SVU- International Journal of Veterinary Sciences, 6(1): 100-115, 2023
Print ISSN: 2535-1826 **Online ISSN: 2535-1877**

Research Article Open Access

The effect of some essential oils against biofilm producing *Pseudomonas aeruginosa* of meat sources

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Abstract

Pseudomonas aeruginosa is one of the biofilm-forming pathogens in the food industry. Biofilms can be formed on both animate and inanimate surfaces throughout the food chain. This poses a tough challenge resulting in changes in the food industry's cleaning and disinfection dynamics. In this study, *Pseudomonas aeruginosa* (*P. aeruginosa*) was detected, using the morphological and biochemical methods, in 12 out of 32 isolates of Gram-negative motile rod-shaped isolates *"Pseudomonas* spp." isolated from meat products, and 5 of the 12 (41.7%) *P. aeruginosa* strains were strong biofilm-producers using crystal violet microtiter plate assay. The PCR results revealed that 5 isolates of *P. aeruginosa* were encoding the aminoglycoside regulator response (*arr*) gene responsible for the biofilm production, with an incidence of 41.7 %; 2 (40%) of which were detected in the minced beef strains, 1 (20%) for each of the sausage and burger strains while it could not be identified in luncheon strains. Thymol and cinnamon essential oils were used to control *P. aeruginosa* strains and their biofilm in vitro using the microtiter plate assay. Applying the EO caused a reduction in *P. aeruginosa* biofilm formation. While clove EO did not inhibit the growth of *P. aeruginosa* using the agar well diffusion method. The application of thymol and cinnamon Eos in meat contact surfaces or meat products could inhibit the growth of *P. aeruginosa* as well as reduce the biofilm formation resulting in preventing serious health and economic problems.

Keywords: *Pseudomonas aeruginosa*, biofilm production, *arr* gene, essential oils.

DOI: 10.21608/svu.2023.174609.1239 Received: November 13, 2022 Accepted: March 10, 2023 Published: March 26, 2023

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Citation: Mohammed et al., The effect of some essential oils against biofilm producing *Pseudomonas aeruginosa* of meat sources**.** SVU-IJVS 2023, 6(1): 100-115.

Competing interest: The authors have declared that no competing interest exists.

Introduction

Pseudomonas is a heterotrophic, motile, Gram-negative rod-shaped bacterium. Pseudomonads are generally ubiquitous psychrotrophic spoilage organisms that are often found in food processing environments, including floors and drains, and also on fruit, vegetables, and meat surfaces, and in low-acid dairy products (Rivas et al., 2018). *P. aeruginosa* is an opportunistic pathogen that is ubiquitous in the environment. It is responsible for persistent infections due to its peculiar resistance mechanisms to antimicrobials and its ability to produce a biofilm (Artini et al., 2018).

The presence of members of the genus *Pseudomonas* especially *P. aeruginosa* can lead to quick spoilage of the products and reduced shelf life of them. In addition to this, the ability of these spoilage bacteria to survive under low temperatures may cause difficulty during the storage of foods (Wang et al., 2017). *P. aeruginosa* causes serious and fatal diseases and frequently causes secondary infections therefore it poses a great risk hazard for both humans and animals (Lopez et al., 2015). *P. aeruginosa* may cause digestive tract infection with symptoms of headache, diarrhea, and in severe cases, fever may occur. In complicated cases, the infection may reach the sterile body sites, such as the bloodstream, respiratory tract, gastrointestinal tract, urinary tract, tendons, pressure abscesses, wounds, and burns, leading to serious complications (Sapkota, 2021).

Bacteria bind to surfaces and form spatially structured communities inside a self-produced matrix, which consists of extracellular polymeric substances known as biofilms. These biofilms composed of immobilized microbial cells and act as an independent ecosystem, are excellent survival techniques for microorganisms, that provide a protective barrier, and allow them to fight against the adverse environmental conditions facing typically the bacteria in man-made, natural settings, and even in food-processing premises such as treatment with sanitizers and other antimicrobial agents (Ordóñez et al., 2019;).

Biofilms imply major challenges for the food industry because they allow bacteria to bind to a range of surfaces, including stainless steel, glass, plastic, polypropylene, rubber, and even food products, within just a few minutes, then mature biofilms could be developed within a few days or even hours, these microorganisms that are capable to attach to surfaces and form mature biofilms represent a great risk to public health (Carrascosa et al., 2021).

Biofilms are believed to be responsible for damaging equipment, more expensive energy costs, outbreaks, and food spoilage. They become more resistant to disinfection in wide-ranging food industries, such as seafood, dairy, meat, and poultry processing (Ordóñez et al., 2019).

The microorganisms' capacity to adhesion and biofilm formation depends on parameters such as surface material, contact time, pH, temperature, the growth phase of cells, and growth medium. Therefore, certain conditions throughout the food chain could enable biofilm formation on biotic and abiotic surfaces (Papazoglou et al., 2016; Bridier et al., 2016).

Pseudomonas aeruginosa is considered a model organism for developing biofilms and their quorum

sensing regulation, it is about 1–5µm long and 0.5–1.0 µm wide a facultative aerobe with nitrate as the terminal electron acceptor. The extracellular filamentous appendices like Flagella and pili of the motile microorganisms help in the attachment process and the interaction with surfaces. *Pseudomonas* spp. produce huge amounts of extracellular polymeric substances and are known to attach and form biofilms on stainless steel surfaces. They can co-exist with other pathogens in biofilms to form multispecies biofilms, which makes them more resistant and stable (Amina and Bensoltane, 2015).

With growing demands for foods with fewer additives, due to their possible toxicity, and consumers demanding healthier foods with fewer preservatives, the search for natural products with antimicrobial properties is also growing. The antimicrobial activity of EOs is highly variable, mainly influenced by their chemical composition and/or concentration. Some EO components act on the lipid bilayer of the cell membrane, and others inhibit protein synthesis and DNA replication (Perrino et al., 2021). Cinnamon EO was reported to be an effective *Pseudomonas* spp and reduced its biofilm production ability (Agha, 2020). This effect is assumed to be owing to phenolic compounds such as eugenol that can disrupt microbial energy production mechanisms (Alessandra et al., 2019).

This work aimed to identify the occurrence of biofilm-producing *Pseudomonas aeruginosa* (*P. aeruginosa*) from meat sources retailed in Sohag Governorate. also, potential application of thyme and cinnamon Eos to the control of biofilm production.

Material and Methods

Bacterial Isolates

32 Gram-negative motile bacteria were previously isolated from 200 retailed meat products (minced beef meat, luncheon, burger, and sausage, 50 of each) in Sohag city, the isolates were identified for the presence of *P. aeruginosa* using conventional biochemical methods. The identified *P. aeruginosa* strains were tested for biofilm production using Congo red and microtiter plate assays. Strains showing positive biofilm production were confirmed by PCR using *arr*- specific primers (Jones et al., 2013).

Phenotypic identification of *P. aeruginosa* (Quinn et al., 2002)

A loopful of inoculated nutrient broth was streaked firstly onto the surface of Cetrimide agar then one colony on nutrient agar for purification and conformation.

Microscopy and Gram stain properties

All purified strains were sub-cultured again for microscopic identification of *Pseudomonas* species using the Gram staining technique (Becerra et al. 2016).

Enzymatic activities of *P. aeruginosa* (PHE, 2015)

The enzymatic activities of *Pseudomonas* species were evaluated using certain biochemical tests, including oxidase, citrate utilization, and indole tests (LaBauve and Wargo 2012). The positive oxidase activity is indicated by the appearance of a purple/ violet color. The indole test is performed by adding a few drops of xylene and 0.5 ml of Kovac's indole reagent, to 5ml of 48 hrs. incubated peptone water (OXOID, CM0509) inoculated with the tested culture, a positive reaction indicated by the formation of a pink colored ring, while a yellow color indicates negative results. *P. aeruginosa* can utilize citrate as a source of energy, this is detected by streaking a freshly purified colony onto a Simmons

citrate agar (OXOID, CM0155) slant, and incubated at 37 °C for 24hrs, negative tubes complete in the incubator for 5-7 successive days. The positive citrate utilization was indicated by the appearance of a sky-blue color.

Congo red binding activity (Berkhoff and Vinal,1986)

The method was performed by Congo red agar medium. Each isolate was cultivated on tryptic soy agar supplemented with 0.03% of Congo red dye and 0.15 bile salt for 24 hours at 37^{o}C. Congo red-positive *P. aeruginosa* was indicated by the development of red colonies. Biofilm production was indicated through the demonstration of black colonies with a dry crystalline consistency **(Freeman et al., 1989)**.

Quantitative method for biofilm-forming capacity (Lee et al., 2011)

Using 150 µl of the bacterial suspensions in TSB medium was inoculated duplicate separate wells of a flat-bottomed 96-well polystyrene microtiter plate and duplicate wells of uninoculated media as a control. The edges of the plate were then sealed with parafilm, and the plate was incubated for 24h at 37^{o}C without shaking, after that, the liquid was removed, and the plates were rinsed three times with 200 µl of Phosphate Buffer Saline (PBS). The biofilms were fixed by adding 100 µl of 99% methanol for 15 min, then removed and the plate was air–dried in an inverted position for 30 min . To each well 150 µl of a 0.1% crystal violet solution (in sterile water) was added and incubated for 20 min at room temperature. Unbound dye was removed by rinsing three times with 200 µl of PBS. The bounded dye was released by adding 150 µl of 33% acetic acid. The contents of each well (100 µl) were then transferred to a sterile polystyrene microtiter plate and the optical density (OD $_{595\ nm}$) of each well was measured by using a micro ELISA auto reader. The isolates were classified into; weak biofilm producers (Mean OD=0.1-0.2), moderate biofilm producers (0.2-0.5), and strong biofilm producers (<0.5).

Molecular identification of P. aeruginosa isolated from meat products

DNA extraction

The DNA of the suspected strains was extracted using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) following the manufacturer's recommendations as follows; in a 1.5 ml microcentrifuge tube, 200 µl of the culture suspension was mixed with 10 µl of proteinase K and 200 µl of lysis buffer then incubated for 10 min at 56°C. After that, 200 µl of 96% analytical grade-ethanol was mixed with the lysate using a pulsing vortex for 15 seconds. In a 2ml collecting capped tube, the mixture was carefully applied to the QIAamp mini spin column and centrifuged for 1 min. at 8000 rpm. The DNA was eluted with 100 µl of AE elution buffer. Then the QIAamp mini spin column was incubated at 15-25˚C for 1 min, after which, it was recentrifuged at 8000 rpm for 1 min.

Polymerase chain reaction (PCR) amplification using oligonucleotide primers

The oligonucleotide primers with sequences shown in Table1 were supplied from Midland Certified Reagent Company_ oligos (USA), and used for the amplification of the *P. aeruginosa arr* gene as the following; 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of the forward and reverse primers (20 pmol) for each. 5 µl of template DNA and complete up to 25 µl with nuclease-free

water (5.5 µl) were added and thoroughly vortexed, then PCR tubes were cycled using an applied biosystem thermal cycler according to Jones et al. (2013) as the following; an initial denaturation step at 95˚C for 5 minutes, 35 cycles at 94˚C for 30 sec., then 52˚C for 40 sec., 72˚ C for 45 sec., and the final extension step at 72˚C for 10 minutes**.**

Table 1: Sequences of the *arr gene* oligonucleotide primers of *P.aeruginosa*

Gene		Primer sequence (5'-3')	Product size	Reference
arr	Forward	AGCGCATCACCCCCAGCAAC	685	**Jones et al., 2013**
	Reverse	**CGCCAAGTGCGAGCCACTGA**		

Analysis of the PCR products

The amplified PCR products were visualized using agarose gel electrophoresis with the aid of ethidium bromide (Applichem, Germany) and running buffer (1× TBE). A 15 µl of each PCR product along with control positive of *P. aeruginosa* (ATCC 27853), and control negative (distilled water, Merck, Germany) were numbered and loaded into the gel wells as well as 6 µl of a 100 bp DNA Marker (Qiagen, Germany, GmbH) to determine the product sizes. The electric current was adjusted at 1-5V/cm of the tank length (80 V/30 min.). The gel was photographed using a gel documentation system (Alpha Innotech, Biometra).

Essential Oils

The following plant essential oils were assayed in the current study; thyme EO, cinnamon EO, and EO were purchased from the National Research Center, Egypt.

Antibacterial activity of essential oils using agar well diffusion assay (CLSI, 2014)

The antibacterial activity was assessed using the agar well diffusion method. 0.1 ml broth of an overnight culture of each bacterial isolate was spread on the surface of Mueller-Hinton agar plates using a sterile L shaped- glass rod. 6 mm wells were made in the inoculated agar plates, and loaded with different concentrations of the tested oil, -ve control as well as +ve control of QACs. Plates were left for 10 min, saturated with the essential oil added to the inoculated agar plate, and tested organisms incubated at 37°C. After 24 hours of incubation, the diameters of the inhibition zone were measured in millimeters (mm).

Determination of biofilm production

Tube method (Deka et al., 2014)

The tube method was used for detecting biofilm production. Different concentrations of each essential oil were prepared in polystyrene test tubes containing brain heart infusion broth, inoculated with 0.1 ml of a 24 hours old culture for 24 h at 37°C. A loopful of colony suspension from an overnight culture was inoculated into 10 ml brain heart infusion broth and incubated for 24 h at 37°C. Then, the tubes were inverted to discard the culture and washed with PBS (pH 7.3). Tubes were stained with 0.1% crystal violet, Crystal violet-stained test tubes were rinsed twice with PBS to remove the stain. Post drying, the occurrence of visible film-lined walls, and the bottom of the tube indicated biofilm production, and biofilm formation was scored as 0-absent, 1: weak, 2: moderate, and 3: strong.

Modified Crystal Violet Microtiter Plate Assay (Djordjevic et al., 2002)

It is a semi-quantitative biofilm formation analysis, based on crystal violet binds to extracellular molecules that are negatively charged, including polysaccharides in biofilms and is used to determine the density of the attached cells. 96-well microtiter plates were inoculated with 200 µl of 24 h old liquid culture containing approx. 10^8 CFU ml^{-1} (180 µl Brain heart infusion broth inoculated with 20 µl bacterial culture). Following 4 h of cell adhesion at corresponding temperatures, the supernatant was removed from each well, and the plates were rinsed with physiological saline. Subsequently, 200 µl of fresh medium containing the EO or the component to be examined was added in 75 & 100 µg/ml concentration (µl/ml) to each well, and the plates were further incubated at 37°C for 24 h. Positive controls contained only the inoculated growth medium and negative controls contained EOs or components in the growth medium (Peeters et al., 2008). The dye was discarded, and the plates were dried for 15 min. Strains were efficient in biofilm formation if absorbance at OD630 nm was equal to or greater than 0.15 (Di Martino et al., 2003). Experiments were repeated at least two times, and six parallel measurements were made each time.

Results

Table 2 and Fig. 1 showed that *Pseudomonas* spp. was identified in 32 (16%) of the examined meat product samples with the highest incidence in minced beef meat 30 % followed by 18%, 10 %, and 6 % in luncheon, sausage, and burger, respectively. The preliminary identification showed that there are 12 isolates of *P. aeruginosa*, 8 of which confirmed as *P. aeruginosa*. All the 8 *P. aeruginosa* isolates showed positive biofilm production activity. By using microtiter plate semi-quantification assay, of 12 biofilm-producing strains; 5 isolates (41.7%) were strong, 3 isolates (25%) were moderate, and 4 isolates (8.3%) were weak biofilm producers (Table 3 and Fig.2)

Table 2: Morphological and biochemical identification of *P. aeruginosa* isolated from some meat products.

	Minced meat	Luncheon	Burger	Sausage	Total
Tested isolates	15	9	3	5	32
Gm -ve rods	15	9	3	5	32
Non-fermenter	15	9	3	5	32
Motility	12	8	3	5	28
Catalase +ve	10	6	3	4	23
Indole -ve	9	6	3	4	22
Oxidase +ve	4	5	3	4	17
MR -ve	6	5	3	3	17
H2S -ve	4	4	2	2	12
Citrate +ve	4	4	2	2	12
Urease -ve	4	4	2	2	12
Gelatin hydrolysis +ve	4	4	2	2	12
Growth at 41ºC	3	1	2	2	8

Fig. 1: Colonies of *Pseudomonas aeruginosa* on nutrient agar.

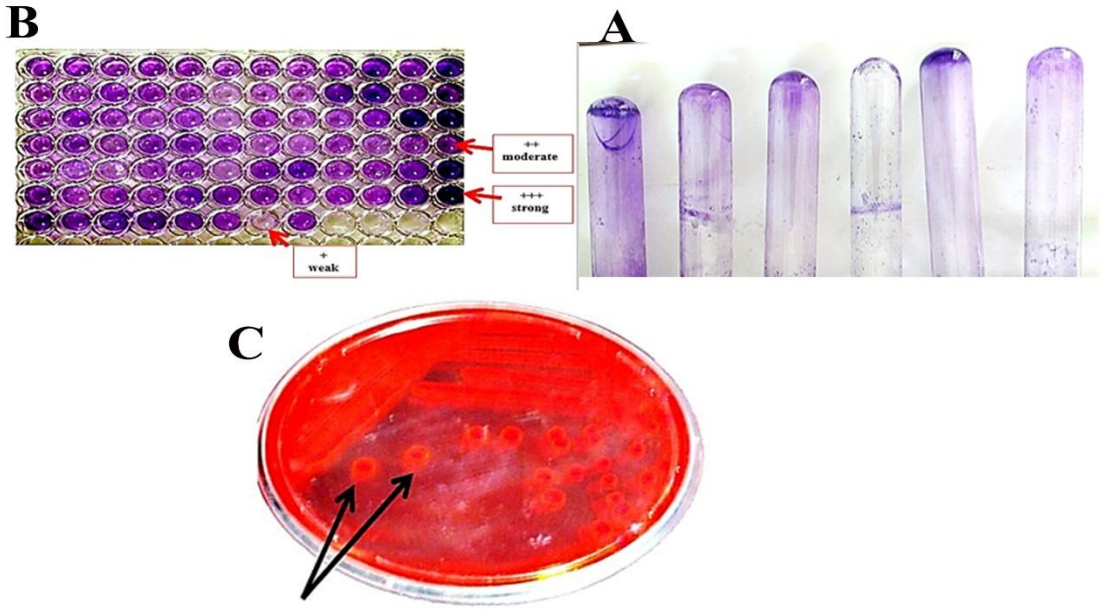

Fig.2: Biofilm production of *P. aeruginosa* strains: (a) Detection of biofilm production by *P. aeruginosa* strains using tube assay (b) Detection of biofilm production by *P. aeruginosa* strains using a microplate assay. (c) Biofilm production of *P. aeruginosa* strains on Congo red agar.

Table 3: Detection of biofilm production of *P. aeruginosa* using Congo red and microtiter plate assay.

Samples	*Pseudomonas* isolates	Tested strains		Congo red binding		Biofilm production	
		+ve	%	+ve	%	+ve	%
Minced beef	15	4	26.7	2	50	2	50
Luncheon	9	4	44.4	1	25	1	25
Sausage	3	2	67.7	1	50	1	50
Burger	5	2	40	1	50	1	50
Total	32	12	37.5	5	41.6	5	41.6

Table 4 and Figs. 3 & 4 revealed the incidence of PCR- confirmed strains of *P. aeruginosa* 8 of 12 PCR-tested isolates were confirmed as *P. aeruginosa* using the *arr* gene-specific primer (the sequence as described by Jones et al. (2013) was shown in Table 1. *P. aeruginosa* was detected in 5 of 12 strains with an incidence of 41.7 %; the highest incidence was detected in the strains obtained from minced beef and sausage 2/5 (40 %) for each, burger strains as 1 (20%), while Luncheon strains did not carry the *arr* gene.

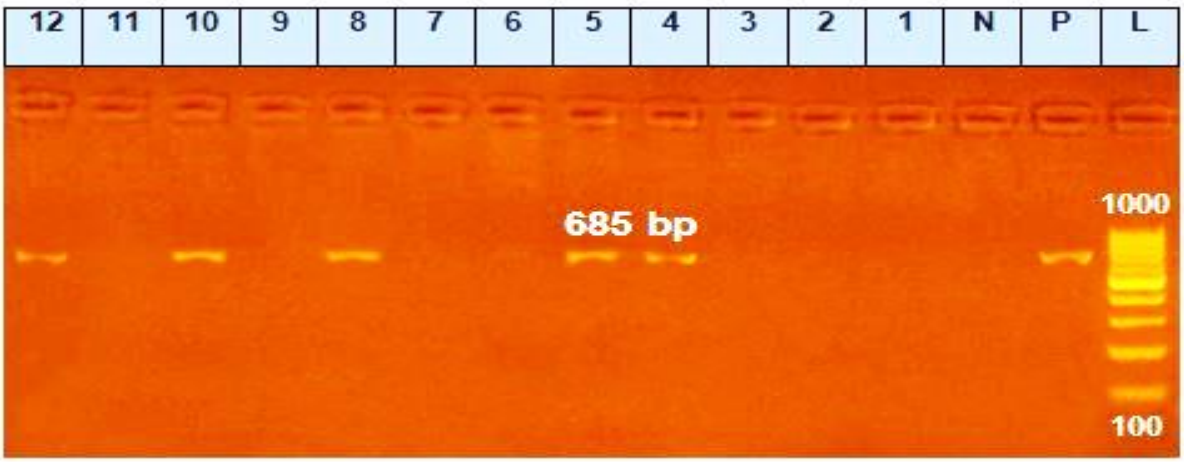

Fig. 3: PCR results for *P. aeruginosa arr* gene (685 pb). Lane L: 100 bp DNA marker; Lane P: Control positive; Lane N: Control negative; Lanes 1, 2 &3: negative results for *arr* gene of *P. aeruginosa* strains isolated from luncheon. Lanes 4 & 5: positive results for *arr* gene of *P. aeruginosa* strains isolated from sausage. Lanes 6, 7, 9 & 11: negative results for *arr* gene of *P. aeruginosa* strains isolated from minced meat. Lanes 8 &10: positive results for *arr* gene of *P. aeruginosa* strains isolated from minced meat. Lane 12: positive results for *arr* gene of *P. aeruginosa* strains isolated from a burger sample.

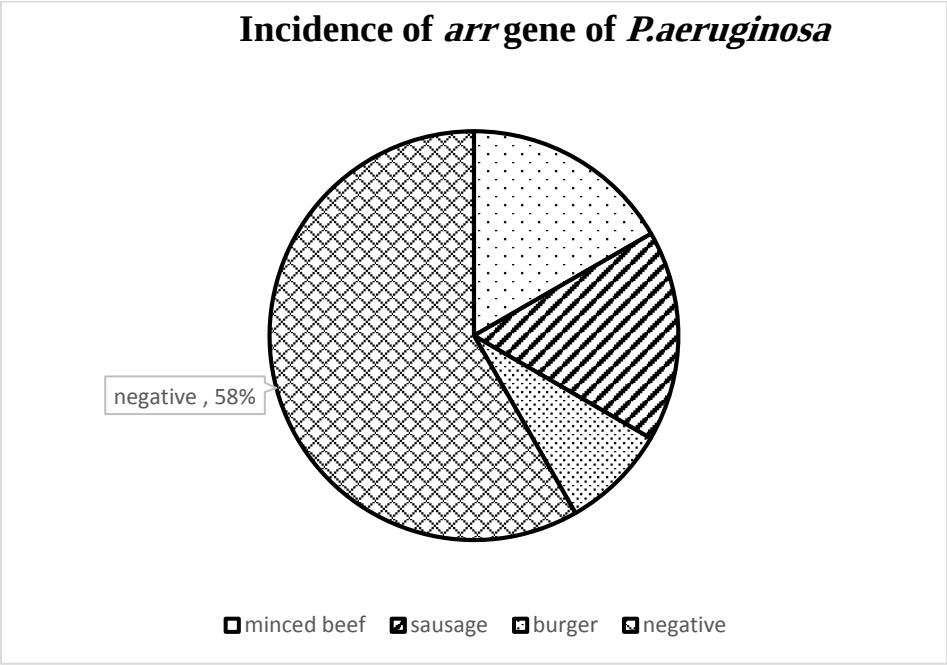

Fig. 4: Incidence of *arr* gene of *P. aeruginosa* strains isolated from some meat products.

Table 4: Degree of biofilm formed by *P. aeruginosa* using microtiter plate assay

	Tested strains*	Strong		Moderate		Weak	
		+ve	%	+ve	%	+ve	%
Minced beef	**5**	**2**	**40**	**2**	**67.7**	**1**	**25**
Luncheon	**3**	**0**	**0**	**0**	**0**	**3**	**75**
Sausage	**2**	**2**	**40**	**0**	**0**	**0**	**0**
Burger	**2**	**1**	**20**	**1**	**33.3**	**0**	**0**
Total	**12**	**5**	**41.7**	**3**	**25**	**4**	**33.3**

*Crystal violet tube assay revealed that 12 of the tested strains were biofilm producers.

Table 5: PCR results of the *arr* genes of *Pseudomonas* spp. isolated from meat products.

Sample	Tested isolates	*P. aeruginosa*		*P. putida*		*P. flurescens*		arr gene	
		+ve	% *	+ve	% *			+ve	% *
Minced beef	**6**	**4/8**	**50**	**1**		**1**		**2/5**	**40**
Luncheon	**3**	**1/8**	**25**	**0**		**2**		**0/5**	**0**
Sausage	**2**	**2/8**	**25**	**0**		**0**		**2/5**	**40**
Burger	**1**	**1/8**	**12.5**	**0**		**0**		**1/5**	**20**
Total (n=+ve)	**12/32**	**8/32**	**5/8**	**0**		**0**		**5/8**	**5/12**
Total %	**37.5%**	**25%**	**62.5%**					**62.5%**	**41.7%**

***P. aeruginosa* (8 strains; 4 minced beef, 2 sausages, 1burger, 1 luncheon), *P. putida* (1 minced meat isolate), and *P. flurescens* (3 isolates; 2 luncheons, 1 minced meat)**

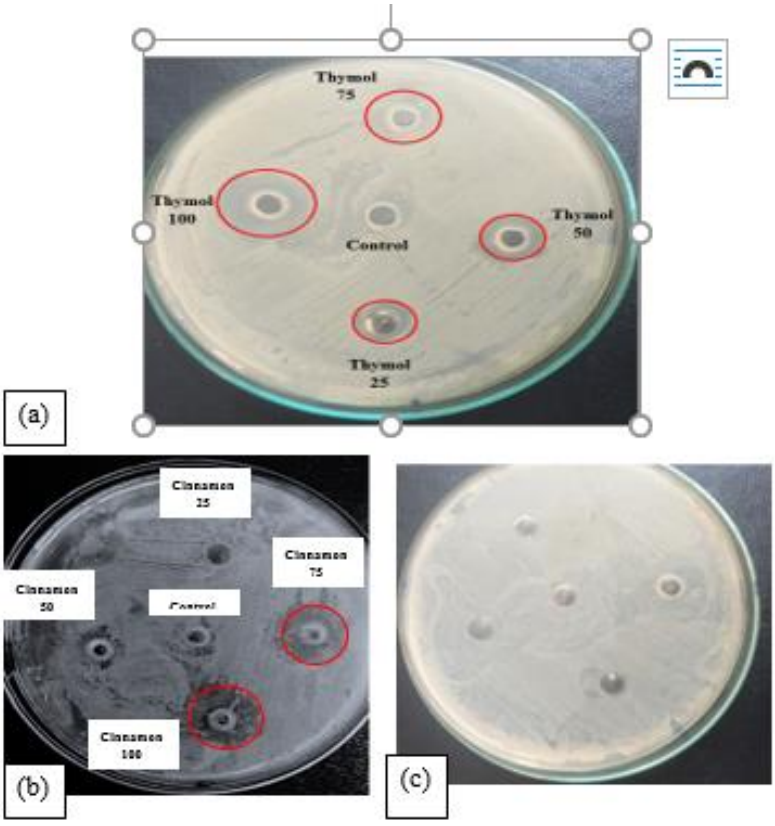

Fig.5: Evaluation of the inhibitory effect of thymol, cinnamon, and clove on *P. aeruginosa*. (a) inhibitory zones produced by different concentrations of thymol EO. (b) inhibitory zones produced by different concentrations of cinnamon EO. (c) agar well diffusion showed that clove EO failed to inhibit the growth of *P. aeruginosa*.

Using the agar well diffusion assay thymol and cinnamon Eos inhibited the growth of *P. aeruginosa* at concentrations of 50, 75, and 100 µg/ml for each, with 50 µg/ml as the minimum inhibitory concentration (MIC) for both Eos. On the other hand, clove EO did not inhibit the *P. aeruginosa* growth (Table 6 & Fig. 6).

Table 6& Fig.7 showed that there is a significant difference between the control and Eos treatment, especially at a concentration of 100 µg/ml of both thyme and cinnamon Eos. While a nonsignificant difference between the low (75 µg/ml) and the high concentration(100µg/ml) of each EO.

Table 6: Antibacterial effect of some essential oils against *P. aeruginosa* isolated from meat products.

Essential oil	Eos Concentrations	Inhibitory zone on agar well diffusion (mm)
Cinnamon	25	R
	50	≥8±2 mm
	75	≤14 ±1mm=MIC
	100	16±2 mm
Thymol	25	R
	50	10±1mm
	75	15±1mm
	100	≤18±1 mm
Clove	25	R
	50	R
	75	R
	100	R

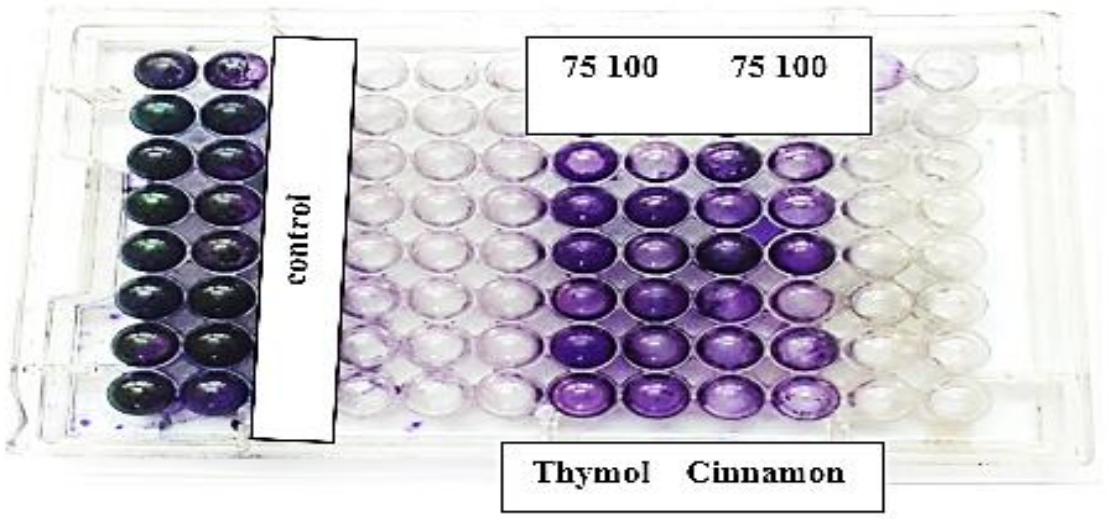

Fig.6: Inhibitory effect of Thymol and Cinnamon Eos on biofilm produced by *P. aeruginosa*

Table 7: Antibiofilm effect of cinnamon EO against *P. aeruginosa* using microtiter plate assay

EOs / Days		Control	Cinnamon		Thymol	
			75	100	75	100
Immature biofilm	1st 2nd 3rd	+ ++ +++	0- weak	weak	0- weak	weak
Mature biofilm	1st 2nd 3rd	+++ +++ +++	weak	weak	weak	weak

DISCUSSION

Morphological and biochemical characteristics confirmed that *P. aeruginosa* is a motile (unipolar), gram-negative, facultative, catalase positive, oxidative positive, nitrate reduction positive, non-fermentative, and rod-shaped bacterium measuring 0.5 to 0.8µm by 1.5 to 3.0µm in size. Its optimum temperature for growth is 37°C but retains a growth potential at temperatures as high as 42°C (PHE, 2015).

Table 2 showed that among 32 *Pseudomonas* spp. 8 out of the 32 strains were identified as *P. aeruginosa* using morphological and biochemical methods. *P. aeruginosa* was found in 4 (50%) minced beef, 2 (25%) sausage, 1(12.5%) for each of the burger and luncheon isolates, while 4 isolates of the 12 suspected strains were distinguished from *P. aeruginosa* as they didn't grow at 41^{o} C, defined as *P. putida* (1 in minced beef strains) and *P. fluorescens* (1minced beef strain and 2 luncheon strains). A lower result was obtained by Elbayoumi et al. (2021) who identified *Pseudomonas* spp. in 10 of the minced meat samples; *P. aeruginosa* was detected in 2/10 (20%), *P. fluorescens* 4/10 (40%) while, *P. putida* could not be detected.

These results are higher than the result obtained by Elbayoumi et al. (2021) who identified *P. aeruginosa* was detected in 4/21beef burgers (19 %), Finally, in sausage *P. aeruginosa* with an incidence of 2/8 (25%), a lower incidence 3/23 (13%) was reported by Elbayoumi et al. (2021). These results agree with Bukhari and Aleanizy (2019) who isolated *P. aeruginosa*., Akan and Gürbüz (2016) who isolated *P. aeruginosa*, *P. fluorescens, and P. putida*. These results also are consistent with those found by Virupakshaiah and Hemalata (2016) who identified *Pseudomonas aeruginosa* from food isolates.

The occurrence of *P. aeruginosa* in meat products indicates that hygienic conditions should be checked in the production, packaging, transport, storage, and marketing of meat and meat products (Akan and Gürbüz, 2016). The presence of *Pseudomonas* spp. in food samples is of great significance as the organism is considered a pathogenic bacterium for man and as an indicator of food quality (Yagoub, (2009). Apart from being a spoilage microorganism, *Pseudomonas* spp. could cause urinary and bloodstream infections (Kotra, 2008).

It was shown that the subinhibitory concentrations of aminoglycoside antibiotics induce biofilm formation in *P. aeruginosa*. In *P. aeruginosa*, the aminoglycoside response regulator (*arr*) gene, is essential for the induction and contribution of biofilm-specific aminoglycoside resistance. The *arr* gene is predicted to encode an inner-membrane phosphodiesterase whose substrate is cyclic di-guanosine monophosphate (c-di-

GMP)-a bacterial second messenger that regulates cell surface adhesiveness (Hoffman et al.,2005).

Determination of biofilm production of *P. aeruginosa*

Table 4 showed that 12 of the tested isolates produced biofilms using tube stained with crystal violet; 8 of which were identified as *P. aeruginosa* and 2 for each of *P. putida* and *P. fluorescens*. There were 5 out of 8 *P. aeruginosa* isolates showed a positive reaction (62.5%) on CRA (Table 4 and Fig. 1). Our results are constant with Rewatkar and Wadhar (2013) that the tube method was easy to apply and accurately detected the ability of *P. aeruginosa* to form biofilm on the glass test tube. Also, it is more qualitative and reliable if compared to Congo red assay. However, there is hard to distinguish weak biofilm and non-biofilm production using tube assay.

Our results shown in Tables 3&4 and Fig. 2 revealed that there were 5 of 8 *P. aeruginosa* strains produce biofilms with an incidence of (62.5%). They could be distinguished into 3 degrees; the strong, the moderate, and the weak biofilm producers as follows; 5 (41.7%), 3 (25%), and 4 (33.3%), respectively. A higher result was reported by Hisham et al. (2012); Saffari et al. (2017) and Abd El-Galil et al. (2013) who reported that all *P. aeruginosa* isolates were biofilm producers. Our results are lower than those obtained by Hisham et al. (2012) who found that 16 isolates (80%) were strong biofilm producers, 2 isolates (10%) were moderate biofilm producers and 2 isolates (10%) were weak biofilm producers. Moreover, Abd El-Galil et al. (2013) recorded that 42 isolates (84%) were strong biofilm producers, 4 isolates (8%) were moderate biofilm producers and 4 isolates (8%) were weak biofilm producers.

The *arr* phosphodiesterases are responsible for the breakdown of c-di-GMP. In addition, the *arr* gene is required for biofilm development, as a high level of c-di-GMP promotes biofilm formation whereas phosphodiesterases diminish c-di-GMP concentration and cause biofilm dispersal (Romling et al., 2013). Tobramycin and gentamicin at subinhibitory concentrations of 0.5 and 0.6 g/ml, respectively, had an impact on biofilm development. They concluded that the *arr* gene is not present in all *P. aeruginosa* strains. PAK responds to subinhibitory concentrations of antibiotics and forms biofilm independently of the *arr* gene (Jones et al., 2013).

As shown in Table 5 and Figs..3&4 there were 5 of 8 *P. aeruginosa* strains encoding the *arr* gene with an incidence of (62.5%), the *arr* gene was detected in 5 strong biofilm producers (100 %) while the *arr* gene was not detected in moderate and weak biofilm producers as shown in Table 4 and Fig. 5, this result is lower than those obtained by Danielle et al. (2010) who found the *arr* gene was detected in 69 % of the biofilm-producing isolates.

Results shown in Table 6 and Fig.5 revealed that thymol and cinnamon Eos caused zones of inhibition at a concentration as low as 50 µg/ml with a maximum zone of inhibition of about 18± 1 at a concentration of 100 µg/ml. Also, thymol and cinnamon EOs caused a reduction in biofilm formed by *P. aeruginosa* (Table 7 and Fig.6). On the other side, clove EO did not show any inhibitory effect on *P. aeruginosa*. These results agree with those obtained by (Agha, 2020) who demonstrated that cinnamon showed antibiofilm effectiveness against

the tested strains of *Pseudomonas* spp. This effect is assumed to be owing to phenolic compounds such as eugenol that can disrupt microbial energy production mechanisms (Alessandra et al., 2019). Similarly, Agha (2020) revealed that clove EO did not inhibit the biofilm production of *P. aeruginosa*, moreover it stimulated the biofilm formation.

Numerous factors influence the yield and composition of essential oils including the plant, seasonal variations, and maturity (Wissal et al., 2016). Essential oils and (E)-cinnamaldehyde inhibit Gram-positive and negative bacteria in planktonic form. Also, they inhibit the formation of biofilms, which are directly related to infections. Bioactivity is associated with the presence of high content of (E)-cinnamaldehyde (Firmino et al., 2018). Other reported antimicrobial agents could stimulate biofilm formation by different bacteria as a self-protective reaction (Shemesh et al., 2010; Maryam et al., 2013).

CONCLUSION

Pseudomonas spp. are of the most prominent meat spoilage bacteria, that cause spoilage under refrigeration conditions, resulting in causing a big problem for the meat industry, overbearing financial losses, food waste, and loss the consumer trust. The ability to produce biofilms on food contact surfaces and resist disinfectants form constant points of cross-contamination that can result in spoilage of meat and meat products This research highlights the importance of using herbal extracts such as EOs as potential disinfectants and food preservatives against food spoilage and biofilm producing bacteria especially *P. aeruginosa* in meat products.

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