **the incubator and measured for color development or turbidity by an optical system allows interpretation of test reaction using different wave lengths.**



- **the test reactions are compared to the database of the system to determine a close value.**

III.7.Molecular biology section:

III.7.1. Genomic DNA extraction:

A total of twenty-two (22) DNA samples, inclusive of APEC strain serving as an positive control, explored from the university of Khemis-Meliana, were employed within the PCR program titled "*E. coli* Virulence Factors." This PCR program was evidently designed to investigate specific genetic elements pertinent to biofil formation.

The investigation was focused on 4 specific genes associated with *avian Escherichia coli*, with each gene outlined within the established protocols. These genes were systematically assigned distinct numerical identifiers in accordance with their respective roles in the study, Different protocols exist for extracting DNA, but the basic principle is nearly always the same (Surzycki, 2000)[7].

- Lysis of the cell and nuclear membranes

- Removal of proteins, nucleic acids (RNAs), and other contaminants such as secondary metabolites
- Treatment with a precipitation solution, usually ethanol or isopropanol.

⇒ **DNA Extraction Protocol:**

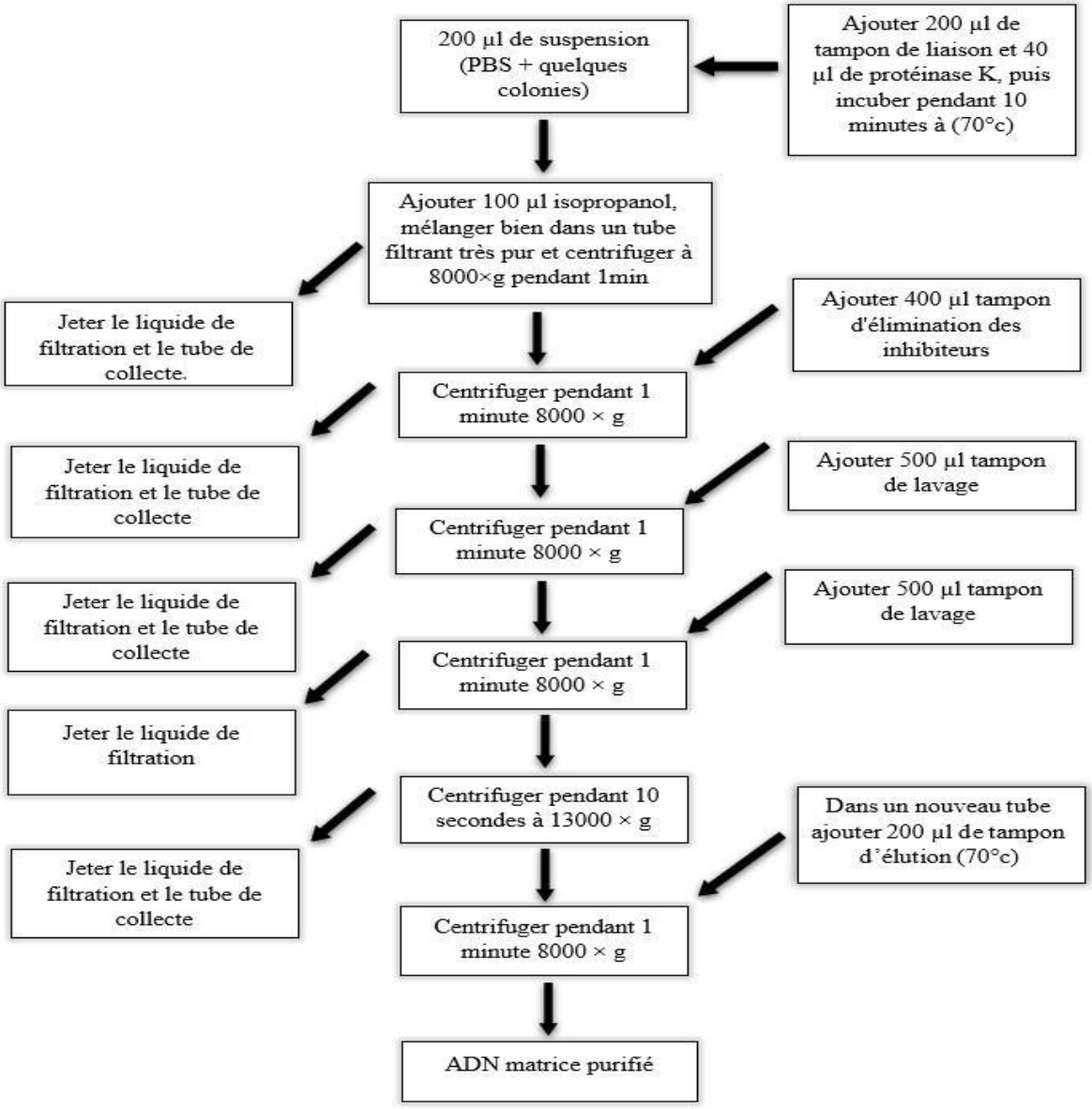


Figure III .7: Extraction of DNA Protocol.

III.7.2. PCR protocol:

E. coli was probed by the Polymerase Chain Reaction (PCR) for the presence of genes responsible for the *pap-G II* biofilm adhesion gene, *fliC* flagellin for filament formation gene, *tsh* temperature sensitive hemaggulation gene, csgA cell signal genetic analysis gene.

Table (4): Primers of genes encoding virulence factors specific to APEC isolates		
Gene	Primer sequences (3′–5)	Size(bp)
csgA - F *csgA - R*	3’-CC ATT ATC AGT TCA GTC TCA-5’ 3’-CAA CTG GTC TGA CGT AGA-5’	200
fliC - F *fliC - R*	3’-GC AGT TCA ACG GAC GTA GCA-5’ 3’-CC AAG ACG TTG CTG GTT GGG-5’	547
tsh – F *tsh -R*	3’-GGT CGT AAG TCC AGT AAA GGG-5’ 3’-CAC CAT GAC TGA CTA CTC GCC-5’	420
papG_II - F *papG_II - R*	3’-TA GTT TCC GGG CGA GTA GGG-5’. 3’-G CTC AAT GAA CCC CCG GGC-5’	190

⇒ **Protocol:**

The PCR protocol was conducted following protocol number 2024_*Ecoli*_Biofilm_01/*pap-G II +fli C* _02/ csg A _ 03/ tsh on March 17, 2024, using the 2X Your Taq PCR Master Mix (20 µl). A total of 23 samples were processed. The PCR program utilized was "Biofilm Factors".

The targeted genes are:

- The gene *pap-G II* with an expected size of 190 base pairs (bp) - associated with biofilm adhesion.
- The gene *fli-C* with an expected size of 547 bp - is linked to flagellin for filament formation.
- The gene *tsh* with an expected size of 420 base pairs (bp) - associated with temperature sensitive hemaggulation.
- The gene *csgA* with an expected size of 200 bp - is linked to cell signal genetic analysis.

Preparation of a total volume of 20 µl of 2X Your Taq PCR Master Mix.The PCR reaction components included: water (7 µl), 2X Your Taq PCR Master Mix (10 µl), forward primer (1 µl), reverse primer (1µl), and template DNA (1 µl). To prepare the reaction cocktail, we multiplied all components by 28(23 number of samples+1positive control +1negative control+3 margin of error), Then, we added 1 µl of extracted DNA to each reaction.

Table III.5: genes amplification protocol.

	Genes	Protocol
MUltiplex	*fliC + papG_II*	1- ***95**°C/ 5min* 2- ***94**°C/30s* 3- ***63**°C/30s* *x35* 4- ***72**°C/60s* 5- ***72**°C/5min* 6- ***4**°C*
Monoplex	*Tsh*	1- ***95**°C/5min* 2- ***94**°C/1min* 3- ***55**°C/45s* *x35* 4- ***72**°C/1min* 5- ***72**°C/5min* 6- ***4**°*
	csgA	

The thermocycler was initiated with the following program

-Multiplex *pap-G + fli C :*

1. An initial denaturation at 95°C for 5 minutes
2. Denaturation at 94°C for 30 seconds
3. Annealing at 63°C for 30 seconds
4. Extension at 72°C for 60 seconds "The cycle was repeated 35 times."
5. The final extension step was carried out at 72°C for 5 minutes.

-Monoplex *tsh:*

1.An initial denaturation at 95°C for 5 minutes

2.Denaturation at 94°C for 60 seconds

3.Annealing at 55°C for 45 seconds

4.Extension at 72°C for 60 seconds "The cycle was repeated 35 times."

5.The final extension step was carried out at 72°C for 5 minutes

-Monoplex *csgA:*

1.An initial denaturation at 95°C for 5 minutes

2.Denaturation at 94°C for 60 seconds

3.Annealing at 55°C for 45 seconds

4.Extension at 72°C for 60 seconds "The cycle was repeated 35 times."

5.The final extension step was carried out at 72°C for 5 minutes

The PCR product was analyzed using 1.5% agarose gel electrophoresis. After staining with ethidium bromide, the bands were visualized under UV light, and their sizes were estimated by comparison with a molecular weight marker, applying a voltage of 100V for 30 minutes.



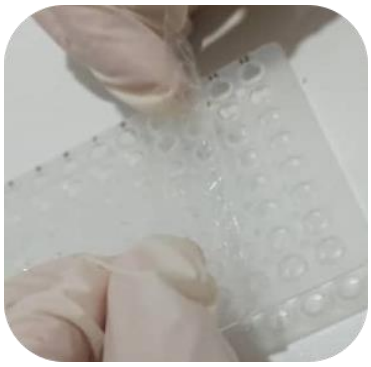
Preparation of PCR cocktail.



Fill the wells of amplification Plate.



Add the extracted DNA.



Cover the amplification plate.



checking the plate.



place the plat in thermocycler



Adjust according to the protocol.

Figure III.8: PCR steps.

III.7.3. Agarose gel electrophoresis :

The visualization of the 23 strains was accomplished using a 1.5% agarose gel. The gel was prepared by dissolving 1.25 g of agarose powder in 100 mL of distilled water, ensuring complete dissolution to achieve a transparent and clear solution. Subsequently, 3 µL/mL of ethidium bromide (EtBr) was added to the mixture. (Garibyan and Avashia 2013)[8].

III.7.3 .1. Preparation of 1.5 % gel :

⇒ **Protocol**:

• Weigh 1.5 g of agarose into a 100 ml Erlenmeyer flask.

• Add 100 ml of distilled water .

• Melt this mixture in a microwave oven, stopping the oven every 10 seconds to stir. The mixture should become completely transparent, without any agarose particles present.

• Add 3 µl of ethidium bromide solution. Homogenize the mixture.

• Place the comb, and pour the gel into the electrophoresis apparatus, being careful to avoid bubble formation. Let the gel cool

The integrity and presence of extracted DNA were verified by electrophoresis on agarose gels at 1.5%, 2%, and 2.5%.

III.7.3.2.Preparation and deposition of samples:

• Place the gel in the tank and ensure it is covered with 1X TBE buffer.

• Deposit 5 µl of extracted DNA + 2 µl bleu of bromophenol into the agarose gel wells and note their order.

• Deposit 5 µl of ladder in gel well number 1

• Connect the wires and power on the power supply at 100V for 30 minutes

At the end of migration, the DNA was visualized under UV light after staining with ethidium bromide.



Figure III.9: Loading the amplified DNA samples into the agarose gel wells.



Figure III.10: Place the agarose gel on the Transilluminator.

III.7.3.3. Visualization of DNA fragments :

The incorporation of ethidium bromide into the agarose gel allows visualization of DNA fragments on the gel. Ethidium bromide has the ability to intercalate between the stacked base pairs of DNA. When illuminated under UV light, the appearance of DNA on the agarose gel is observed as an orange fluorescent band.

Chapter IV

Results and Discussion

IV.1. Results of revival and purity of the strains used :

26 strains responsible for clinical,which were isolated and identified from various provinces in Algeria. After a process of enriching the samples in TSB broth for 24 hours, it was found that 23 samples were succesfully revived and appeared turbid. Subsequently, these isolates were inoculated on MacConkey agar and incubated for 18 to 24 hours at a temperature of 37°C to confirm their purity and vitality.

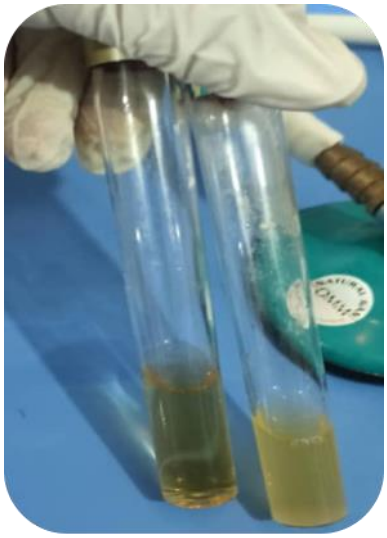


Figure IV.11: observation of turbidity in coli. the TSB medium with the negative control.

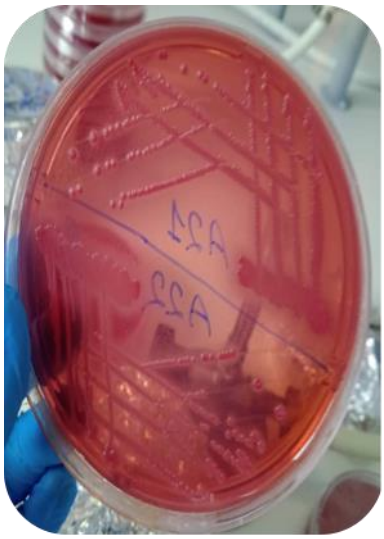


Figure IV.12:observation of the purity of E.

IV.2.Identification of isolates:

IV.2.1.Macroscopic Aspect:

After incubating them for 24 hours, the following characteristics were observed:Round, smooth colonies with regular edges, 2 to 3 mm in diameter, and non-pigmented. It was also observed that the initial color of the medium was retained, confirming that the bacteria do not ferment lactose (no acidification occurred).

IV.2.2.Identification:

The identification of *E.coli* strains was carried out using the MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization Time-Of-Flight) technique.

Table IV.6: Distribution of the isolated and identified E. coli strains

Region	Species	Colibacillosis
Medea	*Escherichia coli*	*9*
Djelfa	*Escherichia coli*	*11*
Chlef	*Escherichia coli*	*2*

The table (06) provides an analysis of the bacterial species involved in cases of colibacillosis reported in poultry, specifically associated with the species Escherichia coli in different regions.

The present analysis showed various avian colibacillosis caused by *Escherichia coli* in the mentioned regions. This also confirms previous studies that have identified Avian colibacillosis as a major bacterial disease problem in the global poultry industry. These diseases pose a significant public health burden and represent a considerable economic cost in many countries(Kabir 2010)[1].These studies have emphasized the importance of implementing preventive and control measures to limit the spread of this bacterium and reduce its impact on poultry farms.

In the regions of Djelfa and Medea, the number of reported cases of colibacillosis is 11 and 9, respectively. All cases were attributed to the bacterial species *Escherichia coli*. These data suggest that *Escherichia coli* is the primary agent responsible for the infection in these regions. In contrast, in the region of Chlef, only 2 cases of colibacillosis were reported.

These results highlight the presence of colibacillosis in different regions, with variations in prevalence. The regions of Djelfa (50%) and Medea (41%) appear to be the more affected by this infection, regarding the year of sampling, while Chlef (9%)reports a relatively low number of cases.

IV.3. Evaluation of biofilm formation:

IV.3.1. Biofilm quantification using TCP:

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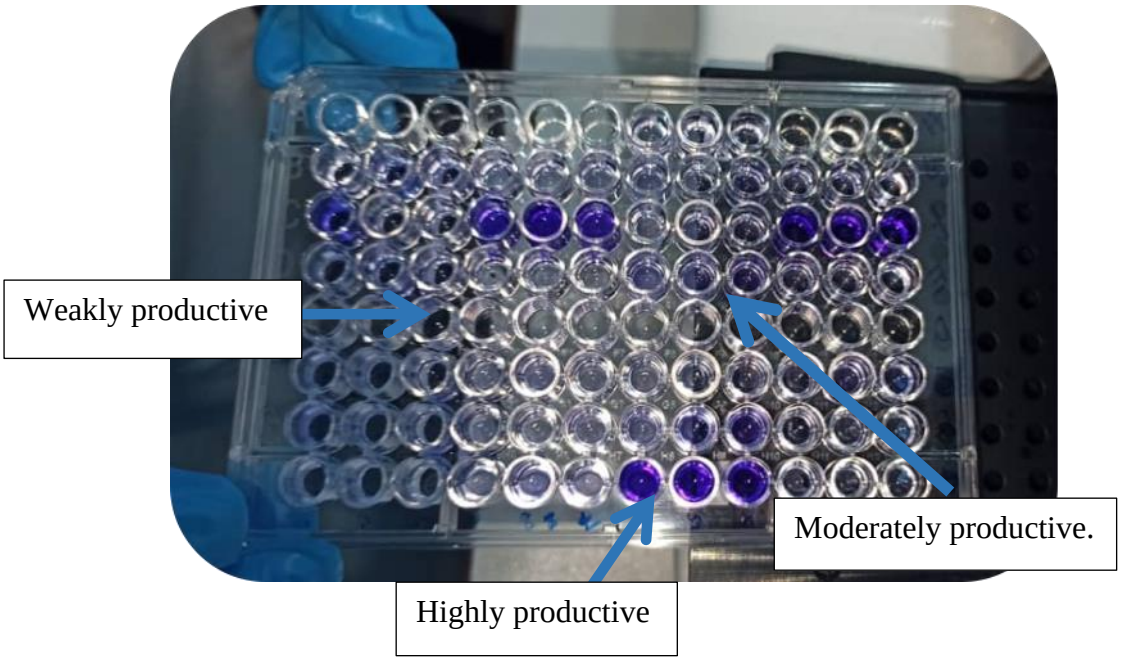


Figure IV.13: Categories of biofilm formation.

This modification of the incubation duration allows us to evaluate the impact of extending the incubation period on the biofilm formation potential. By comparing the results obtained after 24 hours and 48 hours of incubation, we can determine if a longer duration favors greater biofilm formation.

The strains are classified as follows: 1) Strong Biofilm Producers (SBP), 2) Moderate Biofilm Producers (MBP), 3) Non/Weak Biofilm Producers (NBP/WBP) as described in M&M section.

Table IV.7: Comparison of biofilm formation potential after 24 hours and 48 hours.

Samples (ID)	Species	24h average	Interpretation	48h average	Interpretation
c-	-	*0.059*	-	*0.069*	-
A1	*E. coli*	*0.186*	*MBP*	*1.608*	*SBP*
A2	*E. coli*	*0.053*	*NBP*	*1.177*	*SBP*
A3	*E. coli*	*0.295*	*SBP*	*2.340*	*SBP*
A4	*E. coli*	*0.168*	*MBP*	*0.521*	*SBP*
A5	*E. coli*	*0.259*	*SBP*	*2.198*	*SBP*
A6	*E. coli*	*0.190*	*MBP*	*1.317*	*SBP*
A7	*E. coli*	*0.674*	*SBP*	*1.444*	*SBP*
A10	*E. coli*	*3.108*	*SBP*	*0.330*	*SBP*
A11	*E. coli*	*0.188*	*MBP*	*1.252*	*SBP*

A12	*E. coli*	*3.405*	*SBP*	*2.070*	*SBP*
A14	*E. coli*	*0.194*	*MBP*	*0.630*	*SBP*
A15	*E. coli*	*0.184*	*MBP*	*0.274*	*SBP*
A16	*E. coli*	*0.159*	*MBP*	*0.369*	*SBP*
A17	*E. coli*	*0.297*	*SBP*	*3.186*	*SBP*
A18	*E. coli*	*0.229*	*MBP*	*0.214*	*MBP*
A19	*E. coli*	*0.175*	*MBP*	*0.251*	*SBP*
A20	*E. coli*	*0.480*	*SBP*	*0.242*	*SBP*
A21	*E. coli*	*0.161*	*MBP*	*0.260*	*SBP*
A22	*E. coli*	*0.163*	*MBP*	*0.336*	*SBP*
A23	*E. coli*	*0.252*	*SBP*	*0.427*	*SBP*
A24	*E. coli*	*3.532*	*SBP*	*0.322*	*SBP*
A25	*E. coli*	*0.150*	*MBP*	*0.283*	*SBP*
ATCC	*E. coli*	*0.056*	*NBP*	*0.397*	*SBP*

Table 07 presents a Comparison of biofilm formation potential of e.coli strains ,evaluated both after 24 hours and 48 hours of incubation. The samples were identified by their ID

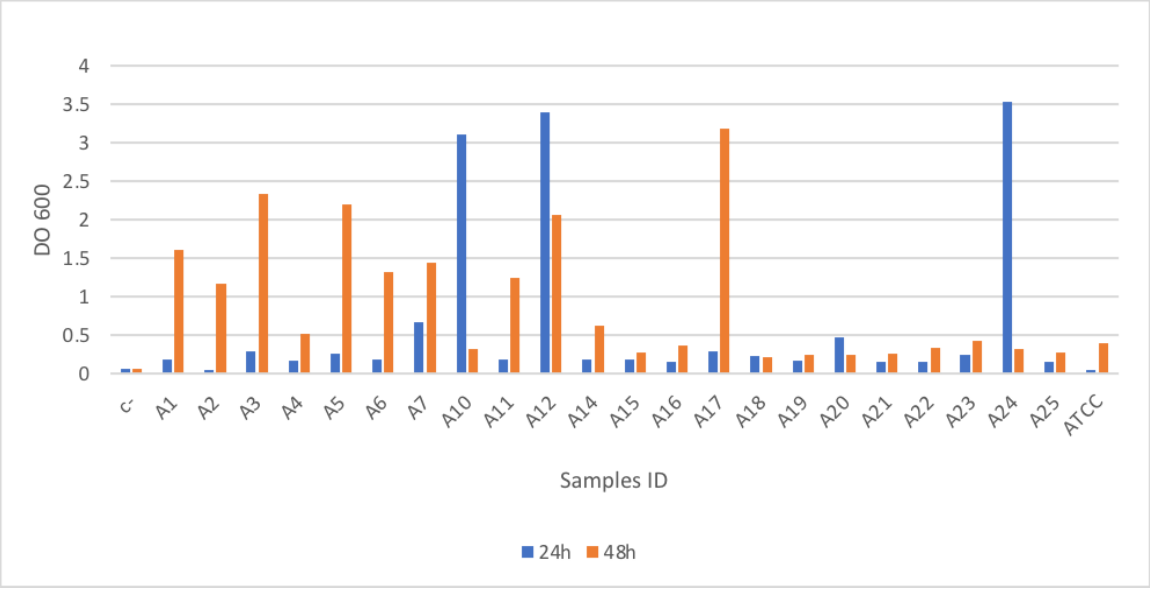


Figure IV.14: Bar chart represent e.coli biofilm formation potential after 24 hours and 48 hours

From table (7)and figure (14), among the 23 *E. coli* isolates from Djelfa (n = 11), Medea (n = 9), and Chlef(n = 2)as mentioned in table (5), 91.30% of isolates were able to form biofilm on polystyrene surface, at 24h,39.13% of isolates formed strong biofilm,52.17%

of isolates formed moderate biofilm and 8.70% weak or non biofilm formation .while at 48h ,95.65% of isolates becomed strong and 4,34% of isolates becomed moderate.

In agreement with the study of EL-baz et al.that reported bioflm formation in 89.3% of *E. coli* isolated from Mansoura hospitals in Dakahlia governorate, with 53.9% grouped as strong/moderate bioflm formers (El-Baz,2022) [2]

Comparing the biofilm averages between 24 hours and 48 hours, we observe a general trend of increasing biofilm potential with longer incubation times. However, some isolated (A1,A2,A4,A6,A11,A14,A15,A16,A18,A19,A21,A22,A25,ATCC) showed interesting variations, shifting from weak to moderate or strong biofilm formations.

These observations highlight the importance of incubation duration in biofilm formation among *ecoli* responsible for colibacillosis. Prolonging the incubation period can promote greater biofilm formation in some samples, which may have significant implications for the virulence and resistance of bacterial strains.

IV.4. Determination of susceptibility to disinfectants (MIC/MBC):

Among the 23strains studied, we've carefully selected three isolates with a strong biofilm-forming capacity(A10-A12-A24), to evaluate the sensitivity of these isolates to two disinfecting agents: chlorhexidine and quaternary ammonium. For this purpose, we used the minimum inhibitory concentration (MIC) method on microtiter plates.

Table IV.8: The Minimum Inhibitory Concentration (MIC) of various disinfectants.

	Disinfectants			
	Chlorhexidine		*Quaternary ammonium*	
Isolates	*MIC Interval*	*MIC 90*	*MIC Interval*	*MIC 90*
***E. coli* strains**	*0.383-1.057*	*1.057*	*0.382-1.090*	*1.090*

MIC = Minimum inhibitory concentration; MIC90 = MIC that inhibits 90% of the isolates

- Interpretation:

The presented table (8) showed the results of the effectiveness tests of disinfectants on E. coli strains. It contained the values of the minimum inhibitory concentration (MIC) for each disinfectant and bacterial isolate, as well as the MIC ranges and MIC 90 values. The MIC values indicated the lowest concentration of disinfectant required to inhibit bacterial growth. The MIC 90 values represent the concentration at which 90% of the isolates are

inhibited. By analyzing the data in the table, it can be observed that Chlorhexidine showed higher inhibitory activity than quaternary ammonium as evidenced by the MIC 90 values.

Table IV.9: The Minimum Bactericidal Concentration (MBC) of the various disinfectants.

	Disinfectants			
	Chlorhexidine		*Quaternary ammonium*	
Isolates	*MBC Interval*	*MBC 90*	*MBC Interval*	*MBC 90*
E. coli strains	*0.382-1.011*	*1.011*	*0.418-0.569*	*0.569*

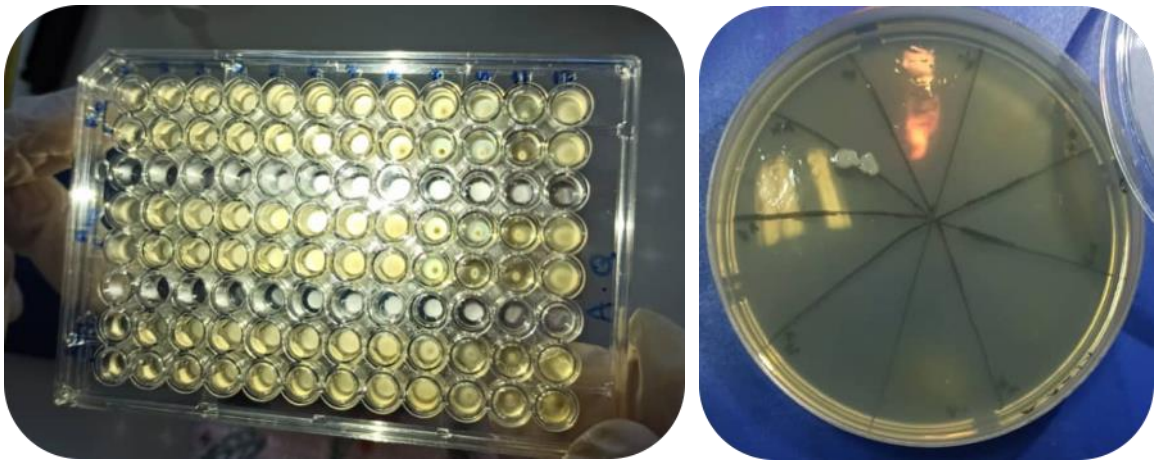
MBC = Minimum Bactericidal Concentration; MBC90 = MBC that inhibits 90% of the isolate

- Interpretation:

The data analysis revealed significant variations in the minimum bactericidal concentration (MBC) required to kill bacterial growth. Chlorhexidine has demonstrated superior bactericidal activity compared to quaternary ammonium, with higher MBC90 values compared to quaternary ammonium.

This difference in efficacy may be related to the specific mechanisms of action of each disinfectant and the unique characteristics of bacterial isolates.

Figure IV.15: The results of MIC and MBC for the Quaternary Ammonium.



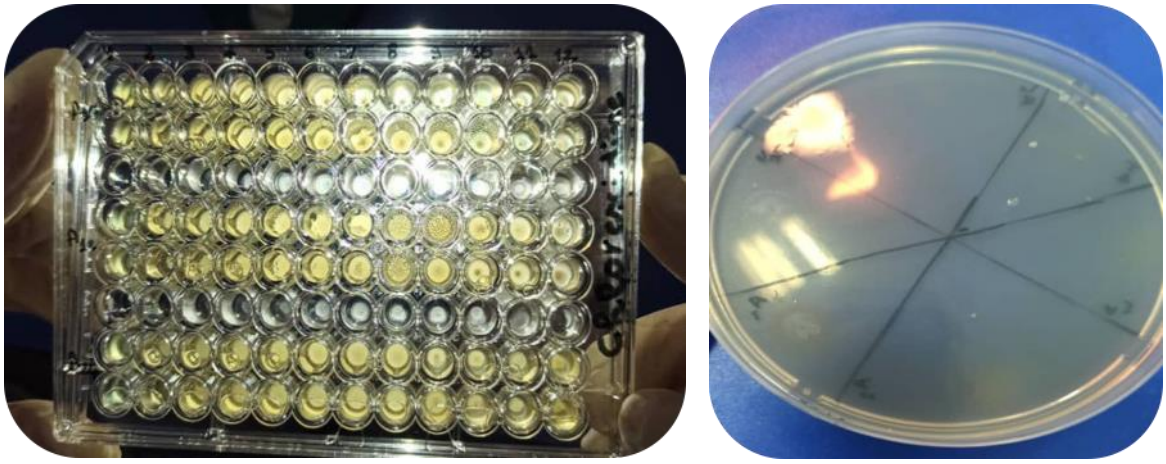


Figure IV.16: The results of MIC and MBC of chlorhexidine.

IV.5. Susceptibility to disinfectants and biofilm production potential:

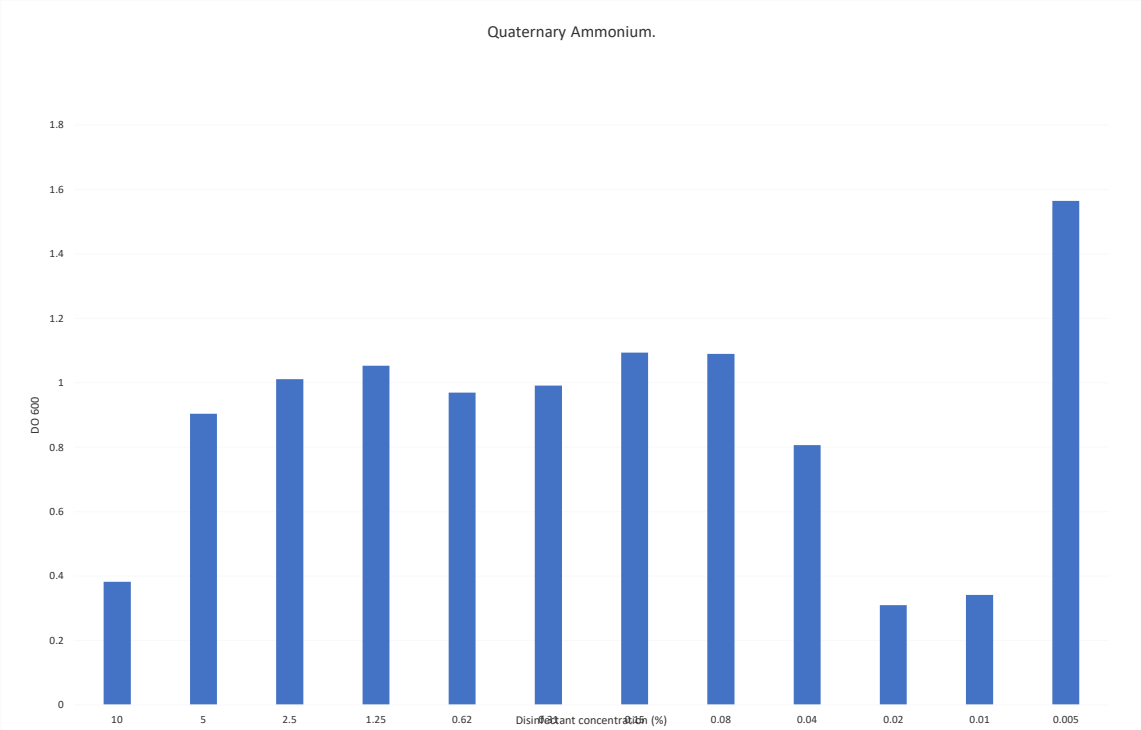


Figure IV.17: The susceptibility to disinfectants (A.Q) on E. coli strains in terms of biofilm production potential.

- Interpretation:

The figure 17 represent a bar chart illustrates the impact of varying concentrations of quaternary ammonium disinfectant on microbial growth, measured through optical density (OD 600). This type of analysis is critical in microbiology and public health to determine the efficacy of disinfectants in controlling microbial growth.

1-Lower Concentrations (0.005% - 0.04%):

- At the lowest concentration (0.005%), the OD 600 is approximately 1.8, suggesting that microbial growth is largely unaffected by this minimal level of disinfectant.
- As the concentration increases to 0.04%, the OD 600 declines, indicating a reduction in microbial growth. This suggests that even at low concentrations, quaternary ammonium begins to exert a bacteriostatic or bactericidal effect.

2-Intermediate Concentrations (0.08% - 1.25%):

- There is a continued decrease in OD 600 values with increasing disinfectant concentration, reflecting enhanced antimicrobial activity. At 0.62%, the OD 600 is significantly reduced, indicating a substantial decline in microbial viability.
- These concentrations demonstrate the effectiveness of quaternary ammonium in a dose-dependent manner, where intermediate levels show considerable microbial inhibition.

3-Higher Concentrations (2.5% - 10%):

- At higher concentrations, the OD 600 values approach zero, especially at the highest concentration tested (10%), where microbial growth is almost entirely inhibited.
- This indicates that quaternary ammonium at these concentrations is highly effective at killing or inhibiting microbial growth, affirming its use as a potent disinfectant in various settings.

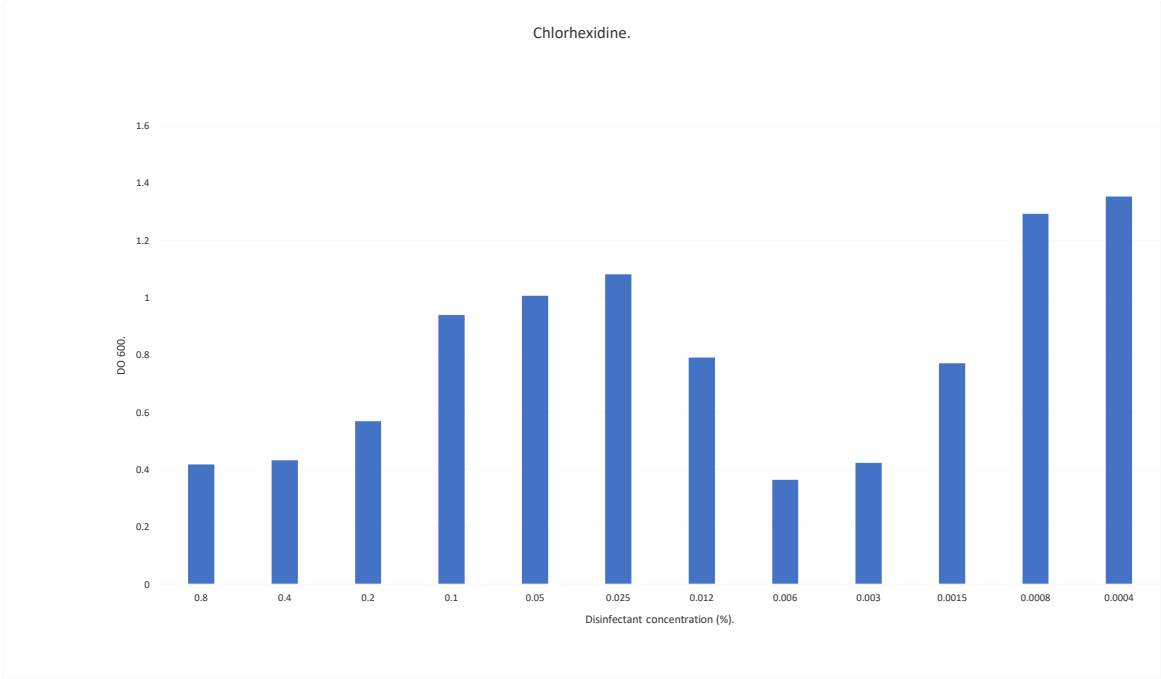


Figure IV.18: The susceptibility to disinfectants (CHX) on E. coli strains in terms of biofilm production potential.

♦ Interpretation:

The figure 18 represent a bar chart examines the effect of different concentrations of chlorhexidine (CHX) on the growth of E. coli strains, measured by optical density at 600 nm (OD 600). Chlorhexidine is a commonly used antiseptic and disinfectant known for its broad-spectrum antimicrobial activity. Understanding its efficacy at various concentrations can inform its application in clinical and hygiene settings to control bacterial growth and biofilm formation.

1-Lower Concentrations (0.0004% - 0.01%):

- At the lowest concentration (0.0004%), the OD 600 is about 1.4 indicating minimal impact on bacterial growth.
- As the concentration increases to 0.01%, there is a noticeable decline in OD 600, suggesting that even at these low levels, chlorhexidine begins to exert inhibitory effects on E. coli growth.

2-Intermediate Concentrations (0.025% - 0.1%):

- The OD 600 values continue to decrease with higher chlorhexidine concentrations, showing enhanced bacterial growth inhibition.
- At 0.05% the OD 600 is significantly reduced, indicating a substantial reduction in E. coli viability.
- These concentrations demonstrate the increasing effectiveness of chlorhexidine in a high dose, with intermediate concentrations showing significant antimicrobial activity.

3-Higher Concentrations (0.2% - 0.8%):

- At these higher concentrations, OD 600 values are substantially lower. For example, at 0.2%, the OD 600 drops to approximately 0.4, indicating a strong inhibitory effect.
- The highest concentration tested (0.8%) shows an OD 600 close to 0.2 suggesting that chlorhexidine at this level is highly effective at killing or significantly inhibiting the growth of *E. coli.*

* Discussion:

The results underscore the efficacy of quaternary ammonium compounds as disinfectants. The dose-dependent decrease in OD 600 values illustrates the progressive antimicrobial activity with increasing concentration. Our results align with the known mode of action of quaternary ammonium and chlorhexidine which disrupt microbial cell membranes, leading to cell death(McDonnell and Russell 1999)[3].

The chart demonstrated a clear inverse relationship between the concentration of (quaternary ammonium-chlorhexidine) disinfectants and microbial growth. Higher concentrations of the disinfectant correspond to lower OD 600 values, indicating less microbial growth. This suggests that those disinfectants are effective at inhibiting microbial growth in a higher concentration.

IV.6. Results of the sensitivity studies of the antibiogram:

The results of the antibiogram performed on E. coli strains using the Vitek2 system reveal the bacteria's response to antibiotics. These results are divided into three categories: resistant(R),intermediate(I),and sensitive(S).

Table: antibio-resistance of E. coli isolated strains.

	Isolated (ID)																					
Antibiotics	A1	A2	A3	A4	A5	A6	A7	A10	A11	A12	A14	A15	A16	A17	A18	A19	A20	A21	A22	A23	A24	A25
Ampicillin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Amoxicillin	S	S	S	I	S	S	S	S	S	S	S	S	S	I	S	S	S	S	S	S	S	S
Piperacillin	S	S	S	S	S	S	S	S	I	S	S	S	S	S	S	S	S	S	S	S	S	S
Cefazolin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Cefoxitin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Cefotaxime	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Ceftazidime	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Ertapenem	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Imipenem	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Amikacin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Gentamicin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Ciprofloxacin	S	R	R	S	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Fosfomycin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Nitrofurantoin	S	S	S	I	S	I	I	S	S	S	I	S	S	S	I	S	S	S	S	S	I	S
Chloramphenicol	S	S	S	R	R	S	S	R	S	I	S	S	I	I	S	S	R	I	I	S	R	S
Colistin																						
trimethoprim	S	R	R	S	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	S

Table IV.10: antibio-resistance of E.coli isolated strains.

Red: Resistant /Green: sensitive /Yellow: Intermediate

The antibiotic resistance patterns of twenty-two *Escherichia coli* isolates were assessed against seventeen antibiotics. The findings, as detailed in Table (10) , indicate that all *E. coli* isolates exhibited a high resistance rate to ampicillin (100%), followed by ciprofloxacin (86.3%) and trimethoprim-sulfamethoxazole (81.8%). Moderate resistance levels were observed for chloramphenicol (22.7%). Conversely, low resistance rates were noted for amoxicillin/clavulanic acid, piperacillin/tazobactam, and nitrofurantoin. Notably, no

resistance was detected for cefazolin, cefoxitin, cefotaxime, ceftazidime, ertapenem, imipenem, amikacin, gentamicin, and Fosfomycin**.**

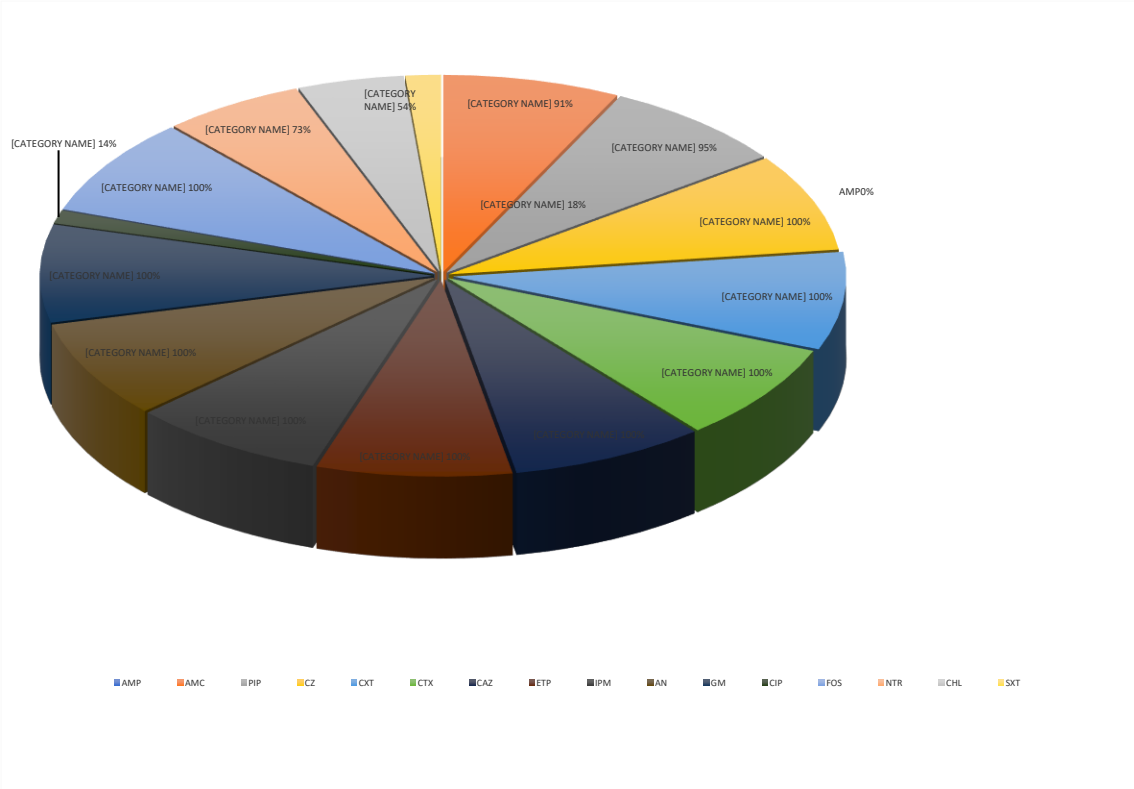


Figure IV.19: pie chart represents the percentage of sensitivity of E. coli strains.

- Interpretation:

The figure 19 represented a pie chart illustrates the percentage of sensitivity of various *E. coli* strains to different antibiotics. Each segment of the pie chart represents the resistance of E. coli strains to a specific antibiotic, measured as a percentage.

1 - Complete susceptibility sensitive (100%):

E. coli strains showed complete resistance (100%) to several antibiotics, indicating that those antibiotics are highly effective against these strains, as indicated by the equal-sized segments. These antibiotics include:

AN (Amikacin) - IPM (Imipenem)- ETP (Ertapenem)- CAZ (Ceftazidime)- CTX (Cefotaxime) - CXT (Cefoxitin) - GM (Gentamicin) - FOS (Fosfomycin)- CZ (Cefazolin).

2 - High sensitive:

- AMC (Amoxicillin) - PIP (Piperacillin)- NTR(Nitrofurantoin).

3 - Moderate sensitive:

-SXT(Trimethoprim-Sulfamethoxazole)-CHL(Chloramphenicol) - CIP(Ciprofloxacin).

4 - Lowersensitive:

- AMP (Ampicillin): 0% (This indicateresistance).

Figure 19 effectively showed the varying degrees of antibiotic sensitivity among E. coli strains. The dominance of the segments for antibiotics with 100% sensitive underscores the effectiveness of these antibiotics against *E. coli*. However, the reduced resectance percentages for certain antibiotics highlight areas of concern where resestance might be prevalent.

The resistance rate of e.coli straines for ampicillin (100%),amoxicillin (10%),cefotaxime (0%),chloramphenicol(46%),cefoxitin(0%),Gentmicin(0%),this results have an agreement with a recent study:Ampicillin (59,09%),Amoxicillin(11,62%),Cefotaxim(4,54%),chloramphenicol(4,04%),Cefoxitin(2.02%),Gentamicin(1.01%)respectetively (Barour,2019)[4].and for the Ciprofloxacine(86%),Trimethoprim Sulfamethoxazole(81%),Imipenem(0%),similar study show Ciprofloxacine (89%),Trimethoprim Sulfamethoxazole (63%) ,Imipenem (0%) (Ait-Mimoune,2022)[5].

Our results are consistent with a previous work conducted in Algeria.

To conclude high sensitivity indicate high antibiotic sensitivity.

IV.6.1.Relationship between biofilm production potential and antibiotic resistance profile:

The study results, which highlight the interaction between the biofilm formation potential of bacterial strains and their antibiotic resistance profile, it was observed from the figure 16 that (81%)of *Escherichia coli* strains exhibited a multidrug resistance (MDR) profile for some antibiotic These strains were resistant to at least three antibiotics belonging to different classes.

E. coli strains exhibit variable biofilm formation potential, ranging from (NBF) to (SBF). These results therefore indicate a weak relationship between antibiotic resistance and biofilm formation capacity. This suggests that other factors may influence biofilm formation independently of antibiotic resistance.

Biofilm formation is recognized as a virulence factor that confers greater aggressiveness to the bacterium, thus prolonging its persistence in the environment. This increased persistence leads to a decrease in the effectiveness of antibiotic therapy. Bacteria

forming biofilms are encased in a protective extracellular matrix that hinders the diffusion and penetration of antibiotic molecules to their specific target (Ito et al, 2009)[6].

IV.7. PCR Technique:

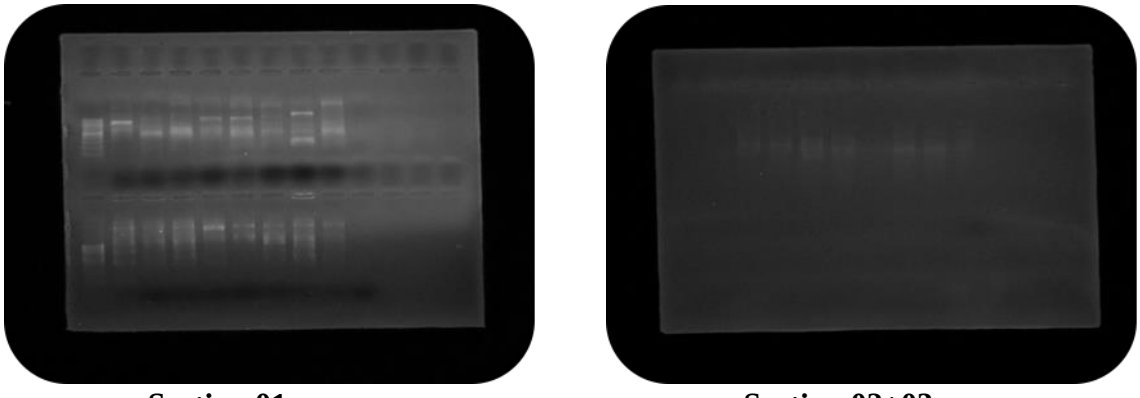
In our study, we performed a collection tubes PCR targeting the *pap-G II, fli-C ,tsh , csgA*

The *pap-G II* gene associated with biofilm adhesion, , while the *fli-C* gene, linked to flagellin for filament formation, the *tsh* gene associated with temperature sensitive hemaggulation, And the *csgA* gene which linked to cell signal genetic analysis,The results of our collection tubes PCR confirmed the presence of the genes already mentioned among certain strains, as evidenced by the appearance of a multiple or single fluorescent band corresponding to the expected DNA size in the agarose gel.

Table: Existence Percentage of the genes in E.coli strains

Genes	Isolates number	Percentages %
fliC	13	57
papG II	6	26
tsh	15	65
csgSA	7	30

Table.IV.11: Existence percentage of the genes in e. coli strains.



Section 01 **Section 02+03**

Figure IV .20: visualization of papG+fliC gene on agarose gel.

* **Results:**

The results showed the presence of specific bands corresponding to the *fliC* genes in 13 samples(57%).the samples are : A2 , A3 , A4 , A5, A7, A10 , A11, A15, A16, A18,A19, A23,ATCC.Compared with a study that detected the fliC gene in 62 strains from 79 by the

PCR assays (78%) which may significantily correspond with the results of our study(Campos ,2008)[7].

However, 11 samples (43%) ;A1, A6, A12, A14, A17,A20,A21,A22,A24,A25 and positive control, do not show specific bands corresponding to the *fliC* gene. These samples may be considered negative for the presence of this gene.

For the *papG II* gene, the results show the presence of specific bands corresponding to the *papG II* genes in 6 samples (26%) : A12,A14,A15,A16,A18, A19.Unlike our study, some research has proven that the band of PapG was the most predominant with (71.4%) (Vandemaele,2003)[8].

However,16samples(74%):A1,A2,A3,A4,A5,A6,A7,A10,A11,A17,A20,A21,A22,A23A24,A25,ATCCand positive control, do not show specific bands corresponding to the papG II gene. These samples may be considered negative for the presence of this gene.

* **Discussion:**

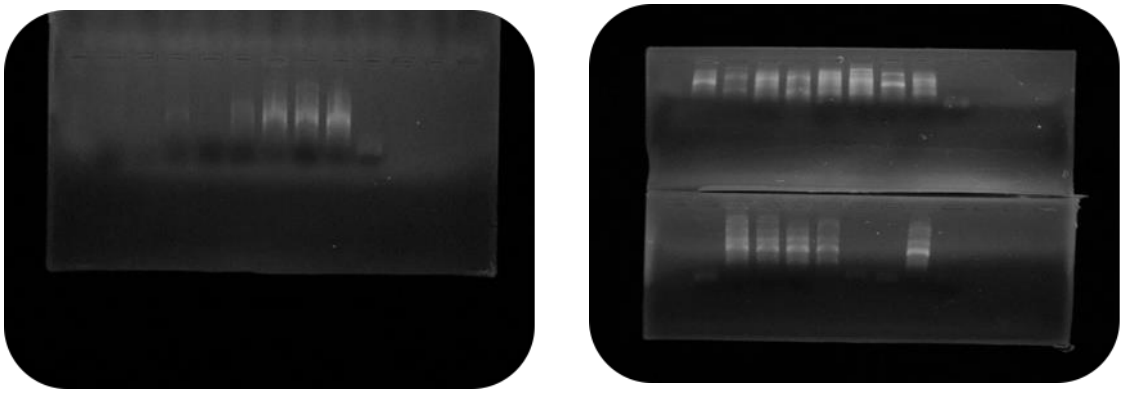
***-FliC* :**

The interpretation of these results suggests that 13 out off 23 samples in our study contain the *fliC* gene. The *fliC* gene encodes for flagellin, the protein that makes up the filament of bacterial flagella. Flagella are crucial for bacterial motility, which is important for the initial stages of biofilm formation. Motility allows bacteria to reach surfaces, adhere, and eventually form biofilms. Studies have shown that the *fliC* gene and its product are essential for the development and maturation of biofilms in various bacteria, including *Escherichia coli* and Salmonella. A study by Pratt and Kolter (1998) demonstrated that mutations in the fliC gene reduced the ability of *E. coli* to form biofilms on abiotic surfaces (Pratt and Kolter 1998)[9].

- papG II:

The interpretation of these results suggests that 6 out off 23 samples in our study contain the papG II gene. PapG II is a crucial adhesin in uropathogenic Escherichia coli (UPEC) that plays a vital role in biofilm formation. It facilitates the initial attachment of UPEC to host tissues, particularly in the urinary tract, by binding to specific receptors on the surface of host cells. This binding is essential for the establishment and colonization of the bacteria, which is a key step in the development of biofilms. These biofilms contribute

significantly to the persistence and chronicity of urinary tract infections caused by UPEC(Kudinha and Kong 2022)[10].



Section 01 Section 02+03

Figure IV .21: visualization of tsh gene on agarose gel.

* **Results:**

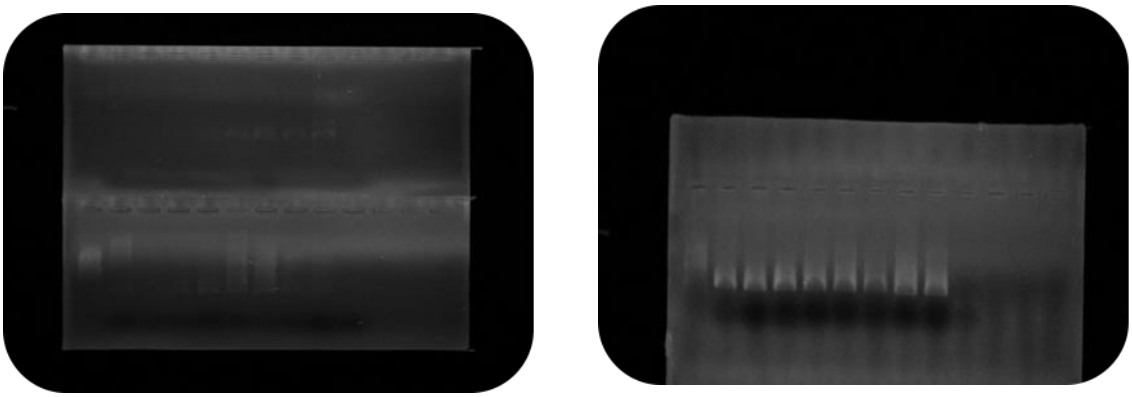
The results show the presence of specific bands corresponding to the tsh gene in 14 samples(65%). For example, samples A6, A7, A10, A11, A14, A15, A16, A17,A18,A19,A21,A22,A23,A24,and positive control, corresponding to the size of amplified *tsh* gene products. In agreement with our results a previous study reported that the tsh gene is present in about 67% of APEC strains(park,2021)[11].

However, 9 sampels(35%) : A1,A2, A3, A4, A5 A12,A20,A25,ATCC do not show specific bands corresponding to the tsh gene. These samples may be considered negative for the presence of this gene.

* **Discussion:**

The interpretation of these results suggests that 14 out off 23 samples in our study contain the tsh gene ,The tsh gene in Escherichia coli (*E. coli*) strains encodes a temperature-sensitive hemagglutinin (T*sh*), which is a large autotransporter protein. This protein is associated with adhesion to host cells, particularly in avian pathogenic E. coli (APEC) strains. Tsh contributes to the bacteria's ability to adhere to and aggregate with epithelial cells, which plays a role in colonization and pathogenicity,tsh gene is regulated by environmental conditions, including temperature, which aligns with its role in infection, as some pathogenic *E. coli* strains are more virulent at the host's body temperature(Dozois et al. 2000)[12].in agreement with our study Research has shown that the tsh gene is present in a significant percentage of APEC isolates. For example, a study reported that the tsh gene is present in

about 67% of APEC strains, highlighting its prevalence and importance in biofilm formation in avian isolates (park,2021)[11].



Section 01+02 Section 03

Figure IV .22: visualization of csgA gene on agarose gel.

* **Results:**.

The results show the presence of specific bands corresponding to the *csgA* gene in 7samples(30%): samples A20, A21,A22,A23,A24,A25,ATCC and positive control exhibit bands of an expected size of 200 base pairs, corresponding to the size of amplified *csgA* gene products. Previous studies have demonstrated the incidence of the *csgA* gene with 63% in a genotypic detection in associated with the biofilm formation which does not correspond with our study(Chapman et al.,2002)[13].

However, 15 sampels (70%):A1,A2, A3, A4,A5,A6,A7,A10,A12,A14,A15,A16, A17, A18 A19,do not show specific bands corresponding to the *csgA* gene. These samples may be considered negative for the presence of this gene.

Discussion:

The interpretation of these results suggests that 7 out off 23samples in our study contain the *csgA* gene ,The *csgA* gene in Escherichia coli (E. coli) strains encodes a major subunit of curli fimbriae, which are extracellular amyloid fibers involved in surface attachment and biofilm formation,Curli fimbriae play a significant role in the adhesion of *E. coli* to surfaces, promoting biofilm formation, which is essential for survival in various environments and contributes to pathogenicity, the csgA gene encodes the major structural protein, *CsgA*, of curli fibers. Curli are important for the bacteria's ability to adhere to surfaces, aggregate, and form biofilms(Chapman et al.,2002)[13].

The study of the *fliC* and papG *II* ,*tsh*,*csgA* genes in our strains has provided important insights into their ability to form biofilms and adhere,attache…,to host tissues. These

findings contribute to our understanding of bacterial interaction mechanisms and may have implications for the development of prevention and treatment strategies for infections associated with them.

-Conclusion:

This study evaluated the ability of Enterobacteriaceae to form biofilms, exploring the tolerance of Enterobacteriaceae isolates responsible for colibacillosis to disinfectants. Additionally, it examined the relationship between biofilm formation in these strains and cross-resistance to disinfectants and antibiotics, and finally, it involved the detection of *fliC, papG II ,tsh* and *csgA* genes in some selected strains.

The results of the TCP method demonstrated that all the strains have the ability to produce biofilm; however, we observed a great variability in this potential among different strains and within the same species. Ten percent (10%) of the isolates showed a low potential for biofilm production, while (90%) exhibited either a moderate to high potential.

Three isolates were selected based on their biofilm formation potential to determine their susceptibilities to two disinfectants (Quaternary Ammonium, Chlorhexidine) by determining their MIC and MBC. The results show that CHX (Chlorhexidine) proves to be the most effective, with lower MIC and MIC90 values for inhibiting bacterial growth.

The results of the antibiotic sensitivity study reveal that all strains (100%) were resistant to at least one tested antibiotic. The resistance rate to Amikacin - Imipenem- Ertapenem- Ceftazidime- Cefotaxime - Cefoxitin - Gentamicin - Fosfomycin- Cefazolin, were the highest (100%) which classified the strains as multidrug-resistant (MDR), followed by Amoxicillin-Piperacillin- Nitrofurantoin, with a rate of 90%-95%-72% respectively. No resistance was observed for the ampicillin.

The study of the *fliC* and *papG II ,tsh,csgA* genes has provided important insights into their ability to form biofilms.

To preserve the quality of poultry meat , it is essential to combat bacterial contamination by resistant bacteria. This involves ensuring hygiene at every stage of the food chain, from farms to consumption of meat, while also monitoring the coordinated evolution of bacteria's resistance to antibiotics.

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Chapter II:

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Annex.

-Annex:

1-Equipment:

The devices and small materials used:

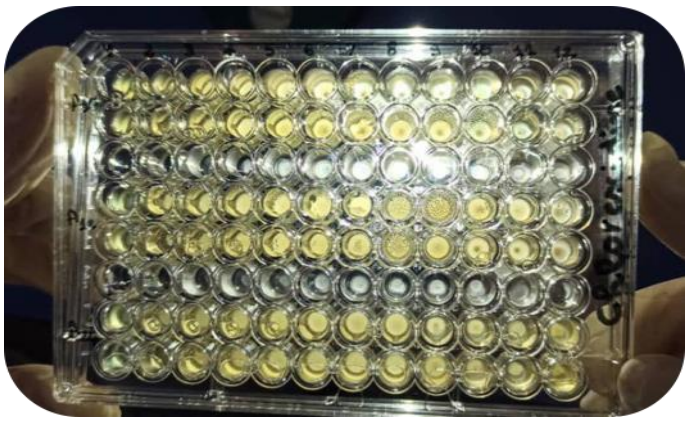
Equipment	Small equipment
-Autoclave (MEMMERT) -Heating magnetic stirrer (SELECTA) -Electrophoresis apparatus -Water bath (MEMMERT) -Balance (Denver Instrument) -Precision balance KERN ALS 220-4N -Centrifuge (Sigma) -Freezer -Densitometer (Grant-bio) -Bacteriological incubator at 37°C (MEMMERT) -Incubator (MEMMERT) -96 well plate reader(Safas Monaco) -Refrigerator at 4°C -Thermo shaker (Optic Evymen System Thermocycler -Vortex -VITEK 2(bioMérieux)	• Platinum loop • Magnetic stir bars • Bunsen burner • Beakers • Plastic Petri dishes • Yellow, blue, and white tips • Test tubes • Erlenmeyer flasks • 200 ml sterile bottles • Eppendorf tubes (1.5 ml; 2 ml) • Micropipettes (200 µl, 1000 µl, 10 µl) • Microplates • Amplification plate • Tube racks • Spatula • Sterile test tubes

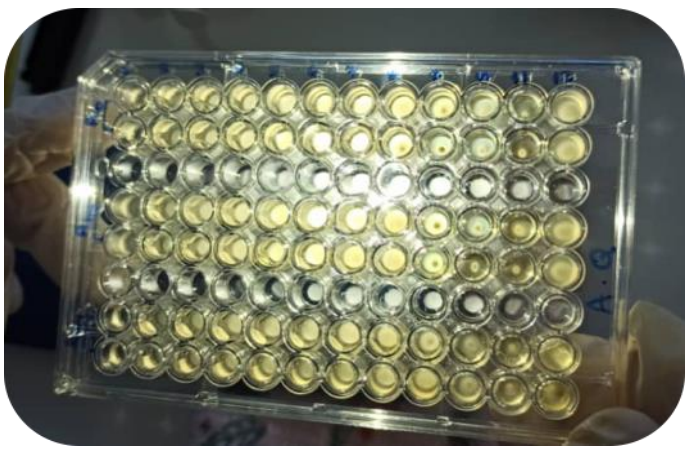
Annex.

2-Media, reagents, and solutions used:

Media	reagents
• 1.5% Agarose gel • MacConkey agar • Mueller-Hinton agar **Liquid Media** • TSB	10% Quaternary ammonium compounds Ethidium bromide 0.01% Chlorhexidine 0.01% Crystal violet DNA ladder Distilled water Physiological water (0.9%) Isopropanol 96% Ethanol 80% Glycerol 2X Your Taq PCR Master Mix (20 µl). Primers PBS buffer TBE buffer

3-MBC/MIC:





Annex.

Electrophoresis Gel Plan

1st Section		
	ID	**Region**
1	*100 bp-Marker*	
2	*A_1*	*Univ-Medea*
3	*A_2*	*Univ-Medea*
4	*A_3*	*Univ-Medea*
5	*A_4*	*Univ-Medea*
6	*A_5*	*Univ-Medea*
7	*A_6*	*Univ-Medea*
8	*A_7*	*Univ-Medea*
9	*A_10*	*Univ-Medea*
10	*-_Kntr*	*H_2O*
11	*+_Kntr*	*Univ -kmis*
12	*100 bp-Marker*	

2nd Section		
	ID	**Region**
1	100 bp-Marker	
2	A_11	Univ-Medea
3	A_12	Univ-Medea
4	A_14	Univ-Medea
5	A_15	Univ-Medea
6	A_16	Univ-Medea
7	A_17	Univ-Medea
8	A_18	Univ-Medea
9	A_19	Univ-Medea
10	*-_Kntr*	*H_2O*
11	*+_Kntr*	Univ -kmis
12	100 bp Marker	

3 rd Section		
1	*100 bp-Marker*	
2	*A_20*	*Univ-Medea*
3	*A_21*	*Univ-Medea*
4	*A_22*	*Univ-Medea*
5	*A_23*	*Univ-Medea*
6	*A_24*	*Univ-Medea*
7	*A_25*	*Univ-Medea*
8	*ATCC*	*Univ-Medea*
9	*-_Kntr*	*H_2O*
10	*+_Kntr*	*Univ -kmis*
12		

Protocol-Nr:*2024_Ecoli_Biofilm* _n°1/ *papGⅡ+fliC* Date: 17.03.2024

with 2X your taq pcr master mix multiplex PCR (20 µl)

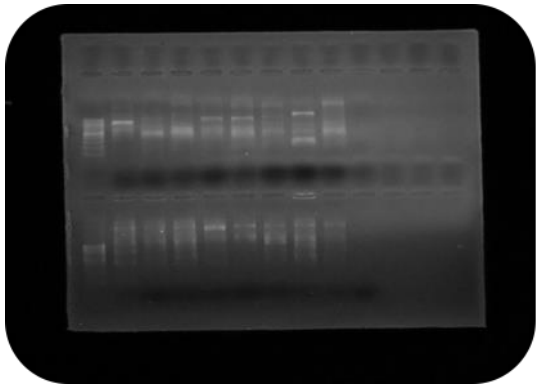
Annex.

PCR 20µl	ID ® / Charge	Concentrat°	µl / React° 20µl	PCR cocktail N=28	Programm:Biofilm Factors
Master Mix	2X your taq pcr master mix	2X	10µl	280µl	190 BP PRIMEE papG II
	28-41-00001				
Forwarde primer **papG II**	EurogenTec ® Belgium	100µM	1	**28µl**	547 BP PRIMER fliC
Reverve primer **papG II**	EurogenTec ® Belgium	100µM	1	**28µl**	
Forwarde primer **fliC**	EurogenTec ® Belgium	100µM	1	**28µl**	
Reverve primer **fliC**	EurogenTec ® Belgium	100µM	1	**28µl**	

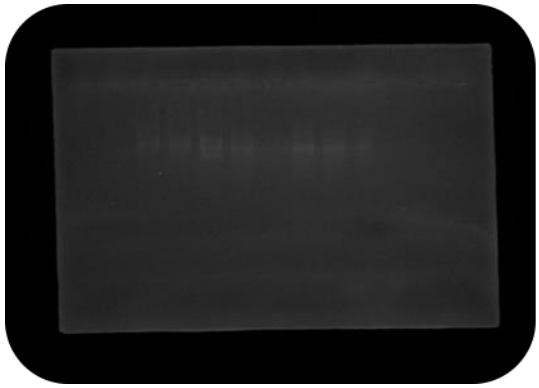
H2O Ultra-Pure			***5µl***	**140**	Init. Denatur.	95°C 5 min
			Total	**532µl**		
Template DNA			**1**		Denatur	94° 30s
					Annealing	63°C 30s
					Extension	72°C 60s
					Cycle	35
					Extension	72°C 5min
					Preservation	4°C
					Agarose-Gel 1.5 %	10 V 30 min

Result:

Section 01:



Section 02+03:



Protocol-Nr:*2024_Ecoli_Biofilm* _n°1/papG II+fliC

Annex.

Protocol-Nr:*2024_Ecoli_Biofilm* _n°2/ tsh Date: 17.03.2024

with 2X your taq pcr master mix multiplex PCR (20 µl)

Electrophoresis Gel Plan

1st Section

	ID	Region
1	*100 bp-Marker*	
2	*A_1*	*Univ-Medea*
3	*A_2*	*Univ-Medea*
4	*A_3*	*Univ-Medea*
5	*A_4*	*Univ-Medea*
6	*A_5*	*Univ-Medea*
7	*A_6*	*Univ-Medea*
8	*A_7*	*Univ-Medea*
9	*A_10*	*Univ-Medea*
10	*-_Kntr*	*H_2O*
11	*+_Kntr*	*Univ -kmis*
12	*100 bp-Marker*	

2nd Section

	ID	Region
1	100 bp-Marker	
2	A_11	Univ-Medea
3	A_12	Univ-Medea
4	A_14	Univ-Medea
5	A_15	Univ-Medea
6	A_16	Univ-Medea
7	A_17	Univ-Medea
8	A_18	Univ-Medea
9	A_19	Univ-Medea
10	*-_Kntr*	*H_2O*
11	*+_Kntr*	Univ -kmis
12	100 bp Marker	

3 rd Section

1	*100 bp-Marker*	
2	*A_20*	*Univ-Medea*
3	*A_21*	*Univ-Medea*
4	*A_22*	*Univ-Medea*
5	*A_23*	*Univ-Medea*
6	*A_24*	*Univ-Medea*
7	*A_25*	*Univ-Medea*
8	*ATCC*	*Univ-Medea*
9	*-_Kntr*	*H_2O*
10	*+_Kntr*	*Univ -kmis*
12		

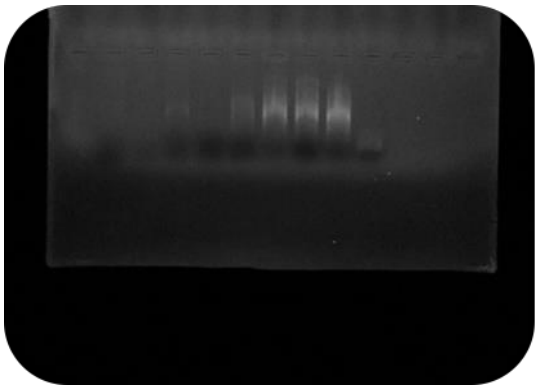
Annex.

PCR 20µl	ID ® / Charge	Concentrat°	µl / React° 20µl	PCR cocktail N=28	Programm:Biofilm Factors
Master Mix	2X your taq pcr master mix	2X	10µl	**280µl**	420 BP PRIMEE tsh
	28-41-00001				
Forwarde primer *tsh*	EurogenTec ® Belgium	100µM	1	**28µl**	
Reverve primer *tsh*	EurogenTec ® Belgium	100µM	1	**28µl**	
H2O Ultra-Pure			***7µl***	**196**	
			Total	**532µl**	
Template DNA			**1**		

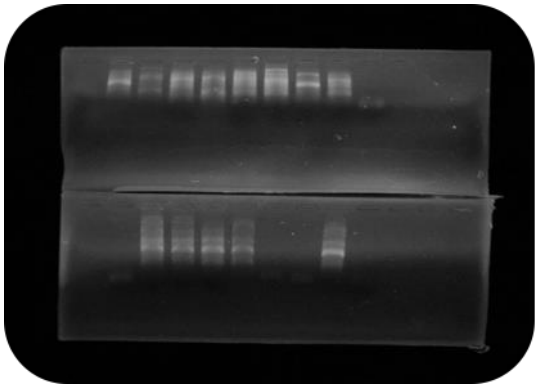
Init. Denatur.	95°C 5 min
Denatur	94°C 1 min
Annealing	55°C 45s
Extension	72°C 60s
Cycle	35
Extension	72°C 5min
Preservation	4°C
Agarose-Gel 1.5 %	100V 30 min

Result :

Section 01:



Section 02 +03:



Protocol-Nr:*2024_Ecoli_Biofilm* _n 2/tsh

Annex.

Electrophoresis Gel Plan

1st Section

	ID	Region
1	100 bp-Marker	
2	A_1	Univ-Medea
3	A_2	Univ-Medea
4	A_3	Univ-Medea
5	A_4	Univ-Medea
6	A_5	Univ-Medea
7	A_6	Univ-Medea
8	A_7	Univ-Medea
9	A_10	Univ-Medea
10	-_*Kntr*	H_2O
11	+_*Kntr*	Univ -kmis
12	100 bp-Marker	

2nd Section

	ID	Region
1	100 bp-Marker	
2	A_11	Univ-Medea
3	A_12	Univ-Medea
4	A_14	Univ-Medea
5	A_15	Univ-Medea
6	A_16	Univ-Medea
7	A_17	Univ-Medea
8	A_18	Univ-Medea
9	A_19	Univ-Medea
10	-_*Kntr*	H_2O
11	+_*Kntr*	Univ -kmis
12	100 bp Marker	

3rd Section

1	100 bp-Marker	
2	A_20	Univ-Medea
3	A_21	Univ-Medea
4	A_22	Univ-Medea
5	A_23	Univ-Medea
6	A_24	Univ-Medea
7	A_25	Univ-Medea
8	ATCC	Univ-Medea
9	-_*Kntr*	H_2O
10	+_*Kntr*	Univ -kmis
12	100 bp-Marker	

Protocol-Nr:*2024_Ecoli_Biofilm* _n°3/ csgA Date: 17.03.2024

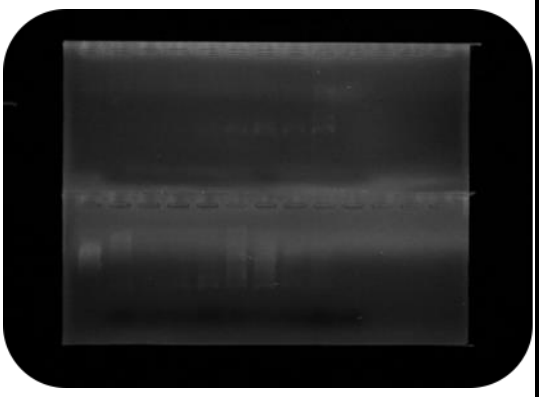
with 2X your taq pcr master mix multiplex PCR (20 µl)

Annex.

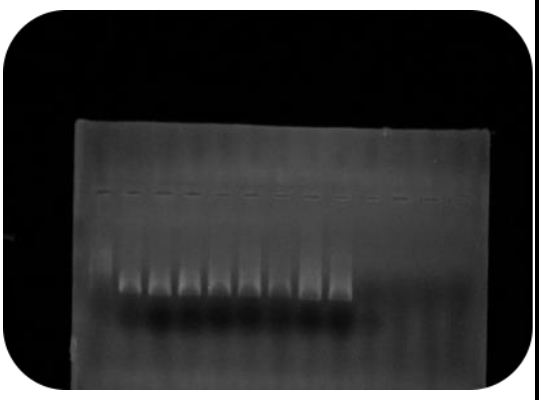
PCR 20µl	ID ® / Charge	Concentrat°	µl / React° 20µl	PCR cocktail N=28	
Master Mix	2X your taq pcr master mix	2X	10µl	**280µl**	**Programm:Biofilm Factors**
	28-41-00001				200 BP PRIMEE tsh
Forwarde primer *csgA*	EurogenTec ® Belgium	100µM	1	**28µl**	
Reverve primer *csgA*	EurogenTec ® Belgium	100µM	1	**28µl**	
H2O Ultra-Pure			***7µl***	**196**	Init. Denatur. 95°C 5 min
			Total	**532µl**	
Template DNA			**1**		Denatur 94°C 1 min Annealing 55°C 45s Extension 72°C 60s Cycle 35 Extension 72°C 5min Preservation 4°C Agarose-Gel 1.5 % 100V 30 min

Result:

Section 01+02:



Section 03:



Protocol-Nr:*2024_Ecoli_Biofilm* _n°3/csgA